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ANALYTICA CHIMICA ACTA

An international journal devoted to all branches of analytical chemistry

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PAPERS PRESENTED AT THE INTERNATIONAL SYMPOSIUM ON HORMONE AND VETERINARY DRUG RESIDUE ANALYSIS, GHENT, BELGIUM, MAY 19–22, 1992

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FOREWORD

The *International Symposium on Hormone and Veterinary Drug Residue Analysis* was organized in Ghent (Belgium), May 19–22, 1992. This Symposium followed two previous Symposia, organized in 1988 and 1990, under the name *International Symposium on the Analysis of Anabolizing and Doping Agents in Biosamples*. The quality of food products of animal origin, and the eventual presence of residues of veterinary drugs and especially of anabolic agents, is indeed an important concern of the consumer. However, the Symposium also again attracted scientists working in doping analysis. The exchange of information between people involved in residue analysis in foods and doping specialists should be encouraged. Only a few years after the illegal use of β-agonists in cattle fattening, they seem to have found their way into sport, as was clearly demonstrated at the latest Olympics in Barcelona. The link between cattle fattening and doping, had been established earlier, and the interference of the consumption of meat contaminated with anabolic steroids on doping tests was further documented at this latest Symposium. So, history teaches that there should be continuing vigilance.

Borders in the European Community disappeared on January 1, 1993. However, as far as the residue problem is concerned, they vanished long before, as well for the scientists in view of the exchange of information, as for the illegal user and the distributor of black-market preparations. Efforts are made to have uniform and consistent controls within the EC Member States and this was documented on the Symposium. There was also a lot of interest from third countries and countries wishing to join the EC in the near future. After all, the problem of residues is a border-crossing one.

The Proceedings of the International Symposium on Hormone and Veterinary Drug Residue Analysis are published in *Analytica Chimica Acta*, "an international journal devoted to all branches of analytical chemistry". And this broadness was indeed needed to cover the papers on analysis for a variety of drugs such as anabolic steroids, β-agonists, antibiotics and sulphonamides. Where immunoassays seem to be extremely useful for screening purposes, chromatography followed by spectroscopy will add the necessary data to confirm the preliminary results. Other trends which were further developed are metabolic studies and the importance of identifying the right target compound, and analysis for natural steroid hormones in order to distinguish illegal administration from endogenous presence. A considerable number of papers was again dedicated to the determination of β-agonists.

The Symposium aimed to make the problems encountered by residue analysts and their latest advances open to discussion. Specialists in the field and interested analytical scientists gathered at this meeting. In these Proceedings, a more than considerable "aliquot" of the presentations is published. We hope that they will contribute to a still better knowledge and exchange of information between scientists.

C. Van Peteghem
(Chairman)

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Certification of the mass concentration of three stilbenes in bovine urine reference materials by gas chromatographic–mass spectrometric methods. Sources of error and their control

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(Received 20th May 1992; revised manuscript received 30th November 1992)

Abstract

In order to confirm the presence of residues in various tissue samples, procedures based on gas chromatography–mass spectrometry (GC–MS) are the methods of choice. The accuracy, repeatability and reproducibility of existing and/or new GC–MS procedures can be checked by certified matrix matched reference materials (CRMs). Reference materials provide support for the quality control in reference laboratories and for the quality criteria of residue analysis. However, to arrive at a certified value several objectives are important such as the elimination of various sources of errors. This paper discusses some analytical errors detected in the certification study on three stilbenes in bovine urine reference materials. The coefficients of variation for the repeatability (within-laboratory), reproducibility (between-laboratory) and for some calibration parameters are compared with those available from the literature. A link to proficiency schemes for laboratory accreditation and laboratory control purposes is also given.

Keywords: Gas chromatography–mass spectrometry; Reference materials; Stilbenes; Urine

Analysis of samples by gas chromatography–mass spectrometry (GC–MS) is obligatory if the presence of residues has to be confirmed [1]. In order to prove and/or check the accuracy, reproducibility and repeatability of existing and/or new procedures, matrix matched reference materials with accurate and traceable certified values are important [2]. Such certified reference materials (CRMs) provide also the basis for quality control in reference laboratories, for quality criteria of residue analysis [1] and for the preparation of secondary reference materials with control values traceable to the certified ones. They allow individual laboratories to establish an internal control system [3] in order to check precision and accuracy of routine as well as newly developed procedures.

In order to arrive at a certified value for matrix matched reference materials it is important to asses the strengths and weaknesses of individual steps of analytical procedures by intercomparison studies, to identify and to eliminate sources of systematic and random errors. Sources of error in trace analysis can generally be classified due to individual steps of the analytical process [4]. This paper discusses some analytical errors detected in the certification study on three stilbenes in bovine urine reference materials. These are the first of a series of reference materials for the analysis of compounds which are prohibited in the European Community by a Council Directive [5]. They are routinely measured in urine. The coefficients of variation (C.V.) for the repeatability (within-laboratory) and reproducibility (between-labora-

Correspondence to: A. Boenke, Commission of the European Communities, Community Bureau of Reference (BCR), Rue de la Loi 200, B-1049 Brussels (Belgium).

tory) are presented together with the C.V.s for some calibration parameters. A comparison of the repeatability and reproducibility C.V.s with the corresponding C.V.s from the quality criteria in the literature [1] and those calculated according to the Horwitz equation [6] are also given.

EXPERIMENTAL

Preparation of urine reference materials

Six urine reference materials, three blanks and three stilbene containing ones (one for each individual stilbene), were prepared in lyophilized form based on incurred urine. The contaminated materials were obtained from three cattle each of which had been treated with one of the stilbenes. After initial analysis of these (source) materials, they were diluted with blank urine obtained from animals which were never treated with stilbenes or other anabolic compounds to form the target mass concentration. Prior to their lyophilization an extensive homogenization was performed. The materials were filled in vials, closed and sealed under nitrogen.

The blank materials were obtained from cattle which were certified by the experimental farm where they were raised as never having been treated with stilbenes or other anabolic compounds. The essential steps employed in the preparation are presented schematically in Fig. 1.

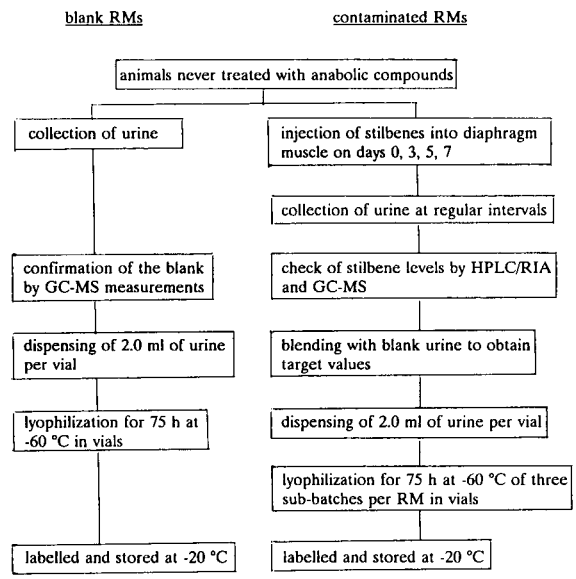

Fig. 1. Preparation of lyophilized bovine urine reference materials.

Homogeneity studies of urine reference materials

The homogeneity studies were carried out on reconstituted liquid urine. The homogeneity of the blank reference materials were evaluated by GC–isotope dilution (ID)MS of the reconstituted contents of 12 vials taken at regular intervals in the filling sequence. Each time two ions were monitored by GC–IDMS, one for each analyte [dienoestrol (DE), hexoestrol (HEX) and *trans*-diethylstilboestrol(*trans*-DES)] and one for the deuterated internal standard.

The homogeneity of the corresponding contaminated reference materials was determined on 24 vials taken at regular intervals from the filling sequence. The within- and between-vial homogeneity was checked by liquid chromatography (LC)–radioimmunoassay (RIA) and the between-vial homogeneity was confirmed by GC–IDMS. The GC–IDMS determinations were carried out over two days.

Stability studies of urine reference materials

A one-year stability study was performed at temperatures of −20°C, +4°C, +20°C and +37°C. The stilbene mass concentrations in the positive reference materials were determined in duplicate and in the blank materials in singular. All measurements of DE, HEX and *trans*-DES at each temperature were performed by GC–IDMS after 1.5, 3, 6 and 12 month time intervals. The results of the homogeneity studies were taken as the starting point (initial time zero).

Certification study

To avoid problems due to the use of calibrants with unknown or inadequate purity or uncertain identity, all calibrants and deuterated internal standard were supplied by the organizing laboratory and were used by all participants. Their purity and identity were checked by LC with multiwavelength detection and GC–MS measurements of their trimethylsilyl derivates in the full scan mode, respectively.

The influence of method variations and losses during sample pretreatment were minimized by using deuterated internal standards. The mass concentrations of the secondary calibration solutions for the determination of the three stilbenes were selected to cover the linear range of the mass response of the single ions monitored at the mass spectrometric detector and to ensure that the response ratios obtained were similar to those to be expected for the samples.

Five samples of each reference material had to be analysed over at least two different days in order to detect any day effects. Only procedures based on GC–IDMS were accepted for certification. Eleven laboratories participated in this certification exercise. Ten analysed the materials with GC–IDMS and one laboratory used LC–RIA as well as LC–radioreceptorassay (RRA).

RESULTS AND DISCUSSIONS

Homogeneity studies of urine reference materials

No substantial within- and between-vial inhomogeneity and no evidence of any sequence effects (drift) of the stilbene mass concentration with the filling sequence was found. No stilbenes were detected in the blank reference materials at the limit of detection of 0.1 $\mu g\ l^{-1}$ (signal to noise ratio of 3:1).

Stability studies of urine reference materials

The results showed that no significant changes in the stilbene mass concentrations occur during storage. The materials allow normal postal shipment.

Certification study (technical aspects)

The analytical errors detected in this study are grouped according to the following individual steps of the analytical process: (i) calibration (i.e. standard stability, purity etc.); (ii) enzymatic hydrolysis and/or extraction; (iii) clean-up; (iv) derivatisation; (v) determination (injection, separation and detection).

The instability of DES standards under UV light and the isomerisation of *trans*-DES caused difficulties in this study. The latter was due to the variation of the *cis / trans* ratio between sub-sample determination. Storage of the standards at +4°C and in the dark is increasing the stability while the measurement of both isomers *cis*- and *trans*-DES in all standards and samples avoid influences of isomerisation.

Insufficient clean-up led to matrix influences for the GC–SIM-MS (SIM = single ion monitoring) determination of the ions: m/z 383 (DES) and m/z 386 (d_6-DES). Poor recovery values for the deuterated DES internal standard was the logical consequence. A clean-up check on spiked samples and the use of CRMs can help to avoid this problem.

Most difficulties were found within the derivatisation/injection step of the analytical procedures. The degradation of heptafluorobutyryl (HFB) derivatives after splitless and on-column injection formed trimethylsilyl (TMS) derivatives. To prevent such difficulties, cleaning of the glass liner, avoidance of TMS derivative injection previous to HFB derivative injection or the use of only TMS derivatives provide a possible solution.

In case of DES, one laboratory used a procedure involving an equilibration step. However, problems due to LC fractionation led only to a value for *trans*-DES. This could be overcome if all LC fractions of interest are collected and stored at −18°C until the analysis is checked.

The application of the criteria for a qualitative analysis by GC–MS led to difficulties. The ion-intensity ratios showed an increase at higher injection levels of the standard. It is therefore suggested to check the linearity of the calibration curve for each of the ions monitored and to dilute or concentrate the sample.

One laboratory reported difficulties to fulfil the criteria for the qualitative GC–MS analysis of HEX with electron impact ionisation (EI). These difficulties were solved by performing GC–MS measurements of the heptafluorobutyryl and the trimethylsilyl derivative. Positive chemical ionisation (PI) may be an additional solution to this problem. In addition, the deuterated internal standards used for these measurements did not control the enzymatic hydrolysis step done by *helix pomatia* juice (an enzyme cocktail).

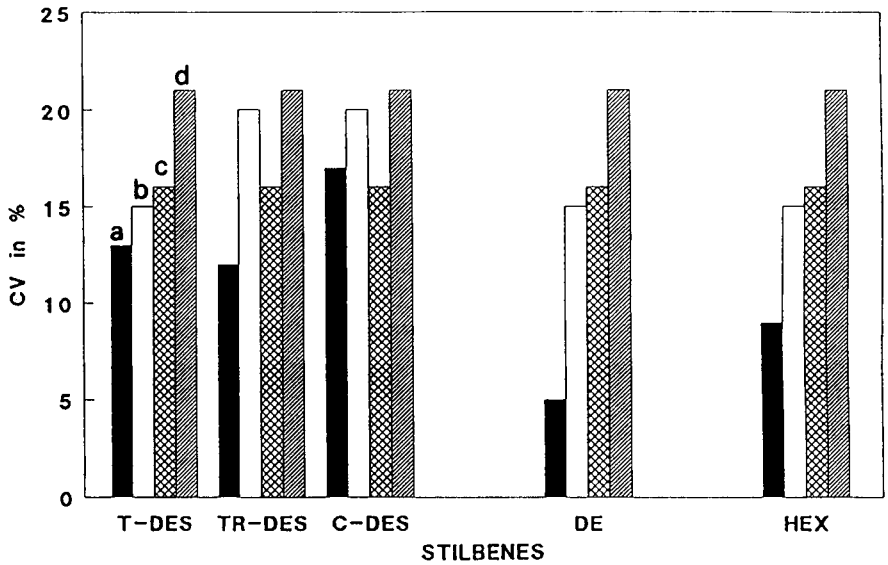

Fig. 2. Comparison of repeatability (within-laboratory) C.V. values based on GC–MS (EI, PI) procedures. T-DES = Total DES; TR-DES = *trans*-DES; C-DES = *cis*-DES. (a) Certification study for urine. (b) Quality criteria from Ref. 1. Typical repeatability values for a compound level of 10 μg kg^{-1} are shown: (c) lower C.V. of the range 16–21%; (d) higher C.V. of the range 16–21%.

Certification study (statistical aspects)

Table 1 shows a summary of the highest coefficients of variation (C.V.) for the calibration parameters. The results are based on within-laboratory calibration data for the slope and the correlation coefficient r. These two parameters were

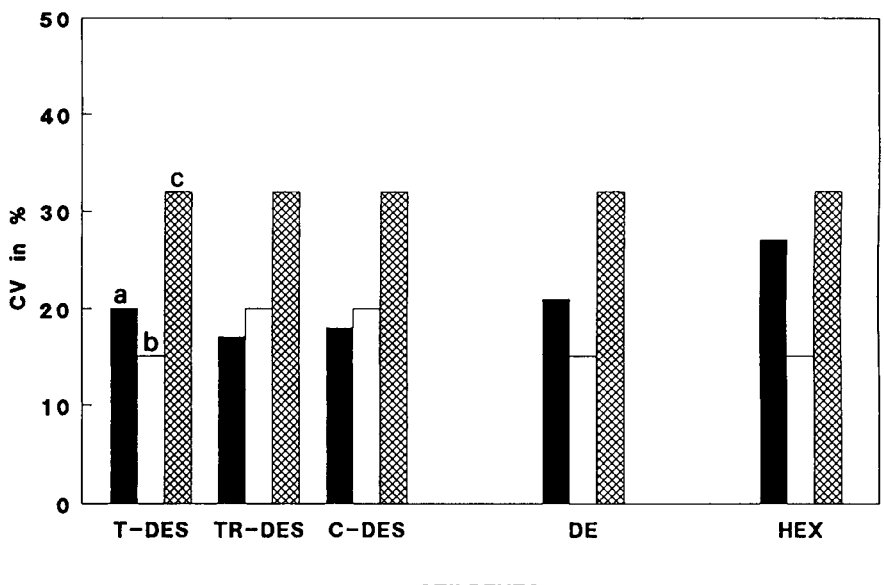

Fig. 3. Comparison of reproducibility (between-laboratory) C.V. values based on GC–MS (EI, PI) procedures. Details as in Fig. 2. (c) Typical reproducibility value for a compound level of 10 μg kg^{-1}.

TABLE 1

Summary of coefficients of variation (C.V.) for two different parameters of the calibration curve (results are grouped by day and analyte)

Stilbene	Effect	C.V.	
		Slope	r [a]
trans-DES	Within-day	⩽10	–
	Between-day	⩽18	⩽2
DE	Within-day	⩽7	–
	Between-day	⩽17	⩽3
HEX	Within-day	⩽8	–
	Between-day	⩽18	⩽5

[a] $n = 6$ (data from four laboratories).

chosen because they are an indication for the quality of the following steps of the GC–MS procedure within one day and over two days: derivatisation, injection, column separation/elution efficiency, ionisation, response behaviour, detection process.

It can be seen in Table 1 that the major variation is caused by day. No substantial difference in C.V.s between the analytes is detected. Concerning HEX some or all of the following difficulties are indicated by the relatively high C.V. for r: derivative stability, injection repeatability, column elution efficiency, ionisation, response stability, the detection process.

Figures 2 and 3 compare the C.V.s for repeatability and reproducibility with their corresponding C.V.s taken from the quality criteria [1] and those calculated according to the Horwitz equation [6]. Such results which were found to be technically incorrect, were excluded from the data before this comparison was made.

The reproducibility C.V.s (between-laboratory) for total-, *trans*- and *cis*-DES agree well with these values from the quality criteria. The reproducibility C.V.s (between-laboratory) for DE and HEX tend to agree better with such values calculated according to the Horwitz equation.

The quality criteria [1] therefore provide a good guideline for the evaluation of collaborative studies such as proficiency schemes on stilbenes in bovine urine samples. The CRMs provide a basis for the preparation of secondary reference materials with control values. These secondary reference materials are useful for proficiency schemes. Their use may lead to a harmonization of European proficiency schemes because their control values are traceable to the CRMs.

The results of this certification study may also be used as guidelines to draft and evaluate protocols and reporting sheets for proficiency schemes for laboratory accreditation and laboratory control purposes.

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Certification exercise

National Institute of Public Health and Environmental Protection, Laboratory for Residue Analysis (RIVM/ARO), Bilthoven, Netherlands (organizing laboratory); State University Gent, Laboratory for Bromatology, Gent, Belgium; Institute for Hygiene and Epidemiology, Brussels, Belgium; State Institute for Quality Control of Agricultural Products, Department for Biopharmaceutical Analysis, Wageningen, Netherlands; BCO Analytical Services B.V., Breda, Netherlands; TNO-Biotechnology and Chemistry Institute, Zeist, Netherlands; Landeshauptstadt Stuttgart, Amt für Umweltschutz, Chemische Untersuchungsanstalt für die Stadt Stuttgart, Stuttgart, Germany; MAFF Central Veterinary Laboratory, Surrey, UK; University of Kaiserslautern, Department of Food Chemistry and Environmental Toxicology, Kaiserslautern, Germany; Bundesgesundheitsamt, Robert von Ostertorg Institut, Berlin, Germany; and Chemisches Landesuntersuchungsamt, Nordrhein-Westfalen, Münster, Germany.

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Pitfalls in selected ion monitoring in gas chromatography–mass spectrometry: a theoretical example

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Abstract

For the routine determination of residues of growth promoters two important types of low-resolution gas chromatograph–mass spectrometer may be distinguished: the ultra-trace full-scan instrument [e.g., the ion trap mass spectrometer (ITS40)] and most other quadrupole apparatus using the selected ion monitoring (SIM) mode for detecting very small amounts (< 1–10 ng). In analysing biological extracts interference between matrix components, present at high concentrations, and analytes, present at low concentrations, should be avoided. In this investigation theoretical examples of pitfalls in SIM due to isotope interference (^{13}C) from matrix components with the analyte were considered. These interferences may lead to false-positive and -negative results and false quantification.

Keywords: Gas chromatography–mass spectrometry; Anabolic steroids; Biological samples; Interferences; Selected ion monitoring; Steroids

For the routine determination of residues of growth promoters in meat-producing animals there is increasing use of coupled techniques. In most instances a low-resolution mass spectrometer coupled to a chromatographic separation is used [1–4]. In low-resolution gas chromatography–mass spectrometry (GC–MS) two important types of apparatus may be distinguished: the ultra-trace full-scan instrument [e.g., the ion trap mass spectrometer (ITS40)] and most other quadrupole apparatus using full scan for large amounts of analyte and the selected ion monitoring (SIM) mode for detecting very small amounts (< 1–10 ng). Both techniques have advantages and disadvantages and also their own supporters [5].

With SIM a limited number of ions (2–4) are monitored during a selected time interval. The presence of the analyte is determined by the presence of these "diagnostic" ions at the correct retention time and with the correct abundance ratio (between certain limits [6]). In the EC guidelines the monitoring of four ions is advised [6]. In practice, the monitoring of four ions at low concentrations (< 1 ng ml^{-1}) does not give satis-

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factory results [7]. As the monitoring of only two ions is certainly not sufficient according to the quality criteria, a decision on three ions seems an acceptable analytical compromise [7]. In this investigation, SIM on three ions was taken as a rule.

With the ion trap mass spectrometer (ITS40) the whole mass spectrum is stored for each point of the chromatogram (e.g., one spectrum per second). Subsequently, full-scan identification of the analyte by a library search may be performed with the data system, while recording a new acquisition. The manufacurers of the ITS40 system claim a full-scan identification of components at (at least) the 10-pg level.

In extracts of biological material (e.g., urine, meat), a wide variety of components with a large range of concentrations are present. Unknown and variable amounts of these matrix components are co-extracted with the analyte. With GC–MS analyses in the SIM mode these interferences are not observed by the highly selective use of the detector. Interference between these matrix components, present at relatively high concentrations (μg ml^{-1} range or higher), and analytes, present at very low concentrations (ng ml^{-1} range), should be avoided when using SIM.

In this investigation a theoretical example of a pitfall in SIM due to isotope interference (^{13}C) from matrix components with the analyte was considered. This theoretical example is intended as a thinking exercise on the qualitative accuracy of SIM. It is the result of research about the origin of the positive signal for nortestosterone by radioimmunoassay (RIA) and SIM in the urine of pregnant cattle [8,9]. This signal, still open to discussion, could be caused by the aspecificity of the antibody against nortestosterone for the RIA. For SIM analysis ^{13}C isotope interference with the high concentrations of estradiol present in the urine of these animals could be possible.

STABLE ISOTOPES: CALCULATION OF ISOTOPE PEAKS

A knowledge of the relative abundances of stable isotopes is essential for the interpretation of mass spectra [10]. A brief explanation is given here for those unfamiliar with this subject. Chlorine has two isotopes, ^{35}Cl and ^{37}Cl, with an abundance ratio of 3:1 (mean atomic mass 35.453). For a molecule containing one Cl atom two peaks separated by 2 u and with an abundance ratio of 3:1 are observed (for the molecular ion and the fragments containing Cl). When a molecule contains two Cl atoms, three peaks are observed in the mass spectrum. The total number of isotope peaks of Cl is $n + 1$ and their relative abundance by the formula $100(1 + 0.333)^n$ where n is the number of Cl atoms. In Table 1 these isotope ratios for molecules containing one to three Cl atoms are summarized.

TABLE 1

Ratios of the isotope peaks of Cl

n [a]	Ratio
1	100:33
2	100:66:11
3	100:100:33:4

[a] Number of carbon atoms.

Isotope peaks may be very specific for the identification of residues (e.g., Cl-containing growth promotors such as clenbuterol). They may also be important for the determination of the fragmentation patterns and metabolization of these molecules.

PEAKS OF LOW RELATIVE ABUNDANCE

For the other elements analogous calculations can be made. In Table 2 the most important

TABLE 2

The most important elements in organic molecules and their natural isotopes

Element	M_r	Isotopes (abundance, %)
H	1.00797	1.00783 (99.985), 2.01410 (0.015)
C	12.01115	12.00000 (98.89), 13.0335 (1.11)
N	14.0067	14.00307 (99.63), 15.00011 (0.37)
O	15.9994	15.99491 (99.76), 16.99913 (0.04), 17.99916 (0.20)
S	32.064	31.97207 (95), 32.97146 (0.76), 33.96786 (4.22)

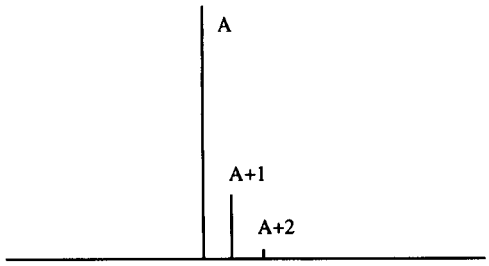

Fig. 1. Spectrum of a component (ion) with 20 carbon atoms.

atoms in organic molecules are given together with their most important natural isotopes.

Isotope interference may occur with any isotope with a relatively high abundance. In this investigation ^{13}C was studied. Carbon has two natural isotopes, ^{12}C and ^{13}C, with a ratio of 98.9:1.1 (the exact figures are rounded for simplicity). In residue analyses two other important parameters should be taken into account: the very large difference in concentration between the analytes and the matrix components, and the fact that the analyte (an organic molecule) contains a relatively large number of carbon atoms. The components co-extracted from the matrix may also contain a substantial number of C atoms, and these components may have similar structures to the analytes (e.g., in anabolizing agents such as steroids 20–30 carbon atoms are present and numerous steroids and metabolites with analogous structures are known).

The number of isotope peaks produced by n carbon atoms is $n + 1$ and the relative abundance of these isotope peaks by the formula $100(1 + 0.011)^n$. The general equation for this formula is given by the Bernoulli binomial distribution:

$$(A+B)^n = A^n + C_n^{n-1}A^{n-1}B + C_n^{n-2}A^{n-2}B^2 + \ldots + C_n^1 AB^{n-1} + B^n$$

For residue analysis the first three terms of this equation are the most important. As an example, the equation is worked out for a component with 20 carbon atoms:

First term:	chance of no ^{13}C: 80.15%	$(0.989)^{20}$
Second term:	chance of one ^{13}C: 17.83%	$C_{20}^{19}(0.989)^{19}(0.011)$ Σ97.98%
Third term:	chance of two ^{13}C: 1.88%	$C_{20}^{18}(0.989)^{18}(0.011)^2$ Σ99.86%

TABLE 3

Relative abundances of the isotope peaks to the generating ion A as a function of the number of carbon atoms, n (%, rounded)

n	A	$A+1$	$A+2$
1	100	1	0
5	100	6	0.1
10	100	11	0.6
15	100	17	1.3
20	100	22	2.3
25	100	28	3.7
30	100	33	5.4

The chances of no, one and two ^{13}C atoms in a molecule containing 20 carbon atoms are equivalent to the relative abundances of the peaks A, $A + 1$ and $A + 2$, where A represents the mass calculated on ^{12}C only. The ratios of these peaks to the "parent" or "generating" peak are 100:22:2.3.

A mass spectrum of a component with 20 carbon atoms is given in Fig. 1. The sum of the first three peaks yields 99.9% of the total abundance. The ion $A + 3$ has a very low abundance for moderate carbon numbers ($< 0.2\%$) and is neglected here together with all the other peaks. Theoretically, the number of carbon atoms of a component could be calculated from the number of the isotope peaks and their relative intensities.

In Table 3 the results of the calculation of the relative abundances of the $A + 1$ and $A + 2$ ions as a function of the number of carbon atoms in a molecule are given. The relative abundance of the ion $A + 1$ is linearly related to the number of

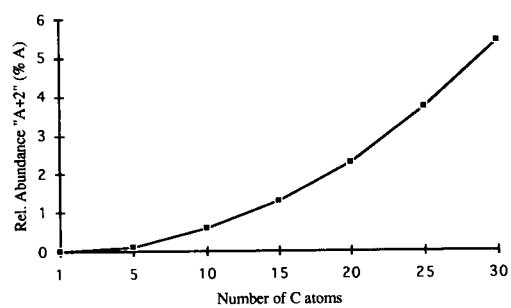

Fig. 2. Relative abundance of the ion $A + 2$ with respect to the generation ion A as a function of the number of carbon atoms n.

carbon atoms n multiplied by 1.11. The relative abundance of the $A+2$ ion as a function of the number of carbon atoms is not linear and is given by $n(n-1)\times 0.00616$. This relationship is expressed in Fig. 2 which shows clearly that the relative abundance of $A+2$ increases considerably at high carbon numbers.

THEORETICAL EXAMPLES IN RESIDUE ANALYSIS

The presence of interferents, co-extracted with the analyte from the matrix, can cause false-positive and -negative results and incorrect quantification. The examples given below are purely theoretical. However, the possibility of their occurrence is not imaginary and may be demonstrated with practical examples.

False-positive results

A laboratory wants to determine the analyte NT in the matrix U at the 0.1–1 ng ml^{-1} level by GC–MS. This is a very realistic situation: different workers claim to be able to determine nortestosterone at this level by GC–MS in the SIM mode [7,8]. In this investigation only a theoretical example is worked out.

The (theoretical) characteristics of the GC–MS SIM analysis are retention time $\approx$ 15 min and three ions are followed during the time interval 14–16 min: m/z 418 (100%), 403 (20%) and 328 (35%). The mass spectrum of the analyte NT reduced to the three diagnostic ions is given in Fig. 3A.

In the matrix U a component E (an interferent) is present at 10 μg ml^{-1} (10^4–10^5 times higher than the concentration of the analyte). The retention time of the interferent E is nearly identical with that of NT (e.g., 14 min 58 s). The mass spectrum of this component is given in Fig. 3B. In this mass spectrum only the ions of m/z 416, 402 and 326 are important for isotope interference with the analyte. The ion of m/z 402 has a very low abundance (ca. 3% of m/z 416) and is nearly invisible in Fig. 3B. This ion of no importance for the mass spectrum of E. However, its influence on the determination of NT is not negligible, as will be demonstrated below.

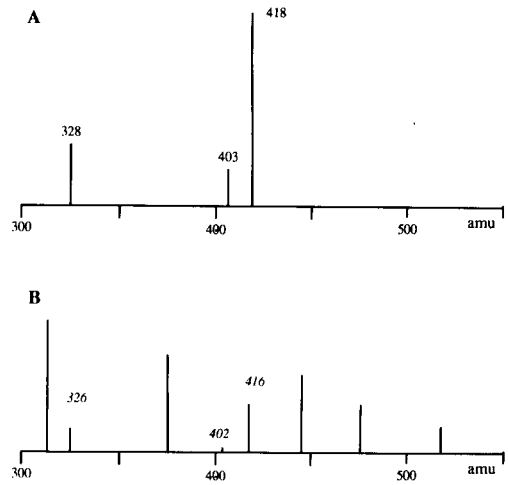

Fig. 3. Mass spectrum of the analyte NT and the interferent E in the full-scan model.

The exact number of carbon atoms in the interferent E is not important, as only the exact number of carbon atoms in the fragment ions is used in the calculation. These (theoretical) numbers of carbon atoms and the relative abundances of the isotope peaks generated are given in Table 4 (as described from Table 3 and Fig. 2). The values in italics represent the abundances that are important for this example.

As the interferent E has a concentration of 10 μg ml^{-1} and the fragment of m/z 416 forms ca. 10% of the total spectrum, this ion is attributed a relative concentration equivalent to 1000 ng ml^{-1}. The ions of m/z 402 and 326 show relative concentrations of 28 and 500 ng ml^{-1}, respectively (their ratios to m/z 416 are 2.8% and 50%, respectively). From these three ions isotope peaks are generated according to the ratios given in Table 4 and shown in Fig. 4.

TABLE 4

Number of carbon atoms of the fragments and the relative abundances of the isotope peaks generated by these fragments (relative to the generating peak)

Ion (m/z)	Carbon number (n)	$A+1$	$A+2$
416	28	31	*4.7*
402	27	*30*	4.3
326	22	24	*2.9*

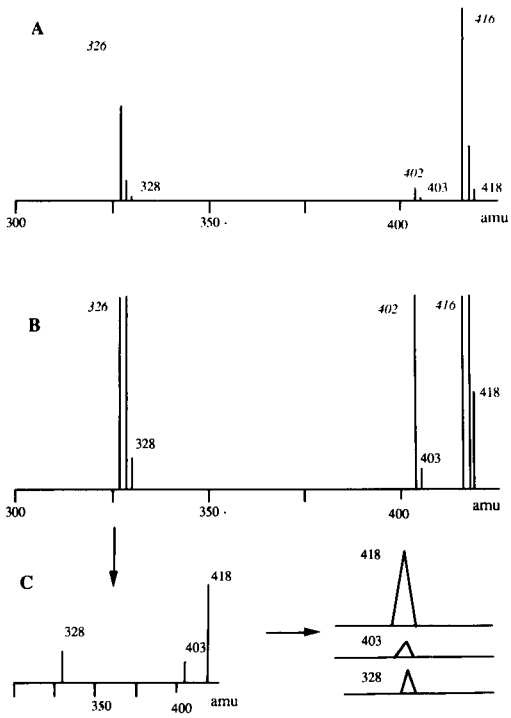

Fig. 4. Isotope peaks of NT generated by E. (A) Full-scan mode, m/z 416 = 100%; (B) full-scan mode, amplified; (C) SIM signal.

In the SIM mode only the ions of m/z 418, 403 and 328 are selected. The relative concentrations of these ions are 46.6, 8.5 and 14.2 ng ml^{-1}, respectively (ratio 100:18:31). Using the SIM method correctly, the analyst will therefore conclude that NT is present at a concentration equivalent to 10–50 ng ml^{-1} (depending on the ion used for quantification) as the three ions are present within the correct retention time windows and with the correct ratios.

Ions with a low relative abundance in a mass spectrum of an interferent may be important when SIM is used. In addition to this theoretical example, more analogous examples may be calculated. The interfering isotope peaks may also be generated by several interferents simultaneously or by stable isotopes of other elements. The hypothesis that nortestosterone (NT) could be present in the urine of pregnant cows [8,9] (in addition to its natural presence in the urine of the stallion and the boar) [11–13] is based on the positive signal obtained with both RIA and SIM. The signal for RIA could be caused by the aspecificity of the antibody against NT. For SIM analysis, ^{13}C isotope interference with the high concentrations of estradiol present in the urine of these animals could be possible.

This phenomenon was studied by full-scan ultra-trace mass spectrometry [14]. It was shown clearly that α-estradiol and β-nortestosterone were not well separated under the chromatographic conditions used. The three peaks at m/z 418, 403 and 328 at the correct retention times and intensity ratios could be due to the high (and strongly variable) concentration of estradiol in urine of pregnant cows. In Fig. 5 the ion chromatogram at m/z 418 of a urine extract of a pregnant cow, spiked with 2 ng ml^{-1} of β-nortestosterone, is shown. This ion chromatogram demonstrates clearly that a high level of estradiol (main ion of m/z 416) generates a signal at m/z 418 at high ng ml^{-1} level owing to ^{13}C isotope interference.

False-negative results

In a laboratory C the analyte EE2 in the matrix M is determined at the 2–10 ng ml^{-1} level by GC–MS. In a slaughtered animal an injection site containing EE2 was found. In the matrix M of the same animal an EE2 value of 3 ng ml^{-1} was found (quantification on the ion of m/z 425). This example also is very realistic: ethinylestradiol may be determined at this level by GC–MS in the SIM mode [15].

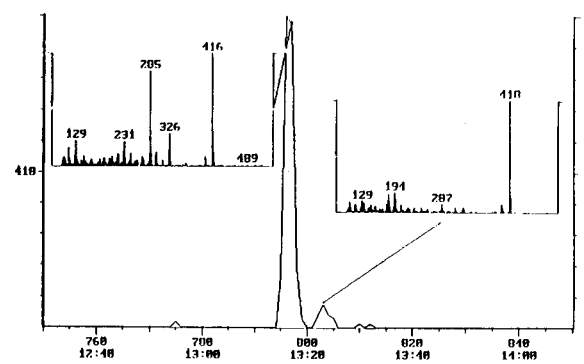

Fig. 5. Ion chromatogram at m/z 418 of a urine extract of a pregnant cow spiked with 2 ng ml^{-1} of nortestosterone.

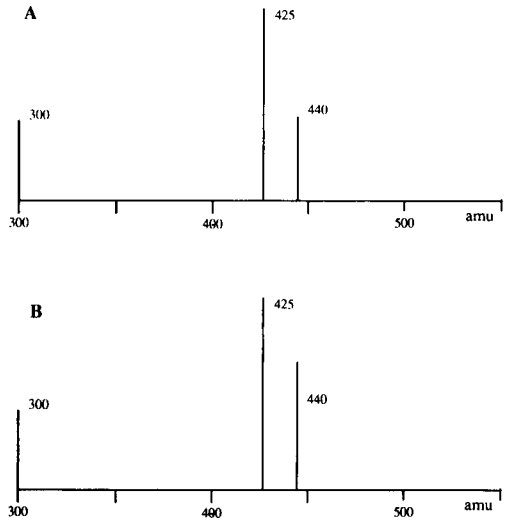

Fig. 6. Mass spectrum of (A) EE2 standard and (B) EE2 distorted by ^{13}C isotope peak of m/z 439.

The characteristics of the GC–MS SIM analysis are retention time $\approx$ 17 min and three ions are followed during the time interval 16–18 min : m/z 440 (45%), 425 (100%) and 300 (45%). The mass spectrum of EE2 reduced to the three diagnostic ions is given in Fig. 6A.

The matrix M contains a component TT at a concentration of 2 μg ml^{-1} with a retention time of 17 min. In the mass spectrum of this component an ion of m/z 439 with a very low abundance is present (ca. 1% of the total spectrum). This ion generates an isotope peak at m/z 440 with a relative abundance of 1.2 ng ml^{-1} (the calculation is analogous to that shown above). The abundance of this m/z 440 ion adds to that of the m/z 440 ion from the analyte (1.35 ng ml^{-1}). The "three-ion" spectrum after isotope interference is shown in Fig. 6B. This spectrum is clearly distorted by the isotope peak and according to the rules of SIM the sample should be considered as negative although all three peaks are present and EE2 was found in the injection site cut from the same animal.

False quantification

Quantification of residues may be very important, especially in the neighbourhood of the decision limit. In an analogous way to that shown above, interferents may influence both the analyte ions and the internal standard ions.

Conclusion

When SIM is used for the determination of residues of analytes by GC–MS at the ng ml^{-1} level, isotope interference should always be kept in mind. Isotope interference could generate the following three effects. First, false-positive results to the presence of three diagnostic ions at the correct retention time and in the correct ratio windows. However, these ions do not originate from the analyte but are generated by one or more interferents present at high concentration in the final extract. The fact that the correct ion ratios can be produced from the interfering endogenous compounds is transparent to the analyst when using GC–MS in the SIM mode. Second, false-negative results owing to disturbance of the normal peak ratios of the ions from the analyte by one or more isotope peaks from one or more interferents. This effect may be of even more importance than the generation of false-positive results as the statistical possibility of its occurrence is higher. In the study of contradictory results (in a second analysis in a second laboratory) this effect must always be considered; using slightly different methods (different columns, reagents, etc.) different interferents from the same matrix may be present in the final extract. Finally, false quantification owing to disturbance of the ions of the analyte or the internal standard.

The above reasonings show that the possibility of isotope interference should be taken into account when using SIM in residue analysis. These isotope interference may be avoided by using apparatus capable of operating in the full-scan mode at low concentration levels. The absence of substantial concentrations of isotope peak generators in the full-scan mass spectrum has to be considered as a quality criterion. With quadrupole apparatus, which is not able to take a full scan at low concentration, the following strategy could be recommended: in the case of a positive result a second full-scan run on the same sample is performed in order to exclude the presence of isotope-generating peaks at the retention time of

the analyte. SIM could also be used for screening purposes only and suspect samples re-chromatographed and fully identified with the other system.

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Coordination of information on residues of veterinary drugs in the European Community

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Abstract

This paper discusses the historical and present day situation to coordinate information on legislation and regulation of residues of veterinary drugs within the European Community (EC). Nowadays three separate Directorates (III, VI & XII) have important inputs and more than forty laboratories from the Member States are involved in the control of residues.

Keywords: Drugs; Veterinary drugs

There is a large amount of information and legislation on the regulation of residues of veterinary drugs within the European Community (EC). It is aimed at harmonising the actions of Member States to improve intra-community trade and provide meat and meat products safe for consumption in the EC. There are many different bodies making contributions to the information, for example at least three Directorates (III, VI and XII) are involved. The aim of this paper is to coordinate the separate information such that those interested in residues will have a clearer picture of the whole process.

The structure of this paper is to summarise the current EC situation on residues by first looking at the recent history and development of the contributing factors and then trying to bring them together and indicate how they might be further coordinated in the future. The key areas of interest are

- Use and abuse of hormones in meat production,
- The emergence of consumer reaction to residues and an EC ban on hormones,
- Development of sensitive assays for residue analysis,
- Development of reference materials (RMs),
- Use of other drugs for meat production,
- The establishment of Maximum Residue Limits (MRLs) by the Committee for Veterinary Medicinal Products (CVMP),
- EC Directive 86/469 (on residues) and other legislation,
- The role of the Community Reference Laboratories,
- The role of the Reference Manual on Residues of Veterinary Drugs.

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Use and abuse of anabolics

The historical landmarks for anabolic agents are:

1950–1970 Use of stilbenes in steer beef and poultry production.

1970s Introduction of new anabolic compounds (anabolics) and their use extended to veal calf production.

1980–1981 Hormone scandal associated with illegal use of diethylstilbestrol (DES) in veal calves.
1981 EC Directive 81/602 [a] restricting the use of anabolics.
1982–1985 EC Committee (Lamming) reviews safety of natural steroids, trenbolone and zeranol.
1985, 1988 EC Directives (85/649 & 88/146) banning the use of anabolics for meat production.
1986 EC Directive for the control of residues in animals and fresh meat.

Anabolics have been used in meat production since the 1950s. Their use was quite different between Member States. In Eire and the UK there was legalised use similar to that in the USA and practised mostly in beef steers. Initially the stilbenes, DES and hexoestrol were widely used but in the seventies the use of 19-nortestosterone derivatives, trenbolone, zeranol and the three natural steroids, gained an increasing share of the market. On mainland Europe anabolics were not applicable to bull beef production but were highly effective in increasing the efficiency of veal production. The legal use of anabolics varied between nations; some countries e.g. The Netherlands, Denmark and Italy prohibited their use while others like France and Germany had restricted use. The illegal use of anabolics in veal calves with their economic advantages to the producer spread across Europe and caused increasing concern to the public. Eventually in 1980 the "Hormone Scandal", which began in Italy and spread to Germany, France and the Benelux countries, changed everything.

Intense consumer pressure led to introduction of EC legislation and other measures for controlling the use of anabolics.

Legislation restricting the use of anabolics

The EC introduced Directive 81/602 which prohibited the use of thyreostats and hormones, except for five "hormones" (three natural steroids, trenbolone and zeranol) for growth promotion in farm animals. The five "hormones" were scientifically examined as to their safety by an EC appointed Committee (Lamming Committee). Although the Committee reported that the three natural steroids were safe and were on the verge of doing the same for the two xenobiotics a new Directive, 85/649 put a total ban on the use of anabolic hormones for growth promotion. The Lamming Committee was never recalled. In 1988 Directive 85/649 was declared invalid on a legal technicality and was replaced by Directive 88/146 imposing a similar ban.

Analytical methods for the control of the use of anabolics

All the Member States are now able to control residues of most of the anabolics. However, in the early seventies much effort was put solely into controlling the use of the stilbenes. The methods used were either histological examination of sex glands in veal calves [1], the effect of meat on the mouse uterus or thin-layer chromatography (TLC). They were useful for the control of calves or poultry receiving very high doses of stilbenes but their lack of sensitivity meant they were not suitable for controlling cattle implanted with anabolics or receiving the increasing number of new anabolics entering the market. The multiresidue TLC method developed by Verbeke [2] was used in many countries to control anabolics but it too lacked sensitivity, especially for compounds like zeranol and hexoestrol.

Beginning in 1975 a group of European scientists from industry, university and government laboratories met regularly in Paris to share their experiences in analytical methodology. The problem to solve was sensitivity and specificity. Immunoassay became the method of choice because it possessed the sensitivity ($\ll 1\ \mu g\ kg^{-1}$) and its specificity could be modified between that for a single analyte and a group of similar substances (e.g. the stilbenes). Within a few years radioimmunoassay had largely replaced TLC.

Following the hormone ban of 1985 industry withdrew from the group and the group was reformed as an EC ad hoc Committee of DGVI. They addressed the difficulties of trying to up-

[a] References to EC legislation are given at the end of the reference section.

grade the routine screening methods to Community Reference methods as required by EC Directive 86/649. The problems were three fold:

(1) The immunoassay methods were not suitable candidates for reference methods except when they are combined with high resolution chromatography.

(2) Every delegate came to the table with different methods.

(3) The spectroscopic methods were not sufficiently developed for residue analysis.

The task was probably too daunting and the emphasis was changed to producing criteria which methods must fulfil for hormones and thyrostats (Commission Decision, 87/410).

In the period of setting the criteria, 1986–1992, methods for residue analysis have progressed. Sensitivity is no longer a problem for mass spectrometry while radioimmunoassay has been joined by enzyme immunoassay. There has been a steady development of the use of liquid chromatography (LC) and solid phase extraction (SPE), for clean-up procedures. These methods have been adopted by all the Member States as suitable methods for routine screening and confirmation of residues of anabolics. Many of the gas chromatography–mass spectrometry (GC–MS) methods are for multiresidues and examples are given in the Reference Manual [3].

The main hurdles still to be crossed are in the validation of the assays. At present there is a lack of reference materials and calibrants and for many immunoassays the supply of antibodies is not guaranteed.

Other veterinary drugs

Nowadays public concern is not confined to anabolic hormones but includes other veterinary drugs. Several new groups of substances have received publicity, particulary, the sulphonamides, tetracyclines, β-agonists, tranquilizers and genetically engineered bovine- and porcine-growth hormone (BST, PST). Some of these drugs are allowed but the MRLs are violated due to misuse of the drug or poor animal management. The EC Directive 86/469 (see below) expanded the control of residues to all veterinary drugs. The criteria document 87/410 was extended in Commission Decision 89/610 to include all veterinary residues. This latter document is now undergoing further revision to cover both screening and confirmatory methods of analysis. Reliable analytical methods are available for most drugs and RMs for several antibiotics and β-agonists are in preparation through the Measurement and Testing Programme (BCR).

EC Directive 86 / 469 - The Residues Directive

The EC Directive 86/469 "concerning the examination of animals and fresh meat for the presence of residues" provides the basis for a general solution of the controls of residues in the Community. The relevant points for this paper are:

(1) Member States are required to submit a National Plan for:

- information on licensed and prohibited drugs,
- list of "approved laboratories",
- sampling plan (numbers of samples and sampling point),
- list of substances investigated,
- methods of analysis for residues,
- measures to be taken when violation occurs.

(2) The appointment of Community Reference Laboratories for each group of residues is proposed (see below).

(3) The role of the National Reference Laboratories with respect to coordinating standards in residue methodology is defined.

(4) Reference methods of analysis for the confirmation of positive samples and for settling disputes are required.

The Member States are now carrying out their approved National Plans. They have introduced National Surveillance schemes and are controlling residues of a large number of veterinary drugs. Some States cover more compounds than others and there is a wide variety of methodology. There is a keen exchange of information between the NRL and already several workshops for training in residue analytical technology have been held.

Four CRLs have been designated (Council Decision 89/187), one for anabolics, two will share

antibiotics and other drugs and one will cover heavy metals.

The functions of CRL include:

- Coordinating the application in National Reference Laboratories of good laboratory practice.
- Advising NRLs of method detail and improvements.
- Carrying out training programmes for methodology (Training programmes organised through DGVI have already been very successful).
- Coordinating with BCR for the preparation of RMs.

So far it has not been possible to establish Community Reference Methods, although there are criteria for such methods [EC Decision 87/410 (under revision)]. This lack of methods is possibly due to: no peer group is formed to give approval, the methods in use by Member States for confirmation of positives have not been adequately ring tested throughout the Community Laboratories, there are insufficient Reference Materials.

Development of reference materials for veterinary drugs

Pure calibrants and suitable reference materials and reagents are essential for the development and validation of methods. The Measurement and Testing Programme (BCR) is a research programme of the Directorate of Science (DGXII). About ten years ago the BCR discussed with scientists in residue technology the need for Reference Materials (RMs). RMs with incurred residues of veterinary drugs were needed for the development and validation of residue methods of analysis.

The initial programme was to produce certified RMs for the stilbenes in bovine tissues and urine. These RMs are just becoming available as lyophilised material with certified or indicative values for the concentration of incurred stilbene in the samples.

The preparation of RMs with the BCR programme has expanded greatly and more than twenty are under preparation. In deciding the target values for the residues in the incurred lyophilised samples, the BCR takes into account the legislation from DGVI, the Maximum Residue Limits (MRL) set by DGIII (see below) and the quality of the analytical methods. Thus for those substances which are not allowed, RMs are prepared which are suitable for positive identification by the routine methods. For allowed drugs at least one of the RMs for a particular drug will have a target value close to the MRL.

Maximum Residue Limits (MRLs)

Drugs are licensed for use either by a Member State or at the EC level under Directives 81/851-2 and its later amendments. New legislation permits MRL to be set for the EC. The Maximum Residue Limits (MRL) are proposed by the Committee on Veterinary Medicine Products (CVMP) which is part of DGIII. MRLs are based on the Acceptable Daily Intake calculated from the no toxic effect levels of a drug.

Because the MRL is the legal level of safe exposure of the consumer to residues of the drug it is natural that this level is monitored in animal products. In turn this implies that the methods of analysis can reliably measure and confirm MRLs. Thus a key integral pathway of residue science at EC level is the relationship between the analyses, methods, RMs and MRLs. Analyses are carried out to reliably measure and confirm MRLs using methods which have been developed and validated using RMs.

Coordination of residue programmes

There are at least three means of coordinating information about residues in the EC.

(1) By organised and informal scientific discussion and participation between those interested. This symposium is one such example. The BCR also hold regular meetings of experts from Member States to transfer information on the programme for preparation of RMs.

(2) Through the functions of the Community Reference Laboratories. These are early days but clearly they should be able to play a major role in coordinating residue technology through their designated functions of advising and training the NRL in residue methodology. Also they will coor-

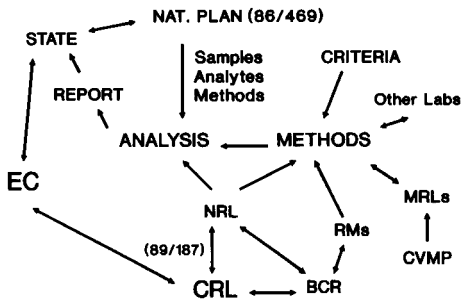

Fig. 1. The relationship of EC Activities on Residues.

dinate more with the BCR in the preparation of RMs.

(3) Through the Reference Manual on Residues of Veterinary Drugs.

The veterinary legal division of DGVI and the BCR have coordinated to support the publication of a Reference Manual [3]. The manual covers in much more detail all of the topics discussed above. The aim of the manual is to bring together information on some of the following: legislation, reference laboratories, residue surveillance in Member States, methods/procedures and criteria, RMs and MRLs, information on individual drugs and their residues.

Because of the dynamic nature of residue technology the information in the manual is to be continuously updated.

General scheme

A general scheme showing the relationships between residue activities in the EC is shown in Fig. 1.

The EC legislation requires each Member State to have in place a National Plan for controlling residues in animals, fresh meat and animal products. The plan includes a surveillance scheme with indicated numbers of samples taken from live and slaughtered animals and analyzed by approved methods for residues of a wide range of veterinary drugs. The results of the surveillance are communicated to the Commission.

The analyses are carried out in NRLs or laboratories approved by the NRLs. There are four CRLs. They inspect and advise on standards, methods, facilities and resources of the NRL and report their findings to the Commission. The CRLs function as trainers and advisers for maintaining the standards and uniformity of methodology throughout the NRLs.

The methods used for analysis must meet the criteria laid down in the Commission Decision 89/610, which is now under further revision.

The methods are developed by the CRLs, NRLs and other laboratories. The methods must be able to reliably measure residues at the MRLs. The MRLs are set by the CVMP (DGIII). The RMs are prepared through the Measuring and Testing Programme (DGXII) and take into account the priorities for RMs, the analytical methods available and the MRLs. There is increasing coordination between the BCR and the CRLs.

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Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites

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Abstract

The use of anabolic steroids was banned by the International Olympic Committee for the first time at the Olympic Games in Montreal in 1976. Since that time the misuse of anabolic steroids by athletes has been controlled by analysis of urine extracts by gas chromatography–mass spectrometry (GC–MS). The excreted steroids or their metabolites, or both, are isolated from urine by XAD-2 adsorption, enzymatic hydrolysis of conjugated excreted metabolites with β-glucuronidase from *Escherichia coli*, liquid–liquid extraction with diethyl ether, and converted into trimethylsilyl (TMS) derivatives. The confirmation of an anabolic steroid misuse is based on comparison of the electron impact ionization (EI) mass spectrum and GC retention time of the isolated steroid and/or its metabolite with the EI mass spectrum and GC retention time of authentic reference substances. For this purpose excretion studies with the most common anabolic steroids were performed and the main excreted metabolites were synthesized for bolasterone, boldenone, 4-chlorodehydromethyltestosterone, clostebol, drostanolone, fluoxymesterone, formebolone, mestanolone, mesterolone, metandienone, methandriol, metenolone, methyltestosterone, nandrolone, norethandrolone, oxandrolone and stanozolol. The metabolism of anabolic steroids, the synthesis of their main metabolites, their GC retention and EI mass spectra as TMS derivatives are discussed.

Keywords: Gas chromatography; Gas chromatography–mass spectrometry; Anabolic steroids; Metabolism

The misuse of testosterone and anabolic steroids by athletes to improve their performance has led to a ban on anabolic steroids by the International Olympic Committee (IOC) and national and international sports federations. Anabolic steroids were banned for the first time at the Olympic Games 1976 in Montreal and testosterone at the Olympic Games 1984 in Los Angeles. To control the misuse of an anabolic steroid the urines of athletes are collected directly after competition or out of competition and have to be analysed by IOC accredited laboratories. The anabolic steroids and their metabolites are isolated from urine, derivatized with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and analysed by gas chromatography (GC) and mass spectrometry (MS) using electron impact (EI) ionization at 70 eV. Because most anabolic steroids are completely metabolized and no parent steroid is excreted, the metabolism of the anabolic steroids must be known. Papers on the metabolism of anabolic steroids in man were available for boldenone [1], 4-chlorodehydromethyltestosterone [2,3], metandienone [4,5], methyltestosterone [6], nandrolone [7] and norethandrolone [8]. The identification of these metabolites was based on the isolation and purification of urinary excreted metabolites after application of large amounts of

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the anabolic steroid and characterization by melting points and infrared (IR), ^{1}H nuclear magnetic resonance (NMR) and mass spectrometry. At least the proposed structure of the metabolite was elucidated by its synthesis.

GC with fused-silica capillary columns combined with MS with EI ionization allows very fast and relatively simple sample preparation to isolate small amounts of anabolic steroids and their metabolites from urine [9–11]. The isolated metabolites are characterized by their GC retention times and EI mass spectra. A metabolite is identified when the comparision with an authentic reference substance shows the same GC retention time and EI mass spectrum.

As most of the anabolic steroid metabolites were not commercially available in earlier times, so-called "reference substances" were obtained from urine in excretion studies with anabolic steroids. A criticism of this method was the low accuracy of a urinary "reference substance" for which the structure is not exactly known and the possible impurity of the urinary extract.

To circumvent this disadvantage we started in 1987 the synthesis of the main metabolites of those anabolic steroids which were mainly misused in sports. The synthesis of metabolites of boldenone [12], metandienone [13], stanozolol [14] and the synthesis of 17-epimeric steroids [15] have been published. Further publications are in preparation.

This paper gives an overview of the main metabolic pathways, the synthesis of the main metabolites of anabolic steroids and their trimethylsilyl (TMS) derivatization for GC–MS and presents the GC retention indices and EI mass spectra of the anabolic steroids excreted unchanged and their main metabolites, which are routinely used for screening and confirmation.

The pharmacokinetics of the excreted steroids and metabolites will not be discussed.

EXPERIMENTAL

Steroids and chemicals

K-Selektride (1 M potassium tri-*sec*.-butylborohydride in tetrahydrofuran), lithium aluminium hydride, chromium trioxide, platinum dioxide and 10% palladium on charcoal were purchased from Aldrich (Steinheim). Bolasterone was purchased from Upjohn; 4-chlorodehydromethyltestosterone was a gift from Jenapharm (Jena); fluoxymesterone was a gift from Ciba-Geigy; furazabol was a gift from Daiichi (Tokyo); oxandrolone was a gift from Searle, oxymesterone was a gift from Carlo Erba; mesterolone, metenolone and metenolone acetate were gifts from Schering (Berlin); formebolone was a gift from A.H. Beckett (London); drostanolone propionate was a gift from Grünenthal (Aachen); boldenone, clostebol acetate, mestanolone, metandienone, methandriol, methandriol dipropionate, methyltestosterone, nandrolone and stanozolol were purchased from Sigma (Deisendorf); norethandrolone, 6β-hydroxytestosterone and 6β-hydroxyandrostenedione were purchased from Steraloids via Paesel (Frankfurt).

N-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was synthesized in the laboratory. All other reagents and solvents were of analytical-reagent grade.

Metabolism studies

Metabolism studies were performed by oral application of the anabolic steroids **I–XX** to male volunteers and by self-administration. Usually a single dose of 20 mg of steroid was applied and the following urine samples were collected and stored at 4°C.

Isolation of anabolic steroids and their metabolites

The steroids and/or their metabolites were isolated as described by Donike et al. [9] with some modifications. The actual sample preparation is described.

Isolation of unconjugated excreted steroids

To 5 ml of urine are added 0.5 g of sodium hydrogencarbonate–potassium carbonate (2 : 1, w/w) solid buffer and 50 ng of 4α-hydroxystanozolol as internal standard and the unconjugated steroids are extracted with 5 ml of diethyl ether (distilled over calcium hydride). The ether

layer is transferred after centrifugation and evaporated to dryness under vacuum.

See also the isolation of conjugated steroids.

Isolation of conjugated excreted steroids and metabolites

To 2 ml of urine is added 1 μg of methyltestosterone (internal standard) and the steroids are adsorbed on Amberlite XAD-2 polystyrene resin. The XAD-2 columns (Pasteur pipette, closed with a glass pearl, bed height 2 cm) are washed with 2 ml of doubly distilled water and eluted with 2 ml of methanol. The methanolic eluate is evaporated to dryness and the residue is dissolved in 1 ml of 0.2 M sodium phosphate buffer (pH 7).

At this stage a separation of conjugated and unconjugated excreted steroids is possible. The unconjugated steroids can be extracted with 5 ml of diethyl ether. The remaining ether must be removed by evaporation of the buffer solution for a short time. To the buffer solution 50 μl of β-glucoronidase from *E. coli* (K12, Boehringer, Mannheim) are added and after 1 h at 50°C the buffer solution is made alkaline (pH 9–10) with 250 μl of 5% potassium carbonate solution and the steroids are extracted with 5 ml of diethyl ether on a vibro mixer for 30 s. After centrifugation the ether layer is transferred and evaporated to dryness under vacuum.

Derivatization for GC–MS analysis

Unconjugated fraction. The dry residue is derivatized with 50 μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide–imidazole (MSTFA–Imi) (100:2, v/w) and heated for 30 min at 80°C [9].

Conjugated fraction. The dry residue is derivatized with 100 μl of MSTFA–ammonium iodide–dithioerythritol (1000:2:4, v/w/w) and heated for 15 min at 60°C. This reaction mixture is equivalent to a mixture of MSTFA–trimethyliodosilane (TMIS) (1000:2, v/v) [16].

GC–MS parameters

Unconjugated fraction. A Hewlett-Packard HP 5890 gas chromatograph and an HP 5970 mass spectrometer were used. The carrier gas was helium at 1 ml/min at 180°C, splitting ratio 1:10. Column C was used (see GC–FID parameters). The column temperature was programmed from 200°C at 40°C/min to 320°C (held for 3 min). The injector temperature was 300°C and the interface temperature 320°C.

Conjugated fraction. The instruments and carrier gas were the same as above. Column A was used (see GC–FID parameters). The column temperature was programmed from 180°C at 4°C/min to 240°C and at 15°C/min to 320°C. The injector temperature was 300°C and the interface temperature 320°C.

GC–FID parameters

An HP 5880 gas chromatograph was used. The carrier gas was helium at 1.5 ml/min at 180°C, splitting ratio 1:10, pressure 27 psi. The column temperature was programmed from 180°C at 5°C/min to 320°C. Column A was Hewlett–Packard Ultra-1 fused silica, cross-linked methylsilicone (OV-1), 17 m × 0.2 mm i.d., film thickness 0.11 μm. Column B was Hewlett–Packard HP-1 fused silica, cross-linked methylsilicone (OV-1), 17 m × 0.25 mm i.d., film thickness 0.33 μm. Column C was Chrompack WCOT fused silica CP-SIL 8CB, cross-linked 5% phenyl–methylsilicone (SE-54), 17 m × 0.25 mm i.d., film thickness 0.25 μm. Column D was Macherey–Nagel Permabond, fused silica, cross-linked 5% phenyl–methylsilicone (SE-54), 17 m × 0.25 mm i.d., film thickness 0.25 μm. Column E was Hewlett–Packard HP-5, fused silica, 5% phenyl–methylsilicone (SE-54), 17 m × 0.2 mm i.d., film thickness 0.33 μm.

Synthesis of reference substances (general description)

Oxidation of secondary hydroxy groups with chromium trioxide. The substance which was to be oxidized was dissolved in 96% acetic acid and with stirring a solution of 10% chromium trioxide in 96% acetic acid was added within 10 min. In general an equimolar addition of chromium trioxide was sufficient, yielding quantitative oxidation. After 30 min of reaction the reaction mixture was neutralized and then made alkaline (pH 12–14) with a cooled aqueous solution of 20% potassium hydroxide. The reaction products were extracted

with a tenfold volume of diethyl ether. The ether layer was washed with water, dried over sodium sulphate and evaporated to dryness under vacuum.

Reduction of the 4,5-double bond. Reduction of the 4,5-double bond in 3-keto-4-ene steroids was performed with hydrogen using methanol or methanol–6 M potassium or sodium hydroxide (10:1, v/v) as solvent and platinum dioxide or 10% palladium on charcoal as catalyst.

Reduction of 3-keto groups with lithium aluminium hydride. Reduction with lithium aluminium hydride was performed in absolute dry diethyl ether or in tetrahydrofuran (both freshly distilled over calcium hydride) depending on the solubility of the substance which was to be reduced. The reaction mixture was held under argon and while stirring solid lithium aluminium hydride was added (1 equimolar excess). After a further 15 min the reaction mixture was poured into the same volume of water and the reaction products were extracted with a tenfold volume of diethyl ether. The ether layer was washed with water, dried over sodium sulphate and then evaporated to dryness under vacuum.

Reduction of 3-keto groups with K-Selectride. The reaction and sample preparation were performed in the same way as the reaction with lithium aluminium hydride. The K-Selektride was added in an equimolar amount to the stirred solution held under argon within 10 min.

Reduction of 3-keto groups with hydrogen. The reduction of 3-keto groups with hydrogen using platinum dioxide or 10% palladium on charcoal was performed in the same way as the reduction of the 4,5-double bond, but the reaction was prolonged to 3 days.

6β-Hydroxylation. Stereospecific 6β-hydroxylation of androst-4-en-3-ones and androsta-1,4-diene-3-ones was performed with the following procedure. The substance was dissolved in ethyl acetate–MSTFA–ammonium iodide (100:10:0.5, v/v/w) and refluxed for 15 min. The obtained androsta-3,5-dien-3-enol and androsta-1,3,5-trien-3-enol TMS-ethers were extracted with *n*-pentane after addition of potassium carbonate and water to the reaction mixture. The *n*-pentane phase was evaporated to dryness and the residue dissolved in ethanol or isopropanol. The ethanolic solution was exposed to sunlight while stirring for about 4–8 h. The androsta-3,5-dien-3-enol and androsta-1,3,5-trien-3-enol TMS-ethers were autooxidized under these conditions at C-6, yielding a 6β-hydroxyandrost-4-en-3-one or 6β-hydroxyandrosta-1,4-dien-3-one structure. During further sample preparation remaining TMS groups, e.g., 17β-*O*-TMS, were hydrolysed. A detailed description of this synthesis is in preparation.

RESULTS AND DISCUSSION

Synthesis of reference substances

A general description of the often used reaction schemes for the synthesis of anabolic steroid metabolites will be described. The synthesis of boldenone [12], conjugated metandienone [13], stanozolol [14] metabolites and 17-epimerization of 17α-methyl-17β-hydroxy steroids [15] have already been published. The synthesis of other metabolites will be published elsewhere.

All the synthesized metabolites were obtained as pure crystals with a yield between 60 and 2000 mg.

Oxidation of secondary hydroxy groups with chromium trioxide

A selective oxidation of only the 3-hydroxy group or 17β-hydroxy group when both hydroxy groups were present was not possible. As all synthesized metabolites have a 17-keto- or 17-hydroxy-17-methyl group, this unspecific oxidation was chosen followed by a group-selective reduction of the 3-keto group only.

Reduction of the 4,5-double bond

The reduction performed in methanol yielded in general a mixture of 5α- and 5β-isomers in comparable amounts whereas the reaction in methanol–aqueous potassium hydroxide or sodium hydroxide favours the production of the 5β-isomer. To obtain a 5α-isomer methanol was chosen as solvent, yielding 5α- and 5β-isomers, which were then separated by column chromatography

on silica gel followed by repeated crystallization. The commonly used Birch reaction which yields mainly the 5α-isomer was not applied.

Reduction of 3-keto groups with lithium aluminium hydride

Reduction with lithium aluminium hydride is not group selective. If more than one keto group is present, e.g., in 5α-estrane-3,17-dione, both keto groups will react immediately with the reagent. The reduction is stereospecific to 3-keto groups which will be reduced mainly to 3β-hydroxy (Table 1) when the A-ring has a 5α or 4-ene configuration but the 3-keto group will be reduced in over 80% yield to 3α-hydroxy when the A-ring has a 5β configuration (Table 1).

TABLE 1

Reduction yields of 3-keto groups with $LiAlH_4$ and K-Selektride

Substance	$LiAlH_4$		K-Selektride	
	3α-OH (%) [b]	3β-OH (%) [b]	3α-OH (%) [b]	3β-OH (%) [b]
5α-Configuration				
5α-Androstan-17β-ol-3-one	12	88	88	12
5α-Androstane-3,17-dione	11	89	93	7
5α-Estrane-3,17-dione [a]	13	87	93	7
1-Methylene-5α-androstane-3,17-dione [a]	7	93	95	5
1α-Methyl-5α-androstan-17β-ol-3-one	49	51	> 99	< 1
1α-Methyl-5α-androstane-3,17-dione [a]	49	51	> 99	< 1
2α-Methyl-5α-androstan-17β-ol-3-one	13	87	> 99	< 1
2α-Methyl-5α-androstane-3,17-dione [a]	8	92	99	1
17α-Methyl-5α-androstan-17β-ol-3-one	11	89	88	12
17β-Methyl-5α-androstan-17α-ol-3-one [a]	11	89	93	7
17α-Ethyl-5α-estran-17β-ol-3-one [a]	14	86	93	7
7α,17α-Dimethyl-5α-androstan-17β-ol-3-one [a]	10	90	87	13
5β-Configuration				
5β-Androstan-17β-ol-3-one	89	11	4	96
5β-Estrane-3,17-dione [a]	90	10	6	94
17α-Methyl-5β-androstan-17β-ol-3-one	87	13	9	91
17β-Methyl-5β-androstan-17α-ol-3-one [a]	88	12	6	94
17α-Ethyl-5β-estran-17β-ol-3-one [a]	90	10	5	95
7α,17α-Dimethyl-5β-androstan-17β-ol-3-one [a]	80	20	4	96
5α-Androst-1-ene-configuration				
1-Methyl-5α-androst-1-en-17β-ol-3-one	9	91	10	90
1-Methyl-5α-androst-1-ene-3,17-dione [a]	10	90	10	90
Androst-4-en-3-one-configuration				
Androst-4-en-17β-ol-3-one	13	87	30	70
Androst-4-en-17α-ol-3-one	14	86	38	62
Estr-4-en-17β-ol-3-one	19	81	42	58
17α-Methylandrost-4-en-17β-ol-3-one	10	90	28	72
17β-Methylandrost-4-en-17α-ol-3-one [a]	14	86	36	64
Androst-4-ene-6β,17β-diol-3-one	14	86	15	85
Androst-4-en-6β-ol-3,17-dione	19	81	16	84
9α-Fluoro-17α-methylandrost-4-ene-11β,17β-diol-3-one	28	72	90	10
9α-Fluoro-17α-methylandrost-4-ene-6β,11β,17β-triol-3-one [a]	27	73	72	28
4-Chloroandrost-4-en-17β-ol-3-one	11	89	14	86
4-Chloroandrost-4-ene-3,17-dione [a]	13	87	19	81

[a] Synthesized reference substance. [b] Percentage values were calculated from GC–FID analysis after reduction (see Experimental) of 3 mg of each reference compound.

TABLE 2

Screening for anabolic steroids (Status Cologne Laboratory, 1st March 1992)

Anabolic steroid	Main excreted substance: parent and/or metabolite [a]	Origin of substance used for confirmation [b]	Excretion in urine [c]
Bolasterone	7α,17α-Dimethyl-5β-androstane-3α,17β-diol (**I**)	Synthesized	Conjugated
Boldenone	Boldenone (**II**)	Parent	Conjugated
	5β-Androst-1-en-17β-ol-3-one (**III**)	Synthesized	Conjugated
4-Chlorodehydro-methyltestosterone	6β-Hydroxy-4-chloro-dehydromethyl-testosterone (**IV**)	Synthesized	"Free"
Clostebol	4-Chloro-androst-4-en-3α-ol-17-one (**V**)	Synthesized	Conjugated
Drostanolone	2α-Methyl-5α-androstan-3α-ol-17-one (**VI**)	Synthesized	Conjugated
Fluoxymesterone	9α-Fluoro-18-nor-17,17-dimethyl-androsta-4,13-dien-11β-ol-3-one (**VII**)	Synthesized	"Free"
	9α-Fluoro-17α-methyl-androst-4-ene-3α,6β, 11β,17β-tetrol (**VIII**)	Synthesized	"Free"
Formebolone	2-Hydroxymethyl-17α-methyl-androsta-1,4-diene-11α,17β-diol-3-one (**IX**)	Synthesized	"Free"
Furazabol	16z-Hydroxyfurazabol	Urine ex. study	Conjugated
Mestanolone	17α-Methyl-5α-androstane-3α,17β-diol (**X**)	Synthesized	Conjugated
Mesterolone	1α-Methyl-5α-androstan-3α-ol-17-one (**XI**)	Synthesized	Conjugated
Metandienone	17-Epimetandienone (**XII**)	Synthesized	"Free"
	6β-Hydroxymetandienone (**XIII**)	Synthesized	"Free"
	17α-Methyl-5β-androstane-3α,17β-diol (**XIV**)	Synthesized	Conjugated
	17β-Methyl-5β-androst-1-ene-3α,17α-diol (**XV**)	Synthesized	Conjugated
Methandriol	17α-Methyl-5β-androstane-3α,17β-diol (**XIV**)	Synthesized	Conjugated
Metenolone	Metenolone (**XVI**)	Parent	Conjugated
	1-Methylene-5α-androstan-3α-ol-17-one (**XVII**)	Synthesized	Conjugated
Methyltestosterone	17α-Methyl-5α-androstane-3α,17β-diol (**X**)	Synthesized	Conjugated
	17α-Methyl-5β-androstane-3α,17β-diol (**XIV**)	Synthesized	Conjugated
Nandrolone	5α-Estran-3α-ol-17-one (**XVIII**)	Synthesized	Conjugated
	5β-Estran-3α-ol-17-one (**XIX**)	Synthesized	Conjugated
Norethandrolone	17α-Ethyl-5β-estrane-3α,17β-diol (**XX**)	Synthesized	Conjugated
Oxandrolone	Oxandrolone (**XXI**)	Parent	"Free"
	17-Epioxandrolone (**XXII**)	Synthesized	"Free"
Oxymesterone	Oxymesterone (**XXIII**)	Parent	Conjugated
Oxymetholone	17α-Methyl-5α-androstane-3α,17β-diol (**X**)	Synthesized	Conjugated
	2z-Hydroxymethyl-17α-methyl-5α-androstane-3z,z,17β-triol	Urine ex. study	Conjugated
Stanozolol	3′-Hydroxystanozolol (**XXIV**)	Synthesized	Conjugated and "free"
	3′-Hydroxy-17-epistanozolol (**XXV**)	Synthesized	"Free"
	4β-Hydroxystanozolol (**XXVI**)	Synthesized	Conjugated
	16β-Hydroxystanozolol (**XXVII**)	Synthesized	Conjugated

[a] z = Configuration not identified. [b] Urine ex. study = metabolite obtained from an excretion study. [c] "Free" = unconjugated.

Reduction of 3-keto groups with K-Selectride

The use of K-Selectride [17,18] instead of lithium aluminium hydride shows group selectivity, e.g., the 3-keto group in 5α-estrane-3,17-dione will be reduced first and then with excess of reagent the 17-keto group reacts. Moreover, the reduction of 3-keto groups is stereoselective, but different to lithium aluminium hydride reduction. The 3-keto group reacts in over 85% yield to give 3α-hydroxy when the A-ring has a 5α configuration (Table 1) and it is reduced in more than 85% yield to 3β-hydroxy when the A-ring has a 5β configuration (Table 1); in both instances the reaction yields the opposite configuration to reduction with lithium aluminium hydride. When a 3-keto-4-ene configuration is reduced with K-Selektride the 3-keto group reacts mainly to give 3β-hydroxy similarly to the reaction with lithium aluminium hydride (Table 1), but the yield of the 3α-isomer is much higher than in the reduction

TABLE 3

Temperature programme Kováts indices of anabolic steroids and their metabolites [a]

Steroid	Column				
	A OV-1 0.11 μm	B OV-1 0.33 μm	C SE-54 0.25 μm	D SE-54 0.25 μm	E SE-54 0.33 μm
5α-Estran-3α-ol-17-one bis-TMS (**XVIII**)	2440	2453	2451	2454	2468
5β-Androst-1-en-17β-ol-3-one bis-TMS (**III**)	2452	2467	2469	2471	2488
17β-Methyl-5β-androst-1-ene-3α,17α-diol bis-TMS (**XV**)	2454	2468	2472	2474	2492
5βEstran-3α-ol-17-one bis-TMS (**XIX**)	2490	2505	2502	2504	2520
2α-Methyl-5α-androstan-3α-ol-17-one bis-TMS (**VI**)	2555	2574	2563	2565	5686
1-Methylen-5α-androstan-3α-ol-17-one bis-TMS (**XVII**)	2583	2604	2599	2602	2623
9α-Fluoro-18-nor-17,17-dimethyl-4,13-dien-11β-ol-3-one bis-TMS (**VII**)	2600	2621	2619	2621	2641
17α-Methyl-5β-androst-1-ene-3α,17β-diol bis-TMS (epi-**XV**)	2607	2628	2626	2630	2649
1α-Methyl-5α-androstan-3α-ol-17-one bis-TMS (**XI**)	2607	2630	2619	2623	2644
17α-Methyl-5α-androstane-3α,17β-diol bis-TMS (**X**)	2611	2632	2625	2628	2650
17α-Methyl-5β-androstane-3α,17β-diol bis-TMS (**XIV**)	2617	2636	2628	2631	2654
17-Epimetandienone TMS (**XII**)	2625	2657	2674	2680	2713
Boldenone bis-TMS (**II**)	2648	2671	2671	2674	2698
17-Epioxandrolone TMS (**XXII**)	2673	2707	2735	2741	2777
7α,17α-Dimethyl-5β-androstane-3α,17β-diol bis-TMS (**I**)	2692	2713	2706	2710	2730
4-Chloroandrost-4-en-3α-ol-17-one bis-TMS (**V**)	2693	2712	2720	2724	2746
Metenolone bis-TMS (**XVI**)	2694	2718	2716	2721	2743
17α-Ethyl-5β-estrane-3α,17β-diol bis-TMS (**XX**)	2695	2715	2710	2714	2733
Methyltestosterone bis-TMS (internal standard)	2754	2778	2775	2779	2803
Oxandrolone TMS (**XXI**)	2778	2816	2845	2851	2893
6β-Hydroxymetandienone bis-TMS (**XIII**)	2846	2873	2877	2882	2911
9α-Fluoro-17α-methylandrost-4-ene-3α,6β,11β,17β-tetrol tetra-TMS (**VIII**)	2854	2855	2855	2854	2860
Oxymesterone tris-TMS (**XXIII**)	2952	2977	2968	2972	2993
6β-Hydroxy-4-chlorodehydromethyl-testosterone bis-TMS (**IV**)	3007	3039	3044	3048	3081
3′-Hydroxy-17-epistanozolol tris-TMS (**XXV**)	3100	3113	3119	3122	3137
2z-Hydroxymethyl-17α-methyl-androsta-1,4-diene-11α-,17β-diol-3-one tris-TMS (**IX**)	3163	3187	3203	3206	3232
3′-Hydroxystanozolol tris-TMS (**XXIV**)	3219	3238	3242	3245	3266
4β-Hydroxystanozolol tris-TMS (**XXVI**)	3219	3245	3244	[b]	3277
4α-Hydroxystanozolol tris-TMS (internal standard)	3238	3262	3268	[b]	3296
16β-Hydroxystanozolol tris-TMS (**XXVII**)	3334	3360	3368	3402	3397

[a] Indices were determined on columns A–E (see Experimental). Temperature programme: from 180°C at 5°C/min to 320°C.
[b] Substance showed strong tailing.

with lithium aluminium hydride; in particular, fluoxymesterone and 6β-hydroxyfluoxymesterone react to give the 3α-hydroxy isomers with 90 and 72% yields (Table 1).

Reduction of 3-keto groups with hydrogen

The catalytic reduction of 3-keto groups was group selective because the 3-keto group reacts much faster than the 17-keto group. A stereoselective reduction yielding preferentially a 3α- or 3β-hydroxy configuration was not obtained.

6β-Hydroxylation

6β-Hydroxylation of steroids was first described for cortisol in 1954 by Burstein et al. [19] and in 1956 by Nadel et al. [20] for cortisol. In 1967 Cardi and Lusignani [21] published a very simple procedure for the synthesis of 6β-hydroxy steroids. They converted androst-4-ene-3-ones into their corresponding *n*-alkyl-3,5-diene enol ethers, especially ethyl enol ethers, and exposed them, dissolved in ethanol, to direct sunlight. The autooxidation yielded 6β-hydroxyandrost-4-en-3-one steroids in high yield. This reaction was chosen here to obtain 6β-hydroxy derivatives of testosterone, metandienone, 4-chlorodehydromethyltestosterone and fluoxymesterone. As the formation of 3-enol alkyl ethers of 17α-methyl-17β-hydroxy steroids by commonly described methods led to dehydration of the acidic lable 17β-hydroxy group, trimethylsilyl enol ethers were used and these reactions were easily performed using MSTFA–ammonium iodide. The androsta-3,5-dien-3-enol and androsta-1,3,5-trien-3-enol TMS-ethers were stable in ethanol and were autooxidized when exposed to sunlight, yielding 6β-hydroxy-androst-4-en-3-one and 6β-hydroxy-androsta-1,4-dien-3-one steroids. Testosterone was used as a reference and was autooxidized to 6β-hydroxytestosterone, which was confirmed using authentic reference substance.

17-Epimerization of 17α-methyl-17β-hydroxy steroids

17-Epimers were synthesized for the first time by Macdonald et al. [5] in 1971, confirming 17-epimetandienone as a metabolite of metandienone. Edlund et al. [22] in 1989 published a simple method for the synthesis of 17-epimetandienone. They dissolved metandienone-17-sulphate in water. The sulphate decomposed in water, yielding several dehydration products and 17-epimetandienone. In this work, 17-sulphates of 17α-methyl-17β-hydroxy steroids were synthesized using sulphur trioxide–pyridine complex in dimethylformamide, and then dissolved in water, yielding the 17-epimers.

Screening of anabolic steroids

Routinely 20 anabolic steroids were controlled by a screening method for 29 steroids (4 parent steroids and 25 metabolites; see Table 2), 27 of which (**I–XXVII**) were available as pure reference substances (4 parent compounds and 23 synthesized metabolites). Two metabolites (furazabol and oxymetholone) were obtained from urinary excretion studies. Table 2 shows further which anabolic steroids and metabolites are excreted unconjugated or conjugated. The hydrolysis of conjugates was performed with β-glucuronidase from *E. coli*. The screening samples were analysed by GC–MS (see Experimental) with selected ion monitoring (SIM) using two or three of the most abundant ions for each substance.

Kováts indices (methylene units)

Temperature-programmed Kováts indices (methylene units) were determined for the trimethylsilylated anabolic steroids and synthesized metabolites to be screened (Table 3). A column temperature programme from 180°C at 5°C/min to 320°C was used. This programme was chosen to determine all the steroids within a 25-min programme. Five different columns were tested, namely OV-1 and SE-54 with different film thicknesses and from different companies. All the columns had a length of 17 m. The anabolic steroids and their metabolites were determined as TMS derivatives (the same derivatives as used in routine control); the conjugated excreted steroids and fluoxymesterone metabolites were trimethylsilylated to per-TMS derivatives whereas the unconjugated steroids were derivatized with MSTFA–Imi, which does not derivatize androsta-1,4-diene-3-ones to enol TMS derivatives.

The results for column B (OV-1, 0.33 μm) and column C (SE-54, 0.25 μm) show for almost all the steroids almost identical indices (differences less than 10 methylene units). Oxandrolone (**XXI**) and epioxandrolone (**XXII**) both show a difference of about 30 methylene units and the metabolite of formebolone a difference of 16 methylene units.

Mass spectrometry

All anabolic steroids and their metabolites were analysed as TMS derivatives by mass spectrometry with EI ionization at 70 eV. General EI fragmentation patterns were registered for 17α-methyl-17β-hydroxy steroids and their 17-epimers which show as TMS derivatives an abundant D-ring fragment at *m/z* 143 and with less intensity an ion at *m/z* 130 [5,23].

16-Hydroxylated 17α-methyl-17β-hydroxy steroids display the corresponding fragment ions (+88 u) when they are trimethylsilylated, at *m/z* 231 and 218, respectively.

17-Keto steroids show a typical C/D-ring fragment at *m/z* 169 when the 17-keto group is trimethylsilylated to an enol TMS derivative. The fragment at *m/z* 169 is still unexplained and the only information is that deuteration at C-16 gave a fragment at *m/z* 170 (results not shown).

Metabolism of anabolic steroids and synthesis of metabolites

The metabolism of 20 anabolic steroids after oral application was investigated (Table 2). For the following discussion the anabolic steroids were divided according to their structure into five classes: androst-4-en-3-ones, androsta-1,4-dien-3-ones, 5α-androstan-3-ones, 5α-androstanes with special structure and methandriol.

For the investigated anabolic steroids a brief overview of the main excreted metabolites and their synthesis is presented.

Androst-4-en-3-ones

Methyltestosterone. Methyltestosterone (17α-methylandrost-4-en-17β-ol-3-one) was first synthesized in 1935 by Ruzieka et al. [24] and in 1937 by Oppenhauer [25]. The metabolism of methyltestosterone in man was investigated by Rongone and Segaloff in 1962 [6], identifying 17α-methyl-5α-androstane-3α,17β-diol (**X**) and 17α-methyl-5β-androstane-3α,17β-diol (**XIV**) as the main metabolites (Fig. 1), a similar A-ring metabolism to that for testosterone [26].

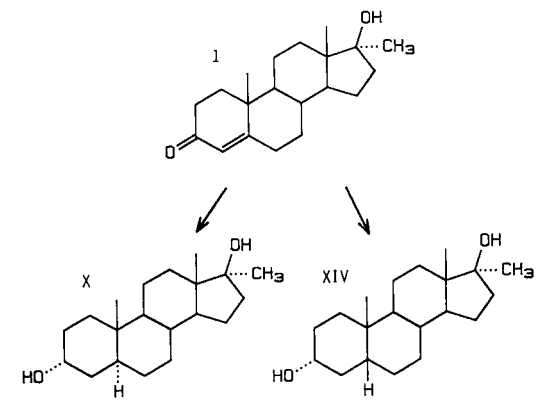

Fig. 1. Metabolism of methyltestosterone (1) to 17α-methyl-5α-androstane-3α,17β-diol (**X**) and 17α-methyl-5β-androstane-3α,17β-diol (**XIV**).

Compared with the metabolism of testosterone, the ratio of the urinary excreted 17α-methyl 5α/5β-isomers is different to the ratio of androsterone to etiocholanolone (5α/5β), the main metabolites of testosterone. Excretion studies with a male volunteer showed a 5α/5β (**X**/**XIX**) ratio of 1:6.4 after oral application of 100 mg of testosterone (0–60 h after application) and a ratio of 1:5.1 when 10 mg of methyltestosterone were applied orally. The same subject showed in both excretion studies an androsterone/etiocholanolone ratio of 1:1.1.

Both **X** and **XIV** were synthesized by a reaction of the Grignard reagent methylmagnesium iodide with 5α-androstan-3α-ol-17-one (androsterone) and 5β-androstan-3α-ol-17β-one (etiocholanolone). The 17-keto group reacts in about 95% yield to give a 17α-methyl-17β-hydroxy structure.

The EI mass spectrum of **X** bis-TMS is shown in Fig. 2 and that of **XIV** bis-TMS in Fig. 3. Both derivatives show an abundant D-ring fragment at *m/z* 143 with 100% relative intensity (base peak).

Nandrolone. Nandrolone (estr-4-en-17β-ol-3-one) was first synthesized in 1950 by Birch [27] and by Wilds and Nelson in 1953 [28] The

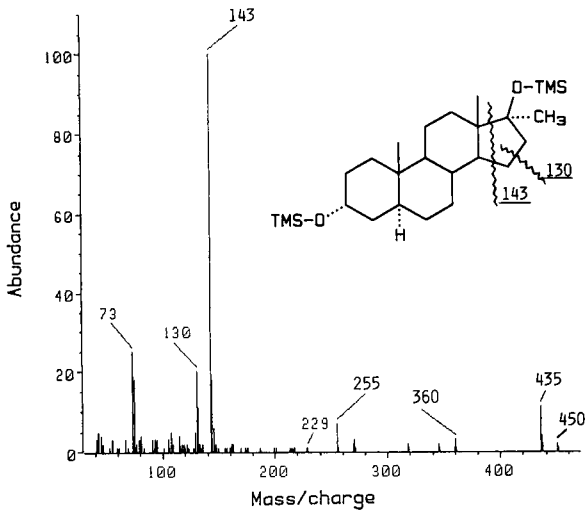

Fig. 2. EI mass spectrum of 17α-methyl-5α-androstane-3α,17β-diol bis-TMS (**X**), molecular ion at m/z 450.

metabolism was investigated by Engel et al. in 1958 [7], who isolated two metabolites 5α-estran-3α-ol-17-one (**XVIII**) and 5β-estran-3α-ol-17-one (**XIX**) (Fig. 4). Both metabolites were synthesized in 1960 by Kupfer et al. [29] and in 1961 by Counsell [30].

Both metabolites were synthesized here starting with nortestosterone which was oxidized with

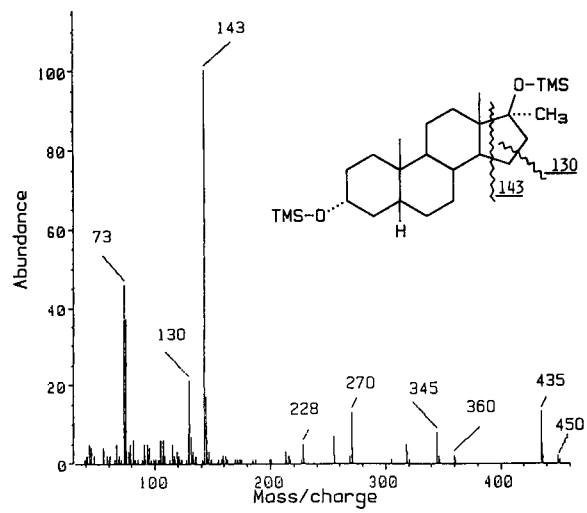

Fig. 3. EI mass spectrum of 17α-methyl-5β-androstane-3α,17β-diol bis-TMS (**XIV**), molecular ion at m/z 450.

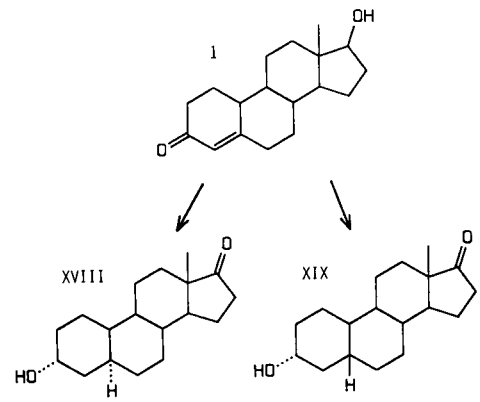

Fig. 4. Metabolism of nandrolone (1) to 5α-estran-3α-ol-17-one (**XVIII**) and 5β-estran-3α-ol-17-one (**XIX**).

chromium trioxide to yield estr-4-ene-3,17-dione. Subsequent reduction with hydrogen in methanol using platinum dioxide as catalyst yielded 35% of 5α-estrane-3,17-dione and 65% of 5β-estrane-3,17-dione. Both isomers were separated by repeated crystallization and could be easily distinguished by their melting points, which are 73–75°C for 5α-estrane-3,17-dione [30] and 179–181°C for 5β-estrane-3,17-dione [31]. Reduction of 5α-androstane-3,17-dione with K-Selektride yielded **XVIII** in about 93% yield (Table 1). Reduction of 5β-estrane-3,17-dione with hydrogen using platinum dioxide as catalyst yielded about 64% of **XIX** and 36% of the 3β-isomer, whereas the reduction with K-Selektride yielded **XIX** in about 6% and the 3β-isomer in 94% yield (Table 1).

The EI mass spectrum of **XVIII** bis-TMS is shown in Fig. 5 and that of **XIX** bis-TMS in Fig. 6.

Norethandrolone. Norethandrolone (17α-ethyl-estr-4-en-17β-ol-3-one) was first synthesized in 1957 by Colton et al. [32].

17α-Ethyl-5α-estrane-3α,17β-diol and 17α-ethyl-5β-estrane-3α,17β-diol (**XX**) were reported as main metabolites by Brooks et al. [8] in 1971.

When an excretion study was made of norethandrolone, only **XX** could be identified and no 5α-isomer of **XX** was detected (Fig. 7). Both isomers were synthesized and are well separated in GC as bis-TMS derivatives [difference of 40 methylene units on an OV-1 capillary column

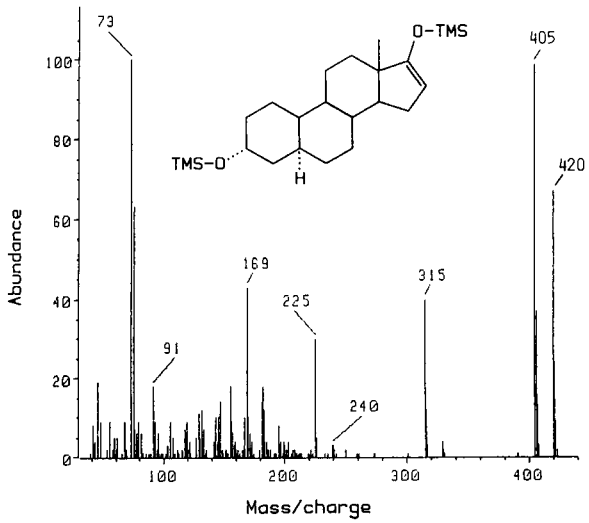

Fig. 5. EI mass spectrum of 5α-estran-3α-ol-17-one bis-TMS (**XVIII**), molecular ion at m/z 420.

(column A, see Experimental)]. A further metabolite was detected which is suggested to be the hydroxy metabolite of **XX**, hydroxylated at the 17α-ethyl group (Fig. 7).

The synthesis of **XX** started with norethandrolone, which was reduced with hydrogen in methanol–6 M sodium hydroxide (10 : 1, v/v) using 10% palladium on charcoal as catalyst, yielding 82% of 17α-ethyl-5β-estrane-17β-ol-3-one, which was further reduced with lithium aluminium hydride to 17α-ethyl-5β-estrane-3α,17β-diol (**XX**) in 90% and the 3β-isomer in 10% yield (Table 1).

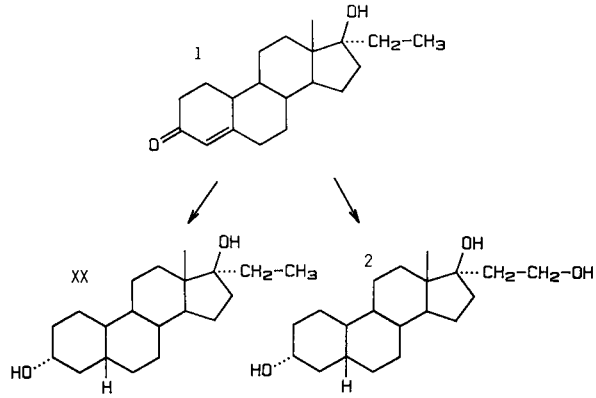

Fig. 7. Metabolism of norethandrolone (1) to 17α-ethyl-5β-estrane-3α,17β-diol (**XX**) and 17α-ethyl-5β-estrane-3α,17β, 20-triol (2).

Figure 8 shows the EI mass spectrum of **XX** bis-TMS. The spectrum shows no molecular ion (M^+, m/z 450) but the highest ion at m/z 435 ($M^+ - 15$) and 421 ($M^+ - 29$). Abundant fragments are the basic ion at m/z 157 and a fragment ion at m/z 144, both D-ring fragments. The

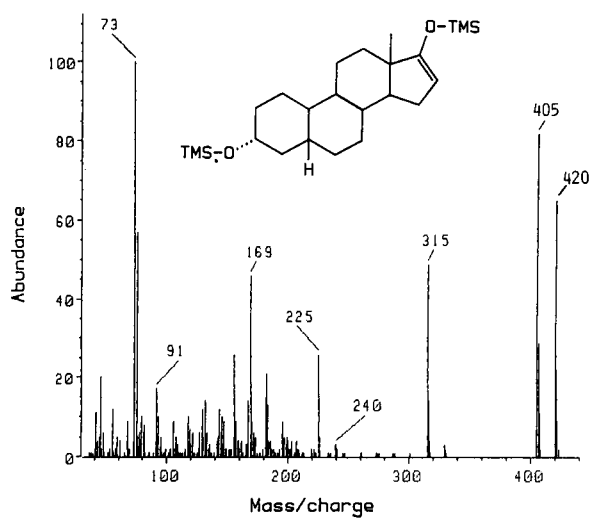

Fig. 6. EI mass spectrum of 5β-estran-3α-ol-17-one bis-TMS (**XIX**), molecular ion at m/z 420.

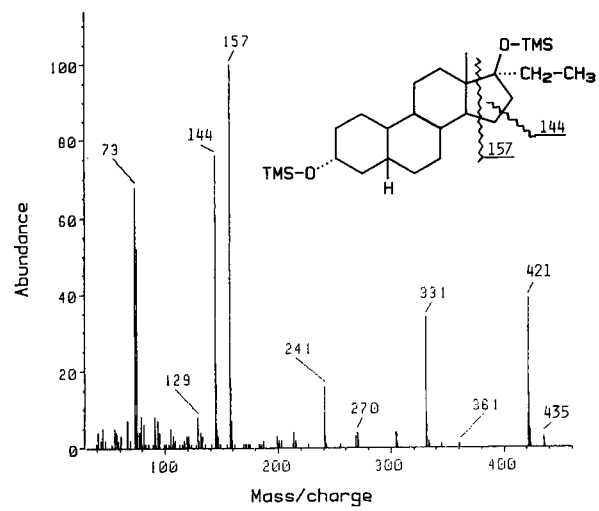

Fig. 8. EI mass spectrum of 17α-ethyl-5β-estrane-3α,17β-diol bis-TMS (**XX**), molecular ion at m/z 450.

origin of these ions can be explained similarly to the D-ring fragmentation of 17α-methyl-17β-hydroxy trimethylsilylated steroids (*m/z* 130 and 143, as shown in Figs. 2 and 3), both with an additional 14 u.

Bolasterone. Bolasterone (7α,17α-dimethyl-androst-4-en-17β-ol-3-one) was synthesized by Campell and Babcock [33]. There appears to be no report of the metabolism of bolasterone in man.

Excretion studies with oral administration of 20 mg of bolasterone to two male volunteers show two metabolites excreted conjugated into urine. Both metabolites show a molecular ion 4 u higher than bolasterone. Synthesis of 7α,17α-dimethyl-5β-androstane-3α,17β-diol (**I**) elucidated that this steroid was the main metabolite. 17-Epimerization of **I** [15] confirmed the second metabolite as 7α,17β-dimethyl-5β-androstane-3α,17α-diol (Fig. 9).

The synthesis of **I** started with bolasterone, which was reduced with hydrogen in methanol using 10% palladium on charcoal. The reaction yielded only one isomer, 7α,17α-dimethyl-5β-androstan-17β-ol-3-one. This configuration could be confirmed by ^{13}C NMR and synthesis of the 5α-isomer, which will be published elsewhere. The 5β-isomer obtained was reduced with lithium aluminium hydride, giving **I** in about 80% and the 3β-hydroxy isomer in 20% yield (Table 1).

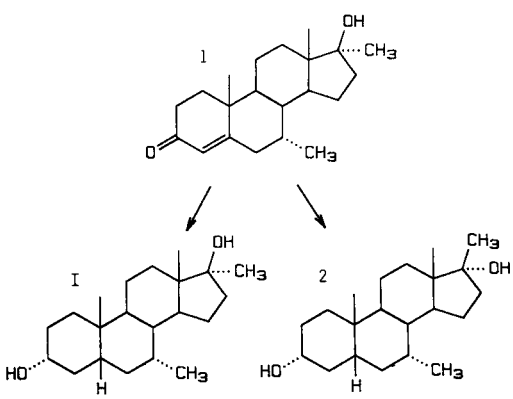

Fig. 9. Metabolism of bolasterone (1) to 7α,17α-dimethyl-5β-androstane-3α,17β-diol (**I**) and 7α,17β-dimethyl-5β-androstane-3α,17α-diol (2).

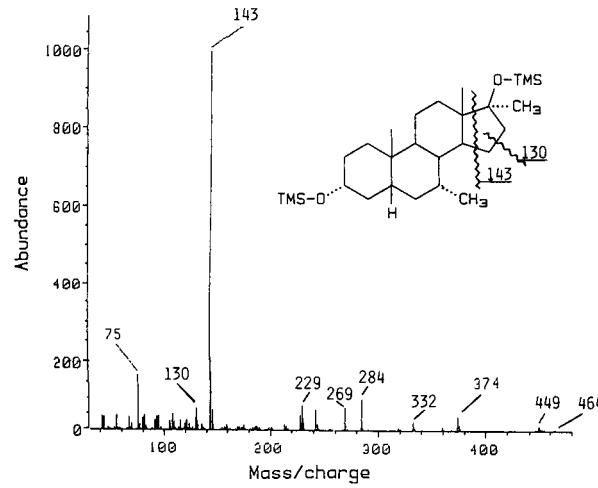

Fig. 10. EI mass spectrum of 7α,17α-dimethyl-5β-androstane-3α,17β-diol bis-TMS (**I**), molecular ion at *m/z* 464.

The EI mass spectrum of **I** bis-TMS (Fig. 10) displays a strong D-ring fragment at *m/z* 143 (100%) and a less intense molecular ion at *m/z* 464 (1%).

Clostebol. Clostebol (4-chloroandrost-4-en-17β-ol-3-one or 4-chlorotestosterone) was synthesized in 1956 by Camerino and co-workers [34,35] and by Ringold et al. [36]. In 1969 Starka et al. [37] investigated the metabolism of clostebol by liver homogenates of man. Oxidation of the 17β-hydroxy group to 17-keto and reduction of the A-ring to dihydro and tetrahydro metabolites were the main metabolic pathways, but the exact identification of the A-ring configuration of the reduced metabolites was not possible.

The present investigations showed after oral administration of 20 mg of clostebol acetate four main metabolites. The main metabolite identified and synthesized was 4-chloroandrost-4-en-3α-ol-17-one (**V**) (Fig. 11). Two further metabolites showed complete reduction of the A-ring and a 17-keto group. It is assumed that the 4-chloro analogues of androsterone and eticholonolone are produced, but the exact configuration of the A-ring has not yet been elucidated. The fourth metabolite is a hydroxylation product of which the exact configuration also was not confirmed.

The results show that the chlorine atom at C-4 inhibits the 5α- and 5β-reductase to a great ex-

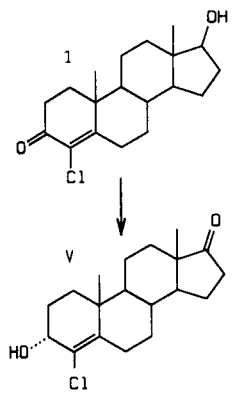

Fig. 11. Metabolism of clostebol (1) to 4-chloroandrost-4-en-3α-ol-17-one (V).

tent and the 3α-hydroxydehydrogenase can reduce clostebol-17-one in the presence of the 4,5-double bond to yield **V**, which is conjugated and excreted as the main metabolite.

The synthesis of **V** started with clostebol, which was oxidized with chromium trioxide to 4-chloroandrost-4-ene-3,17-dione followed by reduction with K-Selektride to yield a mixture of 19% of **V** and 81% of the corresponding 3β-isomer (Table 1).

The EI mass spectrum of **V** bis-TMS is shown in Fig. 12.

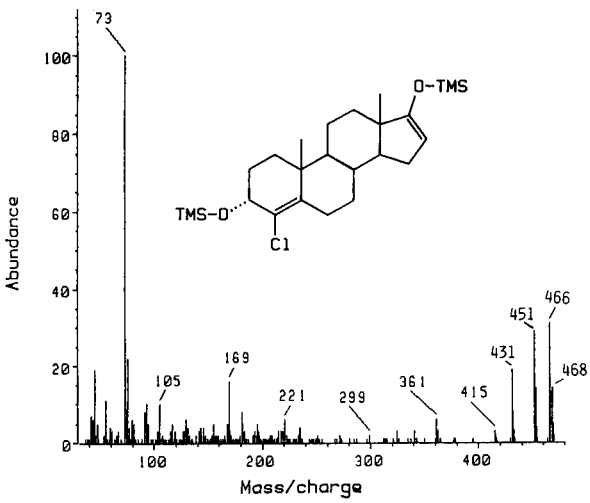

Fig. 12. EI mass spectrum of 4-chloroandrost-4-en-3α-ol-17-one bis-TMS (**V**), molecular ion at *m/z* 466.

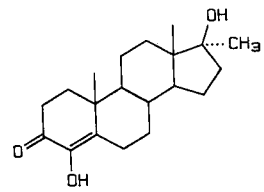

Fig. 13. Structure of oxymesterone (**XXIII**).

Oxymesterone (XXIII). Oxymesterone (**XXIII**) (17α-methylandrost-4-ene-4,17β-diol-3-one; Fig. 13), which was synthesized in 1965 by Camerino [38], is less metabolized and mainly excreted unchanged as a conjugate. The hydroxy group at C-4 may hinder the reduction of the 4,5-double bond and/or allow a very rapid conjugation of **XXIII** at the 4-hydroxy group and excretion into urine.

The EI mass spectrum of **XXIII** tris-TMS (Fig. 14) is dominated by an abundant molecular ion at *m/z* 534.

Fluoxymesterone. Fluoxymesterone (9α-fluoro-17α-methylandrost-4-ene-11β,17β-diol-3-one) was synthesized in 1956 by Herr et al. [39]. In 1990 Kammerer et al. [40] published a GC–MS investigation of fluoxymesterone metabolism in man. They excluded excretion of A-ring reduced metabolites by using reference substances.

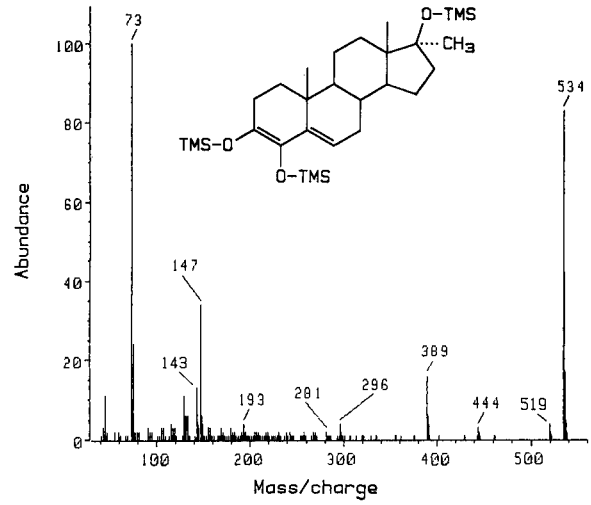

Fig. 14. EI mass spectrum of oxymesterone tris-TMS (**XXIII**), molecular ion at *m/z* 534.

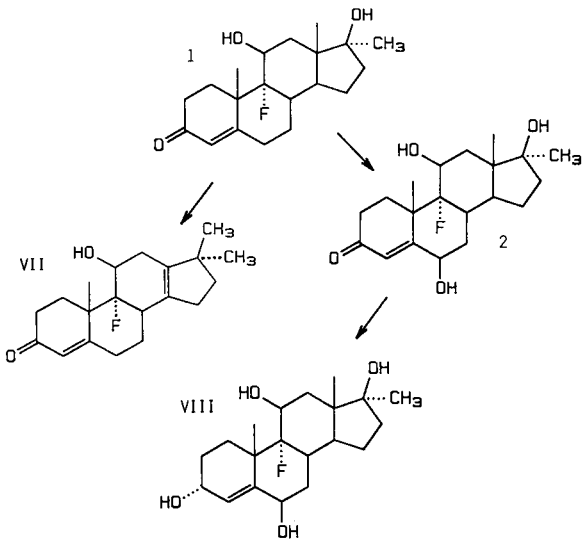

Fig. 15. Metabolism of fluoxymesterone (1), 6β-hydroxyfluoxymesterone (2), 9α-fluoro-18-nor-17,17-dimethyl-4,13-diene-11β-ol-3-one (**VII**) and 9α-fluoro-17α-methylandrost-4-ene-3α,6β,11β,17β-tetrol (**VIII**).

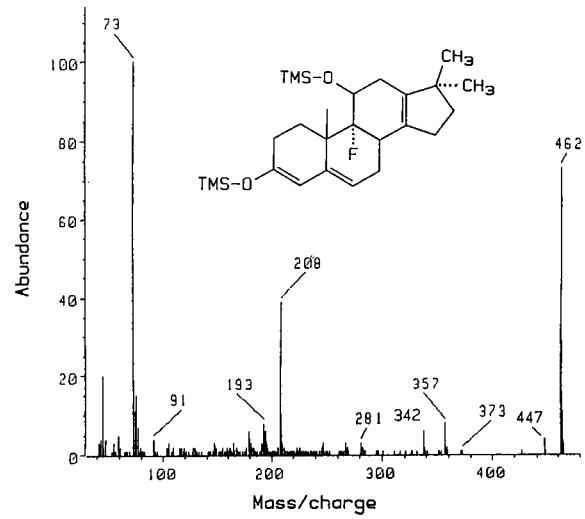

Fig. 16. EI mass spectrum of 9α-fluoro-18-nor-17,17-dimethyl-4,13-diene-11β-ol-3-one bis-TMS (**VII**), molecular ion at *m/z* 462.

The present investigations confirmed 9α-fluoro-18-nor-17,17-dimethyl-4,13-diene-11β-ol-3-one (**VII**), 6β-hydroxyfluoxymesterone and 9α-fluoro-17α-methylandrost-4-ene-3α,6β,11β,17β-tetrol (**VIII**) as the main metabolites (Fig. 15). All three metabolites were synthesized. A detailed publication is in preparation. Compound **VII** was obtained by refluxing fluoxymesterone in acetonitrile–trifluoroacetic acid (10:1, v/v). 6β-Hydroxyfluoxymesterone was synthesized via direct oxidation of the enol-TMS derivative of fluoxymesterone in ethanol catalysed by sunlight. The reduction of 6β-hydroxyfluoxymesterone with K-Selektride yielded **VIII** in about 72% and the 3β-isomer in 28% yield (Table 1).

The EI mass spectrum of **VII** bis-TMS is shown in Fig. 16 and that of **VIII** tetra-TMS in Fig. 17.

The derivatization of **VII** and **VIII** is satisfactory with MSTFA–ammonium iodide as described above; **VII** reacts within 5 min at 80°C completely to a bis-TMS derivative whereas trimethylsilylation of the 11β-hydroxy group in **VIII** is sterically hindered by the fluorine atom at C-9α and the reaction needs 2 h at 80°C, yielding a tetra-TMS derivative. Derivatization with MSTFA–Imi (100:1, v/w) is unsatisfactory. This should be considered when fluoxymesterone metabolites are screened in the unconjugated urine fraction.

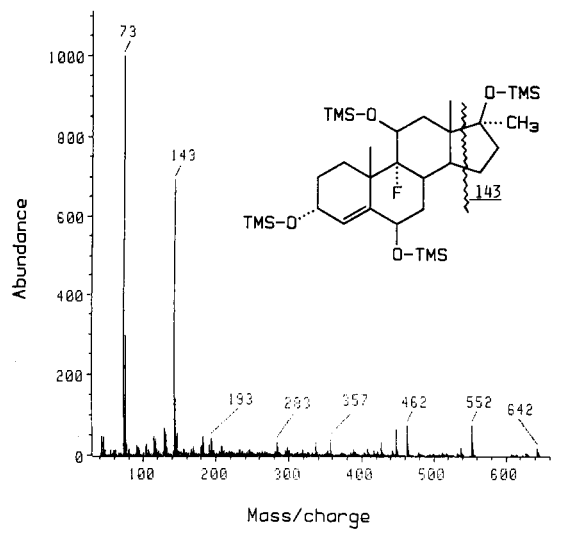

Fig. 17. EI mass spectrum of 9α-fluoro-17α-methylandrost-4-ene-3α,6β,11β,17β-tetrol tetra-TMS (**VIII**), molecular ion at *m/z* 642.

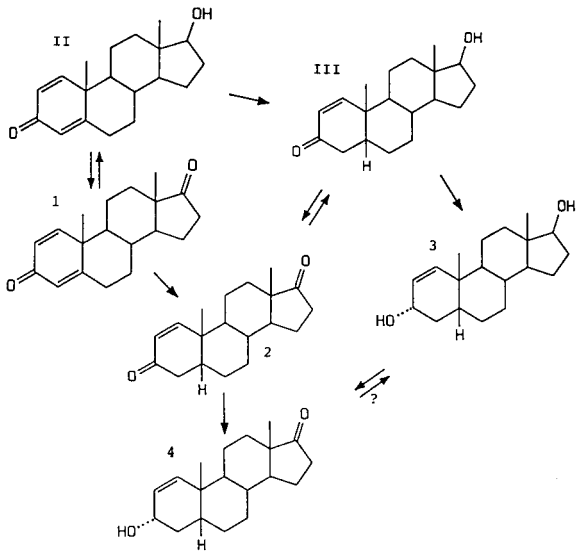

Fig. 18. Metabolism of boldenone (**II**) to 5β-androst-1-en-17β-ol-3-one (**III**), androsta-1,4-diene-3,17-dione (1), 5β-androst-1-ene-3,17-dione (2), 5β-androst-1-ene-3α,17β-diol (3) and 5β-androst-1-en-3α-ol-17-one (4).

Androsta-1,4-dien-3-ones

Androsta-1,4-dien-3-ones are reduced by the 5β-reductase only, but to a smaller extent than testosterone. In this instance 6β-hydroxylation is favoured for metandienone, 4-chlorodehydromethyltestosterone and fluoxymesterone.

Boldenone. Boldenone (**II**) (androsta-1,4-dien-17β-ol-3-one) was synthesized in 1956 by Meystre et al. [41].

The metabolism of **II** was investigated in 1971 by Galetti and Gardi [1]. GC–MS of boldenone and its main metabolites was reported by Schänzer and Donike in 1992 [12].

Boldenone itself is excreted as a conjugate in urine. The main metabolites are 5β-androst-1-en-17β-ol-3-one (**III**), 5β-androst-1-en-3α-ol-17-one and 5β-androst-1-ene-3α,17β-diol (Fig. 18). In the proposed screening procedure **II** and **III** are used to detect boldenone misuse.

The synthesis of **III** started with **II**, which was selectively reduced with hydrogen in methanol–potassium hydroxide using 10% palladium on charcoal as catalyst [12].

The EI mass spectrum of **II** bis-TMS is shown in Fig. 19 and that of **III** bis-TMS in Fig. 20.

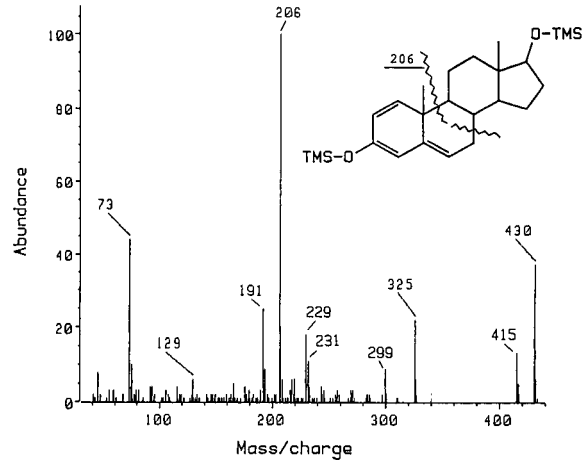

Fig. 19. EI mass spectrum of boldenone bis-TMS (**II**), molecular ion at m/z 430.

Metandienone. Metandienone (17α-methyl-androsta-1,4-dien-17β-ol-3-one) was synthesized in 1955 by Vischer et al. [42] by microbiological dehydrogenation of methyltestosterone. In 1956 Meystere et al. [41] reported the dehydrogenation of methyltestosterone with selenium dioxide. 6β-Hydroxymetandienone (**XIII**) was identified in 1963 by Rongone and Segaloff as the main metabolite [4], which was excreted unconjugated in urine. A further metabolite is 17-epimetan-

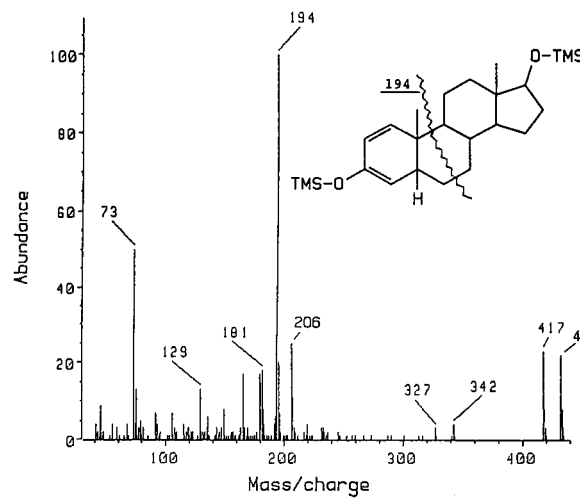

Fig. 20. EI mass spectrum of 5β-androst-1-en-17β-ol-3-one bis-TMS (**III**), molecular ion at m/z 432.

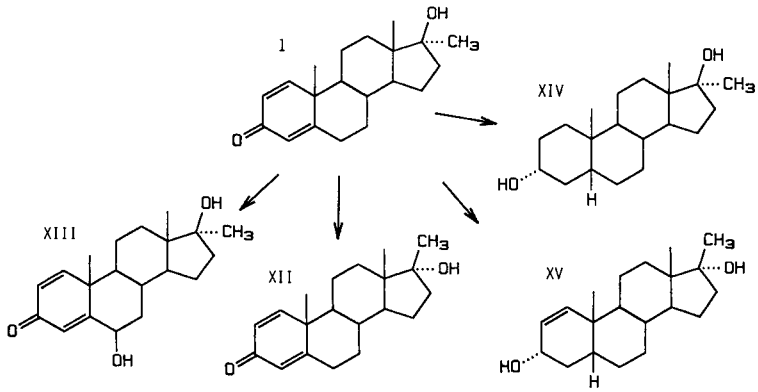

Fig. 21. Metabolism of metandienone (1) to 17-epimetandienone (**XII**), 6β-hydroxymetandienone (**XIII**), 17α-methyl-5β-androstane-3α,17β-diol (**XIV**) and 17β-methyl-5β-androst-1-ene-3α,17α-diol (**XV**).

dienone (**XII**) obtained from the unconjugated fraction which was identified and synthesized in 1971 by Macdonald et al. [5]. In 1980 Dürbeck and Bücker [23] investigated the unconjugated urine fraction after application of metandienone by GC–MS and discussed the EI fragmentation of the trimethylsilylated metabolites. In 1991 Schänzer et al. [13] published the identification of A-ring reduced metabolites excreted as conjugates in urine, 17α-methyl-5β-androst-1-ene-3α,17β-diol, its 17-epimer 17β-methyl-5β-androst-1-ene-3α,17α-diol (**XV**) and 17α-methyl-5β-androstane-3α,17β-diol (**XIV**) (Fig. 21); **XIV** is also a metabolite of methyltestosterone and methandriol.

The synthesis of 6β-hydroxymetandienone (**XIII**) was performed as described above by autooxidation of the enol-TMS derivative in ethanol catalysed by sunlight. The synthesis of **XII** was performed via the 17β-sulphate of metandienone which spontaneously decomposed in water giving several dehydration products and **XII** in about 30% yield. Synthesis of **XV** started with 17-epimetandienone (**XII**), which was selectively reduced with hydrogen in methanol–potassium hydroxide using 10% palladium on charcoal giving 17β-methyl-5β-androst-1-en-17α-ol-3-one in about 20% yield. The reduction of this 3-ketone with lithium aluminium hydride gave **XV** in more than 85% yield. For the synthesis of **XIV**, see the discussion of methyltestosterone.

EI mass spectra are shown for **XII** TMS in Fig. 22, **XIII** bis-TMS in Fig. 23, **XIV** bis-TMS in Fig. 3 and **XV** bis-TMS in Fig. 24. All these EI mass spectra are dominated by the D-ring fragment at *m/z* 143.

4-Chlorodehydromethyltestosterone. 4-Chlorodehydromethyltestosterone (4-chloro-17α-methyl-androsta-1,4-dien-17β-ol-3-one) was synthesized in 1960 by Schubert et al. [43]. The metabolism of 4-chlorodehydromethyltestosterone in man was reported by Schubert and co-workers [2,3]. In addition to the excretion of 4-chlorodehydromethyltestosterone itself they also identified 6β-hydroxy-4-chlorodehydromethyltestosterone (**IV**), 16β-hydroxy-4-chlorodehydromethyltestosterone

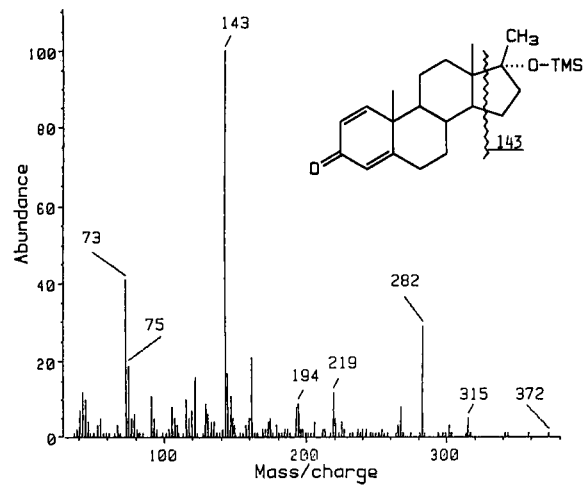

Fig. 22. EI mass spectrum of 17-epimetandienone TMS (**XII**), molecular ion at *m/z* 372.

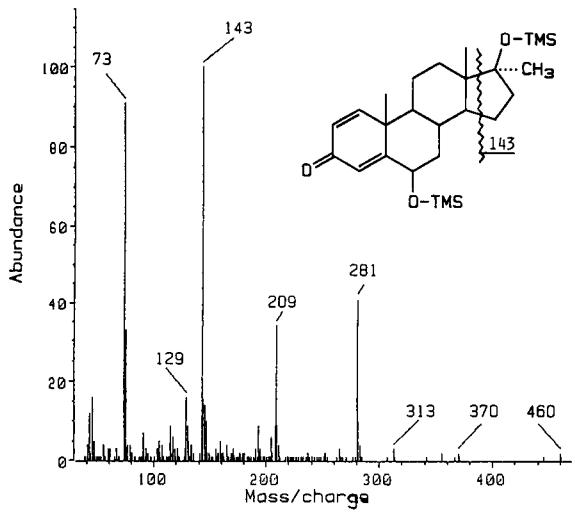

Fig. 23. EI mass spectrum of 6β-hydroxymetandienone bis-TMS (**XIII**), molecular ion at m/z 460.

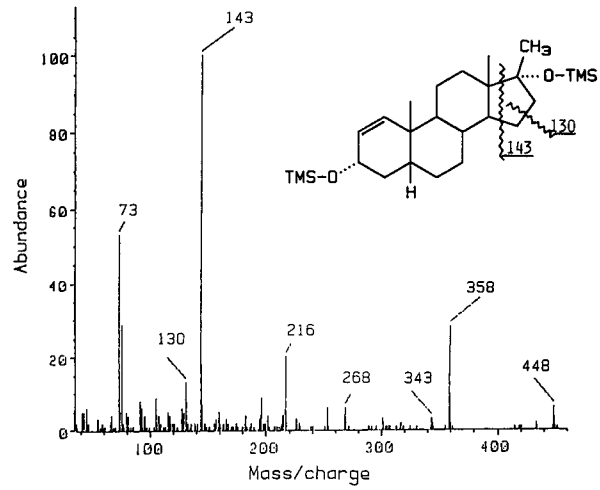

Fig. 24. EI mass spectrum of 17β-methyl-5β-androst-1-ene-3α,17α-diol bis-TMS (**XV**), molecular ion at m/z 448.

and 6β,16β-dihydroxy-4-chlorodehydromethyltestosterone as the main metabolites. In 1983 Dürbeck et al. [44] studied the metabolism of 4-chlorodehydromethyltestosterone by GC–MS. They were able to confirm 6β-hydroxy-4-chlorodehydromethyltestosterone (**IV**) and the 6β,16β-dihydroxy metabolite. They did not detect 4-chlorodehydromethyltestosterone and the 16β-hydroxy metabolite, but a substance showing the same EI mass spectrum as the parent steroid and it was therefore assumed to be a 17-epimer. They further recorded a bishydroxy metabolite which was considered to be a 6β,12-dihydroxy metabolite (Fig. 25).

In an excretion study with 22 mg of orally administered 4-chlorodehydromethyltestosterone, it was possible to identify in the unconjugated urine fraction a 17-epimer, which was synthesized [15], 6β-hydroxy-4-chlorodehydromethyltestos-

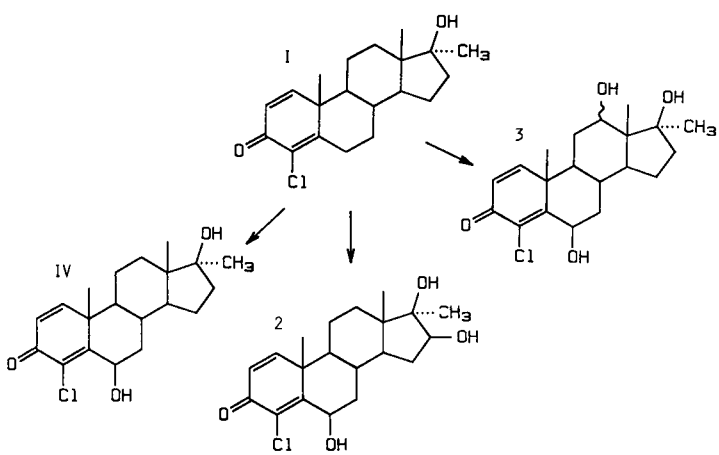

Fig. 25. Metabolism of 4-chlorodehydromethyltestosterone (1) to 6β-hydroxy-4-chlorodehydromethyltestosterone (**IV**), 6β,16β-dihydroxy-4-chlorodehydromethyltestosterone (2) and 6β,12z-dihydroxy-4-chlorodehydromethyltestosterone (3) (z = unidentified configuration).

terone (**IV**), which was also synthesized, a 6β,16-dihydroxy metabolite and a 6β,12-dihydroxy metabolite, as discussed by Dürbeck et al. [44]. The 12-hydroxy group could be confirmed using the EI mass spectra of the tris-TMS derivative of this metabolite, displaying an abundant fragment at *m/z* 170. This fragment was previously reported [13] to originate from a 12,17-dihydroxy-17-methyl-bis-TMS structure.

Compound **IV** was synthesized by the above-described synthesis. The enol-TMS ether was autooxidized when dissolved in isopropanol and exposed to sunlight, yielding **IV**. A detailed description of this synthesis is in preparation.

The EI mass spectrum of **IV** bis-TMS is shown in Fig. 26. The D-ring fragment at *m/z* 143 is the most abundant ion. The fragments at *m/z* 243 and 315 are the 4-chloro analogue fragments of the ions at *m/z* 209 and 281 of 6β-hydroxy-metandienone bis-TMS (Fig. 23).

Formebolone. Formebolone (2-formyl-17α-methylandrosta-1,4-diene-11α,17β-diol-3-one) was synthesized in 1965 by Canonica et al. [45].

A GC–MS investigation of formebolone metabolism in man was published by Masse et al. [46].

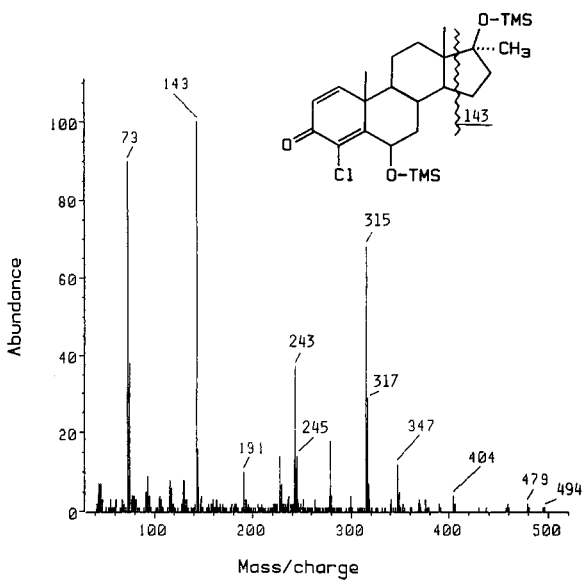

Fig. 26. EI mass spectrum of 6β-hydroxy-4-chlorodehydromethyltestosterone bis-TMS (**IV**), molecular ion at *m/z* 494.

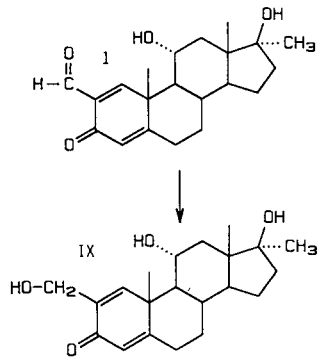

Fig. 27. Metabolism of formebolone (1) to 2-hydroxymethyl-17α-methylandrosta-1,4-diene-11α,17β-diol-3-one (**IX**).

In the present investigation with 20 mg of formebolone applied orally to a male volunteer, a reduced metabolite was identified in the unconjugated fraction (Fig. 27) after basic extraction. This polar metabolite was identified by synthesis as 2-hydroxymethyl-17α-methylandrosta-1,4-diene-11α,17β-diol-3-one (**IX**).

Compound **IX** was synthesized by reduction of formebolone with K-Selectride in tetrahydrofuran. The K-Selectride showed a selectivity to the formyl group which was first reduced and the 3-keto group remained intact when the reagent was not used in excess. Isolation of **IX** by preparative HPLC yielded a pure reference substance.

The EI mass spectrum of **IX** tris-TMS is displayed in Fig. 28.

5α-Androstan-3-ones

Drostanolone. Drostanolone (2α-methyl-5α-androstan-17β-ol-3-one) was synthesized in 1959 by Ringold et al. [47]. Drostanolone is orally applied as propionate.

After oral application of 20 mg of drostanolone propionate to a male volunteer, 2α-methyl-5α-androstan-3α-ol-17-one (**VI**) was identified as the main metabolite (Fig. 29), which is excreted as a conjugate in urine. No drostanolone or drostanolone propionate was excreted.

The synthesis of **VI** started with drostanolone, which was oxidized with chromium trioxide to 2α-methyl-5α-androstane-3,17-dione and then reduced with K-Selektride yielding about 99% of 2α-methyl-5α-androstan-3α-ol-17-one (**VI**) and

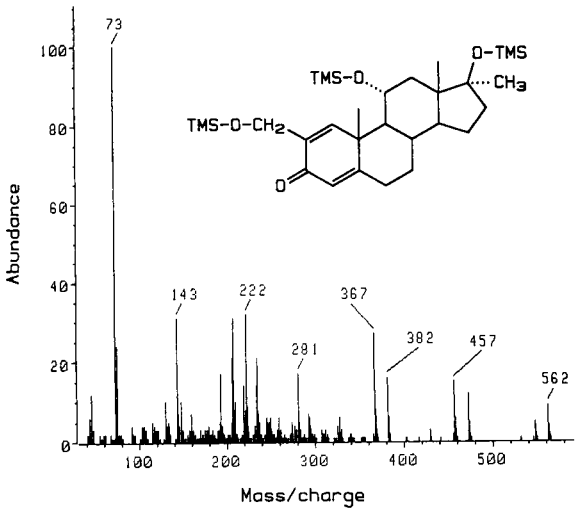

Fig. 28. EI mass spectrum of 2-hydroxymethyl-17α-methyl-androsta-1,4-diene-11α,17β-diol-3-one tris-TMS (**IX**), molecular ion at m/z 562.

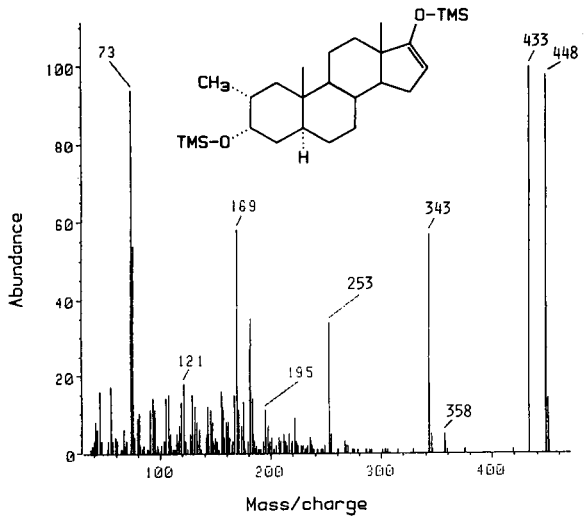

Fig. 30. EI mass spectrum of 2α-methyl-5α-androstan-3α-ol-17-one bis-TMS (**VI**), molecular ion at m/z 448.

1% of the 3β-isomer (Table 1).

The EI mass spectrum of **VI** bis-TMS is presented in Fig. 30.

Mesterolone. Mesterolone (1α-methyl-5α-androstan-17β-ol-3-one was synthesized by Wiechert [48].

It is metabolized in the same way as drostanolone. After oral application of 20 mg of mesterolone the main metabolite was identified as 1α-methyl-5α-androstan-3α-ol-17-one (**XI**), excreted conjugated in urine (Fig. 31). No parent drug was detected.

The synthesis of **XI** was carried out in the same way as that of the drostanolone metabolite **VI**. Starting with the chromium trioxide oxidation of mesterolone 1α-methyl-5α-androstane-3,17-dione was obtained, which was reduced with K-Selektride to 99% of 1α-methyl-5α-androstan-3α-ol-17-one (**XI**) and 1% of the 3β-isomer (Table 1).

The EI mass spectrum of **XI** bis-TMS is shown in Fig. 32.

Mestanolone. Mestanolone (17α-methyl-5α-androstan-17β-ol-3-one) was synthesized in 1935 by Ruziecka et al. [24].

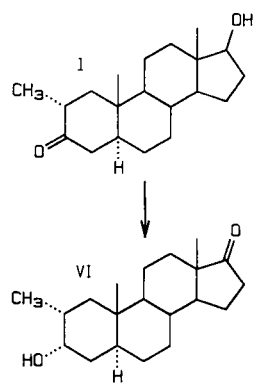

Fig. 29. Metabolism of drostanolone (1) to 2α-methyl-5α-androstan-3α-ol-17-one (**VI**).

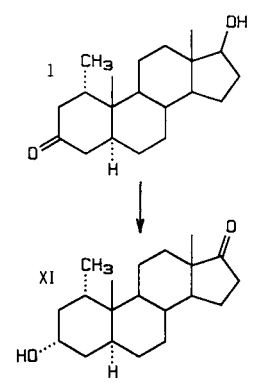

Fig. 31. Metabolism of mesterolone (1) to 1α-methyl-5α-androstan-3α-ol-17-one (**XI**).

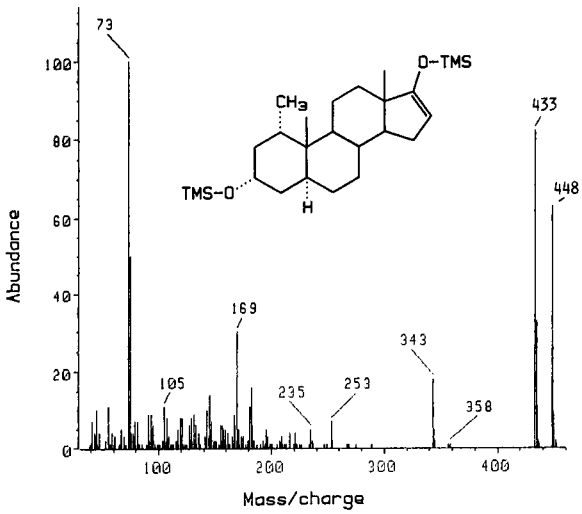

Fig. 32. EI mass spectrum of 1α-methyl-5α-androstan-3α-ol-17-one bis-TMS (**XI**), molecular ion at m/z 448.

After oral administration of 20 mg of mestanolone to a male volunteer, 17α-methyl-5α-androstane-3α,17β-diol (**X**) was identified as the main metabolite, excreted as a conjugate in urine (Fig. 33). No parent drug was detected. The same metabolite was obtained in the metabolism of methyltestosterone and oxymetholone.

For the synthesis of **X** see the discussion of methyltestosterone and for the EI mass spectrum of **X** bis-TMS see Fig. 2.

Metenolone (XVI). Metenolone (1-methyl-5α-androst-1-en-17β-ol-3-one) (**XVI**) was synthesized

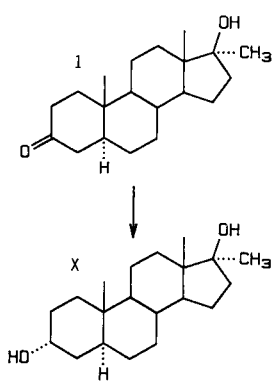

Fig. 33. Metabolism of mestanolone (1) to 17α-methyl-5α-androstane-3α,17β-diol (**X**).

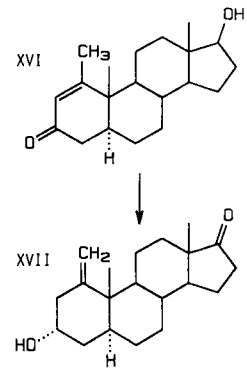

Fig. 34. Metabolism of metenolone (**XVI**) to 1-methylen-5α-androstan-3α-ol-17-one (**XVII**).

in 1960 by Wiechert and Kaspar [49]. Metenolone is applied orally as the acetate or by intramolecular injection as the enanthate. The metabolism of **XVI** was investigated by Gerhards et al. in 1965 [50], identifying 1-methylen-5α-androstan-3α-ol-17-one (**XVII**) as the main metabolite and unchanged metenolone (**XVI**), both excreted as conjugates (Fig. 34). Recently Goudreault and Masse [51] described a GC–MS investigation of **XVI** and its metabolites.

In this work the synthesis of **XVII** started with metenolone (**XVI**), which was oxidized to 1-methyl-5α-androst-1-ene-3,17-dione. The latter was isomerized to 1-methylen-5α-androstane-3,17-dione as described by Wiechert et al. [52] using the method of Ringold and Malhotra [53], who converted α,β-unsaturated ketones into β,γ-unsaturated ketones with potassium *tert*-butoxide in *tert*-butanol.

The reduction of the isomerized product with K-Selektride yielded 80% of **XVII**, 10% of the corresponding 3β-isomer and about 10% of 1-methyl-5α-androst-1-ene-3β,17β-diol (Table 1).

The EI mass spectrum of **XVI** bis-TMS is presented in Fig. 35 and that of **XVII** bis-TMS in Fig. 36.

Oxymetholone. Oxymetholone (2-hydroxymethylene-17α-methyl-5α-androstan-17β-ol-3-one) was first prepared by Ringold et al. in 1959 [47]. Oxymetholone is substantially metabolized and several metabolites can be detected by GC–MS. In the neutral and basic fraction used for screen-

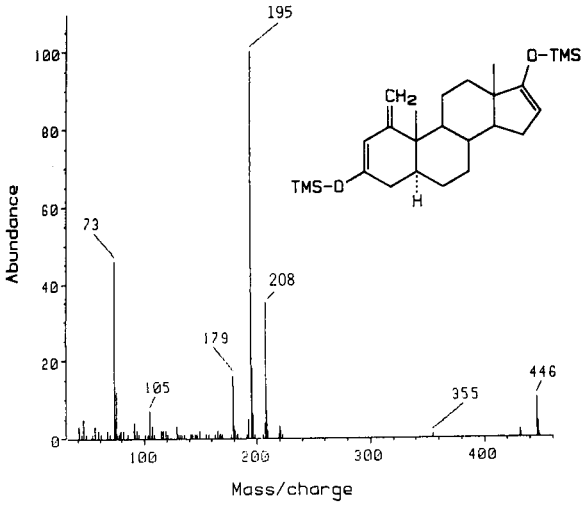

Fig. 35. EI mass spectrum of metenolone bis-TMS (**XVI**), molecular ion at m/z 446.

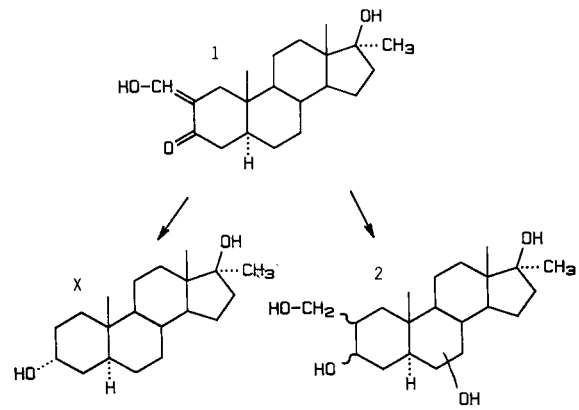

Fig. 37. Metabolism of oxymetholone (1) to 17α-methyl-5α-androstane-3α,17β-diol (**X**) and 2z-hydroxymethyl-17α-methyl-5α-androstane-3z,z,17β-triol (2) (z = unidentified configuration).

ing of anabolic steroids, 17α-methyl-5α-androstane-3α,17β-diol (**X**) was identified as a metabolite. The origin of this metabolite can be explained by oxidation of the 2-hydroxymethylene group to a 2-carbonic acid. This oxidation product is a β-ketocarbonic acid, which is less stable and decomposes to 17α-methyl-5α-androstan-17β-ol-3-one. Subsequent reduction of the 3-keto group yields **X** with a 3α-hydroxy configuration. A further metabolite is detected in excretion studies with oxymetholone with a molecular ion at m/z 640, which is explained as a hydroxy derivative of the completely reduced oxymetholone (Fig. 37). This metabolite is further used for screening of an oxymetholone abuse even though its structure is not completely known.

The synthesis of **X** is described in the discussion of methyltestosterone and the EI mass spectrum of **X** bis-TMS is shown in Fig. 2.

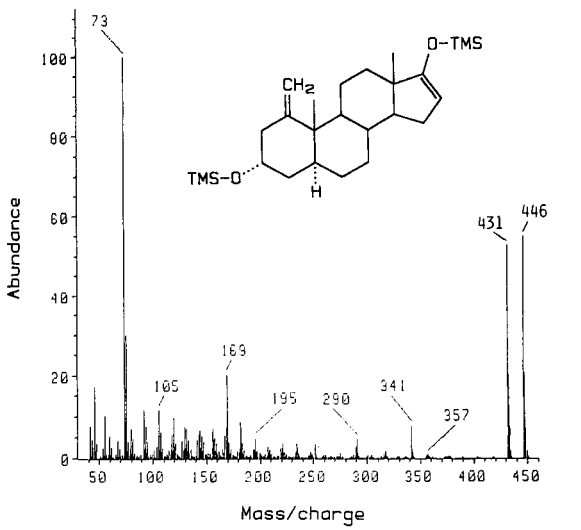

Fig. 36. EI mass spectrum of 1-methylen-5α-androstan-3α-ol-17-one bis-TMS (**XVII**), molecular ion at m/z 446.

5α-Androstanes with special structure

Oxandrolone (XXI). Oxandrolone (**XXI**) (17α-methyl-2-oxa-5α-androstan-17β-ol-3-one) was synthesized in 1962 by Pappo and Jung [54]. In 1989 Masse et al. [55] published a GC–MS investigation of oxandrolone metabolism in man. Compound **XXI** is excreted unchanged to a great extent. As the main metabolite a 17-epimer (**XXII**) of oxandrolone (**XXI**) was described (Fig. 38). Further metabolites hydroxylated at C-16 were detected by GC–MS but in low concentration.

In this work **XXII** was synthesized by the above described method for 17-epimerization [15].

The EI-mass spectra of **XXI** TMS in Fig. 39 and **XXII** TMS in Fig. 40 show that these spectra are identical and dominated by the abundant D-ring fragment at m/z 143.

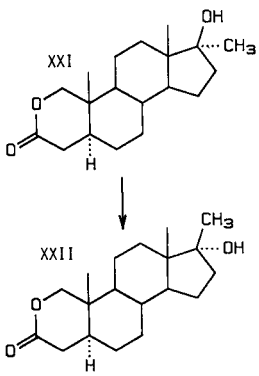

Fig. 38. Metabolism of oxandrolone (**XXI**) to 17-epioxandrolone (**XXII**).

Furazabol. Furazabol was synthesized in 1965 by Ohta et al. [56]. Metabolism studies of furazabol with rats were published by Takegoshi et al. [57], but no study of the metabolism of furazabol in man has been reported.

After oral administration of 20 mg of furazabol in man furazabol itself and a metabolite hydroxylated at C-16 were detected in the conjugated urine fraction (Fig. 41). The EI mass spectrum of the TMS derivative of this metabolite in Fig. 42 show fragments at m/z 218 and 231, confirming a D-ring with a 17-methyl-16,17-dihydroxy-bis-TMS structure.

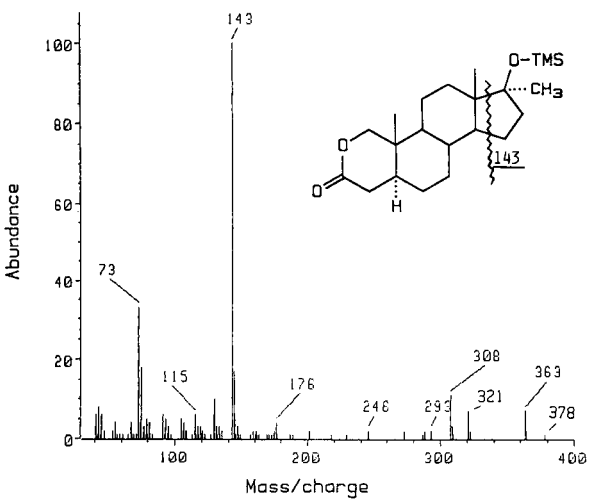

Fig. 39. EI mass spectrum of oxandrolone TMS (**XXI**), molecular ion at m/z 378.

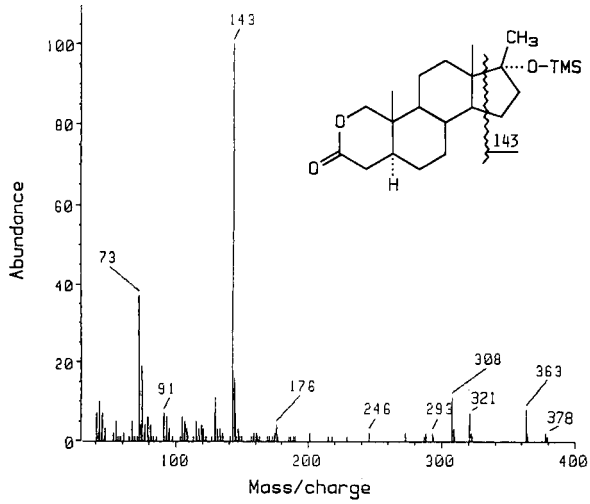

Fig. 40. EI mass spectrum of 17-epioxandrolone TMS (**XXII**), molecular ion at m/z 378.

Because no misuse of furazabol has been reported by any IOC accredited laboratory, this metabolite was not synthesized but a reference obtained from an excretion study with furazabol was used for screening purposes.

Stanozolol. Stanozolol (17α-methyl-17β-hydroxy-5α-androst-2-eno[3,2-*c*]pyrazole) was synthesized in 1959 by Clinton and co-workers [58,59]. The metabolism of stanozolol in man was investigated in this laboratory and published in 1990 [14].

About eleven metabolites were confirmed and the main metabolites were synthesized, namely

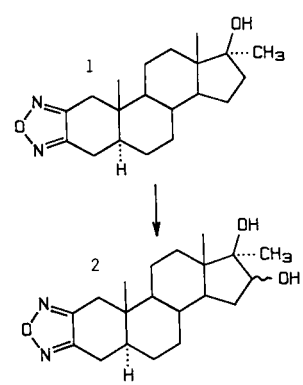

Fig. 41. Metabolism of furazabol (1) to 16z-hydroxyfurazabol (2) (z = unidentified configuration).

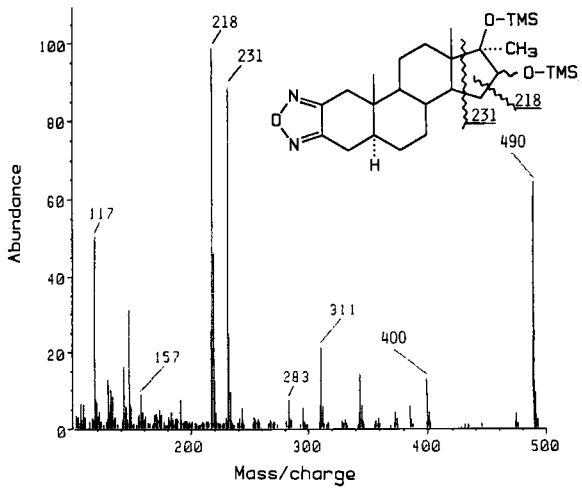

Fig. 42. EI mass spectrum of 16z-hydroxyfurazabol bis-TMS (z = unidentified configuration), molecular ion at m/z 490.

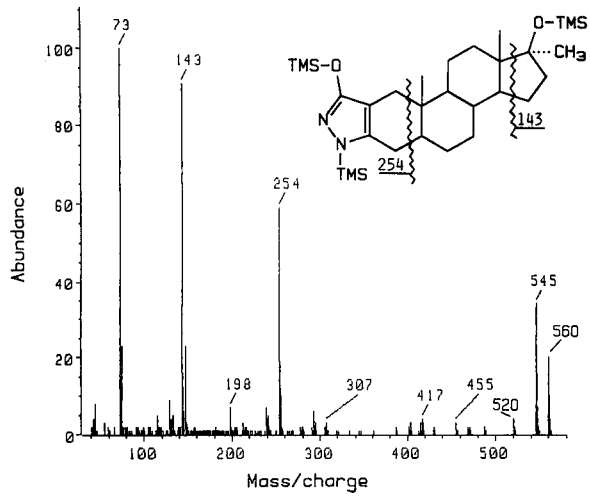

Fig. 44. EI mass spectrum of 3′-hydroxystanozolol tris-TMS (**XXIV**), molecular ion at m/z 560.

3′-hydroxystanozolol (**XXIV**), 3′-hydroxy-17-epistanozolol (**XXV**) [15], 4β-hydroxystanozolol (**XXVI**) and 16β-hydroxystanozolol (**XXVII**) (Fig. 43).

EI mass spectra are displayed for **XXIV** tris-TMS in Fig. 44, **XXV** tris-TMS in Fig. 45, **XXVI** tris-TMS in Fig. 46 and **XXVII** tris-TMS in Fig. 47.

Methandriol. Methandriol (17α-methylandrost-5-ene-3β,17β-diol) was synthesized in 1935 by Ruziecka et al. [24]. It is applied orally as the dipropionate.

The metabolism of methandriol and methandriol dipropionate was investigated and after 30 mg of orally applied methandriol dipropionate excreted methandriol was identified in very low concentration in the sulphate fraction which is routinely not controlled. The main metabolite was identified as 17α-methyl-5β-androstane-3α,17β-diol (**XIV**) (Fig. 48) in the conjugated urine

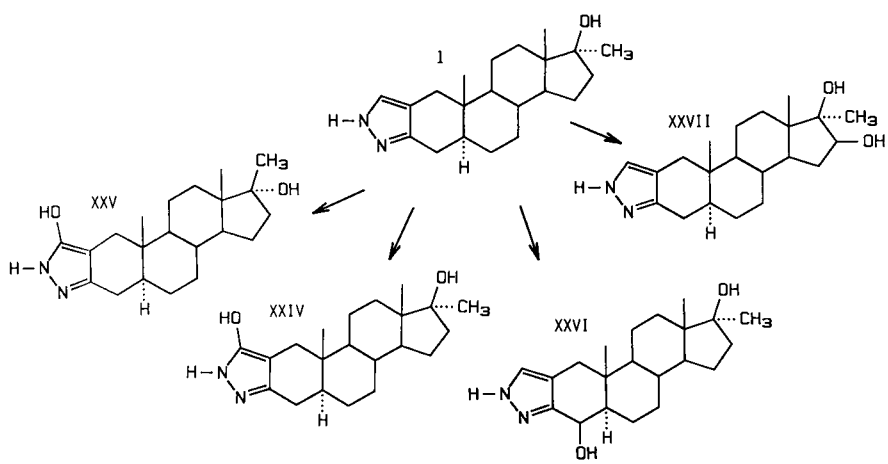

Fig. 43. Metabolism of stanozolol (1) to 3′-hydroxystanozolol (**XXIV**), 3′-hydroxy-17-epistanozolol (**XXV**), 4β-hydroxystanozolol (**XXVI**) and 16β-hydroxystanozolol (**XXVII**).

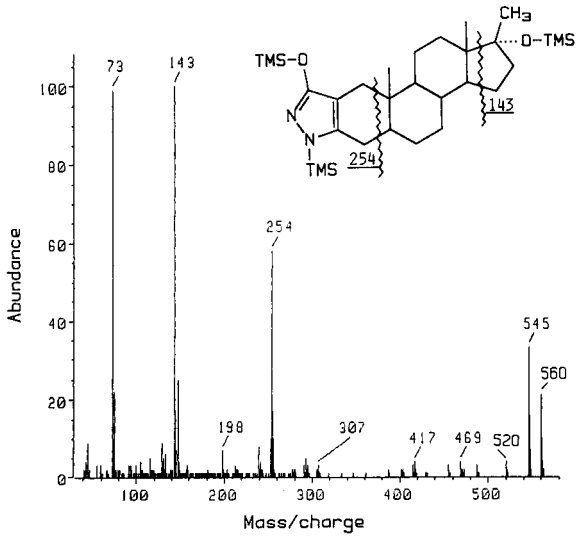

Fig. 45. EI mass spectrum of 3′-hydroxy-17-epistanozolol tris-TMS (**XXV**), molecular ion at m/z 560.

fraction, but in low concentration. An excretion study with 20 mg of methandriol confirmed metabolite **XIV** as the main metabolite and a possible 5α-isomer was not detected.

This result can only be explained by a metabolic pathway of methandriol in man including 3-oxidation and isomerization of the 5,6-double bond to

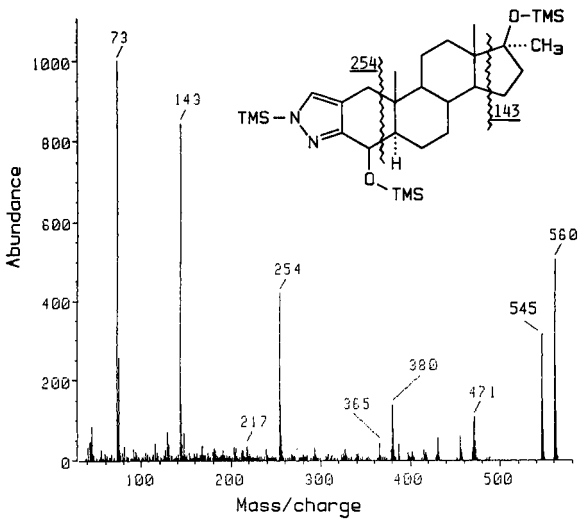

Fig. 46. EI mass spectrum of 4β-hydroxystanozolol tris-TMS (**XXVI**), molecular ion at m/z 560.

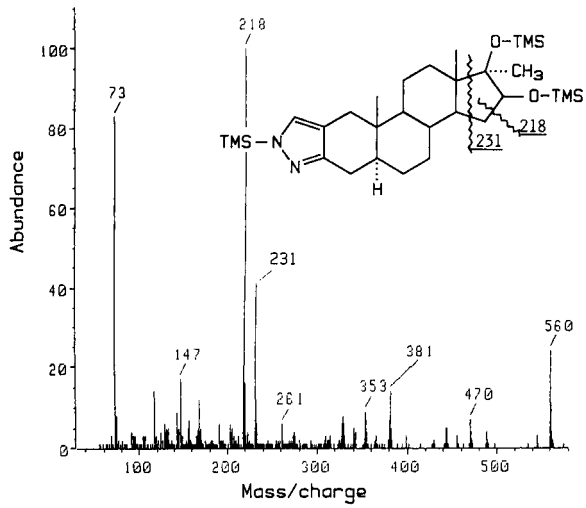

Fig. 47. EI mass spectrum of 16β-hydroxystanozolol tris-TMS (**XXVII**), molecular ion at m/z 560.

C-4,5, similarly as the metabolism of dehydroepiandrosterone (androst-5-en-3β-ol-17-one). The oxidation and isomerization product is methyltestosterone following the above-described metabolic pathway with the excretion of mainly **XIV**, a metabolite with a 3α-hydroxy configuration.

For the synthesis of **XIV** see the discussion of methyltestosterone and for the EI mass spectrum of **XIV** bis-TMS see Fig. 3.

The authors are grateful to the Bundesinstitut of Sportwissenschaft, Cologne, and the Interna-

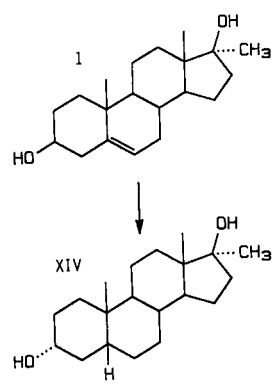

Fig. 48. Metabolism of methandriol (1) to 17α-methyl-5β-androstane-3α,17β-diol (**XIV**).

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Influence of the consumption of meat contaminated with anabolic steroids on doping tests

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Abstract

In 1989, 3 cases out of 20 were found where gas chromatography–mass spectrometry revealed the presence of nandrolone metabolites in the urine of untreated persons. Although the origin of the nandrolone metabolites was uncertain at that time, the presumption was that such findings could be related to the consumption of meat from nandrolone-treated animals. Two years later, in a feeding experiment with volunteers, 1 out of 10 persons was positive for clostebol due to the accidental consumption of meat contaminated with clostebol acetate. In 1992, a similar random experiment was carried out ($n = 50$), yielding 2 positive cases (1 nandrolone case and 1 clostebol case). The urinary concentrations found in the volunteers who had accidentally consumed contaminated meat were compared with the levels found in volunteers after a controlled intake of a minor dose of the anabolic steroid concerned. The above levels were also compared with those after intake of a therapeutic dose of the compound.

Keywords: Gas chromatography; Mass spectrometry; Anabolic steroids; Clostebol; Doping; Meat; Nandrolone

Anabolic–androgenic steroids have pharmacological properties that qualitatively are similar to those of the male hormone testosterone. Mainly because of the anabolic effect they are intensively used in human medicine for the restoration of muscle size and strength in persons emaciated from starvation for whatever reason [1]. This practice is at the origin of the relatively frequent use of anabolic steroids by athletes competing in sports where added strength is an advantage. For medical and ethical reasons, these substances are banned by the International Olympic Committee and by most national sports federations. Despite their almost general prohibition, their prevalence is high in sports that require bodybuilding [2]. In Belgium, doping analysis results have revealed that cyclists also seem to believe in the benefits of hormonal substances [3].

The same anabolic–androgenic steroids are important constituents of many black-market preparations intended for growth stimulation in animal husbandry. These "cocktails" mostly contain one or more estrogens, combined with androgens and/or progestagens. They have optimum efficacy in veal calves, heifers and steers owing to the negligible endogenous hormone production. The European Economic Community (EEC) has prohibited the use of hormones in

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animal breeding [4]. In the USA a number of substances with hormonal activity have been legalized.

Setting aside all political and ethical considerations regarding the use of prohibited substances, there are a few consequences that directly affect the consumer of the products from treated animals. The first is that, because the administration is prohibited, many ways to disguise it are tried. This means that almost all parts of the animal are eligible for injection and thus certain edible tissues, such as tail base and neck muscle. The latter tissues may be processed into minced raw beef meat or stewed meat. A second consideration pertains to the dosage. As the illegal preparations have not been monitored for bioavailability, as is required for registered trade-name preparations produced by the pharmaceutical industry, there is a tendency for overdosage. This means that when the animal is slaughtered, there might be remnants of the injection, encapsulated by an immunological reaction of the animal. Finally, little is known about the pharmacokinetics of xenobiotics in animals. It was concluded that under these conditions biological effects on the consumer cannot be excluded, especially when orally active hormones have been administered [5]. A special aspect of these biological effects might be that the hormones or their metabolites appear in the urine, which as such indicates an infringement of the ban on hormonal substances for sportsmen. The possibility of interferences from the consumption of hormone-contaminated meat in urine doping control was raised in 1989 [6] and was confirmed in 1991 [7]. In this study, further evidence is provided to support this suggestion.

EXPERIMENTAL

The experiments consisted in the consumption by volunteers of randomly purchased raw minced beef meat samples (random experiments) and also in the controlled administration to volunteers, orally or intramuscularly, of known amounts of known substances (control experiments).

Random experiments

Raw minced beef meat samples (200 g) were purchased in 50 different butchers' shops selected at random in the city of Ghent (Belgium). Five laboratory staff members (male, 42, 46, 46, 48 and 51 years old) volunteered to consume a 150-g portion with bread at libidum every Tuesday at noon for ten consecutive weeks. They were asked not to consume any other beef meat within 24 h after the experimental meal. Urine was collected immediately before the meal and at different intervals afterwards, up to 24 h. Urine samples from the volunteers, later referred to as volunteers 1–50, were frozen at −20°C pending analysis. The remainder of the meat was also stored at −20°C pending analysis of only those samples the consumption of which gave positive results in urinary doping control.

Control experiments

Three healthy male staff members volunteered to consume a single dose of 10, 50 and 100 μg of nandrolone (19-nortestosterone) with their lunch, which did not contain any beef meat. Urine samples, later referred to as volunteers 51, 52 and 53, were collected and stored as in the random experiment.

A 26-year-old healthy man was injected intramuscularly with the pharmaceutical preparation Steranabol (Farmitalia, Freiburg, Germany), which contained 40 mg of clostebol acetate. Urine was collected the day before the injection and 1, 2, 3, 6, 9, 12, 15, 20, 25, 30, 40, 50 and 60 days after injection.

Standards

4-Chloroandrost-4-en-3α-ol-17-one (CLOSMET) was synthesized at the laboratory of M. Donike (Cologne, Germany) by W. Schänzer and kindly donated. Nandrolone (19-nortestosterone, 19-NT) was obtained from Aldrich (Milwaukee, WI) and 19-norandrosterone (5α-estran-3α-ol-17-one, 19-NA) from Steraloids (Wilton, NH). 19-Norepietiocholanolone (5ß-estran-3ß-ol-17-one, 19-NEE) was donated by Schering (Berlin, Germany).

Sample pretreatment

The solid-phase extraction of the urine samples, as a modification of the method of Schmidt et al. [8], has already been described [7]. The pretreatment of the meat was described by Daeseleire et al. [9].

Derivatization

N-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and dithioerythritol (DTE) were purchased from Aldrich and ammonium iodide from Merck (Darmstadt, Germany). A 50-μl volume of an MSTFA–NH_4I–DTE (1000 : 2 : 4, v/w/w) mixture was used for derivatization at 60°C for 30 min [10]; 2 μl were injected into the gas chromatograph.

Gas chromatography–mass spectrometry (GC–MS)

GC–MS analyses were carried out on a Model HP 5890 gas chromatograph equipped with a DB5 capillary column (20 m × 0.18 mm i.d.), film thickness 0.4 μm (J&W Scientific, Folsom, CA). The carrier gas was high-purity helium (N60) (L'Air Liquide, Liège, Belgium) at a column head pressure of 80 kPa and a linear velocity of 26 cm^{-1}. The injection temperature and transfer line temperature were 290°C. The splitting ratio was 1 : 10. The oven temperature was held at 100°C for 1 min, then programmed from 100 to 230°C at 40°C/min and from 230 to 280°C at 5°C/min, the final temperature being maintained for 15 min. Electron impact ionization was done at an electron beam voltage of 70 eV. Screening of parent compounds and/or major metabolites was achieved by selected ion monitoring (SIM). Six groups of ions, which had been selected from the mass spectra of reference standards, were monitored. Identification was based on the correspondence of the retention times of unknowns and reference standards, expressed as methylene unit (M.U.) values, and on the simultaneous appearance of the traces of the selected ions. For quantitative analysis, 19-NEE was used as an internal standard [7].

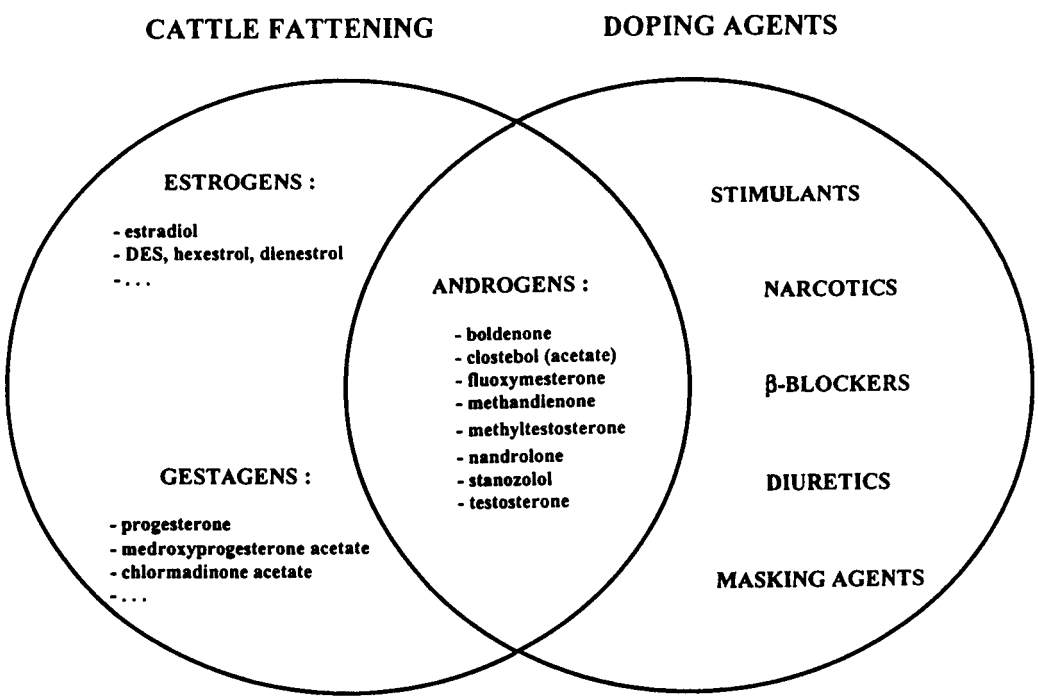

Fig. 1. Overview of the groups of anabolic hormones used in cattle fattening and the products on doping lists.

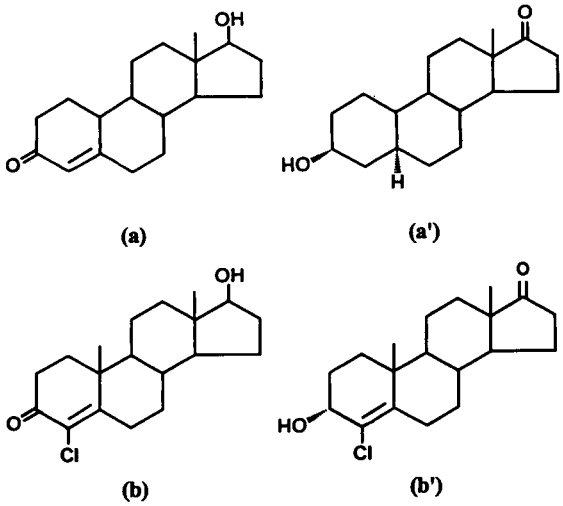

Fig. 2. Structures of (a) nandrolone and (b) clostebol and their most important human urinary metabolites, (a') 19-norandrosterone (19-NA) and (b') 4-chloroandrost-4-en-3α-ol-17-one (CLOS-MET), respectively.

RESULTS

The first two post-meal urine samples from each volunteer in the random experiments were screened for anabolic–androgenic steroids, as shown in Fig. 1. Fluoxymesterone, being detected only very recently in injection sites [11], was screened in the samples from the last 31 volunteers.

In 2 of the 50 random experiments, positive urine samples were detected. Volunteers 27 and 29 were positive for nandrolone and clostebol, respectively. The presence of nandrolone was revealed via the detection of its major metabolite 19-norandrosterone (19-NA) [12], while the major metabolite of clostebol, i.e., 4-chloroandrost-4-en-3α-ol-17-one (CLOS-MET) [13], indicated the presence of clostebol. The structures of these metabolites and the parent compounds are shown in Fig. 2. Nandrolone is available in pharmaceutical preparations and in illegal cocktails as the cyclohexylpropionate, decanoate, hemisuccinate laurate and phenylpropionate esters. The only clostebol derivative encountered so far is the acetate [14].

For the two positive volunteers all available urine samples (i.e., pre-meal and all post-meal samples) were subjected to quantitative analysis. Table 1 summarizes the results for volunteer 27. It has not been reported that 19-NA is an endogenous compound in man. 19-NA could be detected up to 9.17 h after the meal. The total amount excreted was 0.71 μg.

TABLE 1

Determination of 19-NA in the urine samples from volunteer 27

Time (h) [a]	19-NA concentration ($\mu g\,l^{-1}$)	Excreted amount (μg)	Excretion rate ($\mu g\,h^{-1}$)
1.50	0.25	0.03	0.02
3.67	3.79	0.36	0.17
7.00	1.66	0.26	0.08
9.17	0.22	0.06	0.03

[a] Time after consumption of nandrolone-contaminated meat.

The analysis of an aliquot of the raw minced beef meat that had been ingested by volunteer 27 revealed the presence of nandrolone, in the form of the free alcohol. Identification was achieved by monitoring three main ions at m/z 666, 453, 306 (heptafluorobutyrate derivative) [9] at the correct retention time compared with that of a reference standard. A quantitative measurement of the

TABLE 2

Determination of 19-NA in urine samples from volunteers 51, 52 and 53

Volunteer	Time (h) [a]	19-NA concentration ($\mu g\,l^{-1}$)	Excreted amount (μg)	Excretion rate ($\mu g\,h^{-1}$)
51	2.50	9.8	2.5	1.0
	6.00	3.0	0.7	0.2
52	2.25	14.6	5.6	2.5
	4.50	19.5	5.7	2.5
	7.00	6.6	2.5	1.0
	10.25	1.1	0.4	0.2
	19.00	0.4	0.2	0.1
53	4.50	94.1	21.3	4.7
	8.00	29.3	5.7	1.6
	10.75	7.7	1.0	0.4
	18.00	1.9	0.8	0.1
	19.75	0.9	0.1	0.1

[a] Time after consumption of the nandrolone-fortified food.

TABLE 3

Determination of CLOS-MET in urine samples from volunteer 29

Time (h) [a]	CLOS-MET concentration ($\mu g\,l^{-1}$)	Excreted amount (μg)	Excretion rate ($\mu g\,h^{-1}$)
4.67	16.0	4.13	0.88
6.67	22.7	2.25	1.12
11.17	5.7	1.39	0.31
18.25	1.0	0.27	0.04

[a] Time after consumption of the clostebol acetate-contamined meat.

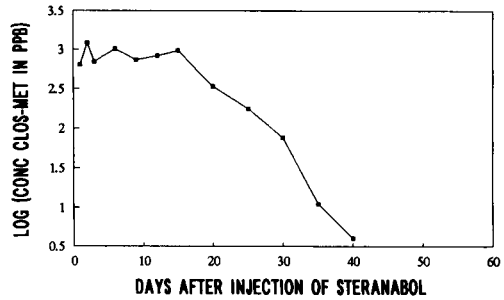

Fig. 3. Concentrations of CLOS-MET in urine after injection of Steranabol.

amount of nandrolone in order to establish an eventual correlation with the concentration of metabolites in urine was not considered as nandrolone will probably occur in the form of an ester. From the Belgian monitoring programme on the composition of the cocktails present in injection sites, it is clear that in almost all instances, when nandrolone esters are detected, a minor fraction is present as the free alcohol nandrolone. The method used for the detection of nandrolone residues in muscle tissue is aimed at measuring the free alcohol, in which form residues of nandrolone undoubtedly occur in tissues. From the control experiments, in which volunteers ingested 10, 50 and 100 μg of nandrolone, 31.2, 28.3 and 28.7% were excreted as 19-norandrosterone. More pharmakokinetic details are given in Table 2.

On screening of the first two urine samples from volunteer 29, the presence of the major metabolite of clostebol, i.e., 4-chloroandrost-4-en-3α-ol-17-one, could be demonstrated. From Table 3 it can be seen that this metabolite was still detectable 18.25 h after the meal. The analysis of an aliquot of the raw meat that had been ingested by volunteer 29 revealed the presence of clostebol in the form of both the free alcohol and the acetate ester. Identification was achieved by monitoring three main ions for both clostebol bis-TMS (m/z 466, 451 and 431) and clostebol acetate enol TMS ether (m/z 438, 436, 401) at the correct retention times compared with those of reference standards. Controlled experiments with volunteers, ingesting either 10 μg ($n = 1$) or 100 μg ($n = 2$) of clostebol acetate, have been reported previously [7]. The levels found after injection of Steranabol (clostebol acetate, 40 mg) in a 26-year-old volunteer are given in Table 4 and shown in Fig. 3.

TABLE 4

Concentrations of CLOS-MET in urine after injection of Steranabol

Days after injection	CLOS-MET concentration ($\mu g\,l^{-1}$)	Days after injection	CLOS-MET concentration ($\mu g\,l^{-1}$)
0	0	20	339
1	644	25	179
2	1203	30	76
3	695	35	11
6	1004	40	4
9	732	50	0
12	829	60	0
15	967		

DISCUSSION

The previous suggestion that hormone-contaminated meat can lead to positive doping tests [6,7] was reconfirmed in this work. As already described, this is most likely to occur in those instances where injection sites are processed into meat products. The meat that was consumed in the random experiments was raw minced beef meat, which is mostly produced by mincing lower quality muscle tissue such as the neck from cattle. This part of the animal frequently contains the injection site. Experience with injection sites has been gained in this laboratory, which is one of

those involved in the Belgian national monitoring programme, as laid down by the EEC Council Directive 86/469 concerning the examination of animals and fresh meat for the presence of residues [4].

The frequency distribution of each of the encountered substances over the period January 1989–April 1992 is given year by year in Fig. 4. It can be seen that over that period the relative incidence of nandrolone decreased from 68.6% to 21.8% of all positive injection sites. Clostebol, which was not found in 1989 because it was not known to be used, has since 1990 become the most frequently used representative of the xenobiotic hormones. It is therefore not a coincidence that the 3 cases where the positive control had to be blamed on ingestion of contaminated meat concerned clostebol (1 case in 1991 and 1 case in 1992) and nandrolone (1 case in 1992). The 3 positive controls in 1989 also concerned nandrolone. Although the link to contaminated meat could only be presumed at that time, as no dietary control was performed, Fig. 4, showing the prevalence of nandrolone in 1989, strengthens this presumption.

The levels that were found after non-deliberate intake of hormones via contaminated meat are of the same order of magnitude as those which can be measured 30 days after injection of 40 mg of clostebol acetate. The same conclusion has been reported for nandrolone [6]. The main difference between a non-deliberate ingestion and a deliberate injection is that a positive urine sample, originating from the ingestion of contaminated meat, is only a random indication. It does not last longer than 1–2 days and a new urine sample will give negative results. With deliberate administration with a view to improving athletic performance, the effect on the urine composition will last for weeks or even months. Another urine sample taken 24 or 48 h after the first will under those circumstances most certainly give a positive result.

There are no data available on to the chances of encountering a minced injection site in a meat meal. Although as it is presumed that most cattle are being treated with one or another growth-promoting agent or mixture of agents, this has never been expressed in figures. Our own results indicate that of all the injection sites that were analysed in this laboratory, 62.2% were positive

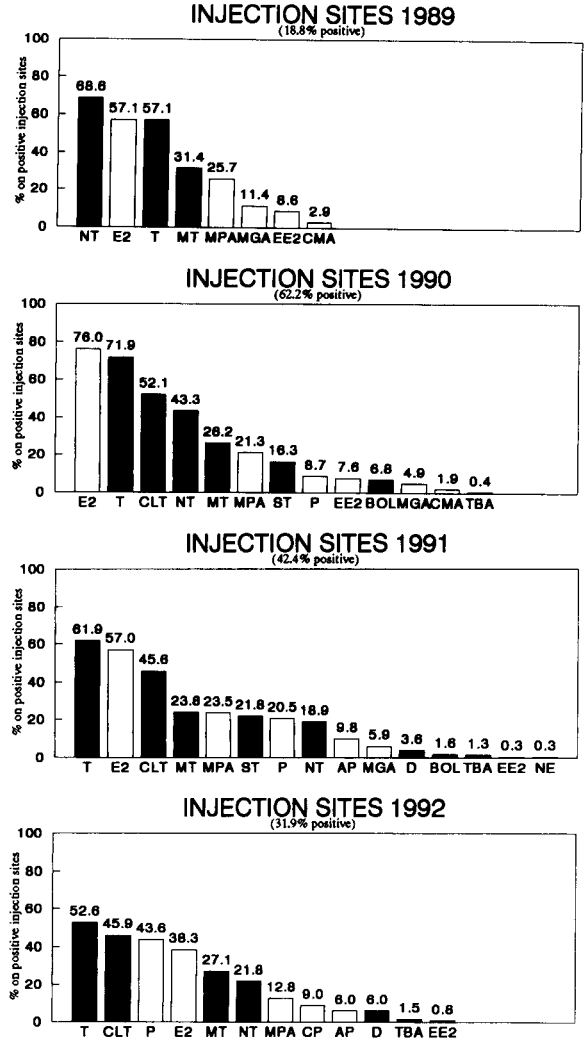

Fig. 4. Statistical evaluation per year of the results of the high-performance thin-layer chromatographic analysis of the injection sites from January 1989 to April 1992. Products that are also used as doping agents in sports are shown in black on the histograms. Abbreviations: AP = 17α-acetoxyprogesterone; BOL = boldenone; CP = 17α-hydroxyprogesterone caproate; CLT = clostebol and/or acetate; CMA = chlormadinone acetate; D = methandienone (Dianabol); E2 = estradiol and/or esters; EE2 = ethinyl estradiol; MGA = megestrol acetate; MPA = medroxyprogesterone acetate; MT = methyltestosterone; NE = norethisterone; NT = nandrolone and/or esters; P = progesterone; ST = stanozolol; T = testosterone and/or esters; TBA = trenbolone acetate.

for one or more anabolic agents in 1990. This level decreased to 42.2% in 1991 and even to 31.4% for the 4 four months of 1992. It is not always clear whether sampling was done at random or was directed at suspicious animals.

There is, however, another approach for estimating the average risk of being exposed to non-deliberate intake of hormone residues. Taking into consideration that residue levels in first-quality tissues such as steak are much too low to interfere with urinary doping control and assuming that ingestion of megadoses can only be revealed through the consumption of minced injection sites from beef, as the practice of hormone administration is exceptional in other large animal species, one can calculate the average exposure to injection sites, expressed in terms of amounts of minced raw beef meat. From the BIRNH study [15], it is known that, in the Flemish community, the average intake per capita per month was 389 g of minced raw meat and 108 g of stewed meat. The latter generally is prepared with low class muscle tissues. This means that on average the Flemish consumer eats four times a month a portion of 100 g of raw minced meat and only once a month a similar portion of stewed meat. This implies that that person is exposed to lower quality beef meat 60 times per year. In our random experiments in both 1991 and 1992, 3 positive results were obtained in a total of 60 experiments. Hence, roughly, a sportsman could be positive 3 times a year due to consumption of contaminated meat. In practice, a sportsman taking part in a competition should, to be absolutely safe, not eat those types of meat preparations within the 48 h preceding the contest. Moreover, samples for doping control, and particularly for the control of anabolic steroids, are also taken during training periods. The international and national sports federations and particularly the International Olympic Committee should face this problem.

In May 1992, results of a study carried out at the official Flemish Doping Control Laboratory were reported [16]. A total of 870 persons were analysed for nandrolone and clostebol urinary metabolites. A questionnaire concerning the meat consumption during the 24 h before urine collection was filled in. Data concerning the sports activities (including fitness training and bodybuilding) and the particular question of whether the person was vegetarian or not completed the questionnaire. Two of the 870 cases were positive for nandrolone. The researchers ascribed this finding to the fact that both persons were bodybuilders.

The discrepancy between the results of the two laboratories can be explained by the very different set-ups of the experiments. In the laboratory of Professor M. Debackere, the eventual beef meat consumption in the 24 h before consumption was only documented by filling in the questionnaire, only one random urine sample was analysed per volunteer and no meat was sampled or analysed. It is not clear how many of the 870 persons ate "high-risk" meat in the hours before the urine collection. In our laboratory, however, deliberate consumption of beef meat, and more in particular minced beef meat, was preferred, and 24-h urine samples were collected in different portions. A portion of the minced beef meat was kept for analysis. This meat was analysed when the corresponding consumer yielded a positive doping test. The urinary concentration of the metabolite was moreover determined.

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Control of the illegal administration of natural steroid hormones in urine and tissues of veal calves and in plasma of bulls

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Abstract

In the context of the control of the illegal administration of natural steroid hormones in cattle husbandry, decision levels of sex steroid hormones were established, taking into account the effect of the treatment, for the cases of the urine of male and female veal calves treated with estradiol and/or testosterone-containing implants, and the plasma of bulls treated by an injection of an estradiol–testosterone cocktail. At each decision level, a score was assigned, that represents the percentage of treated animals detected when the decision limit is applied. Concerning the veal calves, a maximum decision level is proposed for the 17β-estradiol in urine of 1 ng ml^{-1} in both male and female veal calves, giving a score of 95%. A minimum decision level was set at 2 ng ml^{-1} for testosterone in urine of male veal calves, with a score of 95% (95% of the implanted male veal calves display a urinary testosterone level lower than 2 ng ml^{-1}). For female veal calves, a maximum decision level was set at 0.45 ng ml^{-1} for the urinary testosterone concentration (score of 90%). For bulls, the 17β-estradiol concentration in plasma is a good criterion for detecting injected bulls: a maximum decision level was set at 40 pg ml^{-1}, displaying a score ranging from 100 to 45%, 2 and 7 days after the injection, respectively.

Keywords: Immunoassay; Radiochemical methods; Anabolic steroids; Bovine samples; Bulls; Hormones; Plasma; Tissue samples; Urine; Veal calves

In the European Economic Community (EEC), Directive 88/146/EEC prohibits the use of anabolics as growth promoters for meat production.

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As non-endogenous synthetic hormones, such as zeranol, trenbolone and diethylstilbestrol (DES), can easily be detected in urine, plasma or tissue samples [1], anabolic treatments with natural steroids are increasingly applied [2]. The problem is then to discriminate between physiological concentrations and elevated hormone levels due to the administration of natural anabolics. This is

not an easy task owing to the large variability described in the literature for the concentrations of the various steroids in different matrices.

In very well controlled experiments, involving animals of the same age, fed and housed in the same way, Arts and co-workers [3,4] established reference values for 17β-estradiol, 17α-estradiol, testosterone and epitestosterone concentrations in plasma and urine of male and female veal calves. From these reference values, they determined decision limits on veal calves treated by injection of an estradiol–testosterone cocktail. They concluded that the best criterion to detect treated animals is the urinary or plasma level of 17β-estradiol (which increases after the treatment).

Our approach is modelled on that of Arts and co-workers but is somewhat different. The aim of the control is to determine whether a sample originates from a treated animal or not, without knowing its age or its housing and feeding conditions. In order to achieve to this aim, a statistical study was performed on data from several different experiments. These data are concentrations of natural steroid hormones [determined by radioimmunoassay (RIA)] in urine and tissues of male and female veal calves untreated or treated with sex steroid-containing implants.

The need for reference values also exists for adult animals. We therefore measured plasma levels of various sex steroids hormones and metabolites in untreated bulls and in bulls treated by a single injection of a testosterone–estradiol cocktail. Decision limits were then determined taking into account the treatment effect.

EXPERIMENTAL

Samples

Veal calves. Male and female veal calves were treated in several different experiments (Table 1) with estradiol and/or testosterone-containing implants for various periods of time (15–120 days).

Samples of urine were collected during the treatment period and tissue samples were recovered at slaughter. After hydrolysis with *Helix pomatia* juice, diethyl ether extraction and purification on Bond Elut [5], the samples were analysed using specific RIAs for 17β-estradiol, 17α-estradiol, estrone and testosterone according to procedures described previously [5,6].

Bulls. Eleven bulls, aged 9–11 months and weighting 310–470 kg, were treated by a single injection of a cocktail containing 20 mg of 17β-estradiol benzoate (Intervet) and 200 mg of several esters of testosterone (Durateston, Intervet). Control plasma samples were collected from these bulls 4, 2 and 1 day before the treatment and plasma samples from treated animals were collected 2, 3, 5 and 7 days after the steroid injection.

After diethyl ether extraction of the plasma samples, non-conjugated forms of the sex steroid hormones and metabolites (17β-estradiol, 17α-estradiol, estrone, epitestosterone and testosterone) were measured using specific RIAs. The concentration of conjugated forms of the same steroids were determined after incubation of the aqueous phases, resulting from the first diethyl ether extraction step, with *Helix pomatia* juice (20 μl ml^{-1} plasma) for 24 h at 37°C, and subsequent diethyl ether extraction.

Radioimmunoassay

Antisera against 17β-estradiol, estrone and testosterone were raised in rabbits (Laboratoire d'Hormonologie Animale, Centre d'Economie Rurale, Marloie, Belgium) and anti-17α-estradiol and anti-epitestosterone antisera were from Biogenesis (Bournemouth, UK).

Tritiated 17β-estradiol, estrone and testosterone were obtained from Amersham (Amersham, UK). Tritiated 17α-estradiol and epitestosterone were obtained by conversion of tritiated estrone and androstenedione, respectively, by incubation with bovine erythrocytes at 37°C [7]. Cross-reactivities of these antisera are given in Table 2.

The characteristics of each RIA in urine, tissue and plasma samples are described in Table 3. Control samples containing a low, a median or a high amount of each hormone were introduced into the assay. For each RIA, the accuracy and the reproducibility were checked by performing

repeated analyses of spiked samples. As an example, the data for RIA in urine samples are presented in Table 4. In all instances, the mean recoveries ranged from 88 to 124% of the amount of hormone added. In the same experiment, the intra-assay relative standard deviation (R.S.D.) did not exceed 30% in any case. The recovery after the diethyl ether extraction step was calculated by adding a known amount of tritiated hormone to each sample. The mean recoveries are given in Table 3.

Statistical analysis and decision level determination

As in most instances a non-Gaussian distribution of the hormone concentrations was found, a non-parametric test, the Wilcoxon test, was applied (using Statworks 1.3 software; Cricket Software, Philadelphia, PA) in order to determine if the hormone levels measured in control and treated animals are significantly different (with $p < 0.05$). If this is the case for a hormone concentration, a decision limit is defined by the high-

TABLE 1

Description of the different experiments concerning the veal calves (the animals were treated with implants): each experiment involved control animals, from which samples were collected at the same time as those collected in treated veal calves

Animals	Treatment	Samples	Hormones measured
Male veal calves (2–3 weeks of age at day 0)	Day 3: Implix BM (Roussel-Uclaf, France) (20 mg of 17β-estradiol + 200 mg of progesterone)	Samples of urine collected on days 89, 96, 103, 108 and 109	17β-Estradiol, 17α-estradiol, testosterone
	Day 50: Revalor (Roussel-Uclaf) (20 mg of 17β-estradiol + 140 mg of trenbolone acetate)	Samples of tissues (muscle, liver, kidney and fat) collected at slaughter on days 15, 30, 50, 65, 80, 100 and 120	17β-Estradiol, 17α-estradiol (in liver), testosterone
Male and female veal calves (2–3 weeks of age at day 0)	Day 50: Revalor	Samples of urine collected on days 89, 96, 103, 108 and 109	17β-Estradiol, 17α-estradiol
		Samples of tissues (muscle, liver, kidney and fat) collected at slaughter on days 65, 80, 100 and 120	17β-Estradiol, 17 α-estradiol (in liver)
Male and female veal calves	Day 28: Crestar F (Intervet, France) (20 mg of 17β-estradiol + 200 mg of testosterone) Day 56: Crestar F	Samples of tissues (muscle, liver, kidney and fat)	Testosterone, 17β-estradiol, estrone
Male and female veal calves	Day 56: Crestar F	Samples of tissues (muscle, liver, kidney and fat)	Testosterone, 17β-estradiol
Male and female veal calves	Day 56: Crestar M (20 mg of 17β-estradiol + 200 mg of progesterone)	Samples of tissues (muscle, liver, kidney and fat)	Testosterone, 17β-estradiol
Male and female veal calves	Day 42: Revalor	Samples of urine collected on days 26, 66 and 86	17β-Estradiol, 17α-estradiol

TABLE 2

Specificity (%) of the antisera raised against testosterone, epitestosterone, progesterone, 17β-estradiol, 17α-estradiol and estrone

Cross reactivity with	Antiserum against				
	Testosterone	17β-Estradiol	17α-Estradiol	Estrone	Epitesto-sterone
Testosterone	100	< 0.07	ND [a]	0.015	1.4
17β-Estradiol	< 0.05	100	0.71	0.54	ND
17α-Estradiol	ND	0.47	100	0.39	< 0.3
Estrone	< 0.05	1.4	1.25	100	ND
Dihydrotestosterone	32	ND	ND	ND	< 0.4
Androstenedione	0.15	ND	ND	0.07	3.7
Epistestosterone	1.1	ND	ND	ND	ND
Cholesterol	< 0.05	< 0.07	ND	ND	ND
Pregnelonone	< 0.05	< 0.07	ND	ND	ND
Dihydroepiandrosterone	< 0.05	ND	ND	0.15	ND
Cortisone	ND	ND	ND	< 0.005	ND
17α-Hydroxyprogesterone	ND	ND	ND	< 0.005	ND
Estriol	ND	0.27	0.1	0.012	ND

[a] ND = not determined.

est hormone concentration found in the control animals, if the treatment effect is an elevation of that concentration, or the lowest hormone concentration found in the control animals, if the treatment effect is a diminution of that concentration.

The percentage of positives detected among treated animals is an indication of the score of efficacy of the proposed decision levels.

Concerning the veal calves, each experiment displayed in Table 1 involved control animals. As they were treated by implants, which continuously release hormones, the data obtained from the six experiments (from control or treated animals) were taken together, for each category of sample (urine, muscle, liver, kidney or fat). With the exception of testosterone concentration, no distinction was made between male and female

TABLE 3

Characteristics of the radioimmunoassays (after logit–log transformation) used in this study for urine, plasma and tissue samples [a]

Sample	Steroid hormone	Mean recovery (%)	B_0/T (%)	NS (%)	Slope	Intercept	ED_{50} (pg per tube)
Urine	17β-Estradiol	85	54	1.5	−0.82	2.37	18
	17α-Estradiol	90	46	7.0	−1.02	3.97	45
	Testosterone	85	54	1.5	−0.82	2.37	18
	Estrone	70	53	2.5	−0.77	2.33	22
Plasma	17β-Estradiol	70	34	5.5	−0.91	2.75	21
	17α-Estradiol	89	49	5.5	−1.00	2.86	17
	Testosterone	85	48	1.7	−0.99	2.23	10
	Epitestosterone	85	55	1.3	−0.95	2.91	21
	Estrone	50	44	6.5	−1.24	3.44	16
Tissue	17β-Estradiol	71	70	4.3	−1.16	2.90	12
	17α-Estradiol	61	58	6.3	−1.06	4.45	68
	Testosterone	74	53	2.1	−0.95	2.56	15
	Estrone	51	52	1.9	−0.97	3.30	38

[a] B_0/T = percentage of specific fixation; NS = non-specific; ED_{50} = dose at 50% of fixation.

TABLE 4

Mean recoveries obtained from spiked urine samples

Steroid hormone	n	Added ($\mu g\ l^{-1}$)	Measured ($\mu g\ l^{-1}$) (mean ± S.D.)	Recovery (%)
17β-Estradiol	3	0.5	0.5 ± 0.02	100
	3	1.0	1.24 ± 0.10	124
	3	2.0	2.03 ± 0.1	102
17α-Estradiol	3	0.5	0.44 ± 0.04	88
	3	1.0	0.94 ± 0.08	94
	3	2.0	1.94 ± 0.08	97
Testosterone	3	0.5	0.52 ± 0.02	104
	3	1.0	0.96 ± 0.03	96
	3	2.0	2.01 ± 0.08	100
Estrone	2	0.5	0.51 ± 0.11	102
	2	1.0	1.01 ± 0.02	101
	2	2.0	2.03 ± 0.06	102

TABLE 7

17α-Estradiol in urine and liver of male and female veal calves treated or not with natural steroid-containing implants

	17α-Estradiol in urine ($ng\ ml^{-1}$)	17α-Estradiol in liver ($pg\ g^{-1}$)
Controls:	$n = 51$	$n = 13$
Minimum	0.4	560
Maximum	23	761
Treated	$n = 103$	$n = 56$
Minimum	3	294
Maximum	242	4158
U test (p)	< 0.0005	< 0.0005
Decision level	$\leqslant 23\ ng\ ml^{-1}$	$\leqslant 761\ pg\ g^{-1}$
Score (%)	58	78

TABLE 5

17β-Estradiol in urine and tissue of male and female veal calves treated or not with natural steroid-containing implants

	17β-Estradiol in urine ($ng\ ml^{-1}$)	17β-Estradiol in tissues ($pg\ g^{-1}$)			
		Muscle	Liver	Kidney	Fat
Controls:	$n = 40$	$n = 34$	$n = 24$	$n = 23$	$n = 21$
Minimum	0.2	3.5	5.3	7.9	5.6
Maximum	1	33.2	53	69	50
Treated:	$n = 122$	$n = 80$	$n = 111$	$n = 100$	$n = 103$
Minimum	0.6	11	5	6.4	9.3
Maximum	17.6	280	1650	589	358
U test (p)	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005
Decision level	$\leqslant 1\ ng\ ml^{-1}$	$\leqslant 34\ pg\ g^{-1}$	$\leqslant 53\ pg\ g^{-1}$	$\leqslant 69\ pg\ g^{-1}$	$\leqslant 50\ pg\ g^{-1}$
Score of the decision level (%)	95	81	80	75	80

TABLE 6

17β-Estradiol in plasma of bulls before (controls) and after treatment by an injection of a testosterone–estradiol cocktail

	Unconjugated 17β-estradiol in plasma of bulls ($pg\ ml^{-1}$)				Conjugated 17β-estradiol in plasma of bulls ($pg\ ml^{-1}$)			
Controls ($n = 33$):								
Minimum	< LD [a]				< LD			
Maximum	41				32			
	Day 2 [b]	Day 3 [b]	Day 5 [b]	Day 7 [b]	Day 2 [b]	Day 3 [b]	Day 5 [b]	Day 7 [b]
Treated ($n = 11$):								
Minimum	205	65	< LD	< LD	37	29	< LD	< LD
Maximum	1162	421	117	209	116	75	78	38
U test (p)	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	0.107
Decision level	$\leqslant 40\ pg\ ml^{-1}$				$\leqslant 32\ pg\ ml^{-1}$			
Score (%)	100	100	64	45	100	90	18	ND

[a] LD = limit of detection = 20 $pg\ ml^{-1}$. [b] After injection.

TABLE 8

17α-Estradiol in plasma of bulls before (controls) and after treatment by an injection of a testosterone–estradiol cocktail [a]

	Unconjugated 17α-estradiol in plasma of bulls (pg ml^{-1})				Conjugated 17α-estradiol in plasma of bulls (pg ml^{-1})			
Controls ($n = 33$):								
Minimum	14				12			
Maximum	70				183			
	Day 2 [b]	Day 3 [b]	Day 5 [b]	Day 7 [b]	Day 2 [b]	Day 3 [b]	Day 5 [b]	Day 7 [b]
Treated ($n = 11$):								
Minimum	11	23	30	25	300	171	61	13
Maximum	109	92	51	95	1082	601	480	370
U test (p)	0.043	< 0.005	0.034	0.012	< 0.005	< 0.005	< 0.005	0.012
Decision level	⩽ 70 pg ml^{-1}				⩽ 183 pg ml^{-1}			
Score (%)	36	9	0	27	100	90	45	27

[a] Limit of detection = 8 pg ml^{-1}. [b] After injection.

veal calves. The data are displayed in the results tables (Tables 5–12), as "control" values and "treated" values, without distinction of the sampling time.

Data from injected bulls were analysed separately at each time of sampling.

RESULTS

For each hormone studied, the results are given in Tables 5–13 and illustrated in Figs. 1–5. They show the lowest (minimum) and the highest (maximum) hormone concentration found in control and in treated animals; the result of the statistical test (Wilcoxon test, also called U-test); the decision level established according to the values obtained from the control animals; and the score of the decision level.

When measuring 17β-estradiol, 17α-estradiol and estrone concentrations in both urine and tissue veal calves samples, no significant difference was found between female and male veal calves. No distinction was therefore made between male and female animals in the analysis of these data.

TABLE 9

Estrone in tissues of male and female veal calves treated or not with natural steroid-containing implants

	Estrone in tissues (pg g^{-1})			
	Muscle	Liver	Kidney	Fat
Controls:	$n = 3$	$n = 3$	$n = 3$	$n = 3$
Minimum	15	170	23	80
Maximum	78	198	166	94
Treated:	$n = 18$	$n = 18$	$n = 18$	$n = 18$
Minimum	3	73	34	29
Maximum	72	284 [a]	144	149
U test (p)	0.255	0.021	0.093	0.113
Decision level	ND	⩾ 170 pg g^{-1} [b]	ND	ND
Score (%)	ND	83	ND	ND

[a] Three values out of eighteen were above 170 pg g^{-1}. [b] The decision level determined for the estrone content in liver is a minimum level.

17β-Estradiol

Veal calves (Table 5, Fig. 1A). In urine, the 17β-estradiol concentrations were significantly higher in treated than in control animals. A decision level of 1 ng ml^{-1} is proposed, which allows 95% of the treated animals to be declared positive.

In tissues, the 17β-estradiol levels were significantly higher in treated animals. The normality limits, fixed as the highest levels found in control animals, display scores varying from 75 to 81% depending on the tissue.

Bulls (Table 6, Fig. 1B and C). In plasma of bulls, both free and conjugated forms of 17β-estradiol are significantly higher after the treatment until the seventh day for the free form and

TABLE 10

Estrone in plasma of bulls before (controls) and after treatment by an injection of a testosterone–estradiol cocktail

	Unconjugated estrone in plasma of bulls (pg ml^{-1})				Conjugated estrone in plasma of bulls (pg ml^{-1})			
Controls (n = 33):								
Minimum	< LD [a]				< LD			
Maximum	63				96			
	Day 2 [b]	Day 3 [b]	Day 5 [b]	Day 7 [b]	Day 2 [b]	Day 3 [b]	Day 5 [b]	Day 7 [b]
Treated (n = 11):								
Minimum	< LD	< LD	< LD	< LD	< LD	58	< LD	< LD
Maximum	14	20	17	10	753	110	68	108
U test (p)	0.03	0.461	0.01	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
Decision level	≥ 7 pg ml^{-1}				≥ 19 pg ml^{-1} ⩽ 96 pg ml^{-1}			
Score (%)	64	ND	73	64	82	27	54	72

[a] LD = limit of detection = 7 pg ml^{-1}. [b] After injection.

until the fifth day for the conjugated form. Decision levels were set at 41 and 32 pg ml^{-1} for the free and conjugated forms of 17β-estradiol, respectively. The score of the free 17β-estradiol decision limit is better than that of the conjugated form. It ranges from 100%, 2 days after the treatment, to 45%, 7 days after the treatment.

17α-Estradiol

Veal calves (Table 7, Fig. 2A). A higher 17α-estradiol level was found in both urine and liver from treated animals than from the controls. Decision levels of 23 ng ml^{-1} in urine and 761 pg g^{-1} in liver make possible the detection of 58% and 75%, respectively, of the treated animals.

TABLE 11

Testosterone in urine and tissues of male and female veal calves treated or not with natural steroids containing implants

Veal calves		Testosterone in urine (ng ml^{-1})	Testosterone in tissues (pg g^{-1})			
			Muscle	Liver	Kidney	Fat
Male	Controls:	n = 26	n = 10	n = 5	n = 5	n = 5
	Minimum	3	34	96	580	270
	Maximum	36	773	324	10169	3879
	Treated:	n = 40	n = 10	n = 5	n = 5	n = 5
	Minimum	0.3	42	21	167	292
	Maximum	2.5	418	95	310	2981
	U test (p)	< 0.0005	0.065	0.005	0.005	0.174
	Decision level	⩾ 2.5 ng ml^{-1}	ND	⩾ 96 pg g^{-1}	⩾ 580 pg g^{-1}	ND
	Score (%)	100	ND	100	100	ND
Female	Controls:	n = 4	n = 30	n = 10	n = 10	n = 10
	Minimum	0.2	6	12	58	21
	Maximum	0.4	422	121	375	165
	Treated:	n = 28	n = 20	n = 21	n = 26	n = 26
	Minimum	0.06	64	4	195	263
	Maximum	7.8	310	317	870	1742
	U test (p)	0.004	0.250	0.199	< 0.0005	< 0.0005
	Decision level	⩽ 0.45 ng ml^{-1}	ND	ND	⩽ 375 pg g^{-1}	⩽ 165 pg g^{-1}
	Score (%)	90	ND	ND	73	100

TABLE 12

Testosterone in plasma of bulls before (controls) and after treatment by an injection of a testosterone–estradiol cocktail

	Unconjugated testosterone in plasma of bulls (pg ml^{-1})				Conjugated testosterone in plasma of bulls (pg ml^{-1})			
Controls (n = 33):								
Minimum	< LD [a]				< LD			
Maximum	5752				965			
	Day 2 [b]	Day 3 [b]	Day 5 [b]	Day 7 [b]	Day 2 [b]	Day 3 [b]	Day 5 [b]	Day 7 [b]
Treated (n = 11):								
Minimum	572	349	676	514	< LD	< LD	< LD	< LD
Maximum	5027	4081	3696	3566	911	751	439	501
U test (p)	0.228	0.318	0.119	0.253	0.077	0.398	0.219	0.347
Decision level	ND				ND			
Score (%)	ND	ND	ND	ND	ND	ND	ND	ND

[a] LD = limit of detection = 50 pg ml^{-1}. [b] After injection.

Bulls (Table 8, Fig. 2B and C). 17α-estradiol levels are higher ($p < 0.05$) in the plasma of bulls until 7 days after the steroid injection than before treatment. The score of the decision limit fixed for the conjugated form (183 pg ml^{-1}) is better than that of the non-conjugated 17α-estradiol (70

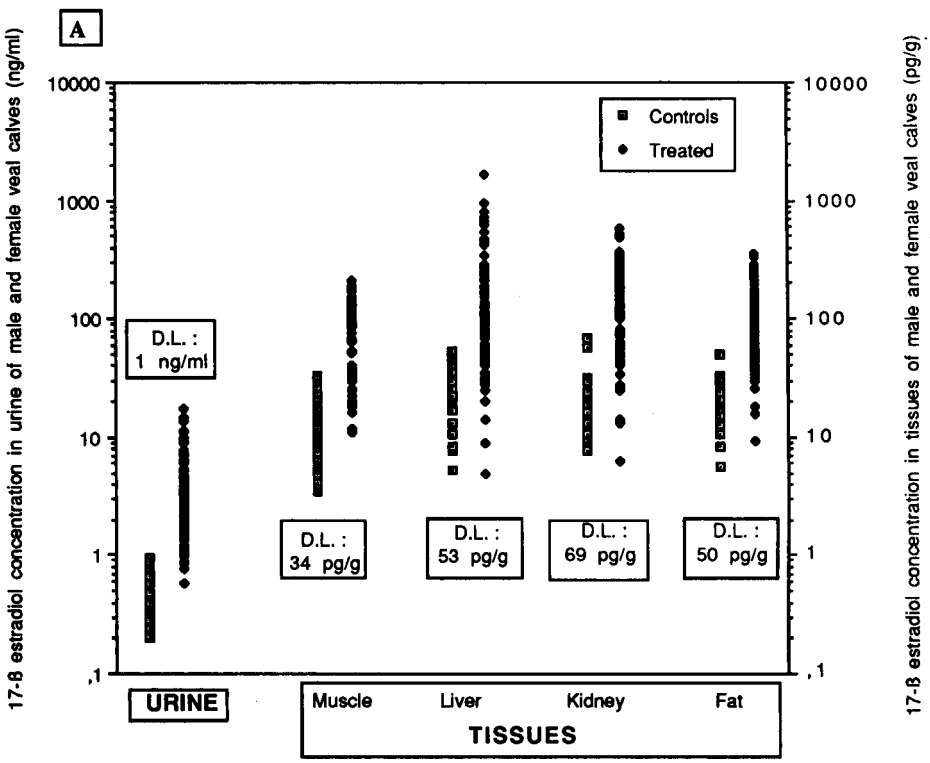
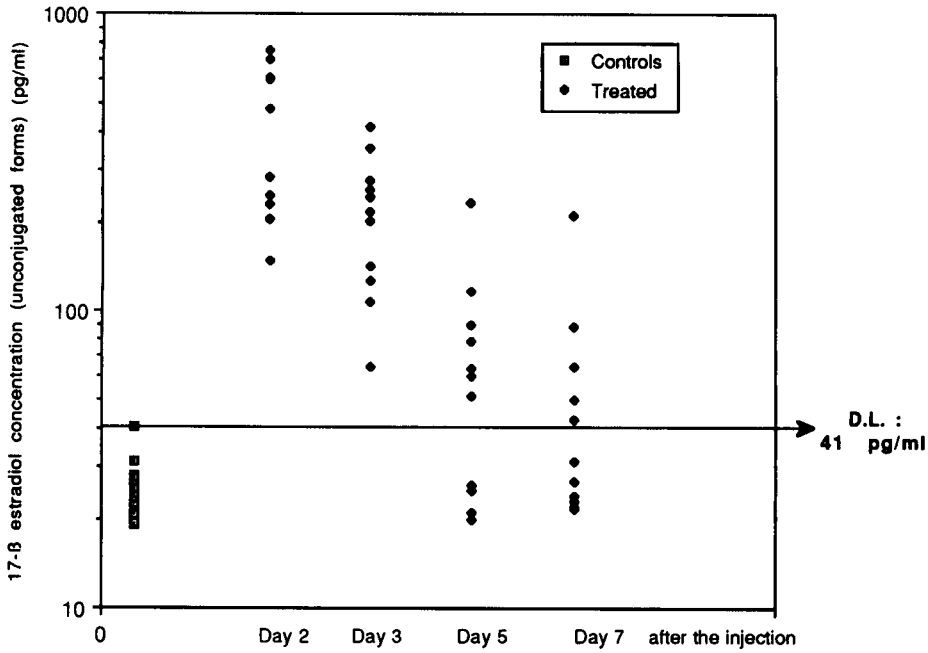


Fig. 1. (A) Distribution of 17β-estradiol concentrations in urine and tissues of treated and untreated veal calves (male and female). (B) Distribution of 17β-estradiol concentrations (unconjugated forms) in plasma of treated and untreated bulls. (C) Distribution of the 17β-estradiol concentrations (conjugated forms) in plasma of treated and untreated bulls. D.L. = decision limit.

B



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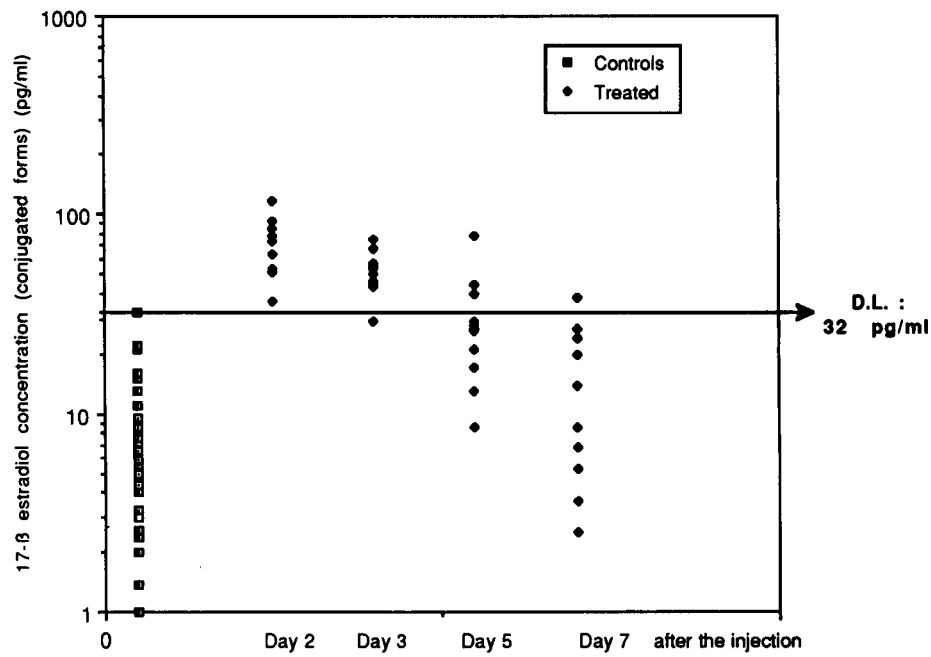


Fig. 1 (continued).

pg ml^{-1}); it ranges from 100%, 2 days after the treatment, to 27%, 7 days after the treatment.

Estrone

Veal calves (Table 9, Fig. 3A). In muscle, kidney and fat, no difference in estrone levels was found between treated and untreated animals. Only in liver were the concentrations obtained from the treated veal calves significantly lower than those observed in the control animals. A decision level established at 170 pg g^{-1} (the minimum hormone concentration found in the control animals) led to a score of 83%.

Bulls (Table 10, Fig. 3B and C). In contrast to the 17β- and 17α-estradiol results, the free estrone levels decrease ($p < 0.05$) after the steroid treatment (with the unexplained exception of the plasma samples on the third day after the injection). The decision limit was then set at the minimum level found in control samples, 7 pg ml^{-1}. The score of the free estrone decision limit ranges from 64 to 73% (64–73% of the bulls display a free estrone concentration lower than 7 pg ml^{-1}).

Conjugated estrone first increases ($p < 0.05$) in plasma of bulls (the second and third days after the injection) and decreases ($p < 0.05$) on the fifth and seventh days after the treatment. Hence a minimum limit of 19 pg ml^{-1} and a maximum level of 96 pg ml^{-1} are proposed. That means that bulls displaying a conjugated estrone concentration in their plasma lower than 19 and higher than 96 pg ml^{-1} would be declared positive. This is the case for 72% of the bulls, 7 days after the steroid injection.

Testosterone

Veal calves (Table 11, Fig. 4A and B) In both male and female veal calves, the testosterone concentrations were significantly different in treated animals and in controls. For male veal calves, the values are lower in the treated ani-

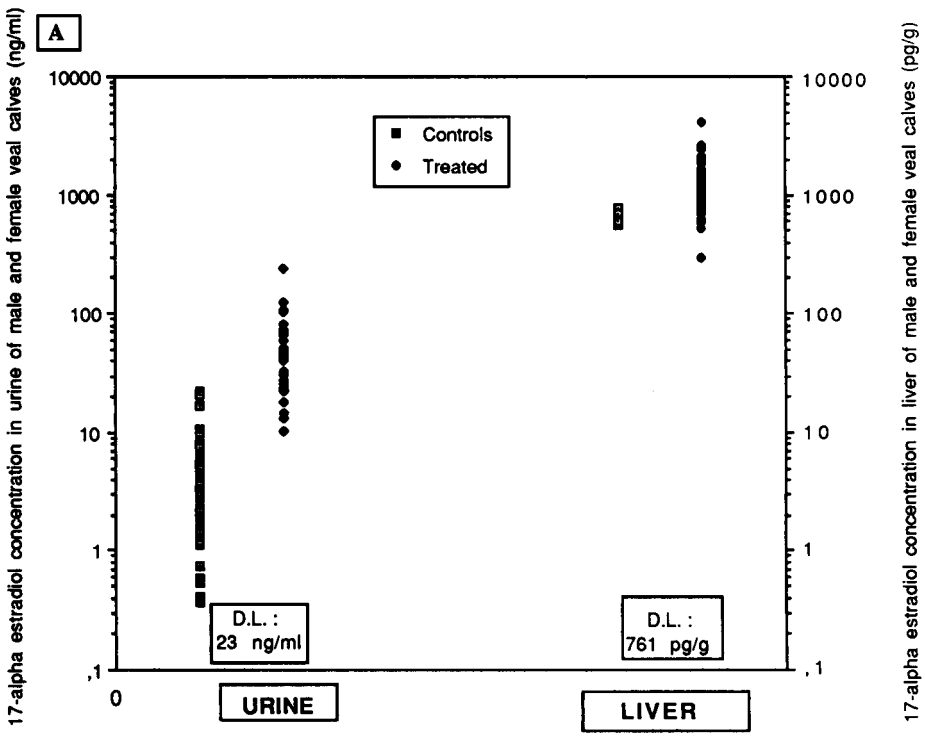
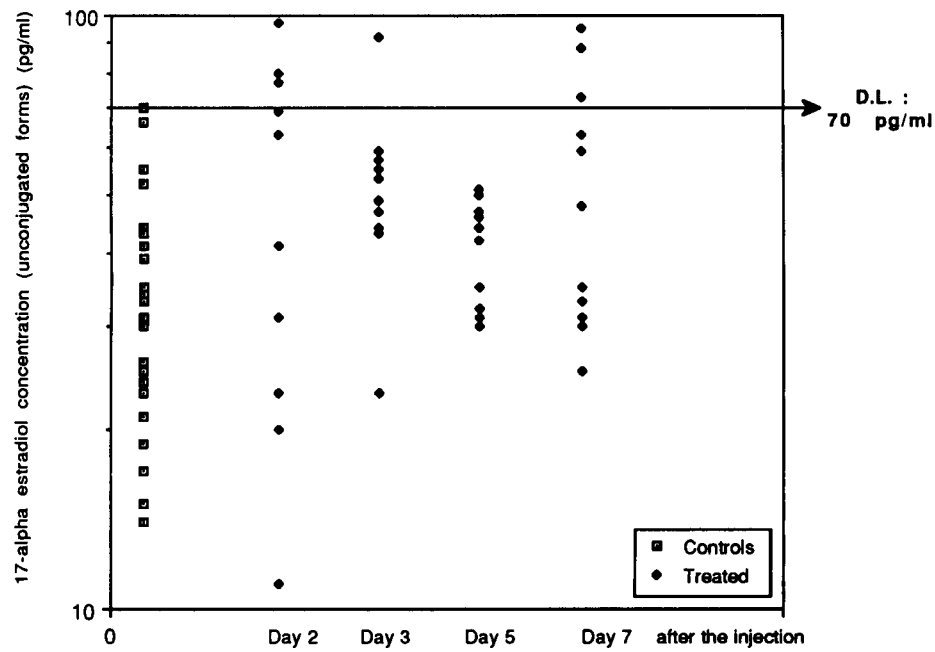


Fig. 2. (A) Distribution of 17α-estradiol concentrations in urine and liver of treated and untreated veal calves (male and female). (B) Distribution of 17α-estradiol concentrations (unconjugated forms) in plasma of treated and untreated bulls. (C) Distribution of 17α-estradiol concentrations (conjugated forms) in plasma of treated and untreated bulls. D.L. = decision limit.

B



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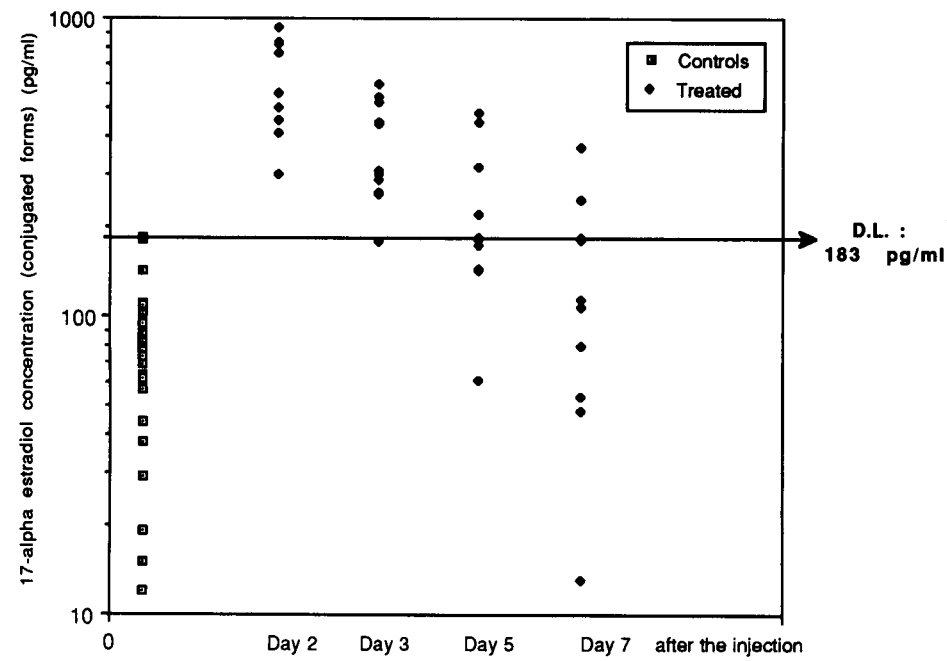


Fig. 2 (continued).

TABLE 13

Epitestosterone in plasma of bulls before (controls) and after treatment by an injection of a testosterone–estradiol cocktail [a]

	Unconjugated epitestosterone in plasma of bulls (pg ml^{-1})				Conjugated epitestosterone in plasma of bulls (pg ml^{-1})			
Controls (n = 33):								
Minimum	134				219			
Maximum	974				1750			
	Day 2 [b]	Day 3 [b]	Day 5 [b]	Day 7 [b]	Day 2 [b]	Day 3 [b]	Day 5 [b]	Day 7 [b]
Treated (n = 11):								
Minimum	361	200	337	309	450	524	382	333
Maximum	1970	1950	820	1196	1386	1297	951	865
U test (p)	0.04	0.01	0.114	0.197	0.196	0.337	0.130	0.053
Decision level	974 pg ml^{-1}				ND			
Score (%)	27	27	ND	ND	ND	ND	ND	ND

[a] Limit of detection = 50 pg ml^{-1}. [b] After injection.

mals, and a decision level of 2.5 ng ml^{-1} (which is a minimum acceptable testosterone concentration) gives a score of 100%. In female veal calves, the values are higher in the treated than in the untreated animals. A decision limit established at 0.45 ng ml^{-1} allows the detection of 90% of the treated animals.

Concerning the tissues, the liver and the kid-

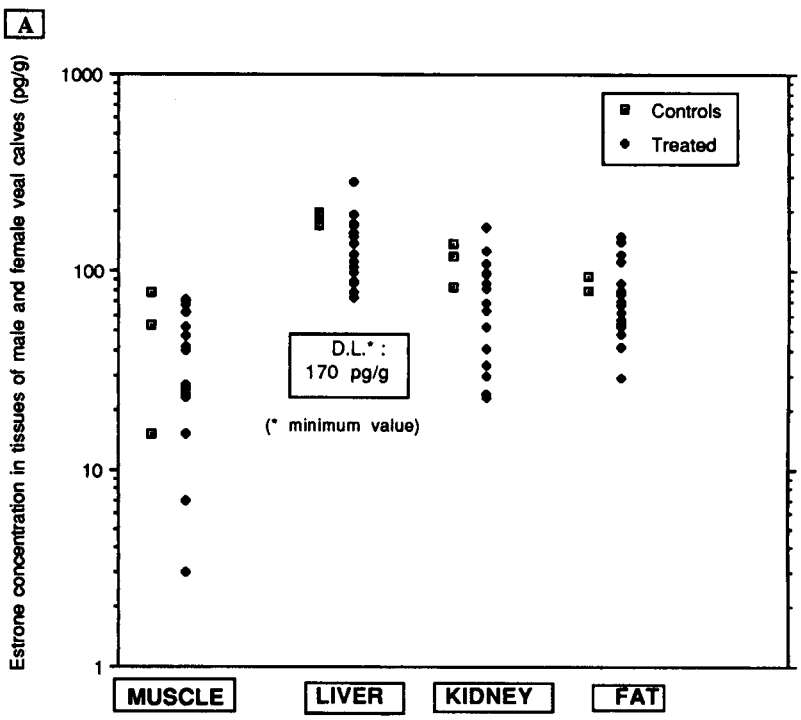
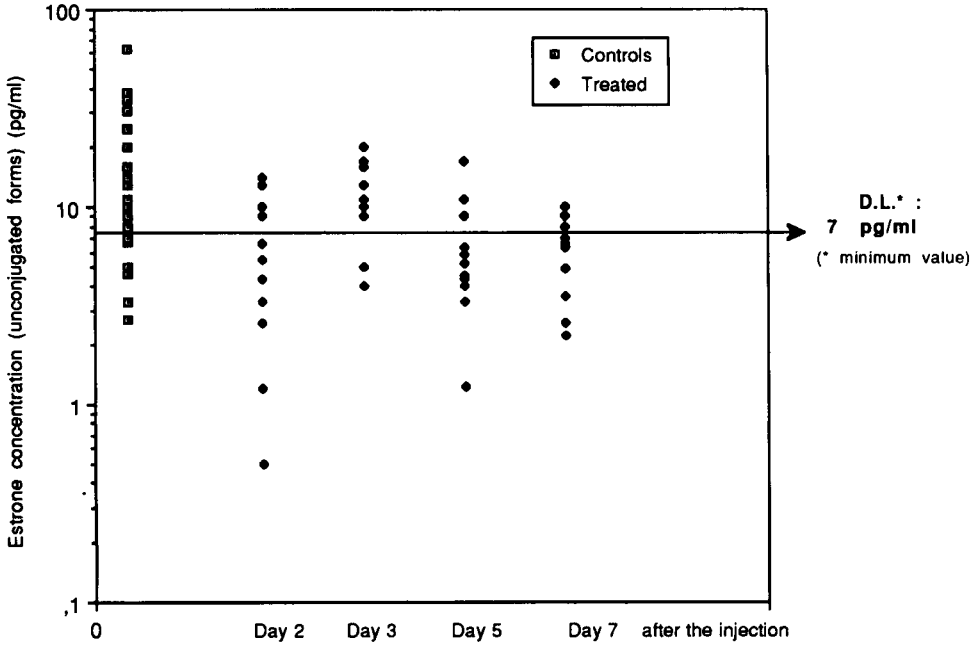


Fig. 3. (A) Distribution of estrone concentrations in tissues of treated and untreated veal calves (male and female). (B) Distribution of estrone concentrations (unconjugated forms) in plasma of treated and untreated bulls. (C) Distribution of estrone concentrations (conjugated forms) in plasma of treated and untreated bulls. D.L. = decision limit.

B



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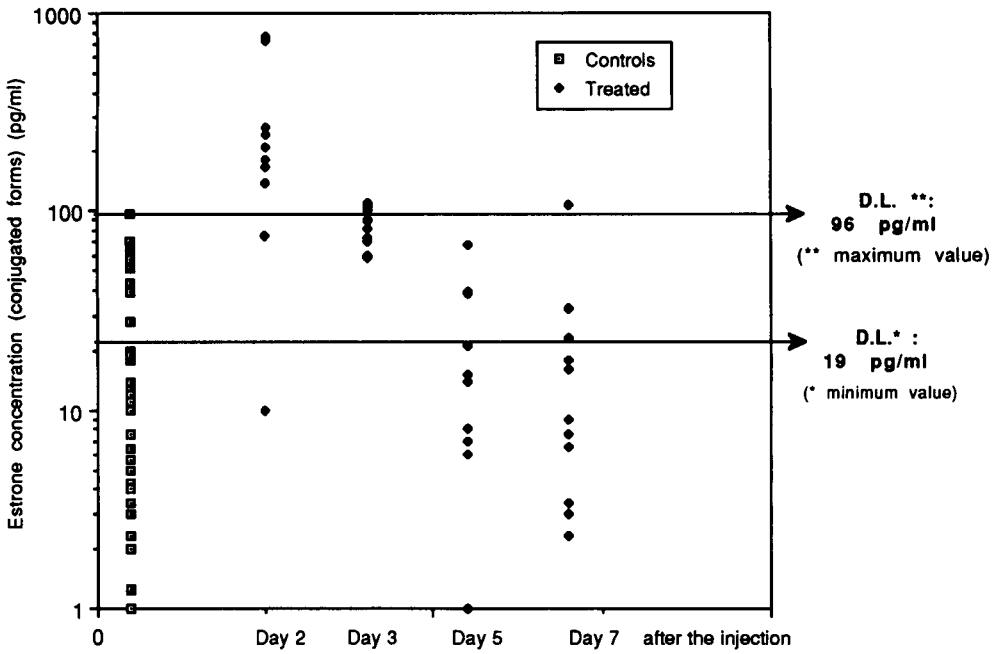


Fig. 3 (continued).

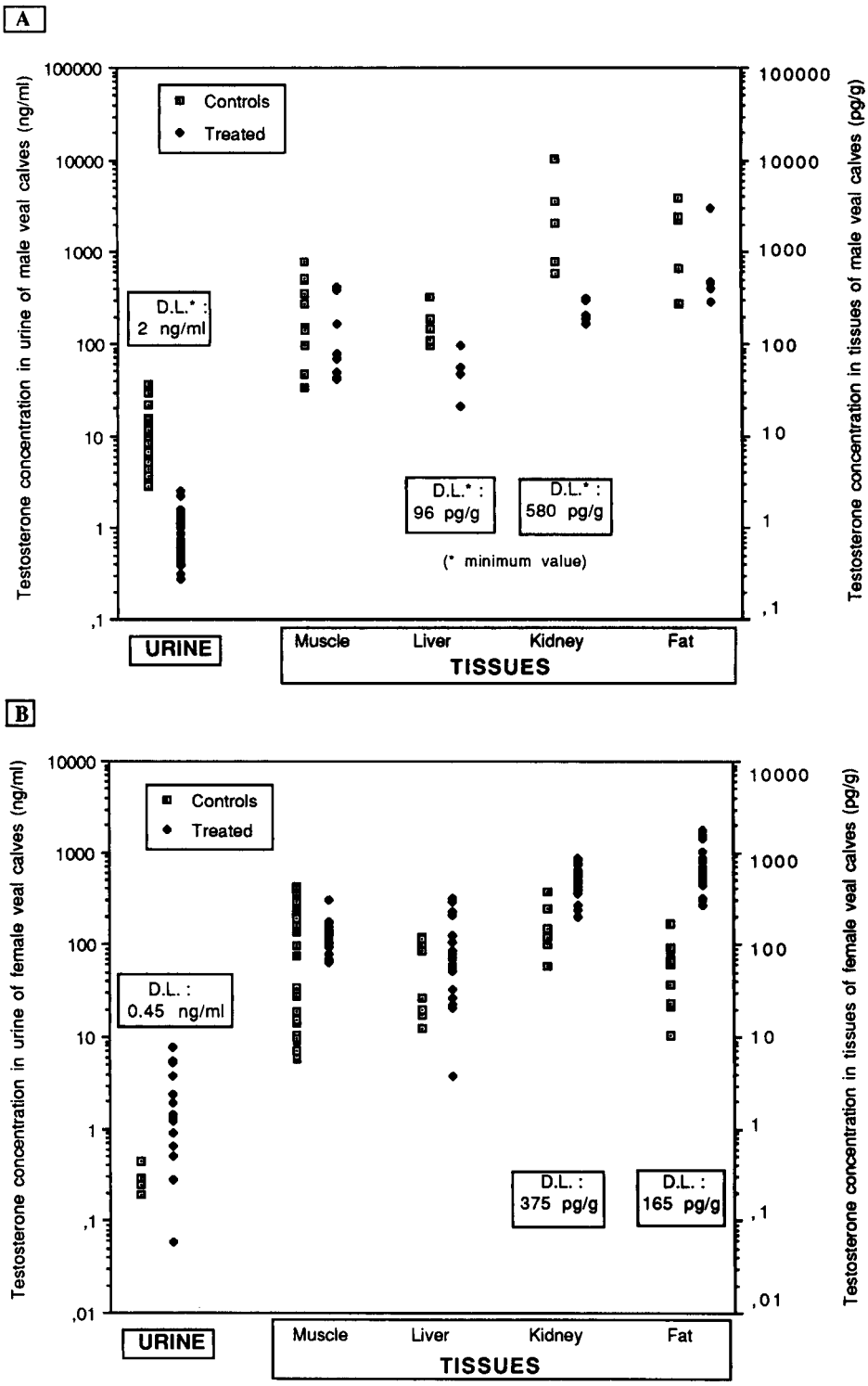
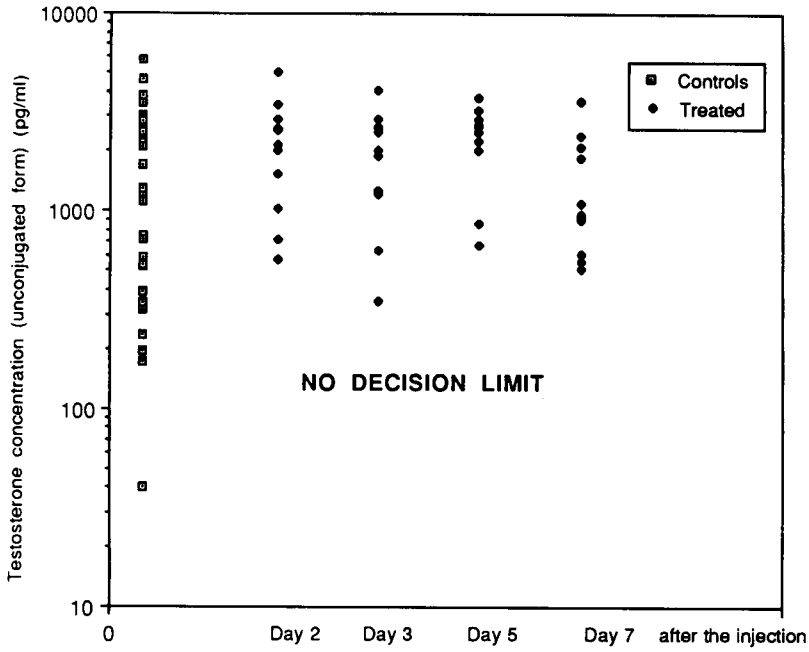


Fig. 4. (A) Distribution of testosterone concentrations in urine and tissues of treated and untreated male veal calves. (B) Distribution of testosterone concentrations in urine and tissues of treated and untreated female veal calves. (C) Distribution of testosterone concentrations (unconjugated forms) in plasma of treated and untreated bulls. (D) Distribution of testosterone concentrations (conjugated forms) in plasma of treated and untreated bulls. D.L. = decision limit.

C



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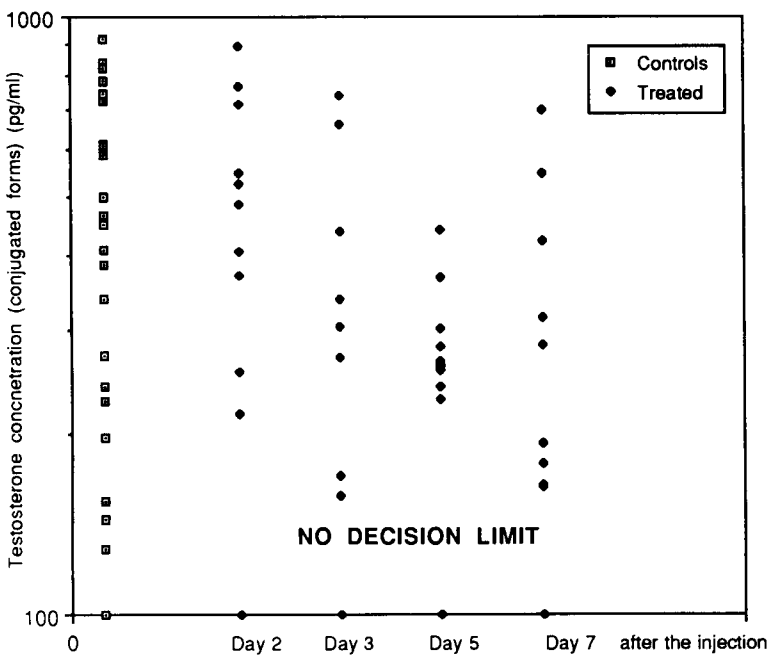

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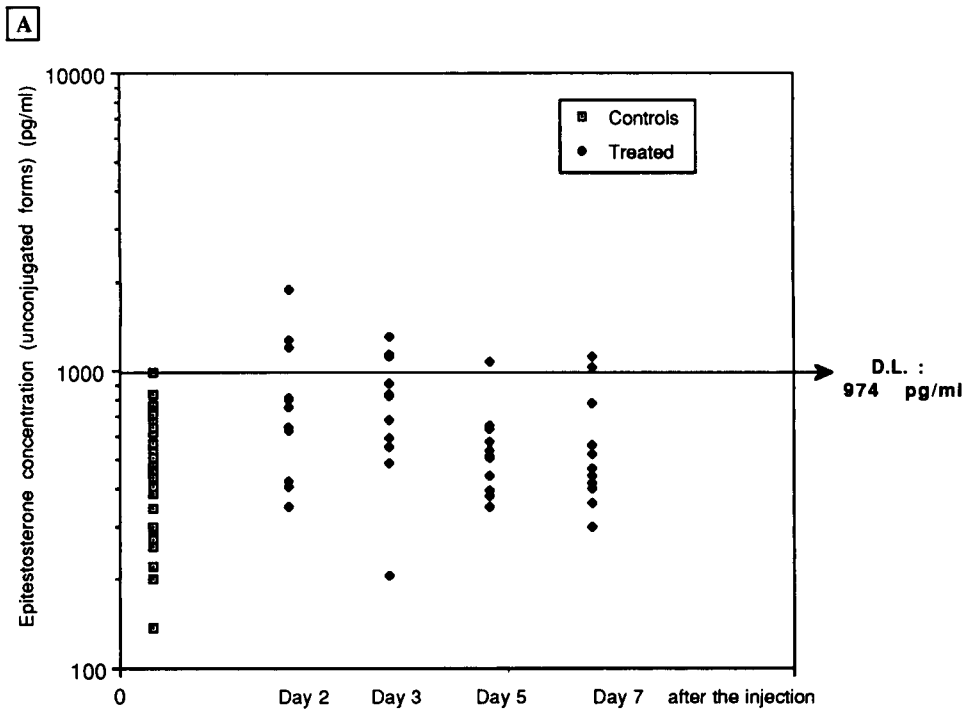

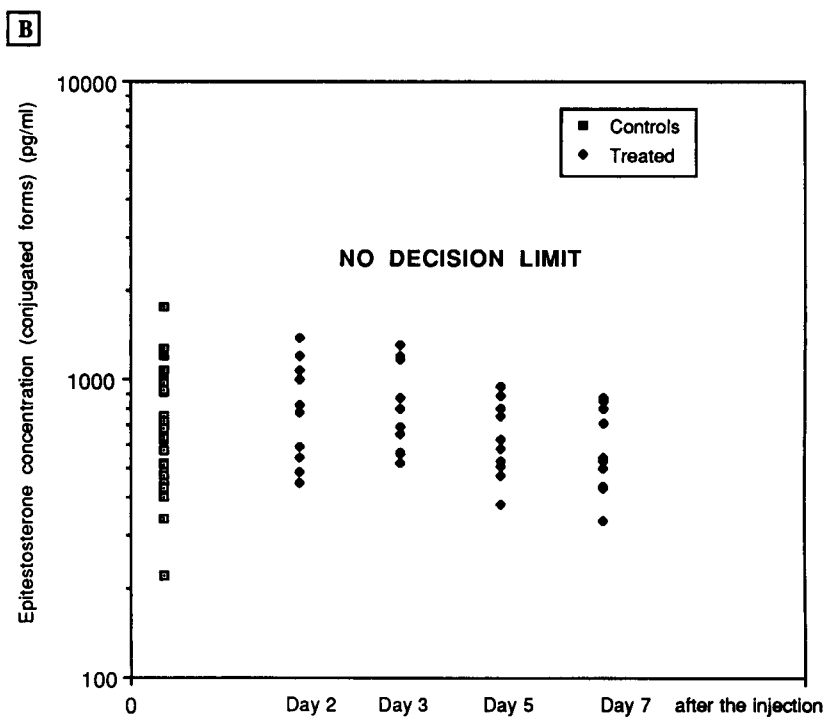

Fig. 5. (A) Distribution of epitestosterone concentrations (unconjugated forms) in plasma of treated and untreated bulls. (B) Distribution of epitestosterone concentrations (conjugated forms) in plasma of treated and untreated bulls. D.L. = decision limit.

ney of treated male animals contain significantly lower amounts of testosterone than those of control male veal calves (Table 11). Decision levels of 96 and 580 pg g^{-1}, respectively, would make it possible to declare positive 100% of the treated animals. However, these values were obtained on a very limited series of animals ($n = 5$). In the female veal calves, only the testosterone contents of fat and kidney are significantly higher in treated than in control animals; 100% of the treated animals could be detected with a decision limit of 165 and 375 pg g^{-1}, respectively (Table 11).

Bulls (Table 12, Fig. 4C and D). The plasma testosterone concentration was not significantly different in bulls before and after the steroid injection.

Epitestosterone in bulls (Table 13, Fig. 5A and B)

A slight increase was obtained 2 and 3 days after the treatment for the non-conjugated epitestosterone only. The decision limit of 974 pg ml^{-1} (the maximum free epitestosterone concentration before the treatment) allows only 27% of the treated animals to be detected.

DISCUSSION

From 1 January, 1988, the use, as growth promoters in meat production, of anabolic steroids and related substances including the stilbenes and zeranol was banned in the EEC. The control of the illegal use of these substances is based on the analysis of blood, tissues or excreta for the presence of synthetic compounds (residues of parent drugs or metabolites) not occurring naturally in the body. The composition of several "hormone cocktails" circulating on the black market involved "natural" sex steroid hormones (17β-estradiol, testosterone, progesterone) or their ester derivatives [2]. These hormones and their metabolites occur naturally in both male and female meat-producing animals. The discrimination between physiological levels and elevated concentrations due to hormone administration

TABLE 14

Decision levels for 17β-estradiol, 17α-estradiol, estrone, epitestosterone and testosterone in urine and tissues of male veal calves and in plasma of bulls [a]

Steroid hormone	Decision level in						
	Veal calves					Plasma of bulls	
	Urine	Muscle	Liver	Kidney	Fat	Unconjugated form	Conjugated form
17β-Estradiol (male and female veal calves)	$\leqslant 1$ ng ml^{-1} (95%)	$\leqslant 34$ pg g^{-1} (81%)	$\leqslant 53$ pg g^{-1} (80%)	$\leqslant 69$ pg g^{-1} (75%)	$\leqslant 50$ pg g^{-1} (80%)	$\leqslant 40$ pg ml^{-1} (100%; 45%)	$\leqslant 32$ pg ml^{-1} (100%; 0%)
17α-Estradiol (male and female veal calves)	$\leqslant 23$ ng ml^{-1} (58%)	ND	$\leqslant 761$ pg g^{-1} (78%)	ND	ND	$\leqslant 70$ pg ml^{-1} (36%; 27%)	$\leqslant 183$ pg ml^{-1} (100%; 27%)
Estrone (male and female veal calves)	ND	ND	$\geqslant 170$ pg g^{-1} (83%)	ND	ND	$\geqslant 7$ pg ml^{-1} (64%; 64%)	$\geqslant 19$ pg ml^{-1} $\leqslant 96$ pg ml^{-1} (82%; 72%)
Testosterone:							
Male veal calves	$\geqslant 2.5$ ng ml^{-1} (100%)	ND	$\geqslant 96$ pg g^{-1} (100%)	$\geqslant 580$ pg g^{-1} (100%)	ND	ND	ND
Female veal calves	$\leqslant 0.45$ ng ml^{-1} (90%)	ND	ND	$\leqslant 375$ pg g^{-1} (73%)	$\leqslant 165$ pg g^{-1} (100%)		
Epitestosterone						$\leqslant 974$ pg ml^{-1} (27%; 0%)	ND

[a] The percentages of treated animals detected (= the score of efficiency of the decision level) are indicated in parentheses (2 and 7 days, respectively, after the injection for the bulls).

requires quantitative analysis and the establishment of limits at which one can decide whether or not an animal has been treated. Nevertheless, physiological levels are influenced by nutrition, breed, sex, age, circardian rythms, environmental factors, stress, etc. [8]. Age is an important parameter when controlling veal calves. As illustrated by Massart et al. [9], large changes in blood plasma testosterone are observed around puberty and delayed puberty can occur.

Many data have already been published, but the large variability reported in the literature needs further studies before the fixation of consensus reference values that could be used as decision levels.

The data presented here for the veal calves were obtained from several experiments in which male and female calves had been submitted to different treatments with estradiol and/or testosterone using implants or injections. This experimental context contrasts with that of Arts and co-workers [3,4], where blood, urine or tissue samples were obtained from animals untreated or injected with oily cocktail containing 17β-estradiol and testosterone or their ester derivatives. In addition, Arts and co-workers used an analytical procedure that was time consuming, namely liquid chromatography (LC) followed by RIA. In our hands, the analytical results on test samples were not significantly different when RIA was applied without preliminary LC to those obtained using the LC–RIA procedure.

Despite these experimental differences, we reached the same conclusion as Arts and co-workers concerning 17β-estradiol in veal calves, namely that its level in urine is a good criterion for detecting female and male veal calves treated with natural steroids. A decision limit of 1 ng ml^{-1} allowed 95% of the implanted veal calves to be detected in this study (Table 14) and 100% of the veal calves, 5 days after a steroid injection, in the study of Arts and co-workers.

It is considered that the testosterone concentration in urine or plasma is not a good criterion for detecting cattle treated by injection.

In this study, it was found that in addition to the 17β-estradiol in urine, the urinary testosterone level is also a good criterion for detecting implanted veal calves; 100% of the implanted male veal calves (aged 16–20 weeks) displayed a urinary testosterone level lower than the minimum value found in untreated animals, 2.5 ng ml^{-1}. In the study of Arts and co-workers, the testosterone concentration was sometimes lower than 2.5 ng ml^{-1} in the urine of male veal calves aged less than 20 weeks. From the age of 20 weeks, 97% of the values were higher than 2.5 ng ml^{-1}.

Because of the restriction of age for the testosterone decision level, the criterion of the 17β-estradiol appears more suitable for detecting natural steroid-treated veal calves.

Table 14 summarizes the decision levels proposed to detect bulls treated by an injection of natural steroids. It can be seen that the best criterion is either the plasma non-conjugated 17β-estradiol concentration or the plasma conjugated 17α-estradiol.

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Bovine blood analysis for natural hormones: an overview of analytical strategies

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Abstract

The results of an inquiry, organized among a large number of European Community (EC) control laboratories, revealed that most EC countries have a control program for the natural hormones 17β-estradiol and 17β-testosterone which is based on the analysis of samples of plasma or serum by a (radio)immunoassay procedure. The diversity within these procedures, however, is large and good quality control programs are not available. As base for such a program a method based on gas chromatography–isotope dilution mass spectrometry was developed and validated for purposes of the simultaneous accurate quantification of low levels of 17β-estradiol and 17β-testosterone. Accuracy, repeatability and within-laboratory reproducibility of this method are adequate. However, to obtain a limit of determination that is low enough for accurate quantification at the EC-recommended action level of 0.04 $\mu g\ l^{-1}$ for 17β-estradiol the use of negative chemical ionisation is necessary.

Keywords: Gas chromatography–mass spectrometry; Isotope dilution methods; Immunoassay; Radiochemical methods; Anabolic steroids; Cattle; Hormones; Quality control

European Community (EC) legislation makes it mandatory that member states have an Annual National Plan describing procedures and methods used during the control on the illegal use of anabolic agents [1]. One of the groups of compounds mentioned are the natural hormones 17β-testosterone, 17β-estradiol and progesterone. For several reasons, however, this group did get relatively little priority until now. Part of these reasons are no doubt related to the analytical problems associated with the accurate quantitative determination and identification of these analytes. A number of studies [2,3] demonstrated that the use of these compounds results in only minor average changes in hormone levels. This, together with high variability in naturally occurring levels, makes it very difficult to state whether or not a single animal has been treated. However, based on the available data the Commission has given guidelines for the control on 17β-estradiol and 17β-testosterone. Upper limits of naturally occurring levels are given for plasma and serum: 17β-estradiol: 0.04 $\mu g\ l^{-1}$; 17β-testosterone: male bovine animal younger than 6 months, 10 $\mu g\ l^{-1}$; older than 6 months, 30 $\mu g\ l^{-1}$, female bovine animal (non-pregnant), 0.5 $\mu g\ l^{-1}$.

To obtain information on the analytical strategies and programmes in use within the respective EC member states we organized an inquiry among EC control laboratories. The results of this inquiry have been evaluated. For reference purposes we developed and validated a method for

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the simultaneous determination of 17β-estradiol and 17β-testosterone in serum.

MATERIALS AND METHODS

Inquiry

In September 1990 all EC National Reference Laboratories were approached with an inquiry which they were asked to distribute among the field laboratories performing the analysis for the natural hormones. The inquiry contained questions with respect to relevant analytical programs, analytical methodology for screening and confirmation, action levels and quality control programs.

Analytical methodology

Deuterated internal standards [17β-estradiol-d_3 (MSD, md-2325) and 17β-testosterone-d_2 (MSD, md-2962) (1 μg l^{-1})] are added to 10 ml of serum. The samples are incubated overnight during which they are slowly rotated. After the addition of 10 ml of water the samples are placed on an Extrelut cartridge (Merck, art. no. 11737). After 15 min the steroids are extracted with 60 ml ethyl acetate (Merck, art. no. 9623). The solvent is removed under vacuum and the residue is dissolved in 0.1 ml of ethanol (Merck, art. no. 893) and 5 ml of water are added. The aqueous extracts are placed on top of a glass column (1 × 10 cm, BioRad, art. 737-1010) containing an immunoaffinity matrix prepared as described before [4] from antisera raised against 17β-testosterone [H148178 immunogen 4-androstene-17β-ol-3-one-17-hemisuccinate–BSA (Steraloids, art. no. A 6986)] and 17β-estradiol [H143826 immunogen 1,3,5,(10)-estratriene-3,17β-diol-17-hemisuccinate–BSA (Steraloids, art. no. E 1077)], respectively. The columns are percollated under gravity and washed with 5 ml of water. The steroids are eluted with a mixture of water and ethanol (1 + 1, v/v). The columns are washed subsequently with 10 ml of a mixture of ethanol and water (4 + 1, v/v), 20 ml of water and 20 ml of phosphate-buffered saline, 0.02 mol l^{-1}, pH 7.4, containing sodium azide, and stored at 4°C. Alternatively the extracts are purified by solid phase extraction (SPE) (Seppak, C_{18} cartridges). The cartridges are pretreated with 2 ml of methanol and 5 ml of water, the extracts dissolved in 5 ml of water and flushed through the cartridges. Subsequently the cartridges are washed with methanol–water (9 + 11, v/v) and eluted with 5 ml of methanol–water (4 + 1, v/v). The eluates are evaporated to dryness and derivatized by adding a mixture of acetone (Merck, art. no. 14) and heptafluorbutyric acid anhydride (HFBA, Pierce, art. no. 63163) (4 + 1, v/v). The reagents are incubated during one hour at 60°C after which they are removed under a stream of nitrogen at 50°C. The dry residue is dissolved in 25 ml of iso-octane (Merck, art. no 2747) and subjected to gas chromatography–mass spectrometry (GC–MS) analysis. GC apparatus: HP 5890 (Hewlett Packard); GC column: Macherey-Nagel Permabond SE 52; injection: 1–5 μl, splitless at 225°C; program: 100°C–280°C at 20°C min^{-1}; temperature transfer line: 280°C; MS apparatus: HP 5970 (Hewlett Packard); ions monitored during selected-ion monitoring (quantification): 17β-estradiol (m/z 664), 17β-estradiol-d_3 (m/z 667), 17β-testosterone (m/z 680) and 17β-testosterone-d_2 (m/z 682).

RESULTS AND DISCUSSION

A total of 32 laboratories, representing 11 EC countries, returned an inquiry.

Analytical programs

Table 1 lists the different purposes of the analytical programs for estradiol. Many member states already have national regulatory control programs, whereas a significant number has re-

TABLE 1

Distribution of analytical programs for estradiol (N = 32) over the different purposes

Purpose	Amount
National regulatory control	21
National surveillance	22
EC monitoring	12
Research and development	12

search and development activities. A number of 24 laboratories used blood (plasma or serum), 15 urine and 14 muscle as sample material.

Analytical methodology

The use of all known analytical procedures was reported. However, radioimmunoassay (RIA) procedures were mentioned most frequently ($N = 20$). The diversity within the group of RIAs, however, is extensive. Over eight different sources of primary antibodies are used and several different radioactive labelled analytes (tracers) and separation principles are being used. The combination GC–MS was reported 13 times, most frequently for urine and tissue analyses. Only a limited number of laboratories reported GC–MS as a method for confirmation for plasma or serum. Strategies that were mentioned were: repeating the analyses, additional sample clean-up [e.g., liquid chromatography (LC)] or using the same method in another laboratory. Most laboratories, however, did not report a method for confirmation.

Action levels

Table 2 summarizes the action levels for 17β-estradiol that were reported. The majority of laboratories have based their action level on the EC recommendation of 0.04 μg l^{-1}. For urine analysis only action levels for the metabolite 17α-estradiol were reported.

TABLE 2

Reported action levels for estradiol

Matrix	Analyte	Level (μg l^{-1})	N
Urine	17α-Estradiol	15	1
Urine	17α-Estradiol	20	1
Blood	17β-Estradiol	0.04	13
Muscle	17β-Estradiol	0.1	1

Quality control programs

Most laboratories reported to employ some sort of internal quality control (QC) program based on home-made, or with a commercial reagent set supplied, QC samples. None of the laboratories participated within an externally organized national or international quality control program. All laboratories were of the opinion that such (EC) programs would be of great benefit to the analytical programs.

Simultaneous determination of 17β-testosterone and 17β-estradiol

The analytical GC–isotope dilution (ID) MS method was developed with the objective of the simultaneous quantitative determination and identification of 17β-estradiol and 17β-testosterone at the recommended EC action levels of 0.04 μg l^{-1} for 17β-estradiol and 0.5 μg l^{-1} for 17β-testosterone (non-pregnant female bovine animals).

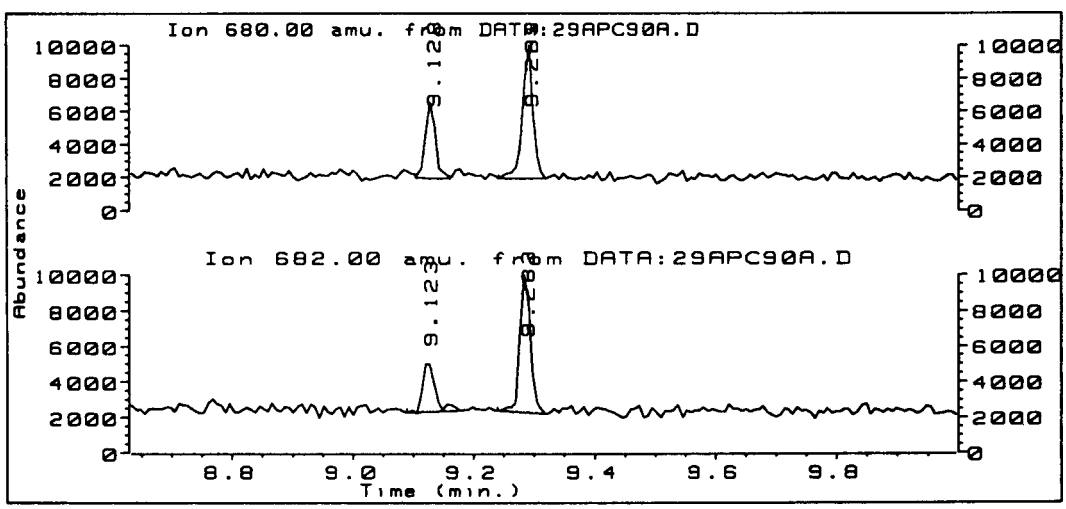

Fig. 1. Analysis of serum for 17β-testosterone with multi-immunoaffinity chromatography (MIAC)-GC–MS. Samples were spiked at a level of 1 μg l^{-1}.

TABLE 3

Repeatability and within-laboratory reproducibility (Samples spiked with 1 $\mu g\ l^{-1}$ of both 17β-testosterone and 17β-estradiol.)

	Mean value ($\mu g\ l^{-1}$)	S.D. (within day)	S.D. (between days)
17β-Testosteron	0.99	0.11	0.25
17β-Estradiol	0.91	0.06	0.15

TABLE 4

Limits of detection and determination for 17β-estradiol

	Detection	Determination
Electronimpact ionisation		
Pure compound (absolute)	60 pg	200 pg
Sample	60 ng l^{-1}	200 ng l^{-1}
Negative chemical ionisation		
Pure compound	2 pg	5 pg
Sample	2 ng l^{-1}	5 ng l^{-1}

Figure 1 shows the selected ion monitoring (SIM) chromatograms for 17β-testosterone [m/z 680 and 682 (internal standard)] for a sample of plasma spiked at a level of 1 $\mu g\ l^{-1}$. Figure 2 shows similar chromatograms for 17β-estradiol [m/z 664 and 667 (internal standard)]. Both chromatograms clearly indicate that the 1 $\mu g\ l^{-1}$ is well above the limits of detection. To assess whether accurate quantification at this level is possible the repeatability and within-laboratory reproducibility were determined (Table 3). A sample of serum in which 17β-estradiol and 17β-testosterone were not detectable was spiked (1.0 $\mu g\ l^{-1}$) with both analytes. Both the trueness, reproducibility and within-laboratory repeatability were acceptable.

In order to determine the effect of protein binding on the extractability of the analytes various protein precipitation procedures were included prior to extraction. No significant changes in the hormone levels determined were observed. Consequently it was concluded that the overnight incubation of the sample with the deuterated internal standards results in identical extractability of the added standards and endogenous analytes.

For 17β-testosterone the limit of determination is well below 0.5 $\mu g\ l^{-1}$, which makes the procedure described suitable for control purposes. However, Table 4 summarizes sensitivity data for 17β-estradiol. From this table it can be concluded that the procedure described has a limit of determination well above 0.04 $\mu g\ l^{-1}$. Previously, however, we demonstrated the suitability of negative chemical ionisation (NCI) for the detection of HFB derivatives of anabolic steroids [5]. Therefore we included NCI measurements for 17β-estradiol. The results clearly confirm the usefulness of this technique for 17β-estradiol as HFB derivative, the limit of determi-

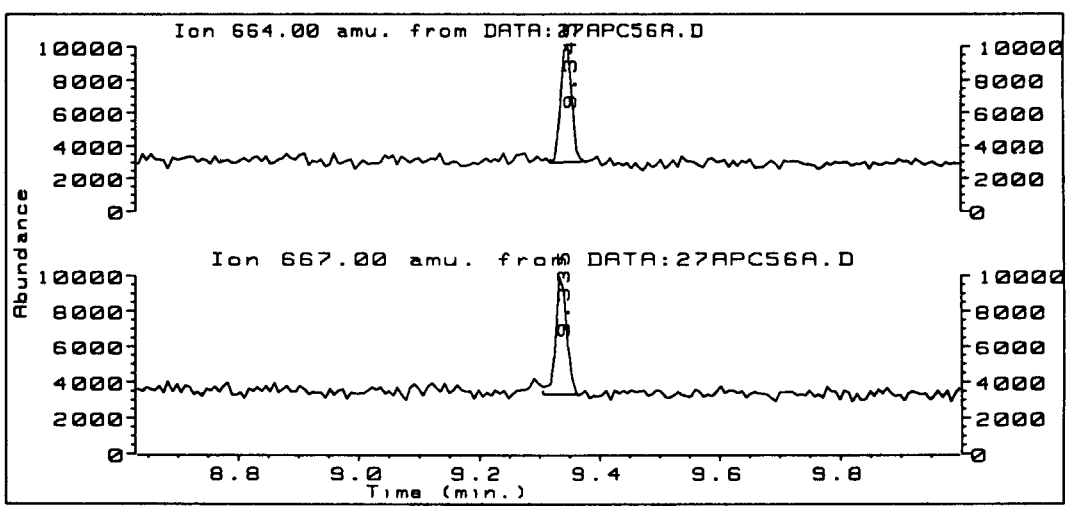

Fig. 2. Analysis of serum for 17β-estradiol with MIAC-GM–MS. Samples were spiked at a level of 1 $\mu g\ l^{-1}$.

nation being clearly below the EC-proposed target level. Full technical details and further validation will be published in the near future. Technical descriptions will be made available through the Community Reference Laboratory.

In the past the advantages of immunoaffinity column (IAC) separation over SPE and LC were clearly demonstrated [4]. To assess whether the same holds for the analyses of serum we analysed the same samples as used for the validation experiments described with SPE instead of an IAC. The results for both 17β-testosterone and 17β-estradiol are shown in Fig. 3. Contrary to other matrices it seems that for serum analyses SPE is a suitable alternative for an IAC for extract clean-up.

In conclusion it can be stated that a large number of laboratories have analytical programs for control for the illegal use of 17β-estradiol and 17β-testosterone. Most laboratories analyse samples of serum or plasma with a radioimmunoassay procedure. The diversity within this group of methods, however, is very large. There clearly is a need for good quality control programs. The

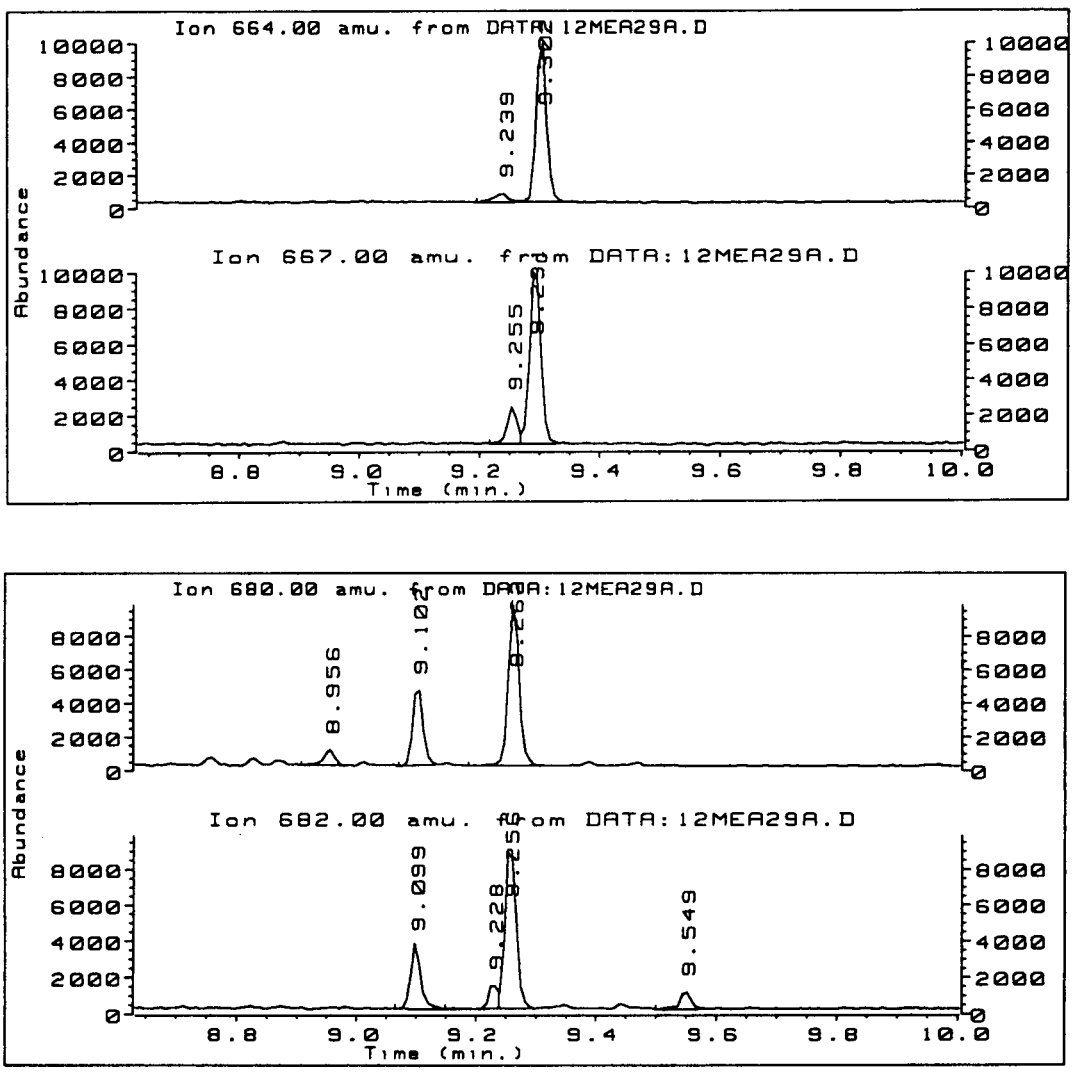

Fig. 3. Analysis of serum with SPE-GC–MS; upper panel, 17β-estradiol; lower panel, 17β-testosterone.

Community Reference Laboratory will undertake actions to organize cooperative studies in the near future. The analytical methodology developed is suitable for reference purposes, the accurate quantification of 17β-estradiol and 17β-testosterone and the recommended action levels, fulfilling EC criteria for both screening [6] and confirmation [7].

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Antibody binding efficiency of differently labelled steroid hormones

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Abstract

The binding of estradiol labelled with ^3H or with external labels (isoluminol and horseradish peroxidase) to anti-estradiol–6-carboxymethyloxime–bovine serum albumin antibodies was compared. The external labels were coupled covalently to the steroid via homologous 6-carboxymethyloxime or heterologous 3- and 17-hemisuccinate bridges. Different factors and conditions influencing this binding and the slope and shape of the calibration graph were studied, including the type of label, the liquid- and solid-phase antibody system, the matrix effect and general incubation conditions, e.g., time and temperature. External homologous labels yielded the most sensitive calibration graphs and the lowest detection limits. Large differences in the binding of the label and in the shape of the calibration graph between the different antibodies were observed.

Keywords: Immunoassay; Radiochemical methods; Anabolic steroids; Antibody binding efficiency; Chemiluminescent labels; Enzyme labels; Estradiol; Steroid hormones

Immunoassays offer convenient and inexpensive means for the screening and determination of many different compounds. In recent years alternative labels such as enzymes and fluorogenic and chemiluminescent compounds have frequently been used for the determination of steroid hormones in biological materials, e.g., tissues and body fluids. Generally, the binding of unlabelled and tritium (^3H)-labelled steroids to specific antibodies occurs with comparable avidity. However, a 50% difference in antibody binding potency of estradiol (E_2) and [^3H]E_2 has been described [1]. More problems were encountered with iodine radioisotopes as these were coupled to the steroid ligand via a chemical bridge. This was most conveniently done using the same bridge structure (e.g., hemisuccinate or carboxymethyloxime) as used in the steroid–protein conjugate that served as the immunogen [2]. However, because antibodies tend to recognize the bridge in addition to the steroid, iodine-labelled steroids generally have a higher affinity for the antibody than does the unlabelled steroid. Consequently, the assay may be less sensitive than the corresponding assay using ^3H-labelled steroid. In some instances the sensitivity may even be completely lost [3]. The

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sensitivity can be restored by changing the structure of the bridge so that the avidity of the antibody for the radioligand is diminished [4]. These hapten immunoassays using external labels have been classified as homologous or heterologous according to whether or not the immunogen and labelled analyte contain an identical bridge [5]. Even heterologous-site steroid immunoassays in which the bridge in the immunogen and in the labelled analyte is attached to different positions on the steroid molecule (e.g., C-3 and C-11 of the ring structure) have been evaluated [4,6].

Other external labels, such as the chemiluminescent compound isoluminol and the enzyme horseradish peroxidase (HRP), have also been coupled to the steroid hormones by means of a chemical bridge. Consequently, the homology and heterology of the bridge and of the site of attachment are important characteristics of these conjugates. Indeed, on several occasions it has been shown that sensitivity of enzyme immunoassay (EIA) could be considerably increased when the steroid derivative used for producing the enzyme conjugate and the immunogen differed slightly [7–10]. On the other hand, with isoluminol-labelled steroids the main problem concerning increased avidity of the antibody for the labelled steroid could be overcome by delayed addition of the conjugate [11]. In fact, sensitive chemiluminescence immunoassays (CIAs) for steroid hormones could be elaborated using homologous combinations of immunogen and labelled steroid [12–15].

Homology and heterology of steroid–immunogen and steroid–label conjugates can thus sometimes be decisive with regard to assay sensitivity, whereas on other occasions they are not. Moreover, other components of the immunoassay system and the assay conditions themselves may strongly influence the slope of the calibration graph. The antibody type, its use in the liquid or solid phase, the nature of the solid phase, the label itself, the matrix and the general incubation conditions, e.g., time and temperature, each on its own or in combination, may all have a great impact on assay sensitivity. Examples of this are given in this paper. In this work, different antibodies to estradiol-17β (a polyclonal antibody and two monoclonal antibodies), different labels [^{3}H, isoluminol and the enzyme horseradish peroxidase (HRP)], liquid- and solid-phase systems, homologous and heterologous combinations of antibody and steroid–label conjugates and different matrices were studied and compared. On all occasions incubation conditions were selected so as to obtain optimum or near-optimum assay sensitivity and still permit comparisons of different assays.

EXPERIMENTAL

Chemicals and reagents

Steroids, microperoxidase (MP-11; EC 1.11.1.7), bovine serum albumin (Cohn fraction V) (BSA), Tween 20 and thimerosal were obtained from Sigma (St. Louis, MO), ethanol, hydrogen peroxide (300 g l^{-1} solution) and activated charcoal from Merck (Darmstadt, Germany), Dextran-T70, Sephadex G-100 and Sepharose–protein A from Pharmacia (Uppsala, Sweden), microtitre plates (Maxisorp) and sealing tape from Nunc (Kampstrup, Denmark), 3,3′, 5,5′-tetramethylbenzidine (TMB) from Boehringer (Mannheim, Germany), second antibody, rabbit anti-mouse immunoglobulins (code Z259) from Dakopatts (Glostrup, Denmark), donkey anti-mouse antibody-coated cellulose suspension (anti-mouse Sac-Cel) from Wellcome (Beckenham, UK) and [2,4,6,7-$^3H_4$]estradiol (specific activity 110 kCi mol^{-1}) from Amersham International (Amersham, Bucks., UK).

Different conjugates of horseradish peroxidase (HRP) with estradiol (E_2) coupled through carboxymethyloxime (CMO) or hemisuccinate (HS) spacer were purchased: E_2-3-HS–HRP and E_2–6-CMO–HRP from Dr. A. Roda (University of Bologna, Bologna, Italy) and E_2–17-HS–HRP from Sigma.

The assay buffer for RIA and CIA was sodium phosphate (50 mmol l^{-1}, pH 8.0), containing 9 g l^{-1} NaCl, 100 mg l^{-1} BSA and 1 g l^{-1} sodium azide. The assay buffer for EIA was sodium phosphate (25 mmol l^{-1}, pH 8.0), containing 9 g l^{-1} NaCl, 250 mg l^{-1} BSA, 12.5 mg l^{-1} thimerosal and 4.15 g l^{-1} EDTA.

The coating buffer was sodium carbonate (50 mmol l^{-1}, pH 9.6) containing 100 mg l^{-1} thimerosal.

The wash solution contained 9 g l^{-1} NaCl, 1 g l^{-1} sodium azide (CIA) or 100 mg l^{-1} thimerosal (EIA) and 0.5 ml l^{-1} Tween 20.

The blocking buffer for CIA was assay buffer containing 1 g l^{-1} BSA and that for EIA was potassium phosphate (50 mmol l^{-1} pH 8.0) containing 1 g l^{-1} BSA and 100 mg l^{-1} thimerosal.

Specific reagent solutions for CIA and EIA were as described previously [16–18]. Dextran-coated charcoal was prepared by mixing 0.5 g of activated charcoal with 50 mg of Dextran T70 in 100 ml of assay buffer.

Apparatus

A Model 2000 Biocounter and a Model 2010 automated Biocounter from Lumac Systems (Basle, Switzerland) were used for light measurement. Photons generated during the chemiluminescent reaction were recorded for 10 s and an integrated photon count was obtained. The absorbance of the coloured product that resulted from the enzymatic conversion of tetramethylbenzidine was measured at 450 nm using an MPR-A4 microplate reader from Eurogenetics (Tessenderlo, Belgium).

Procedures

Preparation of antibodies. Conjugates of steroid hormones convalently coupled to BSA were synthesized as described [19]. Polyclonal anti-E_2–6-CMO–BSA were raised in rabbits [19] and monoclonal anti-E_2–6-CMO–BSA, MoAb clones 15 and 2F9, were prepared as described [20].

Synthesis of conjugates. The chemiluminescent conjugate E_2–6-CMO–aminobutylethylisoluminol (E_2–6-ABEI) was synthesized according to Schroeder et al. [21], as described previously [10]. An E_2–6-CMO–HRP conjugate was synthesized by the mixed anhydride method [22], as modified by Rajkowski et al. [23], and purified by Sephadex G-100 chromatography, followed by extensive dialysis.

Immunoassays for estradiol in serum and in saliva. These were of the direct type, i.e., without prior extraction of the steroid from serum or saliva. Instead, the samples were added directly to the incubation mixture (50 μl of serum and 100 μl of saliva in a final incubation volume of 200 μl).

Radioimmunoassay (RIA). Volumes of 0.1 ml each of diluted antibody clone 15 or 2F9, [^{3}H]E_2 and displacing agents [24] and a standard in steroid-free serum [17] or a serum sample were mixed and incubated overnight at 4°C. Dextran-coated charcoal was used to separate bound and free ligands. The antibody–ligand complex in the supernate was decanted into 3 ml of liquid scintillation cocktail and the radioactivity was counted in a Packard Model 3255 beta counter for 5 min.

Chemiluminescence immunoassay (CIA). Three systems were studied, as follows.

A "liquid" system in test tubes, using soluble antisteroid antibody. After incubation, phase separation was effected by the addition of second antibody-coated cellulose (Sac-Cel), centrifugation and recovery of the antibody bound fraction in the pellet.

A "liquid" system in microtitre plates. At the start of the assay, soluble antisteroid antibody was added to the wells, which had been coated in advance with second antibody. During incubation for the assay, antisteroid antibody could bind to second antibody. Phase separation was effected by inversion of the plates.

A "solid" system in microtitre plates. The wells of the plates were coated with second antibody, after which antisteroid antibody was bound to second antibody, all before the immunoassay was started.

In all CIAs two consecutive incubations (at room temperature) were performed. In the first incubation specific antisteroid antibody was incubated with steroid present in the standards or in the samples. When the samples were saliva, the standards were dissolved in buffer [16]. When the samples were serum, standards were added in steroid-free serum [17], and in all wells displacing agents were added. A mixture of natural and synthetic steroids was used [24]. In the assays with serum samples a wash concluded the first incubation, then the steroid–isoluminol conjugate was added. In the assays with saliva samples the conjugate was added without a prior wash step.

This was followed by the second incubation and subsequently by separation of antibody-bound and -free fractions. Treatment of the steroid–isoluminol-containing fraction and light measurement have been described [16,17].

Enzyme immunoassay (EIA). Both liquid and solid systems on microtitre plates were studied. The liquid system was as described for CIA. The solid system was obtained by either direct coating of specific antisteroid antibody to the wells or binding it to a second antibody as in the CIA system [3].

The enzyme immunoassay was carried out at room temperature in a single incubation. The sequence of additions of the reagents, i.e., steroid, steroid–HRP, buffer or matrix (including displacing agents) did not influence the results. However, in the liquid system, the antisteroid antibody was added last. The assay was stopped by washing the microtitre plate followed by adding substrate and chromogen solutions [18]. The enzymatic reaction was stopped after 10 min by adding 2 M HCl. The absorbance at 450 nm was recorded.

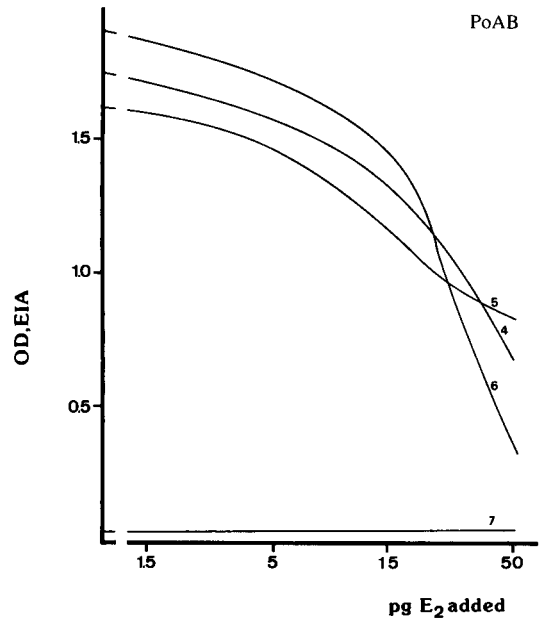

Fig. 1. Calibration graphs for enzyme immunoassays for estradiol, using polyclonal anti-E_2–6-CMO–BSA antibodies. EIAs were of the solid type on MTP. The reaction mixture (200 μl) contained 50 μl of serum and 150 μl of assay buffer. Incubation for 1 h at room temperature.

RESULTS AND DISCUSSION

Calibration graphs covering the range 1.5–50 pg of E_2 of different assays for estradiol are presented in Figs. 1–4. The polyclonal antiserum (Fig. 1) and the monoclonal antibodies 2F9 (Figs. 2 and 3) and 15 (Fig. 4) were obtained by immunization with E_2–6-CMO–BSA. Table 1 identifies the curve numbers in Figs. 1–4.

The polyclonal antibodies (Fig. 1) recognized and bound well both homologous E_2–6-CMO–HRP and heterologous E_2–3-HS–HRP conjugates in the presence of serum (curves 4–6). They did not, however, bind the heterologous E_2–17-HS–HRP conjugate, either in the presence (curve 7) or in the absence (results not shown) of serum. The sensitivities at zero binding, i.e., the 2σ limits of detection of estradiol, were 3.5 pg (curve 5), 2.6 pg (curve 4) and 1.5 pg (curve 6).

Monoclonal antibody 2F9 was used in a variety of types of assay (Fig. 2), including RIA, CIA and EIA in the presence of serum (curves 1, 2, 4 and 6) and CIA in the presence of saliva (curve 3). Comparison of two "liquid" system CIAs, one in test-tubes with separation of bound and free phases by means of Sac-Cel (curve 2) and one in microtitre plates (curve 3), both using the same E_2–6-CMO–ABEI conjugate, revealed that the binding kinetics in microtitre plates were not necessarily slower or less efficient than with soluble reagents. The RIA (curve 1) was also performed with soluble reagents. The incubation time, however, (overnight at 4°C) was much longer than that for the CIA (curve 2). RIA (curve 1) and EIA with homologous conjugate (curve 4) had acceptable slopes and sensitivity, their detection limits being 5 pg per tube (curve 1) and 1 pg per well (curve 4), respectively. However, by far the most sensitive assay was the CIA for salivary E_2 (curve 3). It had a detection limit of 0.2 pg per well, equivalent to 3.8 pmol l^{-1}. The binding of heterologous E_2–3-HS–HRP to 2F9 (curve 6) was weak: the absorbance was 0.3 with no E_2 added. Consequently, the slope of the calibration

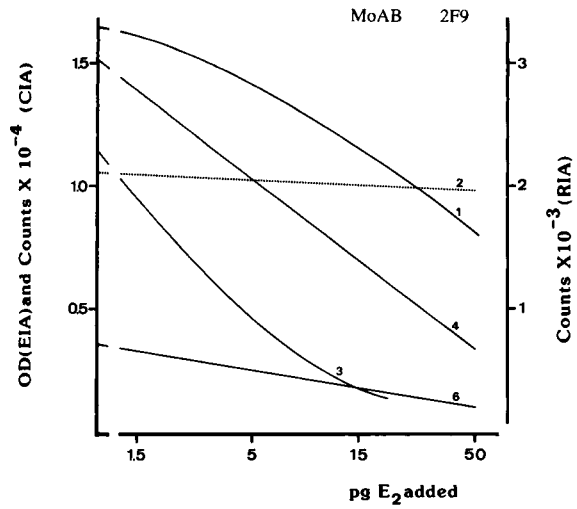

Fig. 2. Calibration graphs for RIA, CIA and EIA for estradiol, using monoclonal anti-E_2-6-CMO-BSA antibody 2F9. The reaction mixture contianed 50 μl of serum and 150 μl of buffer (curves 1, 2, 4 and 6) or 100 μl each of saliva and buffer (curve 3). Incubation conditions: curve 1, liquid, RIA in test-tubes, overnight at 4°C; curve 2, liquid, CIA in test-tubes (Sac-Cel), 1 and 2 h at room temperature; curve 3, liquid, CIA in MTP, 90 min + 30 min; curves 4 and 6, solid EIA in MTP, 1 h at room temperature.

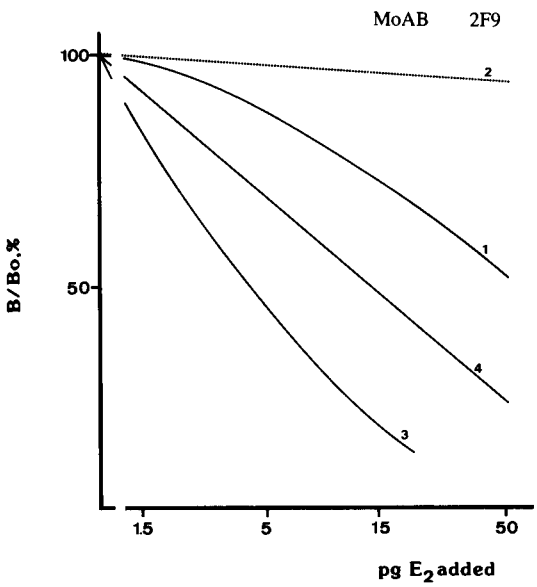

Fig. 3. Calibration graphs for RIA, CIA and EIA for estradiol, using monoclonal antibody 2F9, expressed as percentage relative binding, B/B_0. For explanation of curves, see Fig. 2.

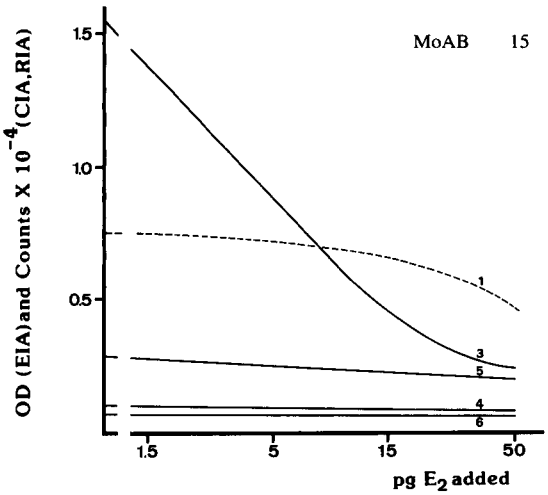

Fig. 4. Calibration graphs for RIA, CIA and EIA for estradiol using monoclonal anti-E_2-6-CMO-BSA antibody 15. The reaction mixture contained 200 μl of buffer (curve 3) or 50 μl of serum and 150 μl of buffer (curves 1, 4, 5 and 6). Incubation conditions: curve 1, liquid, RIA in test-tubes, overnight at 4°C; curve 3, liquid, CIA in MTP, 2 h + 5 min at room temperature; curves 4–6, liquid, EIA and MTP, 1 h at room temperature.

graph was shallow. The heterologous conjugate E_2–17-HS–HRP did not bind to antibody 2F9 (results not shown). In Fig. 3, showing the relative binding (B/B_0) of conjugates by 2F9, the differ-

TABLE 1

Immunoassays for estradiol-17β

Curve No. [a]	Assay type	Labelled steroid [b]	Obtained from [b]	Solid phase for separation of bound from free
1	RIA	[^{3}H] E_2	Amersham	DCC
2	CIA	E_2-6-CMO-ABEI	Synthesized [c]	Sac-Cel
3	CIA	E_2-6-CMO-ABEI	Synthesized [c]	MTP
4	EIA	E_2-6-CMO-HRP	Roda	MTP
5	EIA	E_2-6-CMO-HRP	Synthesized [c]	MTP
6	EIA	E_2-3-HS-HRP	Roda	MTP
7	EIA	E_2-17-HS-HRP	Sigma	MTP

[a] Figs. 1–4. [b] See *Chemicals and reagents* for more details. [c] Synthesized by the authors.

ences between the slopes of these calibration graphs are apparent.

The effect of the matrix on the immunoassay performance is illustrated in Fig. 4. In the CIA (curve 3), incubation was carried out in buffer. In RIA and EIA (curves 1 and 4–6), 50 μl of serum in 200 μl of incubation volume were present. There was a substantial decrease in sensitivity in the presence of serum. The detection limits of CIA (curve 3) and RIA (curve 1) were 0.5 pg per well and 8 pg per tube, respectively. One of the E_2–6-CMO–HRP conjugates (curve 4) and the heterologous conjugate E_2–3-HS–HRP (curve 6) bound very weakly to MoAB clone 15. The heterologous E_2–17-HS–HRP was not bound (results not shown).

From the results, it is apparent that, when setting up an immunoassay, especially one using an alternative, i.e., non-radioactive label, care must be taken not to decide heedlessly on the applicability of either antibodies or labelled hapten for immunoassay. The best and safest way of checking the value of an antibody is still by using a ^{3}H-labelled ligand. Differences between a ligand and the tritiated ligand are obviously not of the kind to bias results. If these results are satisfactory in terms of titre and displacement of the label by unlabelled ligand, other, external labels may be tested for binding to the antibody. Of the three antibodies described here the polyclonal one bound E_2–HRP conjugates better than did the monoclonal antibodies 2F9 and 15. However, extensive testing and comparison of results revealed that MoAb 2F9, in combination with homologous E_2–6-CMO–HRP, offered better sensitivity, a lower detection limit and better specificity than the polyclonal and monoclonal 15 antibodies. It was therefore selected for elaborating an EIA for direct measurement of estradiol in serum [18].

Although the combination of homologous antibody and steroid–enzyme label originally was judged less suitable for developing sensitive enzyme immunoassays for steroid hormones [5,8,10,25], more recent studies indicated that it was possible to develop homologous EIAs for steroid hormones, e.g., estradiol, with very good sensitivity [26]. This has recently been confirmed in several laboratories by the development of sensitive EIAs for E_2 [27–31]. In all instances the homologous combination included the use of E_2–6-CMO–HRP. The problem of bridge recognition has not been raised in chemiluminescence immunoassays. The homologous E_2–6-CMO–ABEI conjugate has been used for the development of sensitive CIAs for E_2 in serum and in saliva [24,32]. Obviously the problem of bridge recognition and bridge binding resides in the use of iodinated estradiol. Recently suggestions were made for practical ways of avoiding these phenomena in estradiol radioimmunoassay [33].

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Liquid chromatographic separation and gas chromatographic–mass spectrometric determination of 17α-methyltestosterone residues extracted from rainbow trout tissues

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Abstract

The residues of [^{3}H]-17-methyltestosterone (17MT) in rainbow trout after a single intragastric dose (200 μg, 0.37 MBq) were investigated. The metabolites were extracted from liver and carcass with chloroform–methanol (2 + 1, v/v). After enzymatic hydrolysis of the glucuronides, unconjugated steroids and aglycones were chromatographed by reversed-phase liquid chromatography. Each labelled fraction was collected and analysed by gas chromatography–mass spectrometry. After 24 h, the liver contained 4% of the administered dose. In the hepatic unconjugated and aglycone fractions the major metabolite was an isomer of 17α-methylandrostane-3ξ,16ξ,17β-triol. 7α-Hydroxy-17α-methylandrostane-3α,17β-diol was also observed in the unconjugated fraction. In addition to these metabolites, unchanged 17MT, 17α-methyl-5β-androstane-3α,17β-diol and 17β-hydroxyandrosta4,6-dien-3-one were identified in the unconjugated fraction extracted from carcass samples. After 72 h, 7α-hydroxy-17α-methylandrostane-3α,17β-diol persisted to a significant extent in carcass samples whereas no trace of 17MT was detected. This metabolite could therefore be used as an indicator of the administration of 17MT in fish.

Keywords: Gas chromatography; Liquid chromatography; Mass spectrometry; Anabolic steroids; Fish; Methyltestosterone; Steroids

17α-Methyltestosterone (17MT) is known to stimulate growth in various animal species including fish. In addition, 17MT is used experimentally for sex reversal in salmonids. However, as for other steroids, the use of this feed additive in livestock breeding has been banned in all EEC countries. Consequently, efforts have been made to develop methods for the detection and determination of 17MT residues in samples of animal origin [1,2].

A prerequisite for the implementation of such a methodology is a thorough knowledge of 17MT metabolic pathways. It has been shown previously that 17MT was extensively metabolized in rainbow trout and that some metabolites persisted in the tissues after the elimination of the parent compound [3]. However, the identification of these metabolites remained to be evaluated.

The purpose of this study was to identify the residues of 17MT isolated from liver and carcass

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of trout treated per os with a single dose of this steroid. Metabolites were detected and characterized by comparison with authentic or structurally related steroids and on the basis of gas chromatography–mass spectrometry (GC–MS)

EXPERIMENTAL

Radiochemicals and chemicals

^{3}H-labelled 17MT was prepared as described previously [3] by the Grignard reaction of [1,2,6,7-^{3}H]dehydroepiandrosterone (purchased from Amersham, Les Ulis, France) with methylmagnesium iodide followed by the Oppenauer oxidation with aluminium isopropoxide.

[^{3}H]-17MT was purified by thin-layer chromatography using silica gel plates (Kieselgel 60; Merck, Darmstadt, Germany) and chloroform–ethyl acetate (4 + 1, v/v) as the solvent. The radiochemical purity, as evaluated by radio-HPLC, was 97%. [^{3}H]-17MT was diluted with non-radioactive 17MT (Sigma, Saint Quentin Fallavier, France) to a final specific activity of 1.85 MBq mg^{-1} prior to preparation of the medicated feed.

17α-Methyl-5β-androstan-3α,17β-ol and 17α-methyl-17β-hydroxyandrosta-4,6-dien-3-one were from Research Plus (Bayonne, NJ).

17α-Methyl-5α-androstane-3α,16α,17β-triol was synthesized from 5α-androstan-3α,16α-diol-17-one (Steraloids, Wilton, NH) by the Grignard reaction.

Animal treatment

Immature rainbow trout (mean weight 190 g) were obtained from the Institut National de la Recherche Agronomique pisciculture at Donzacq (Landes, France) and held in flowing dechlorinated tap water at 16°C for at least 1 week before use. Once a day, the animals were fed ad libitum a pelleted commercial fish diet (Trouvit, Fontaine-les-Vervins, France). Twelve fish were force-fed a gelatin capsule containing 1.5 g of feed and 200 μg of [^{3}H]-17MT (0.37 MBq). The fish were divided into two groups, one group being killed after 24 h and the other after 72 h.

Sample preparation

The entire liver was taken and the gall bladder was carefully excised and discarded. The remaining carcass was minced using a blender.

Extraction of 17MT residues was performed as described previously [3] on pooled livers or carcass homogenates (20–50 g). Samples were homogenized in 100–300 ml of chloroform–methanol (2 + 1, v/v). Water (0.2 volume) was added to the extract and after centrifugation at 1800 *g* for 10 min the organic phase was evaporated. The aqueous phase was extracted again with two volumes of chloroform. The extract was evaporated to dryness, the residue was dissolved in acetonitrile and, after partitioning with isooctane and elution through a Lipidex 5000 column (Packard, Downers Grove, IL) and a Sep-Pak silica cartridge (Waters, Milford, MA), analysed by liquid chromatography (LC).

The aqueous fraction was added to an Amberlite XAD-2 column (Rohm and Hass, Philadelphia, PA) prepared for use according to Bradlow [4]. Labelled compounds were then eluted with methanol as solvent. The methanol phase was evaporated to dryness and the residue was dissolved in water. Conjugated metabolites were hydrolysed enzymatically using β-glucuronidase from *E. coli* (Type VII, Sigma) before analysis by LC.

Equipment

A Waters model 680 controlled gradient LC system equipped with two Waters Model 510 solvent-delivery pumps, a fraction collector (Gilson Model 202) and a liquid scintillation counter (Packard Tricarb 2200) were used for analysis by LC and radioactivity measurements. GC–MS analyses were performed on a Delsi DN 200 gas chromatograph interfaced with a Nermag R10-10 mass spectrometer working in the electron impact mode.

Chromatographic conditions

Analyses by LC were carried out on a 250 × 4.6 mm i.d. (SFCC ODS 1 (5 μm) column.

Free metabolites were developed using a binary gradient system. The solvents used were (A) acetonitrile–methanol–tetrahydrofuran (45 + 15

+ 5, v/v/v) and (B) water containing 0.2% acetic acid. Solvent was delivered to the column at a flow-rate of 1 ml min^{-1} as follows: time 0.0–5.0 min, elution with A–B (4:6, v/v); 5.0–25.0 min, linear gradient from A–B (4 + 6, v/v) to A–B (6 + 4, v/v); 25.0–40.0 min, linear gradient from A–B (6 + 4, v/v) to A–B (8 + 2, v/v); 40.0–45.0 min, elution with A–B (8 + 2, v/v) to A–B (9 + 1, v/v).

Fractions of 150 μl were collected in disposable 3-ml scintillation counting tubes. Aliquots of 10% to which 2 ml of scintillation mixture (Ultima Gold, Packard) were added were used for radio-LC profiles. The remaining volume was stored at −20°C until analysed by GC–MS.

Identification of metabolites

The major metabolites were characterized by co-chromatography with reference compounds using the C_{18} column under the LC conditions described above. Free metabolites were analysed by GC–MS underivatized or as trimethylsilyl (TMS) or methyloxime–trimethylsilyl (MO–TMS) derivatives. Methyloxime derivatives were prepared by the method of Thenot and Horning [5]. TMS ethers were prepared by reacting the isolated metabolites and reference compounds with a mixture of *N,O*-bis(trimethylsilyl)trifluoroacetamide–trimethylchlorosilane (Pierce, Rockford, IL) (8 + 2, v/v).

Electron impact (EI) mass spectra were obtained under the following conditions: column, fused-silica capillary (8.5 m × 0.25 mm i.d.) coated with 0.25-μm BP1 (SGE, Villeneuve Saint-Georges, France); carrier gas, helium at 2 ml min^{-1}, splitless mode; temperature programme, initial temperature 50°C (50 s), increased at 25°C min^{-1} to 230°C and then at 5°C min^{-1} to the final temperature of 280°C; electron energy, 70 eV; ion source temperature, 210°C; emission current, 300 μA.

RESULTS

After 24 h , ca. 4% and 25% of the dose were found in the liver and remaining carcass, respectively (Table 1). In the carcass, 80% of the radioactivity was extracted in chloroform (data not shown), suggesting that most of the residues were free metabolites and/or unchanged 17MT. In contrast, 65% of the hepatic labelled compounds were found in the aqueous fraction (data not shown). Incubation of conjugates from liver and carcass with β-glucuronidase resulted in their complete hydrolysis.

TABLE 1

Fate of radioactivity following administration of [^{3}H]-17MT to rainbow trout

Sample	Radioactivity (%) [a]	
	24 h	72 h
Liver	3.6 ± 2.9	0.19 ± 0.05
Remaining carcass	24.3 ± 3.8	8.45 ± 2.8

[a] Values are means ± S.D. from three fish and are expressed as % of administered dose.

Hepatic residues

LC analysis of 17MT free residues isolated from the trout liver showed three peaks, LI, LII and LIII (Fig. 1 A), while the hydrolysis of the glucuronide fraction resulted in a single peak having the same retention time as peak LII (Fig. 1B).

Peak LI. This fraction was found to contain two major compounds (metabolites 1 and 2) which were separated by GC analysis. Metabolite 1 produced an EI mass spectrum with a molecular ion at *m/z* 320, indicating hydroxylation and partial reduction of 17MT. This was corroborated by the mass spectrum of the corresponding MO–TMS derivative (Table 2), which exhibited a molecular ion at *m/z* 493, suggesting a hydroxymethyldihydrotestosterone structure, but the position of the hydroxyl group was not identified. Metabolite 2 exhibited a molecular ion at *m/z* 322, corresponding to trihydroxymethylandrostane compounds. A prominent fragment ion was observed at *m/z* 178, which is characteristic of the androstane-3,7,17-triol structure [6]. The mass spectrum of the TMS derivative of metabolite 2 was consistent with this structure assignment, as a molecular ion was observed at *m/z* 538 and a diagnostic fragment ion at *m/z* 393, indicative of

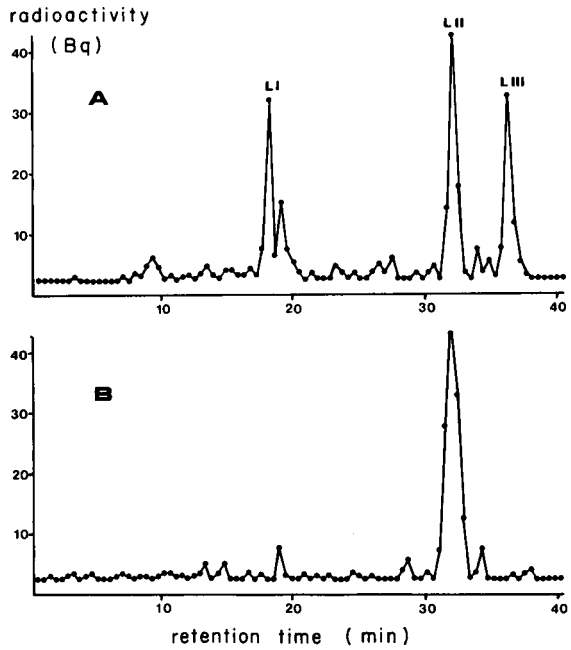


Fig. 1. Radio-LC profiles from six pooled trout livers removed 24 h after [^{3}H]-17MT administration. (A) Unconjugated metabolites; (B) aglycones from glucuronide fraction.

7α-hydroxylation [7]. Moreover, this metabolite was successfully oxidized by 3α-hydroxysteroid dehydrogenase (Sigma) by using the previously described procedure [8], indicating a 3α-isomer. Based on these results, it is suggested that metabolite 2 was 17α-methyl-5ξ-androstane-3α,7α,17β-triol.

Peak LII. One metabolite (metabolite 3) was contained in this fraction. This metabolite yielded a parent molecular ion of m/z 322 and accompanying ions of m/z 304, 279, 261, 243 and 217 (Table 2), which were similar to the reference compound 17α-methyl-5α-androstane-3α,16α, 17β-triol. Nevertheless, different retention times were observed for the two compounds, suggesting that metabolite 3 may be an epimer of the standard.

Peak LIII. The identity of this metabolite is currently under investigation; however, GC–MS data suggest that it corresponds to a 17α-methylandrostanetriol.

Conjugated fraction (Fig. 1B). Treatment of the liver aqueous fraction with β-glucuronidase resulted in the isolation of an aglycone identified by GC–MS as 17α-methylandrostane-3ξ,16ξ, 17β-triol. This aglycone exhibited GC and LC retention times identical with those of metabolite 3.

Carcass residues

The 24-h LC profile obtained from the chloroform extract showed seven major peaks (Fig. 2A).

Peak CI. This was identified as 7α-hydroxy-17α-methylandrostane-3α,17β-ol, based upon its retention time in LC and GC as well as on the comparison of its mass spectrum with that of metabolite 2 isolated from the liver.

Peak CII. GC analysis of this fraction showed three metabolites which were not characterized further.

Peak CIII. Owing to the small amount of this metabolite, no interpretable mass spectrum was obtained.

Peak CIV. This metabolite exhibited a similar mass spectrum to that of authentic 17α-methyl-5α-androstane-3α,16α,17β-triol, but as for metabolite 3 in the liver, the GC retention time did

TABLE 2

GC–MS data for hepatic 17MT metabolites in trout

Peak No. [a]	Metabolite	RRT [b]	M$^+$ [c]	Major ions and intensites [d]
LI	1	1.055	320	263(12), 249(14), 194(100)
	1 [e]		493	462(49), 376(18), 350(21), 143(24)
	2	1.058	322	304(15), 246(100), 215(29), 178(88)
	2 [f]		538	448(11), 393(100), 233(25), 205(22)
LII	3	1.089	322	304(10), 279(22), 261(26), 243(20) 217(53)
LIII	4	1.086	322	294(10), 271(12), 243(14), 217(19)

[a] See Fig. 1A. [b] Retention time relative to that of 17MT (t_R = 10.49 min). [c] Molecular ion m/z values. [d] Values in parentheses are relative intensities expressed in terms of % of base peak. [e] Analysed as MO–TMS derivative. [f] Analysed as TMS derivative.

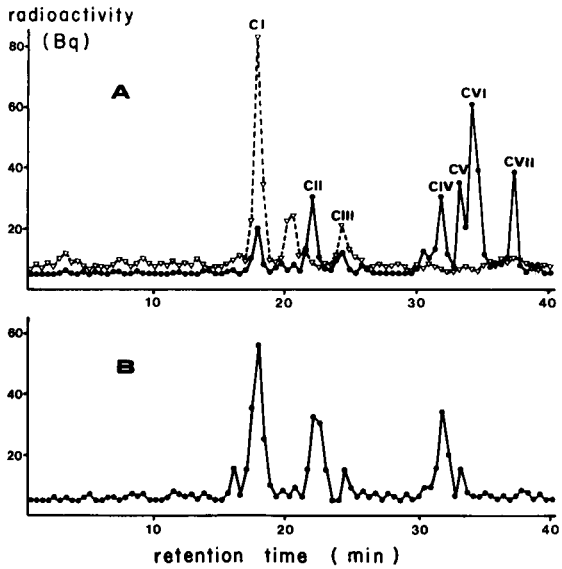


Fig. 2. Radio-LC profiles from the homogenate of six pooled carcasses of trout administered [^{3}H]-17MT. Solid lines, 24-h samples; dashed lines, 72-h samples. (A) Unconjugated metabolites; (B) aglycones from glucuronide fraction.

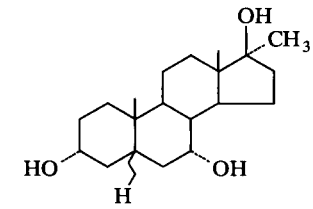
not correspond to this reference compound. Based on this difference, peak CIV is suggested to be an isomer of the standard.

Peak CV. The LC, GC and mass spectral data of this metabolite were the same as those observed for 17α-methyl-17β-hydroxyandrosta-4,6-dien-3-one.

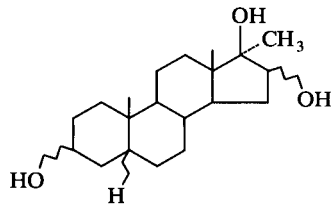
Peak CVI. This was identified as unchanged 17MT, which accounted for 27% of the radioactivity that was present at 24 h in the carcass sample.

Peak CVII. This peak exhibited exactly the same retention time as an authentic standard of 17α-methyl-5β-androstane-3α,17β-diol. The identity of this metabolite was further confirmed by comparison of its mass spectrum with that of corresponding standard.

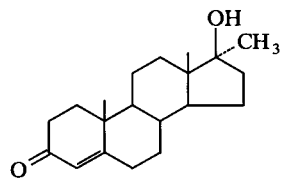
After 3 days (Fig. 2A, broken line), the pattern of metabolites was substantially modified. No traces of 17MT, 17α-methyl-5β-androstane-3α,17β-diol, 17α-methyl-17β-hydroxyandrosta-4,6-dien-3-one or 17α-methylandrostane-3ξ,16ξ,17β-triol were detected but 7α-hydroxy-17α-methylandrostan-3α,17β-ol was still present in



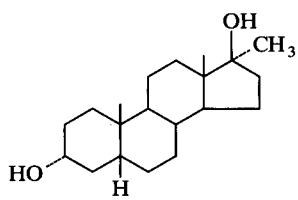
17α-methyl-5ξ-androstane-3α,7α,17β-triol
(isolated from LI and CI)



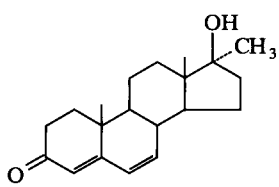
17α-methyl-5ξ-androstane-3ξ,16ξ,17β-triol
(isolated from LII and CIV)



17MT
(isolated from CVI)



17α-methyl-5β-androstane-3α,17β-diol
(isolated from CVII)



17α-methyl-17β-hydroxyandrosta-4,6-dien-3-one
(isolated from CV)

Fig. 3. Structures of the residues (unconjugated and aglycone fraction) isolated from the liver and carcass of trout administered 17MT.

significant amounts. In addition, a new metabolite appeared between peaks I and II, the identity of which remains to be investigated.

As indicated in Fig. 2B, the LC analysis of the glucuronide fraction deconjugated with β-glucuronidase showed three major peaks. Based on LC and GC–MS analyses, these peaks were identified as 7α-hydroxy-17α-methylandrostane-3α,17β-diol, peak CII from the chloroform-extracted fraction showed in Fig. 2A and 17α-methylandrostane-3ξ,16ξ,17β-triol, respectively.

DISCUSSION

Previous studies on the biotransformation of 17MT by various mammal species, including man, have shown the urinary excretion of significant amounts of reduced and hydroxylated metabolites [9–12]. However, in these early studies, no account was taken of the metabolites retained in tissues. In this work on rainbow trout, a number of compounds with either reduced, oxidized or hydroxylated steroid structures were identified as primary metabolites or glucuronic acid conjugates. After analysis by LC and capillary column GC–MS, three and four primary metabolites were identified in the liver and the carcass, respectively. However, in most instances, the stereochemistry of these metabolites could not be determined, owing to the lack of authentic reference compounds.

Four types of metabolites were isolated from tissues. For the major part of the metabolites monohydroxylation of 17MT occurred, followed by full reduction of the double bond and the keto group in the A ring. Several 17α-methylandrostanetriols were identified in the carcass and liver either as the glucuronide or in the unconjugated form.

These metabolites were hydroxylated at C-7 (metabolite 2) or C-6 (metabolite 3). Oxidation at C-16 has been reported previously for 17MT biotransformation in rabbit by Watabe et al. [11] and Templeton and Jackson [12], who found no trace of C-7-hydroxylated compounds. However, 7α-hydroxylated metabolites of testosterone have been identified in fish [13] and other species [14]. In the liver, a second type of monohydroxylated metabolite (metabolite 1) was identified. For this compound the A ring was only partially reduced, as metabolite 1 contained a 3-one group. No similar metabolite has been reported previously for 17MT. As has been found in rabbit [12] and fish [8], a fully reduced metabolite of 17MT was observed in trout carcass. Only the 17α-methyl-5β-androstane-3α,17β-diol epimer was observed in our samples.

Finally, an unusual steroid metabolite was isolated and identified as 17α-methyl-17β-hydroxy androsta-4,6-dien-3-one. This dehydrogenation pathway at the C-6 and C-7 positions was previously demonstrated with testosterone incubated with liver microsomes [15] but has never been observed in vivo. Three days after the administration of 17MT, 7α-hydroxy-17α-methylandrostane-3α,17β-diol was identified as the remaining major residue in the carcass. Consequently, this metabolite may be considered as a marker for 17MT residues in rainbow trout.

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Metabolism of 17β,19-nortestosterone in urine of calves after oral intake and intramuscular administration

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Abstract

The metabolism of 17β,19-nortestosterone was investigated in six calves. Three calves were injected with Laurabolin and the other three were fed 17β,19-nortestosterone-containing food. Urine samples were taken before and at regular time intervals after the injection or oral intake. After enzymatic hydrolysis, the sample clean-up consisted in solid-phase extraction followed by a liquid chromatographic purification. Detection was by gas chromatography–mass spectrometry. Urine samples from pregnant and non-pregnant cows and from steers were also collected and analysed in the same way.

Keywords: Gas chromatography; Mass spectrometry; Anabolic steroids; Sample preparation; Calves; Nortestosterone; Urine

17β,19-Nortestosterone (17β,19-NT) is a frequently used anabolic compound in cattle fattening. It has an anabolic activity comparable to that of testosterone and less androgenic activity. The use of growth-promoting agents, however, is forbidden throughout the European Economic Community (EEC).

The presence of 17β,19-NT as an endogenous compound in stallions [1] and boars [2,3] has been reported. The question of whether 17β,19-NT could be endogenous also in cattle under certain physiological conditions, e.g., gestation has been raised. If this hypothesis is true, it would be impossible to prove the illegal use of 17β,19-NT by analysing urine for nortestosterone.

In this study, 17β,19-NT metabolites were determined in urine from six calves, three of which were injected with Laurabolin and the other three were fed 17β,19-NT-containing feed. Based on a preliminary literature study of 17β,19-NT metabolism in different animal species, urine samples were analysed for 17β,19-NT and its epimer 17α,19-nortestosterone [4–6] and for further possible metabolites, particularly isomers of 3-hydroxyestran-17-one [7–11] and of estrane-3, 17-diol [1,7–12].

Urine samples from pregnant and non-pregnant cows and from steers were also collected on an experimental farm where no anabolic compounds were used.

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EXPERIMENTAL

Animals

Six female calves were divided into two groups. The first group containing calves No. 1 (145 kg), No. 2 (187 kg) and No. 3 (214 kg) received intramuscular injections with Laurabolin (nortestosterone laurate) (Intervet, Brussels) containing 50, 100 and 250 mg of the steroid ester, respectively. The second group, containing calves No. 4

(183 kg), No. 5 (174 kg) and No. 6 (205 kg) was fed for 14 days with food containing 500 μg, 200 μg and 1 mg of 17β,19-NT per day, respectively.

Urine samples were taken during 3 days before the experiments and at regular time intervals after the treatment (for injection, 2, 4, 8, 11, 14, 17, 21, 23, 25 and 28 days after injection; for the oral intake, 3, 7, 9, 11 and 14 days after the beginning of the treatment). The samples were stored at −80°C until analysis.

The urine samples from pregnant and non-pregnant cows and from steers were collected on an experimental farm where no anabolics were used and were stored at −80°C until analysis.

Chemicals, glassware and solvents

Disposable derivatization vials were silanized with a solution of 5% dimethylchlorosilane (Merck, Darmstadt) in toluene (Merck) before use. Methanol and water were of LC grade from Lab Scan (Dublin). Ethyl acetate was obtained from Janssen Chimica (Geel, Belgium).

Standards

19-Noretiocholanolone (5β-estran-3α-ol-17-one), 19-norepiandrosterone (5α-estran-3β-ol-17-one) and 19-norepietiocholanolone (5β-estran-3β-ol-17-one) were kindly donated by Schering (Berlin/Bergkamen). 19-Norandrosterone (5α-estran-3α-ol-17-one), 5α-estrane-3α,17β-diol and 5α-estrane-3β,17α-diol were purchased from Steraloids (Wilton, NH). 17α,19-Nortestosterone was kindly provided by Professor Martens (LUC, Diepenbeek, Belgium).

Instrumentation

Octadecyl (C_{18}) and amino (NH_2) disposable extraction columns were purchased from J.T. Baker (Phillipsburg, NJ). For liquid chromatography (LC) the following system was used: a pump and system controller (Model 600 E), an automatic injector (Wisp 710 B) and a UV detector (Model 484) at 254 nm, all from Waters (Milford, MA) and a fraction collector (2212 Helirac) from Pharmacia–LKB (Uppsala).

The analytical column was a Nova-Pak C_{18} (Waters) column (150 mm × 3.9 mm i.d.; particle size 4 μm) and was protected by a guard column (Hibar, 30 mm × 4 mm i.d.) (Merck). Analyses by gas chromatography–mass spectrometry (GC–MS) were done on a Model HP-5970 mass-selective detector linked to an HP 5890 gas chromatograph, equipped with an HP Ultra 2 fused-silica (cross-linked 5% phenyl–methylsilicone) capillary column (25 m × 0.2 mm i.d., film thickness 0.33 μm) (Hewlett-Packard, Palo Alto, CA).

Derivatization agents

Heptafluorobutyric anhydride (HFBA) was purchased from Macherey–Nagel (Düren), *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) and trimethylsilyl iodide (TMSI) from Aldrich (Milwaukee, WI), pyridine from Pierce (Rockford, IL) and benzene from Merck.

Sample preparation

Steroid extraction was based on the procedure of Schmidt et al. [13]. Free and conjugated steroids, in 10 ml of urine, were extracted on a C_{18} column. A 100-ng amount of trideuterated NT (RIVM, Bilthoven, Netherlands) was added as an internal standard. The conjugated steroids were hydrolysed by means of *Helix pomatia* digestive juice (Boehringer, Mannheim) at 55°C for 1 h. The steroids were extracted on the previously used C_{18} column. Additional purification was done by linking the C_{18} column with an NH_2 column before elution. The extract was dissolved in 100 μl of methanol, of which 75 μl was purified by LC. The mobile phase was methanol–water (65 + 35) at a flow-rate of 1 ml min^{-1}. A fraction containing the two 19-NT epimers, the four 3-hydroxyestran-17-one epimers and the estrane-3,17-diol isomers was collected (3.5–10 min).

Derivatization

Heptafluorobutyrates were prepared by adding 50 μl of HFBA and 200 μl of benzene to the dried extract. The mixture was heated for 1 h at 60°C and then concentrated to dryness under a flow of nitrogen. The derivative was dissolved in 50 μl of hexane. Trimethylsilyl derivatives, used for confirmation, were prepared by adding 50 μl of a mixture containing MSTFA–TMSI (1000 + 2)

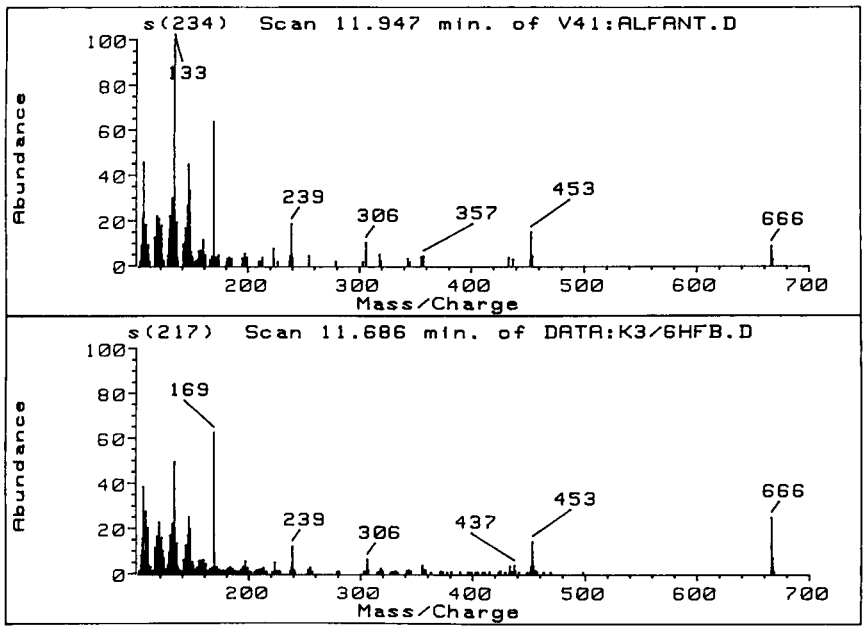

Fig. 1. Mass spectra of 17α,19-NT-diHFB in calf 3 (bottom) and of the reference 17α,19-NT-diHFB (top).

to the dried extract. The mixture was heated for 0.5 h at 60°C and was injected without further treatment.

GC–MS analysis

A 3-μl volume of the derivative was injected into the GC–MS instrument using an all-glass moving-needle injection system. The carrier gas was high-purity helium (N60) (L'Air Liquide, Liège) at a flow-rate of 0.47 ml min^{-1}. The injection and transfer line temperatures were 290°C.

The oven temperature was programmed from 200 to 280°C at 5°C min^{-1}, the final temperature being held for 10 min. The mass spectrometer was operated in the electron impact mode with an electron beam voltage of 70 eV. The analyses were performed in the SCAN and in the selected-ion monitoring (SIM) mode (dwell times 100 ms).

TABLE 1

GC–MS data for the HFB and TMS derivatives

Compound [a]	HFB		TMS	
	MU	Ions (m/z)	MU	Ions (m/z)
17α,19-NT	23.72	666, 453, 306	26.43	418, 194, 182
17β,19-NT	24.52	666, 453, 306	26.95	418, 194, 182
5α,3α	23.58	472, 454, 428	24.92	420, 405, 315
5β,3α	24.16	472, 454, 428	25.38	420, 405, 315
5β,3β	24.26	472, 454, 428	25.41	420, 405, 315
5α,3β	24.61	472, 454, 428	25.70	420, 405, 315
5α,3α,17β	23.15	456, 441, 415	25.10	422, 407, 332
5α,3β,17α	23.35	456, 441, 415	25.40	422, 407, 332

[a] 17α,19-NT = 17α,19-nortestosterone; 17β,19-NT = 17β,19-nortestosterone; 5α,3α = 19-norandrosterone; 5β,3α = 19-noretiocholanolone; 5β,3β = 19-norepietiocholanolone; 5α,3β = 19-norepiandrosterone; 5α,3α,17β = 5α-estrane-3α,17β-diol; 5α,3β,17α = 5α-estrane-3β,17α-diol.

TABLE 2

Results for the non-treated fully grown animals

Animal	Compounds detected [a]
Pregnant cow ($n = 1$)	α- and β-E2
	α-T
	5α,3β,17α
Non-pregnant cows ($n = 3$)	α-E2
	α-T
Steers ($n = 6$)	α- and β-T
	5β,3α (in two animals)

[a] α- and β-T = α- and β-testosterone; α- and β-E2 = α- and β-estradiol; 5α,3β,17α = 5α-estrane-3β,17α-diol; 5β,3α = 19-noretiocholanolone.

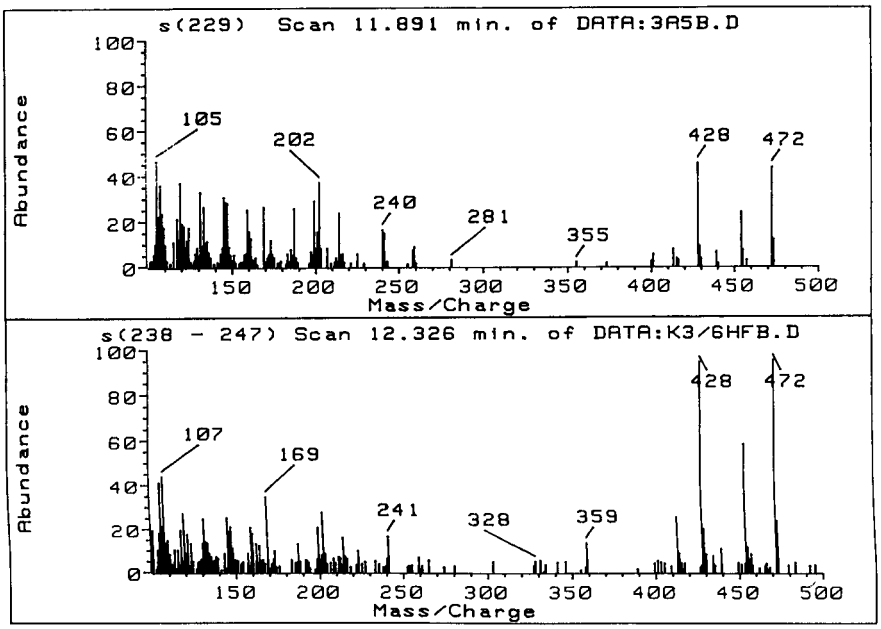

Fig. 2. Mass spectra of 19-noretiocholanolone-mono-HFB in calf 3 (bottom) and of the reference 19-noretiocholanolone-mono-HFB (top).

RESULTS AND DISCUSSION

Recoveries from both purification and fractionation by LC were calculated for 17β,19-NT by means of radioactive tracers and liquid scintillation counting. The results were 97 ± 4% ($n = 5$) after C_{18} extraction and 92% ($n = 1$) after LC.

The urine samples were analysed in the SCAN

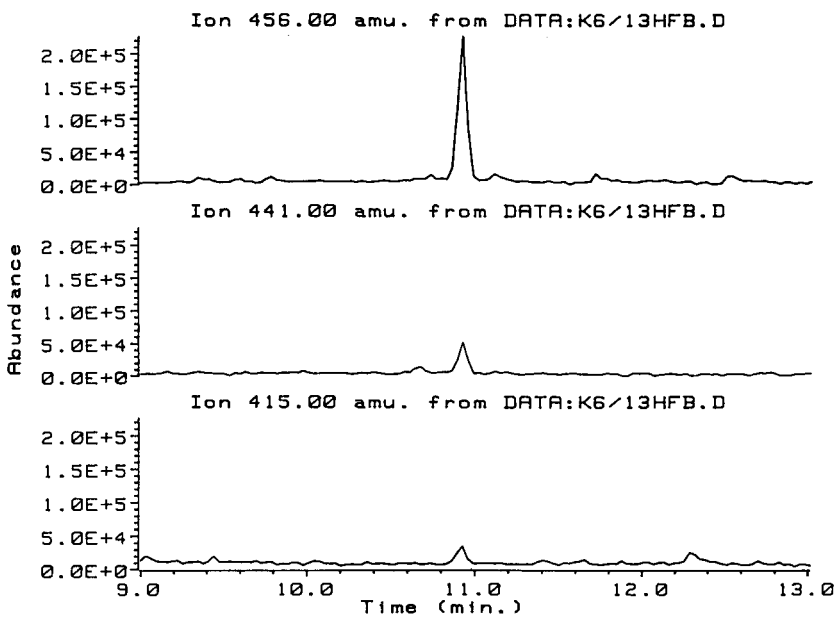

Fig. 3. Selected-ion monitoring of ions of m/z 456, 441 and 415 in calf 3 (5α-estrane-3β,17α-diol-di-HFB).

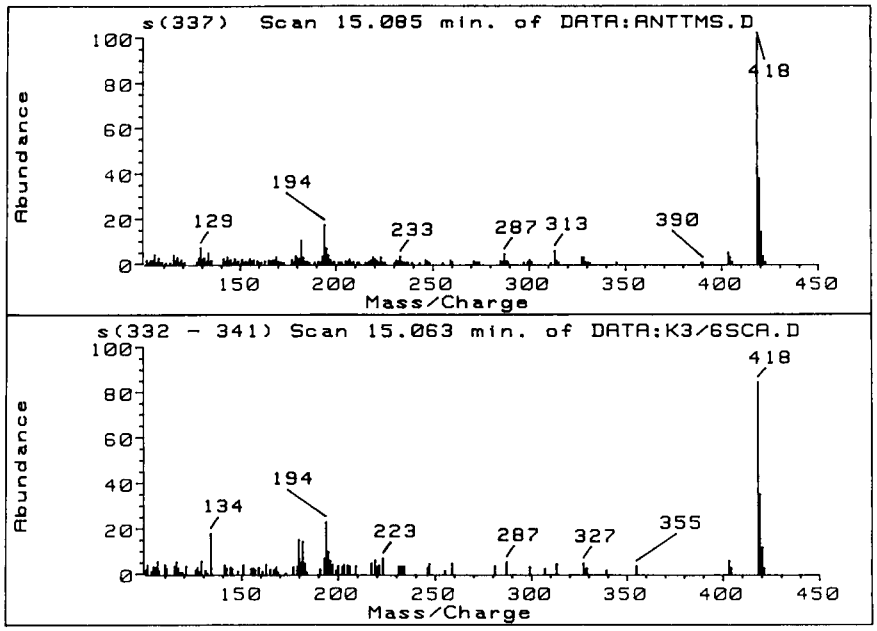

Fig. 4. Mass spectra of 17α,19-NT-di-TMS in calf 3 (bottom) and of the reference 17α,19-NT-di-TMS (top).

and SIM modes. Both GC retention data, expressed as methylene unit values (MU), and MS data based on the characteristic ions were used for identification. For a positive identification, all characteristic ions must appear simultaneously at the correct retention time and the ratio of the ions must correspond to that observed for the standards. Results obtained with HFB derivatiza-

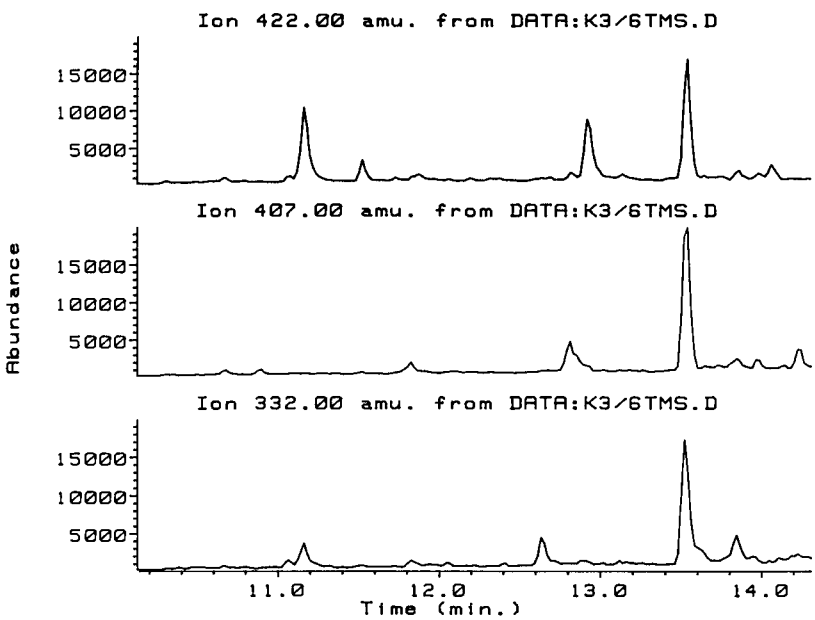

Fig. 5. Selected-ion monitoring of ions of m/z 422, 407 and 332 in calf 3 (5α-estrane-3β,17α-diol-di-TMS).

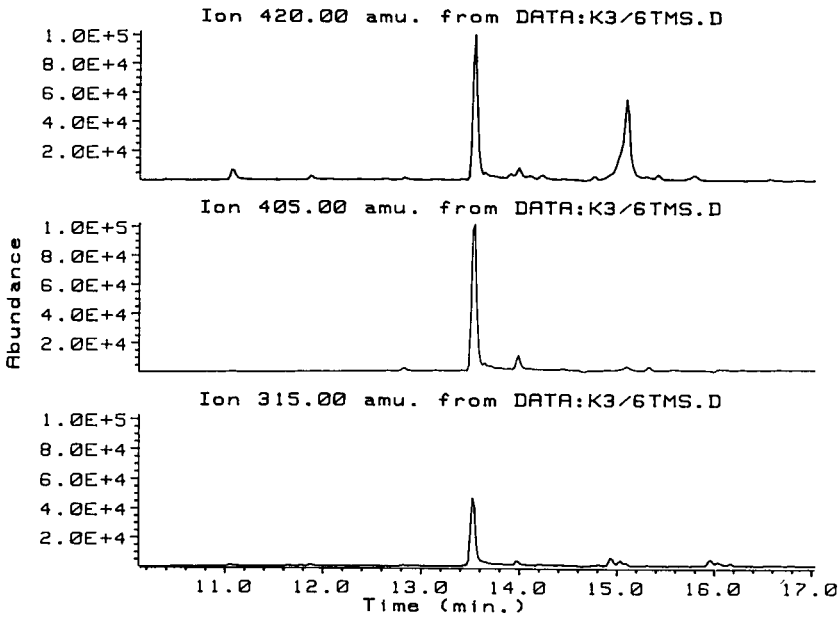

Fig. 6. Selected-ion monitoring of ions of m/z 420, 405 and 315 in calf 3 (19-noretiocholanolone-di-TMS).

tion were confirmed with TMS derivatization. Table 1 gives the identification and confirmation data for 17β,19-NT and its possible metabolites.

The results obtained for the urine samples from the pregnant and non-pregnant cows and from the steers are given in Table 2. In addition to estradiol and testosterone, 5α-estrane-3β, 17α-diol was found in the urine from the pregnant cow and traces of 19-noretiocholanolone in the urine from two steers. 17β,19-NT itself or the 17α-epimer could not be detected. In the blank urine samples from the calves only α-testosterone could be detected.

After intramuscular injection of Laurabolin, the most abundant metabolite in the urine was 17α,19-NT. In all samples from all animals 5α-estrane-3β,17α-diol and 19-noretiocholanolone were detected. The maximum concentrations were found 8 days after injection. It was also observed that high concentrations of 17α,19-NT were coupled with small amounts of 5α-E-3β-ol-17-one (19-norepiandrosterone) and with an estrane diol, which on comparison with the data of Debruyckere and Van Peteghem [7] could be attributed to 5β-estrane-3α,17β-diol.

No 17β,19-NT could be detected in the post-administration samples. In bovine species, C_{17} epimerization is a major pathway of metabolism [6]. Figure 1 shows the mass spectrum of 17α, 19-NT-di-HFB in the urine of the calf which had

TABLE 3

Results for the treated animals

Administration [a]	Nortestosterone		Estrane-3, 17-diols		3-Hydroxyestran-17-ones	
	17β,19-NT	17α,19-NT	3β,5α,17α	3α,5β,17β	5β,3α	5α,3β
Oral	n.d. [b]	+ [c]	+	n.d.	+	n.d.
I.m.	n.d.	+	+	+ [d]	+	+ [d]

[a] I.m = intramuscular [b] n.d. = not detected. [c] Only in the first two samples collected. [d] Only present when the concentration of 17α,19-NT reaches the highest levels.

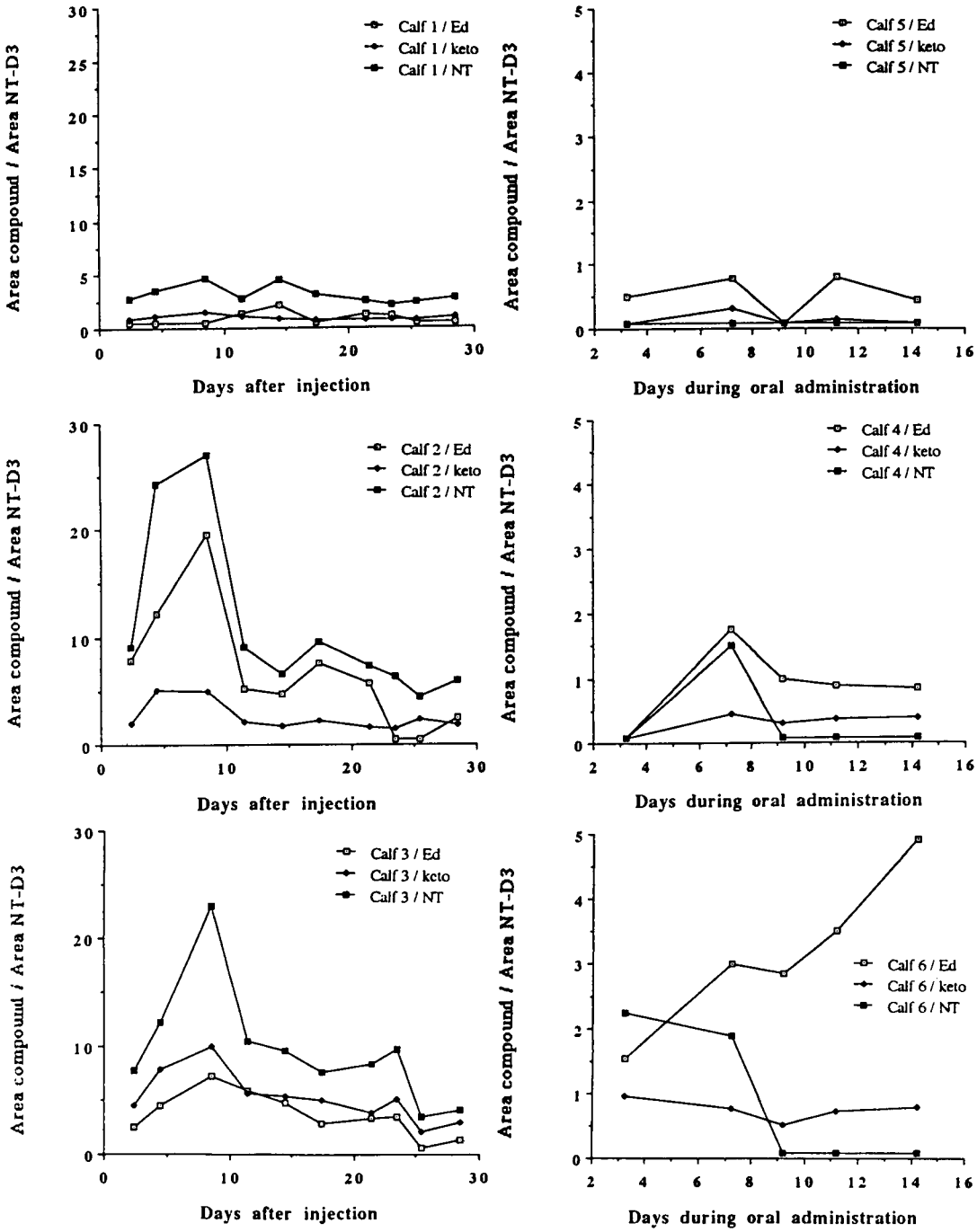

Fig. 7. Elimination curves of 17α,19-NT (NT), 5α-estrane-3β,17α-diol (Ed) and 19-noretiocholanolone (keto) relative to the internal standard.

received 250 mg of Laurabolin compared with that of the reference standard. In contradiction to Benoît et al. [4], HFB derivatives were suitable for the detection of 17-ketosteroids yielding mono-HFB derivatives. Figure 2 shows the mass spectrum of 5β-E-3α-ol-17-one-mono-HFB in the urine from calf 3 compared with that of the reference standard.

For 5α-estrane-3β,17α-diol it was impossible to obtain a reasonable spectrum because there was an interfering peak in the chromatogram. An ion chromatogram of this compound (ions at m/z 456, 441 and 415) in the urine from calf 3 is shown in Fig. 3.

Confirmation of the identity of the compounds was done by TMS derivatization. The identity of 17α,19-NT, 5α-estrane-3β,17α-diol, 19-noretiocholanolone, 19-norepiandrosterone and probably 5β-estrane-3α,17β-diol could be confirmed. Figure 4 shows the mass spectrum of 17α,19-NT-di-TMS in the urine from calf 3 compared with that of the reference standard. Figures 5 and 6 show ion chromatograms of the TMS derivatives of 5α-estrane-3β,17α-diol and 19-noretiocholanolone, respectively.

The metabolites that were detected in the urine after oral intake are qualitatively the same as after intramuscular injection. 17α,19-NT could only be detected in the first and/or the second urine sample. In the other urine samples, 17α,19-NT was not detectable but 5α-estrane-3β,17α-diol and 19-noretiocholanolone were still present. The concentrations were lower than in the urine samples from the injected calves. A summary of the results obtained for the treated animals is given in Table 3.

An attempt was made to construct the elimination curves of 17α,19-NT, 5α-estrane-3β,17α-diol and 19-noretiocholanolone for the six calves (Fig. 7). The ratio of the area of the molecular ion of each of these compounds to that of the molecular ion of 17β,19-NT-d_3 was plotted against the number of days after injection or against the number of days of oral intake. This gives only an idea of the elimination and cannot be considered to be very accurate, as no 24-h urine samples were available because of technical difficulties in the collection of the samples by catheter.

Conclusions

The results presented are comparable to those obtained by Habrioux et al. [14]. They identified the metabolites of 17β,19-NT after incubation with a bovine liver homogenate. The compounds that were identified were 17β,19-NT and its epimer 17α,19-NT, and at least three estranediols with the presumed configurations 5β,3α,17α,5α, 3β,17α and 5β,3α,17β. The urinary metabolites of 17β,19-NT that were detected in this study were identical for both administration modes, namely 17α,19-NT, 5α-estrane-3β,17α-diol and noretiocholanolone. In the urine samples with the highest concentration of metabolites also 19-norepiandrosterone could be detected and probably 5β-estrane-3α,17β-diol.

In the urine samples from the pregnant and non-pregnant cows and from steers, no 17α,19-NT or 17β,19-NT could be detected. In the urine from the pregnant cow 5α-estrane-3β,17α-diol was detected and in the urine from two of the six steers traces of 19-noretiocholanolone were found to be present. Therefore, 17α,19-NT is an ideal target compound in urine for routine analysis.

From a qualitative point of view it is impossible to differentiate between the urinary metabolites of 17β,19-NT after oral intake or after intramuscular administration. Further work is necessary to search for a difference on a quantitative basis.

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Urinary excretion of androstenedione in cattle as an indicator of possible illegal use in livestock breeding

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Abstract

Androstenedione (androst-4-ene-3,17-dione) is a metabolite and a precursor of testosterone (17β-hydroxyandrost-4-en-3-one) and is present in cattle. The features of the molecule, also produced by synthesis, include an anabolysing effect, without or with a moderate androgenic effect. During a period of 2.5 years, within a control project aimed at the production of quality meat, more than 2000 urine samples from cattle and veal calves were analysed by liquid chromatography (detection limit 1 ng ml^{-1}). Confirmations were carried out by gas chromatography–mass spectrometry. In most instances the concentration of androstenedione found was below 1 ng ml^{-1}. Androstenedione contents in muscle and plasma at levels from 0.02 to 0.72 ng g^{-1} or ng ml^{-1} have been reported, but only a few data on urinary excretion have been published. Only 35 samples of 2000 analysed showed the presence of androstenedione at levels exceeding the revealability limit with values from 5.5 to 70.6 ng ml^{-1}. Most of the results showed that amounts excreted in urine are similar to those in tissues, i.e., below 1 ng ml^{-1}. The recovery data indicate that levels of urinary excretion much above 1 ng ml^{-1} are to be considered as anomalous and need further investigation to verify their pathological or fraudulent origin.

Keywords: Gas chromatography–mass spectrometry; Anabolic steroids; Androstenedione; Cattle; Steroid hormones

Androstenedione (androst-4-ene-3,17-dione) is present at various stages of the metabolism of steroids as a natural compound [1]. In fact, it originates from C-3 oxidation of dehydroepiandrosterone (DHEA), from pregnenolone, but in a more complex way, and finally from C-17 oxidation and reduction of testosterone (17β-hydroxyandrost-4-en-3-one).

In a direct way, androstenedione is also a precursor of androsterone, estrone (obtained by aromatization) and testosterone; indirectly, it is the precursor of 17β-estradiol, obtained by the C-17 oxidation and reduction of estrone.

With regard to the anabolysing effects of the molecule, also produced by synthesis like 19-oxoandrostenedione [2], opinions diverge. Skin-

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ner et al. [3] reported an anabolysing effect, but scarcely androgenic, and suggested the possibility of use in meat production. In contrast, Johnson and Wilkinson [4] considered the anabolysing action to be moderate.

In the literature, physiological values of this hormone are mentioned because of its presence in several tissues and biological fluids (Table 1). In muscle the concentration is between 0.13 and 0.6 ng g^{-1}, whereas in fat tissue it is higher, between 2.3 and 17.5 ng g^{-1}. The values are higher in male than in female tissues, for which androstenedione represents the major androgen in both young and adult animals. In the serum of male calves aged less than 6 months, the value lies between 0.02 and 0.72 ng g^{-1}, whereas in bovine follicular liquid it can be up to 31.5 ng ml^{-1}. In cow blood plasma, in oestrus and in pregnancy, values from 0.08 to 0.1 and from 0.2 to 1.4 ng ml^{-1}, respectively, have been recorded.

In contrast, there is little information on the androstenedione content in bovine urine, and only a few data on human urine are available.

EXPERIMENTAL AND RESULTS

Origin of samples

Over a 2.5-year period (1990–92), within the plan of Coop Italia's controls preliminary to the sale of controlled bovine meats in its own supermarkets (sold under the "Prodotti con Amore" trade-mark), samplings of biological fluids were made from more than 500 calves and steers breedings (in Northern and Central Italy) on live and just slaughtered cattle. Over 2000 samples of single and group urines, rapidly frozen after sampling, were analysed using a multiple detection method. Several hormones (including androstenedione) and many β-agonists were sought.

Principle

A method for determining androstenedione in biological fluids was developed. Urine samples were hydrolysed with β-glucuronidase–aryl sulphatase and extracted with diethyl ether. After purification by means of a buffer solution, the extract was reduced to a small volume and puri-

TABLE 1

Physiological levels of androstenedione reported in bovine biological materials

Matrix [a]	Concentration (ng ml^{-1} or ng g^{-1})	Reference
Calf plasma (m) (1–190 days)	0.02–0.72	5
Cow plasma (oestrus cycle)	0.08–0.1	6
Cow plasma (pregnancy)	0.2 –1.4	7
Bovine follicular liquid	31.5	8
Cow milk	3.5	7
Calf muscle (fresh meat)	0.3 –0.6	9
Calf muscle (softened meat)	0.25–0.6	9
Calf muscle (m and f)	0.22–0.43	10
Adult cattle muscle (m and f)	0.13–0.37	10
Calf kidney (m and f)	0.53–0.78	10
Adult cattle kidney (m and f)	1.06–1.3	10
Calf fat (m)	17.5	10
Calf fat (f)	2.3	10
Adult cattle fat (m)	10.3	10
Adult cattle fat (f)	2.6	10

[a] m = Male; f = female.

fied by liquid chromatography (LC). The purified androstenedione-containing fraction was analyzed by LC on a reversed-phase column using gradient elution and UV detection. The identity of the androstenedione was confirmed by gas chromatography–mass spectrometry with ion-trap detection (GC–MS–ITD). The limit of determination was 1 ng ml^{-1}.

Reagents

Analytical-reagent grade chemicals were used (unless indicated otherwise) as follows: acetic acid, stabilized aqueous solution (Merck) of β-glucuronidase–aryl sulphatase (*Helix pomatia*), diethyl ether (peroxides-free), sodium sulphate (anhydrous) (Fluka), doubly distilled water (LC grade), isooctane (LC grade), ethanol, acetonitrile and androstenedione (analytical standard) (Sigma).

Mobile phase 1 consisted of isooctane (solution A) and isooctane–ethanol (80 + 20, v/v) (solution B). Mobile phase 2 consisted of acetonitrile (solution C) and water–acetonitrile (95 + 5, v/v) (solution D).

Sample pretreatment

Transfer into a glass or polyethylene container 25 ml of doubly distilled water and 50 ml of urine. Add acetic acid until the pH is 4.5–5 and then 50 μl of a stabilized solution of β-glucuronidase–aryl sulphatase (*Helix pomatia*) and incubate at 37°C for at least 8 h. After hydrolysis, cool to room temperature and adjust the volume to 100 ml with water.

Extraction and LC clean-up

Extract a fraction of hydrolysed solution with three aliquots of peroxide-free diethyl ether. Add

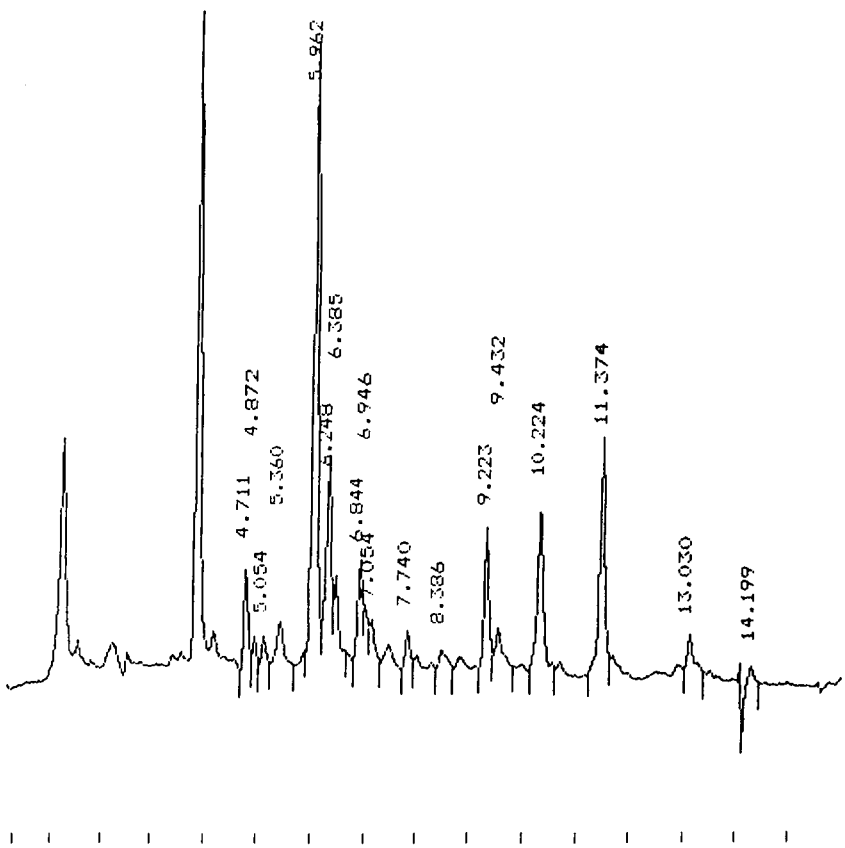

Fig. 1. LC trace of a urine sample not containing residues of androstenedione.

buffer solution (pH 10.3), shake and keep the organic phase. Repeat with an aliquot of water. Dry over sodium sulphate and concentrate to a small volume. Add an equal volume of isooctane, filter the mixture with a cellulose or PTFE filter (0.45 μm) and inject 100 μl into the LC system.

The LC apparatus consisted of a Varian 5000 liquid chromatograph, a Varian UV-100 detector and a Gilson 202 fraction collector. The column was Supelcosil LC-Diol (25 cm × 4.6 mm i.d.), film thickness = 5 μm. The flow-rate was 2 ml min^{-1} and the detection wavelength was 240 nm. Mobile phase 1 was used as follows: 90 + 10 (v/v) solvent A + solvent B from 0 to 1 min and a gradient to 50 + 50 (v/v) A + B after 20 min.

Evaporate to dryness the purified fraction corresponding to the peak of androstenedione by means a stream of nitrogen and reconstitute the residue with 100 μl of acetonitrile–water (1 + 1, v/v). Inject the purified extract into the LC system.

LC detection

A Varian 9010 liquid chromatograph with a Varian UV-100 detector was used. The column was Hypersil ODS (25 cm × 4.6 mm i.d.) with a

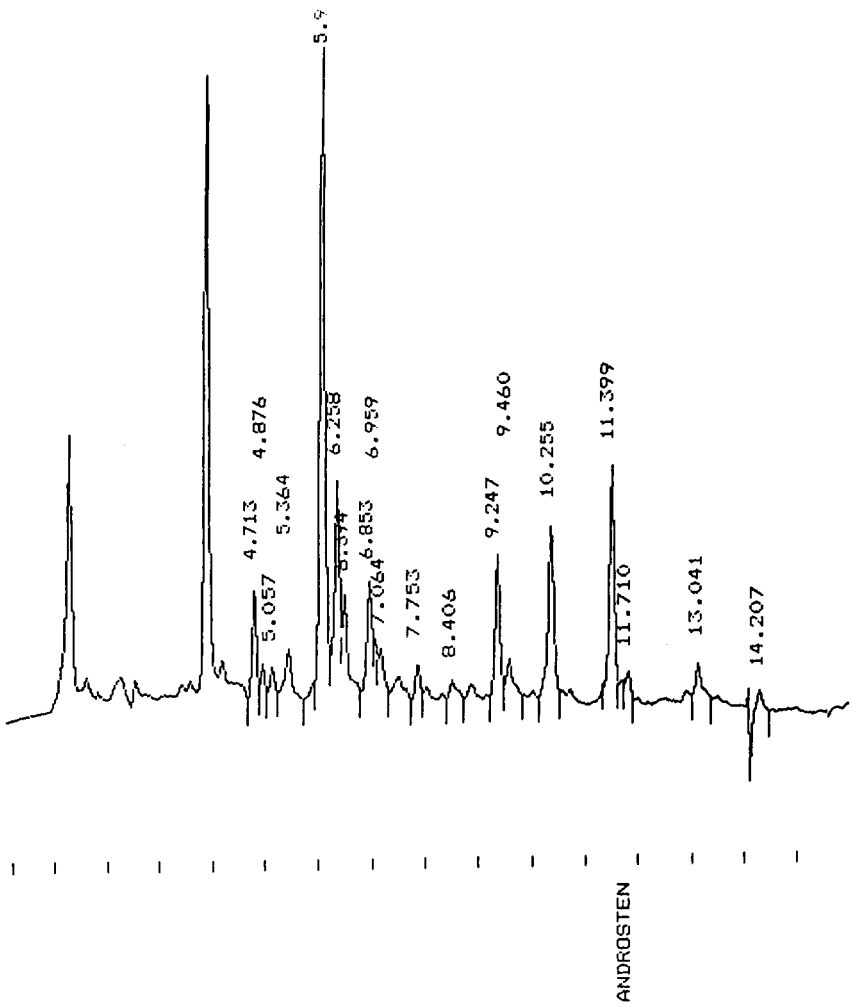

Fig. 2. Recovery test on a urine sample not containing residues of androstenedione and fortified with 1 ng ml^{-1} of standard. Recovery: > 70%.

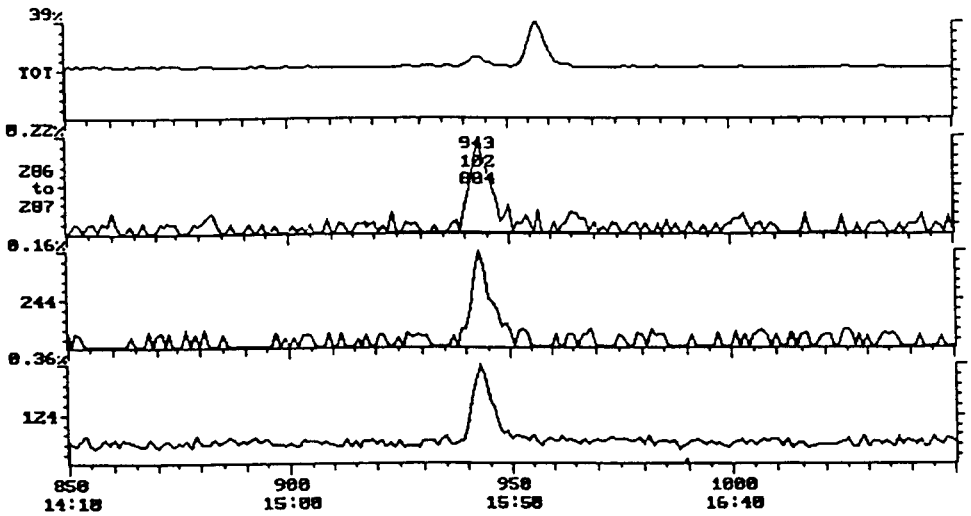

Fig. 3. GC–MS–ITD trace of a standard solution of androstenedione at 50 ng ml^{-1}.

film thickness of 5 μm. The flow-rate was 1.2 ml min^{-1} and the detection wavelength was 242 nm. Mobile phase 2 was used as folllows: 30 + 70 (v/v) solvent C + solvent D from 0 to 1 min and gradient to 70 + 30 (v/v) C + D after 13 min. The injection volume was 20 μl.

Interpolate areas of the peak at 11.7 min on a calibration graph obtained from standard solutions of androstenedione at 20, 100 and 500 ng ml^{-1}. The graph is linear from 0 to 500 ng ml^{-1} ($r = 0.99999$, $n = 3$).

Figure 1 shows the LC trace from a sample of urine without residues of androstenedione and Fig. 2 shows the trace from the same sample fortified with standard at 1 ng ml^{-1}. The recovery is > 70%.

TABLE 2

Urine samples with androstenedione values higher than 5 ng ml^{-1}

Cattle type	Time of sampling	No. of samples	No. of values higher than 5 ng ml^{-1}	Range (ng ml^{-1})
Calves	During breeding	640	21	5.8–70.6
	At slaughter	70	4	7.3–21.2
Steers	During breeding	1042	10	5.5–30.0
	At slaughter	247	–	–

GC–MS–ITD confirmation [11]

Evaporate to dryness by means of a nitrogen stream the purified fraction containing the androstenedione, reconstitute the residue with acetonitrile and inject into GC–MS–ITD system.

The apparatus consisted of a Varian 3400 gas chromatograph with a split–splitless injector, coupled to a Finnigan MAT ITD 40 mass spectrometer. The capillary GC column was Durabond-5 in fused silica (30 m × 0.25 μm i.d.). The following temperatures were applied: injector, 260°C; transfer line, 260°C; column, initially = 150°C for 3 min, then increased at 10°C min^{-1} to 260°C, which was maintained for 12 min. The carrier gas was helium, the injection volume was 2 μl and the mass range covered was 100–300.

Interpolate the peak areas on a calibration graph obtained from standard solutions of androstenedione at 50, 200 and 1000 ng ml^{-1}. The graph is linear over this range ($r = 0.9999$, $n = 3$). Figure 3 shows the trace obtained with standard solution of androstenedione at 50 ng ml^{-1}. Figure 4 shows the mass spectrum and the library search (NIST Library) of the significant peak (scan number = 950) relative to an urine sample

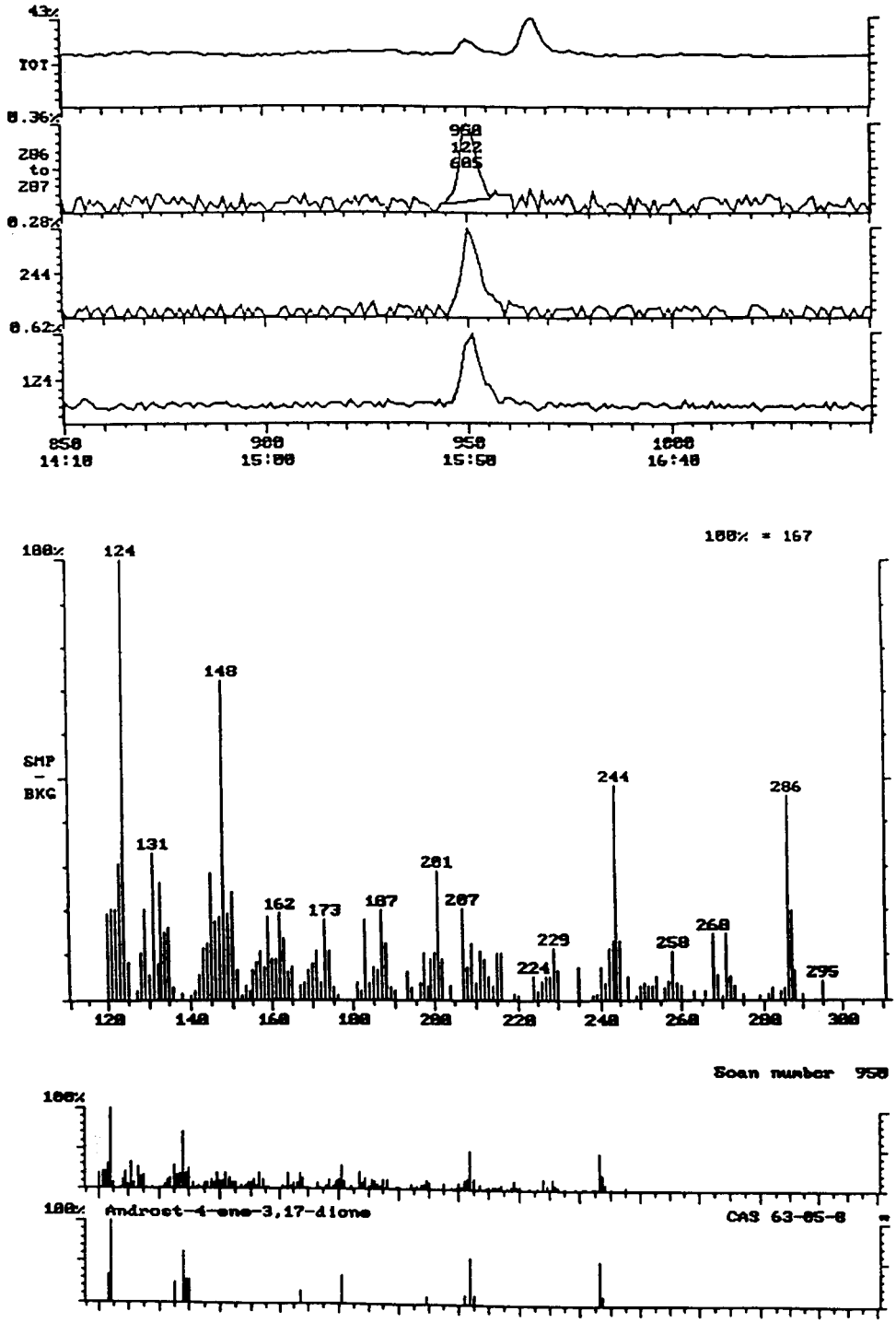

Fig. 4. Recovery test on a urine sample spiked with 1 ng ml^{-1} of androstenedione. Recovery: > 70%.

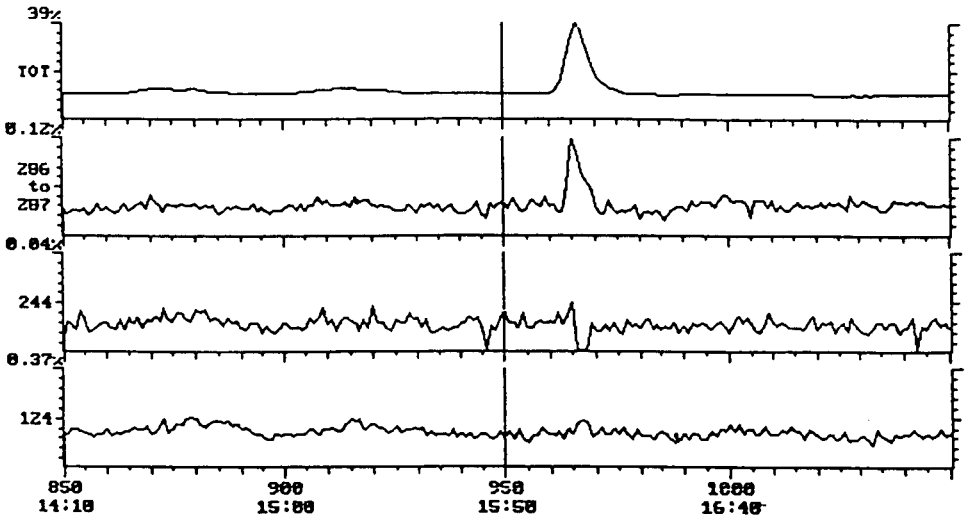

Fig. 5. GLC–MS–ITD trace of a urine sample not containing residues of androstenedione.

spiked with 1 ng ml^{-1} of androstenedione. Figure 5 shows the trace for the same urine sample which was not spiked. The recovery at 1 ng ml^{-1} is > 70%.

DISCUSSION

For most of the samples analysed (ca. 98%), under the conditions used no traces of androstenedione were found. The present results and those reported by other workers for various biological matrices suggest that the average physiological values of androstenedione excreted in the urine are similar to the levels detected in other materials, i.e., lower than 2.5 ng ml^{-1}. In only 35 cases (1.7% of the total) were values higher than that level recorded, with urine concentrations between 5.5 and 70.6 ng ml^{-1} (Table 2).

Most of the values higher than the detection limit (25 out of 35) were found in milk calves' urine, in spite of the fact that the number of samplings from these intensively bred animals was lower than that from adult cattle; only ten positive values were recorded for steers.

Thirty one out of the 1682 samplings from live animals were positive (1.8%), whereas of a total of 317 samplings carried out at slaughter only four positive samples were found (1.3%). Moreover, it must be noted that the average value recorded for slaughter-houses samples (11.4 ng ml^{-1}) is less than half that recorded for live animals (24.8 ng ml^{-1}), although because of the small number of positive samples from slaughtered-animals (only four) no conclusion can be drawn about the hormone concentration at different times.

Conclusions

The almost complete lack of information in the literature about the androstenedione content of cattle urine makes it difficult to interpret the results of this work, but further studies are in progress. It seems, however, especially considering the large number of samples analysed (over 2000), that the average physiological level of androstenedione excreted in the urine is below 2.5 ng ml^{-1}, and the small number of values that were higher (in some instances 20–25 times higher) can be considered to be anomalous. These anomalous values need to be investigated further in depth to establish whether they can be related to aspects connected with feeding, breeding techniques, endocrine pathologies or fraudulent administration during fattening.

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Matrix effects in the radioimmunoassay of estradiol and testosterone in plasma of veal calves and how to avoid them

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Abstract

Within the scope of the control of the illegal use of naturally occurring steroids in veal calves, an evaluation was made of two commercially available kits for the determination of estradiol-17β and testosterone in plasma. Four radioimmunoassay (RIA) kits, two with and two without extraction, used in human medicine were evaluated and eventually adapted for the assay of bovine plasma samples. On measuring estradiol-17β lower values were obtained when the plasma samples were assayed without extraction (^{125}I-RIA). This difference was due to a plasma matrix effect which could be avoided by using either the extraction method (^{3}H-RIA) or by dissolving estradiol standards in bovine plasma instead of human serum. This plasma matrix effect did not interfere as much when testosterone levels were measured. However, the best method for the determination of both steroids in bovine plasma was RIA with extraction (^{3}H-RIA).

Keywords: Immunoassay; Radiochemical methods; Anabolic steroids; Estradiol; Plasma; Testosterone; Veal calves

After the illegal use of stilbenes as growth promoters and their elimination by rigid law enforcement, the use of anabolic steroids as growth promoters in animal production has become common practice. The use of these products leads to a higher retention of nitrogen and a better food conversion. This is why these steroids are used among other products to improve the meat production in veal calves [1]. However, by a decision

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of the European Commission (Directive 85/358), the use of anabolic steroids is forbidden in all European Economic Community (EEC) countries. Therefore, the control of the abuse of anabolic steroids in the production of meat for human consumption is necessary. Several radioimmunoassay (RIA) methods and multi-residue methods such as thin-layer chromatography [2] and gas chromatography–mass spectrometry [3,4] have been developed for the detection of a wide range of synthetic anabolic steroids. Because of the high efficiency of these detection techniques, the use of these synthetic anabolic compounds has been greatly reduced in favour of naturally occurring steroids such as estradiol-17β (E_2-17β), testosterone (T) and progesterone [5]. To make the control of these naturally occurring steroids possible a programme was initiated within the EEC to evaluate the levels of E_2-17β and T in plasma of veal calves (ref. VI/6588/89-EN REV. 3) to determine the normal concentration ranges of these hormones.

The aim of this study was to evaluate reliable commercially available RIA methods for the determination of estradiol and testosterone in plasma of veal calves.

EXPERIMENTAL

Materials

^{125}I-RIA kits for estradiol and testosterone were obtained from Diagnostic Systems Labs. (Webster, TX) and ^{3}H-RIA kits from BioMérieux (Marcy L'Etoile, France). All these kits are commercialized for use in human medicine.

Blood samples

Blood samples from animals in different slaughterhouses were drawn into EDTA tubes and centrifuged immediately. All samples were divided in multiple small aliquots and stored at −20°C until the time of assay.

Spiked pools of plasma

A pool of blood plasma from slaughtered male calves was stripped of endogenous steroids by treatment overnight at room temperature with dextran-coated charcoal (DCC) (plasma/DCC ratio = 1:1, v/v). The suspension of DCC was prepared by mixing 0.5 g of charcoal (Norit A) with 0.05 g of dextran P 60 (both from Serva, Leuven) in 100 ml of 0.02 M phosphate buffer (pH 7.4).

Standard samples of estradiol in plasma were prepared by adding increasing amounts (final concentration 0.02–2 ng ml^{-1}) of estradiol (dissolved in ethanol) to the plasma pool treated with DCC, the final concentration of ethanol in plasma being less than 2.5%.

Standard samples for testosterone were prepared in the same way, except that the final concentrations ranged from 0.2 to 20 ng ml^{-1}. The standards prepared were divided into small aliquots and stored at −20°C until the time of assay.

Pools of plasma from slaughtered female calves and pregnant and non-pregnant cows were prepared in the same way. This means that four different pools spiked with increasing concentrations of estradiol and testosterone were made.

All samples were analysed with the ^{3}H- and ^{125}I-kits for E_2-17β and T and the results were compared. The limit of detection was defined as the concentration of estradiol or testosterone at counts per minute (cpm) value three standard deviations below the cpm value of the specific binding in the absence of standard (B_0). The limit of decision was defined as the concentration of estradiol or testosterone at a cpm value six standard deviations below the cpm value of B_0 (analytical criteria of European Commission Directive 87/410).

Radioimmunoassay kits

Estradiol ^{125}I-RIA kit. The reagents of the kit were used as prescribed by the manufacturer. The estradiol antiserum provided with the kit contains pre-precipitated rabbit anti-estradiol in a protein-stabilized buffer. Cross-reactions with other naturally occurring steroids are negligible. Volumes of 200 μl of standards and samples are needed for assay and no sample preparation is required.

Testosterone ^{125}I-RIA kit. The reagents of the kit were used as prescribed by the manufacturer.

The testosterone antiserum provided with the kit contains rabbit anti-testosterone in a protein-stabilized buffer. Cross-reactions with other naturally occurring steroids are negligible. Volumes of 50 μl of standards and samples are needed for assay and no sample preparation is required.

Estradiol ^{3}H-RIA kit. The reagents of the kit were used as prescribed by the manufacturer. The kit contains an estradiol stock standard solution of 800 ng ml^{-1} of estradiol in pure ethanol. The tracer stock solution contains about 180 mCi mg^{-1} of [6,7-^{3}H]estradiol in ethanol. Cross-reactions with other naturally occurring steroids are negligible. Separation of bound and free radioactive fractions is carried out with DCC.

For sample preparation 1 ml of plasma is extracted with 5 ml of diethyl ether by shaking the mixture on a vortex mixer for 1 min. After separation and freezing, the organic phase is decanted and evaporated. The dry extract is taken up in 500 μl of phosphate buffer and, after vortex mixing, 2 × 100 μl are used for subsequent analysis.

Testosterone ^{3}H-RIA kit. The reagents of the kit were used as prescribed by the manufacturer. The kit contains a stock standard solution of 800 ng ml^{-1} of testosterone in ethanol. The tracer stock solution contains about 180 mCi mg^{-1} of [1b,2b-^{3}H]testosterone in ethanol. Cross-reactivity with other naturally occurring steroids is negligible. Separation of bound and free radioactive fractions is carried out with DCC.

For sample preparation, 200 μl of plasma are extracted with 5 ml of diethyl ether by shaking the mixture on a vortex mixer for 1 min. After separation and freezing, the organic phase is decanted and evaporated. The ether extract is further purified by partition chromatography on a Chromatolithe A column (removal of dihydrotestosterone) as described by the manufacturer. The final extract is taken up in 500 μl of phosphate buffer and, after vortex mixing, 2 × 100 μl are used for subsequent analysis.

Standards and samples were analysed in duplicate and the assay results were corrected for the recovery (^{3}H-RIA). All freshly thawed plasma samples were thoroughly mixed before analysis.

For data reduction a computerized four-parameter logistic curve-fitting analysis was used. Additional calculations to account for the recoveries and extraction volumes (^{3}H-RIA) were done manually.

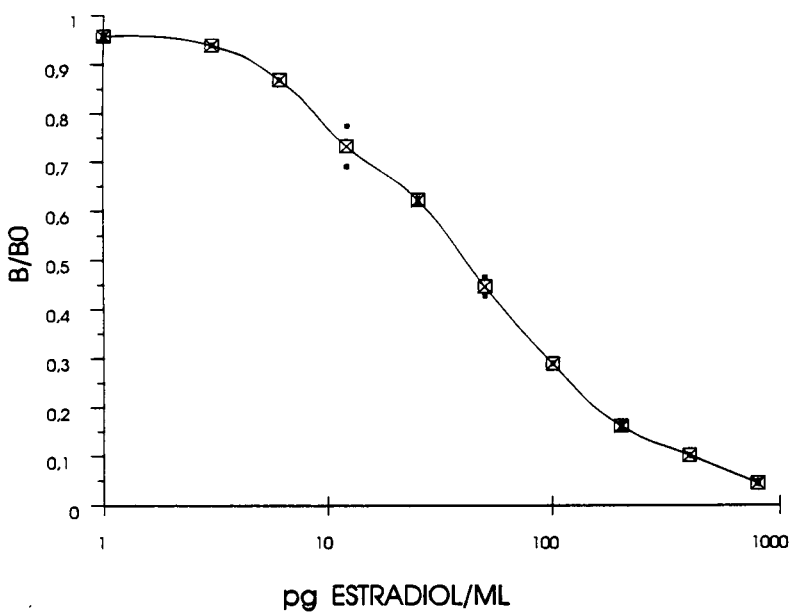

Fig. 1. Calibration graph for the commercial E_2-17β ^{3}H-RIA.

RESULTS AND DISCUSSION

Estradiol

Figures 1 and 2 show typical calibration graphs for the ^{3}H-RIA and ^{125}I-RIA, respectively. The detection limit was 3.30 pg ml^{-1} for the ^{125}I-RIA and 4.42 pg per tube (22 pg ml^{-1}) for the ^{3}H-RIA. The limit of decision was 7.52 pg ml^{-1} for the ^{125}I-RIA and 6.75 pg per tube (34 pg ml^{-1}) for the ^{3}H-RIA. The results for the spiked pools and real samples, assayed with both kits, are given in Table 1.

For the spiked pools, the ^{3}H-RIA gives a very good correlation between the measured values and the theoretical amounts whereas with the ^{125}I-RIA the measured values were always significantly lower (about 50%) than the values of the theoretical amounts. The same conclusion can be drawn for the real samples: the values measured with the ^{125}I-RIA were always significantly lower than those obtained with the ^{3}H-RIA.

These results and the possibility of measuring concentrations below the action level of 40 pg ml^{-1} (decision limit of 34 pg ml^{-1}) with the ^{3}H-RIA suggest that ^{3}H-RIA is the most reliable method for measuring estradiol in plasma of veal calves.

As the values obtained for the human serum controls (only provided with the ^{125}I-RIA kit) were in agreement with those given by the manufacturer and as the experimental results obtained for the bovine plasma samples were significantly lower than expected, the matrix effect of the bovine plasma samples was evaluated. One set of spiked samples (more precisely the standard samples made in the plasma pool of pregnant cows) was used as standard preparations (instead of the human serum kit standards) and the other three spiked pools and real samples were subsequently evaluated. The results obtained are shown in Fig. 3.

These results are in agreement with the experimental values found with the ^{3}H-RIA, which indicates a pronounced matrix effect. On the other hand, the determination of the detection limit by means of a sample blank (plasma pool pregnant cows, DCC treated) gave a value of 12 pg ml^{-1} and a limit of decision of 56 pg ml^{-1}, which is higher than the action level (40 pg ml^{-1}). This low sensitivity is also shown by the shallow

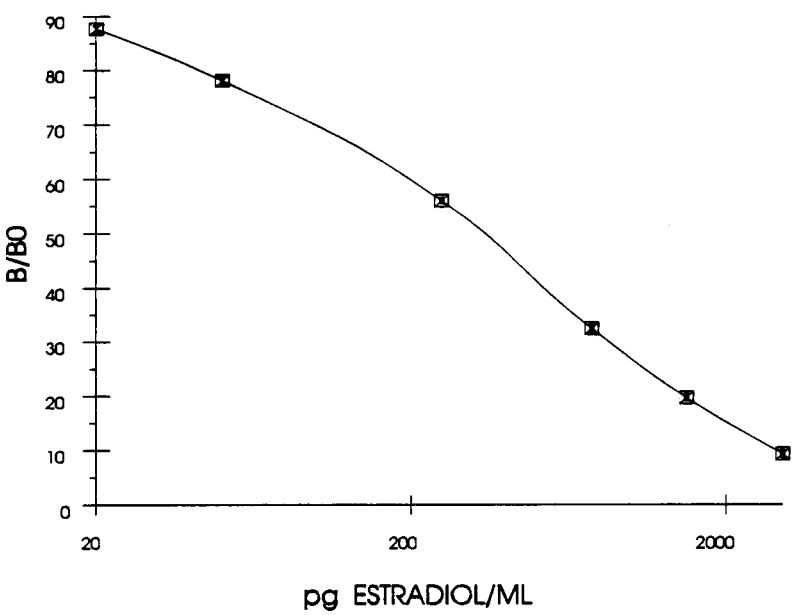

Fig. 2. Calibration graph for the commercial E_2-17β ^{125}I-RIA.

slope of the calibration graph in the low-concentration zone.

To improve the ^{125}I-RIA when used with standards in veal plasma, the tracer and antiserum concentrations provided in the kit should be optimized.

As shown in Table 2, no differences could be observed when different bovine plasma samples were used, which suggests that binding proteins probably do not interfere with the analysis.

The same experiments were carried out with the ^{3}H-RIA. The use of a spiked plasma pool as a standard set to assay the other three spiked pools and the real samples resulted in experimental values which correlated very well with those of the ^{3}H-RIA using the kit standards. As stated earlier, no matrix effect was observed here.

Testosterone

Figures 4 and 5 show typical calibration graphs for the ^{3}H-RIA and ^{125}I-RIA, respectively. The detection limit was 30 pg ml^{-1} for the ^{125}I-RIA

TABLE 1

Experimental E_2-17β concentrations in spiked plasma pools (different matrices) and real samples of veal calves assayed with commercial ^{125}I-RIA and ^{3}H-RIA kits

Samples	Concentration added (theoretical value) (ng ml^{-1})	Concentration measured (ng ml^{-1})	
		^{125}I-RIA	^{3}H-RIA
Standard samples			
Female veal	0 (sample blank)	0	0 (0.02)
calves	2	1.20	1.98
(= pool 1)	0.5	0.25	0.54
	0.05	0.002	0.05
	0.02	0	0.02
Male veal	0 (sample blank)	0	0 (0.03)
calves	2	1.13	2.71
(= pool 2)	0.5	0.25	0.50
	0.05	0	0.04
	0.02	0	0.01
Non-pregnant	0 (sample blank)	0	0 (0.03)
cows	2	1.28	2.19
(= pool 3)	0.5	0.24	0.45
	0.05	0.009	0.09
	0.02	0.004	0.01
Pregnant	0 (sample blank)	0	0 (0.03)
cows	2	1.20	2.04
(= pool 4)	0.5	0.26	0.54
	0.05	0.009	0.05
	0.02	0.005	0.02
Real samples			
1		0.15	0.42
2		0.08	0.32
3		0.02	ND [b]
4		0	0.02
5		0.70	1.32
6		0.05	0.16
7		0.047	0.13
8		0.01	0.09
9		0.27	0.57
10		ND	0.17

[a] Corrected for blank values (given in parentheses). [b] ND = not done.

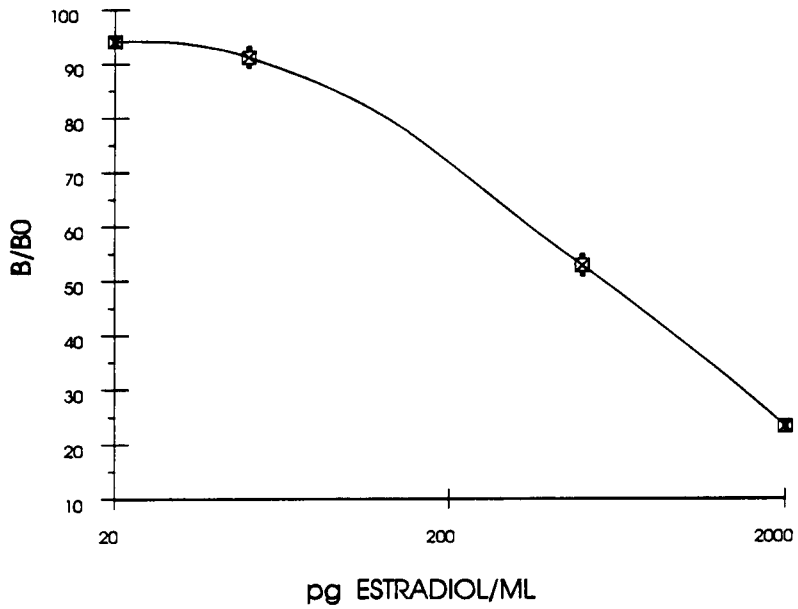

Fig. 3. E_2-17β ^{125}I-RIA calibration graph constructed using a set of spiked samples from a plasma pool of pregnant cows.

and 5.71 pg per tube (180 pg ml^{-1}) for the ^{3}H-RIA. The limits of decision were 60 pg ml^{-1} and 11.83 pg per tube (370 pg ml^{-1}) for the ^{125}I-RIA and ^{3}H-RIA, respectively. The results for the spiked samples and real samples, assayed with both kits, are given in Table 3.

For the spiked pools, on using the ^{3}H-RIA a good correlation is obtained between the meas-

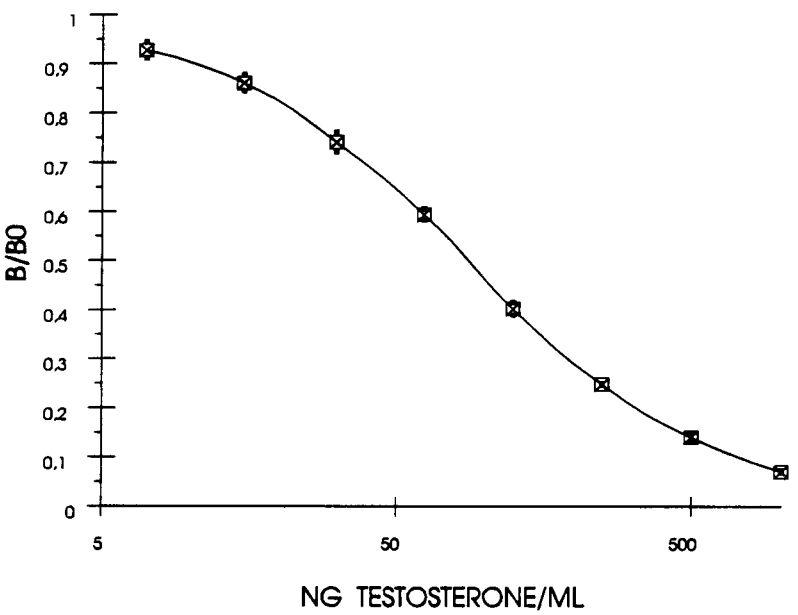

Fig. 4. Calibration graph for the ^{3}H-RIA for testosterone.

TABLE 2

Experimental E_2-17β concentrations in spiked plasma pools and real samples of veal calves assayed with the ^{125}I-RIA using the spiked plasma pool of pregnant cows as standard set[a]

Samples	Concentration added (ng ml^{-1})	Concentration measured (ng ml^{-1})		
		Pool 1	Pool 2	Pool 3
Pool 4	0	0	0	0.006
	2	2.01	1.86	2.19
	0.5	0.49	0.49	0.48
	0.05	0.01	0.005	0.05
	0.02	0.003	0	0.02
Real samples				
1		0.35		
2		0.24		
3		0.09		
4		0		
5		1.09		
6		0.19		
7		0.18		
8		0.05		
9		0.52		
10		ND [b]		

[a] Pool 1 = female calves; pool 2 = male calves; pool 3 = non-pregnant cows; pool 4 = pregnant cows. [b] ND = not done.

ured values and the theoretical amounts (however, the results obtained were slightly lower than the spiked amounts).

With the ^{125}I-RIA the measured values are lower than the values of the theoretical amounts, except for the standard samples in plasma of pregnant cows, where higher values are obtained for the two lowest concentrations (in Table 3 indicated in bold type). For the real samples, the correlation between both kits is much better than for the estradiol assays, but some samples still show discrepant values.

The values obtained for the human serum controls (only provided with the ^{125}I-RIA kit) were also in agreement with those given by the manufacturer, so the lower experimental results with the ^{125}I-RIA kit could also be explained by the differences in the matrix of human and cattle plasma. The overall effect is not as great as for estradiol, but is dependent on the matrix itself (Table 3: pregnant cow plasma pool).

As for the estradiol experiments, three of the spiked pools and the real samples were assayed against the fourth pool and, because of the greater

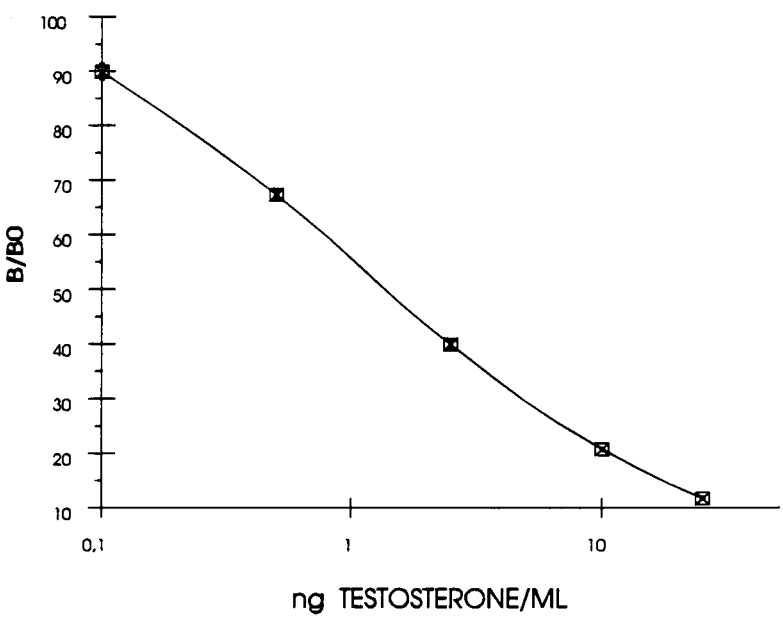

Fig. 5. Calibration graph for the ^{125}I-RIA for testosterone.

TABLE 3

Experimental testosterone concentrations in four spiked plasma pools and real samples of veal calves assayed with commercial ^{125}I-RIA and ^{3}H-RIA kits

Samples	Concentration added (theoretical value) (ng ml^{-1})	Concentration measured (ng ml^{-1})	
		^{125}I-RIA	^{3}H-RIA
Standard samples			
Female veal	0 (sample blank)	0	0 (0.32)
calves	20	15.34	18.42
(= pool 1)	10	7.93	9.07
	0.6	0.45	0.41
	0.2	0.12	0
Male veal	0 (sample blank)	0	0 (0.22)
calves	20	14.36	ND
(= pool 2)	10	6.67	8.46
	0.6	0.35	0.38
	0.2	0.11	0.09
Non-pregnant	0 (sample blank)	0	0 (0.15)
cows	20	13.78	18.89
(= pool 3)	10	7.17	8.76
	0.6	0.43	0.57
	0.2	0.12	0.09
Pregnant	0 (sample blank)	0	0 (0.12)
Cows	20	15.44	19.07
(= pool 4)	10	8.17	8.07
	0.6	**0.73**	0.48
	0.2	**0.22**	0.23
Real samples			
1		0.14	0.34
2		0.17	0.71
3		0.40	0.52
4		8.40	8.70
5		0.49	0.44
6		0.21	0.25
7		0.24	0.43
8		0.23	0.41
9		7.89	10.42
10		0.60	0.49
11		1.78	0.86
12		0.78	0.84
13		0.21	0.42
14		0.58	0.55
15		7.30	8.44

[a] Corrected for blank values (given in parentheses).

matrix effect of pregnant cow plasma, this experiment was repeated with the spiked plasma pool of female calves. Table 4 gives the measured values for the standard and the real samples against the two sets of standard samples. Samples assayed against pool 1 all show higher values compared with the results obtained with pool 4 as a standard set. Hence for the ^{125}I-RIA for testosterone there is a clear difference among the plasma matrices used as standards.

The same experiments were carried out with the ^{3}H-RIA. The use of a spiked plasma pool as a standard set to assay the other three spiked pools and the real samples resulted in experimen-

TABLE 4

Experimental testosterone concentrations in spiked plasma pools and real samples of veal calves assayed with the ^{125}I-RIA using another spiked plasma pool as standard set

Samples	Concentration added (ng ml^{-1})	Concentration measured (ng ml^{-1})		
		Pool 1	Pool 2	Pool 3
Plasma pool of pregnant cows (= pool 4)	0	0	0	0
	20	19.86	18.56	17.78
	10	9.68	7.92	8.60
	0.6	0.37	0.29	0.35
	0.2	0.13	0.12	0.13
		Pool 4	Pool 2	Pool 3
Plasma pool of female calves (= pool 1)	0	0	0	0
	20	> 20	18.70	17.91
	10	10.31	8.37	9.00
	0.6	0.94	0.47	0.57
	0.2	0.32	0.18	0.19
		Against pool 4	Against pool 1	
Real samples				
1		0.15	0.23	
2		0.17	0.26	
3		0.33	0.54	
4		10.32	10.61	
5		0.40	0.65	
6		0.19	0.31	
7		0.21	0.34	
8		0.20	0.33	
9		9.61	9.94	
10		0.49	0.78	
11		1.65	2.25	
12		0.64	1.00	
13		0.19	0.31	
14		0.47	0.76	
15		8.78	9.16	

tal values that correlated very well with those of the ^{3}H-RIA using the kit standards. As was observed for the estradiol ^{3}H-RIA, no matrix effect could be detected.

Conclusions

This study has shown that it is possible to apply commercially available RIA kits for human diagnostic purposes to plasma samples of veal calves.

For estradiol, the ^{3}H-RIA kit tested (with sample extraction and standards in buffered medium) showed very good recoveries for the spiked samples, whereas for the commercial ^{125}I-RIA kit (no sample extraction and human serum standards) the same results could only be obtained when using a calibration graph constructed for plasma of veal calves. Because of the resulting low sensitivity, tracer and antiserum concentrations of the latter kit have to be optimized.

For testosterone, the correlation between the two kits is better, although an effect of binding proteins is observed on the spiked samples with the ^{125}I-RIA, as shown by the kind of plasma matrix used.

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Detection and identification of anabolic steroids in bovine urine by gas chromatography–mass spectrometry

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Abstract

Within the scope of the national plan for hormone control in France, a study was made to develop a system for the control of the illegal use of anabolic steroids for fattening purposes in animal production. A screening procedure for these residues in bovine urine was developed, based on C_{18} solid-phase extraction, silica gel purification and gas chromatographic–mass spectrometric analysis with selected ion monitoring. All the method parameters are discussed, especially the enzymatic and chemical hydrolysis steps and the choice of derivatives.

Keywords: Gas chromatography–mass spectrometry; Anabolic steroids; Bovine urine; Hormones; Urine

Steroids with anabolic properties are applied in both humans and animals to augment muscle size and strength. Many experimental studies on treated cattle have shown an increase in body weight and protein deposition and a decrease in fat deposition. Because of the theoretical risk to public health, the use of anabolic steroids has been prohibited in the member states of the European Economic Community (EEC) since 1988 (EC directive 81/602) [1]. However, controls performed in slaughterhouses and farms by immunoassay (IA) on urine or by high-performance thin-layer chromatography on injection site samples revealed that some of these promoting agents were still being illegally used. Consequently, each country has to develop a programme including efficient analytical methods in order to detect residues of anabolic steroids.

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IA can provide an effective means of screening numerous samples. However, as it is required to screen for many parent steroids and their metabolites, this kind of technique appears to be inadequate. The introduction of glass capillary columns with superior resolving power combined with quadrupole mass spectrometers [2,3] and more sophisticated data systems now makes it possible to perform large-scale steroid analyses under routine conditions with an increase in detection sensitivity and specificity.

A few workers have presented gas chromatographic–mass spectrometric (GC–MS) methods for the routine screening of anabolic steroids in humans [4–7] and horses [8–10], but no effective multi-residue methods for meat-producing animals. The aims of this paper are to present a general analytical approach to isolate and detect these anabolic compounds in urine, to explain the choice of the method parameters and to show that a low-cost electron impact (EI) MS detector is adequate for screening for anabolic steroids in bovine urine.

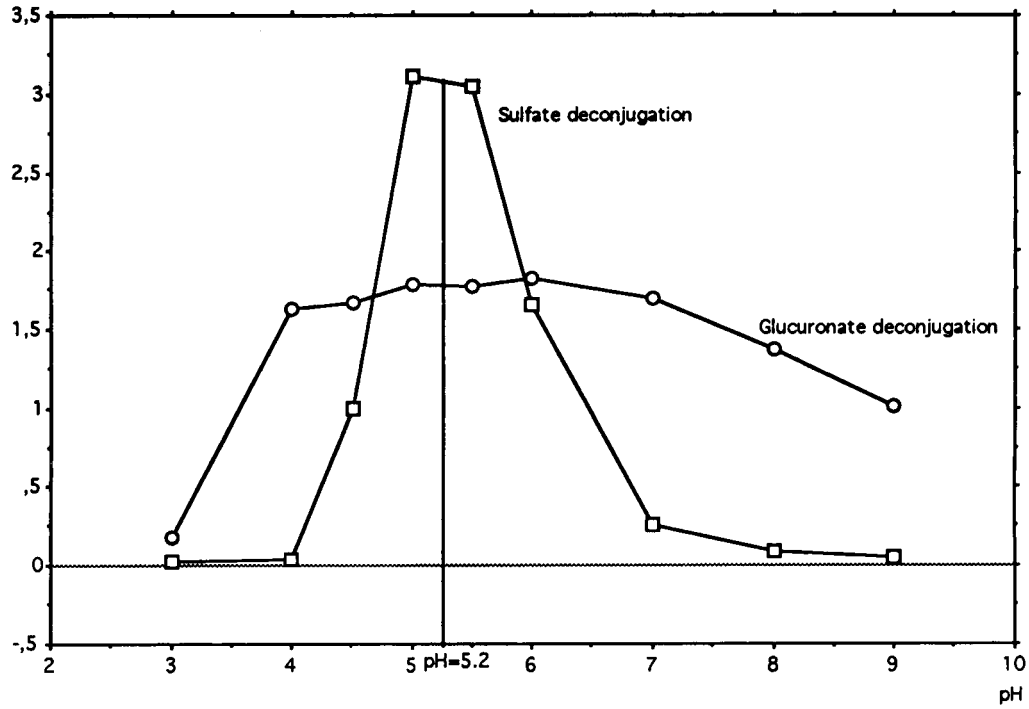

Fig. 1. Study of optimum pH for deconjugation of steroid sulphate and glucuronate. □ = Testosterone 17β-sulphate; ○ = nandrolone 17β-glucuronate. Along the *y*-axis, [deconjugated molecule]/[internal standard] is given.

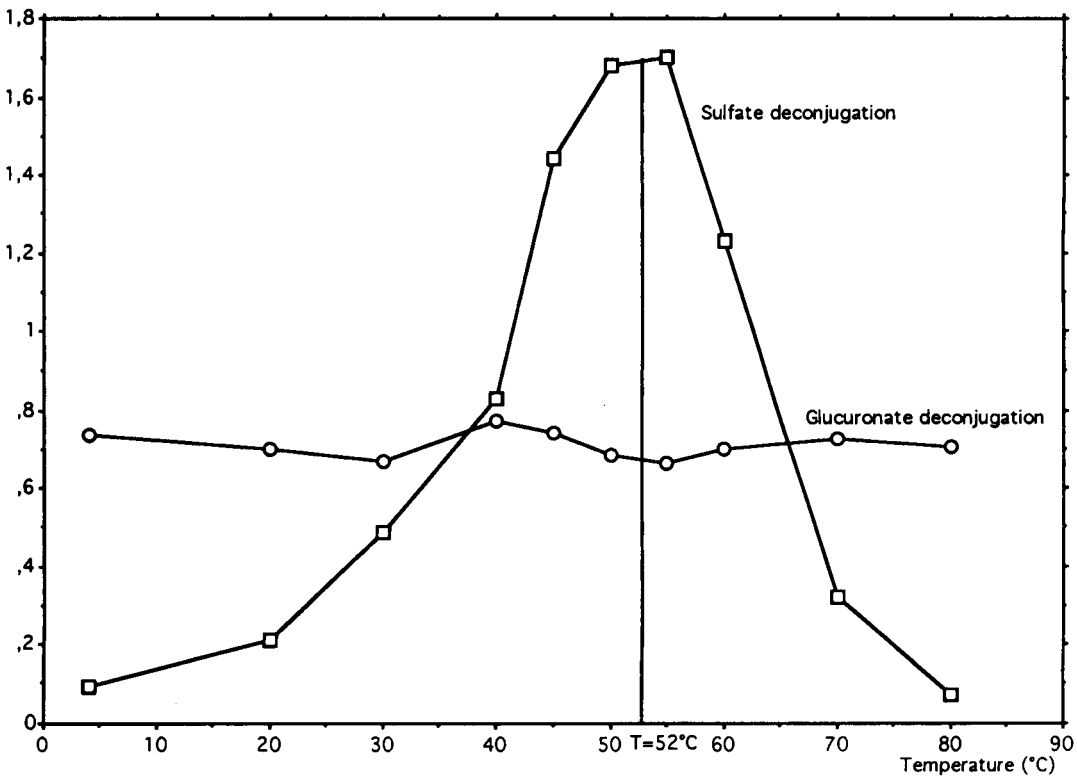

Fig. 2. Study of the optimum temperature for deconjugation of steroid sulphate and glucuronate. ○ = Nandrolone 17β-glucuronate; □ = testosterone 17β-sulphate. Along the *y*-axis, [deconjugated molecule]/[internal standard] is given.

EXPERIMENTAL

Chemicals

All the solvents were of analytical-reagent grade (Merck, Darmstadt, Germany). Dry pyridine was stored over molecular sieve 5 Å. Water was purified (> 14 MΩ cm). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), trimethyliodosilane (TMIS) and *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) + 1% of *tert*-butyldimethylchlorosilane (TBDMSCl) were purchased from Fluka (Buchs, Switzerland). Methyloxime (2%) in dry pyridine (MO) was obtained from Pierce (Rockford, IL). The steroids were obtained from Steraloids (Wilton, NH), Sigma (St. Louis, MO) and Research Plus (Bayonne, NJ). Deuterated methyltestosterone was supplied by Sigma and dichlorophene by Aldrich (Milwaukee, WI). All the solutions were kept refrigerated in the dark until used. Silica gel 60 and *Helix pomatia* digestive juice were obtained from Merck and Mega Bond Elut C_{18} (2 g) from Varian (Les Ulis, France). Glucuronidase (bovine liver) was from Sigma.

Extraction of urine

After centrifugation, urine (20 ml) was adjusted to pH 5.2 with acetate buffer (2 M; pH 5.2) and hydrolysed overnight (15 h) at 52°C with 100 μl of *Helix pomatia*. After addition of 100 ng of methyltestosterone-d_3, the hydrolysed urine was passed down an activated C_{18} cartridge (previously conditioned with 10 ml of methanol and 10 ml of ultrapure water). A 10-ml volume of water was used to eliminate most of the water-soluble urinary compounds. After washing with 10 ml of hexane, the steroids were eluted with 5 ml of ethyl acetate–methanol–sulphuric acid (100 : 40 : 0.5, v/v/v) and incubated for 30 min at 50°C directly in the solvolysis mixture [8,11]. The hydrolysate was cooled to room temperature and washed twice with carbonate sodium [10% (w/v)

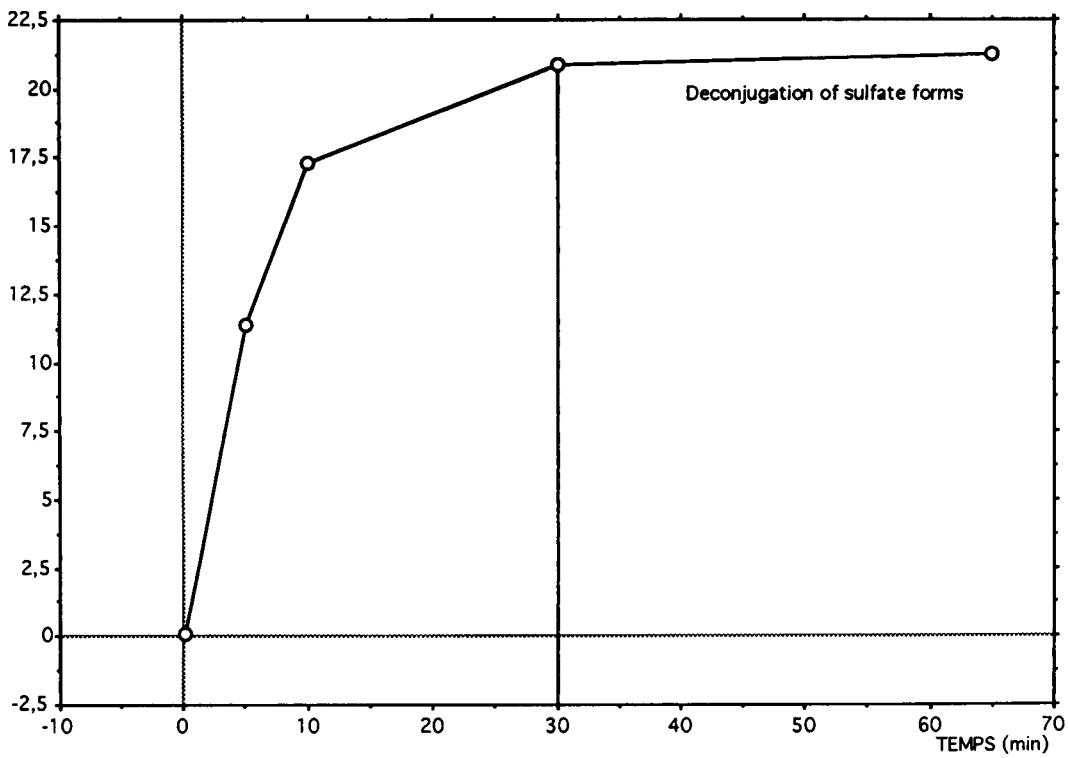

Fig. 3. Solvolysis kinetic of testosterone 17β-sulphate.

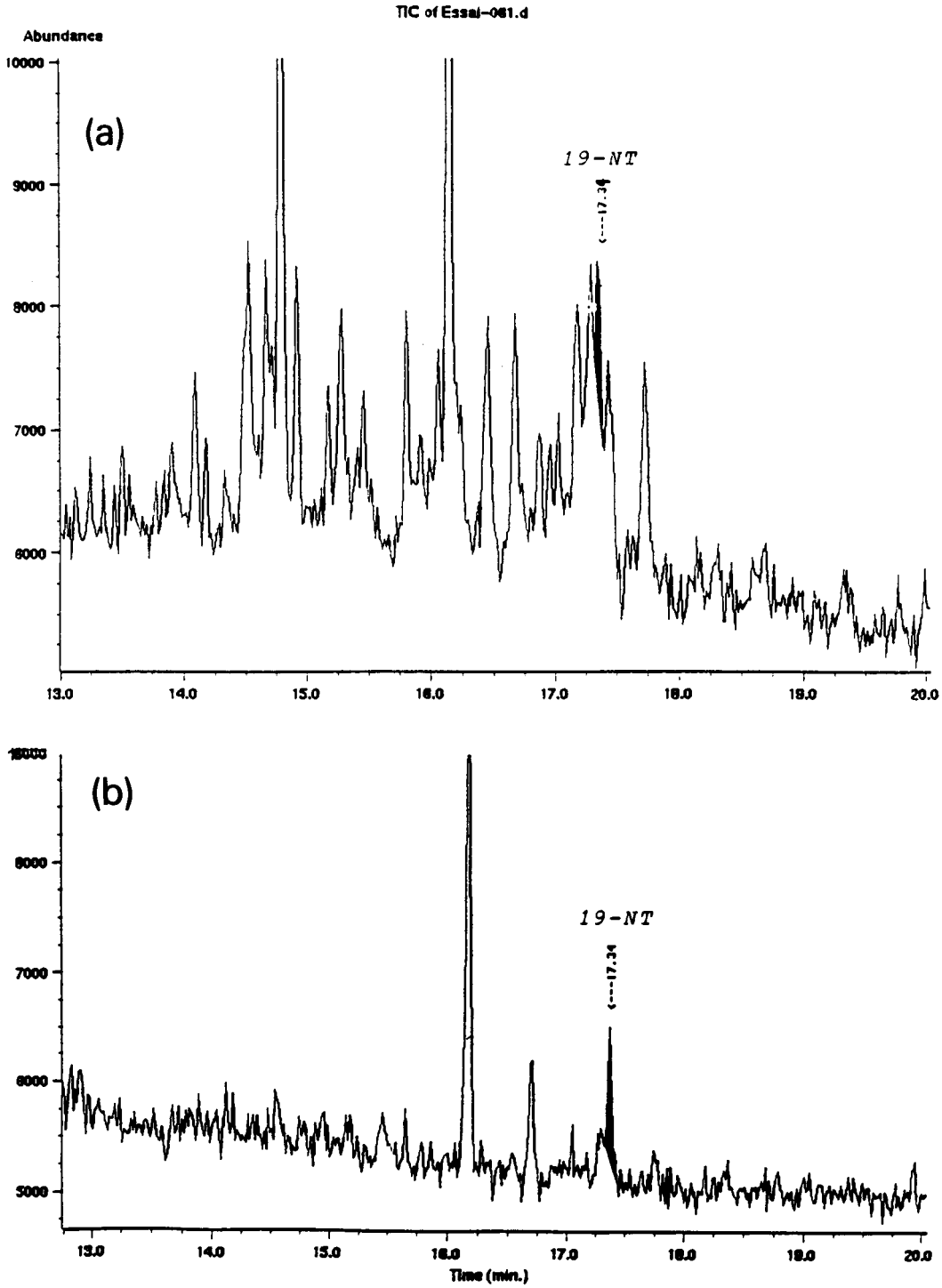

Fig. 4. Comparison between (top) C_{18} SPE alone and (bottom) C_{18} SPE combined with an alkaline liquid–liquid extraction and silica gel 60 purification, for a urine sample spiked with 0.5 $\mu g\ l^{-1}$ of nandrolone.

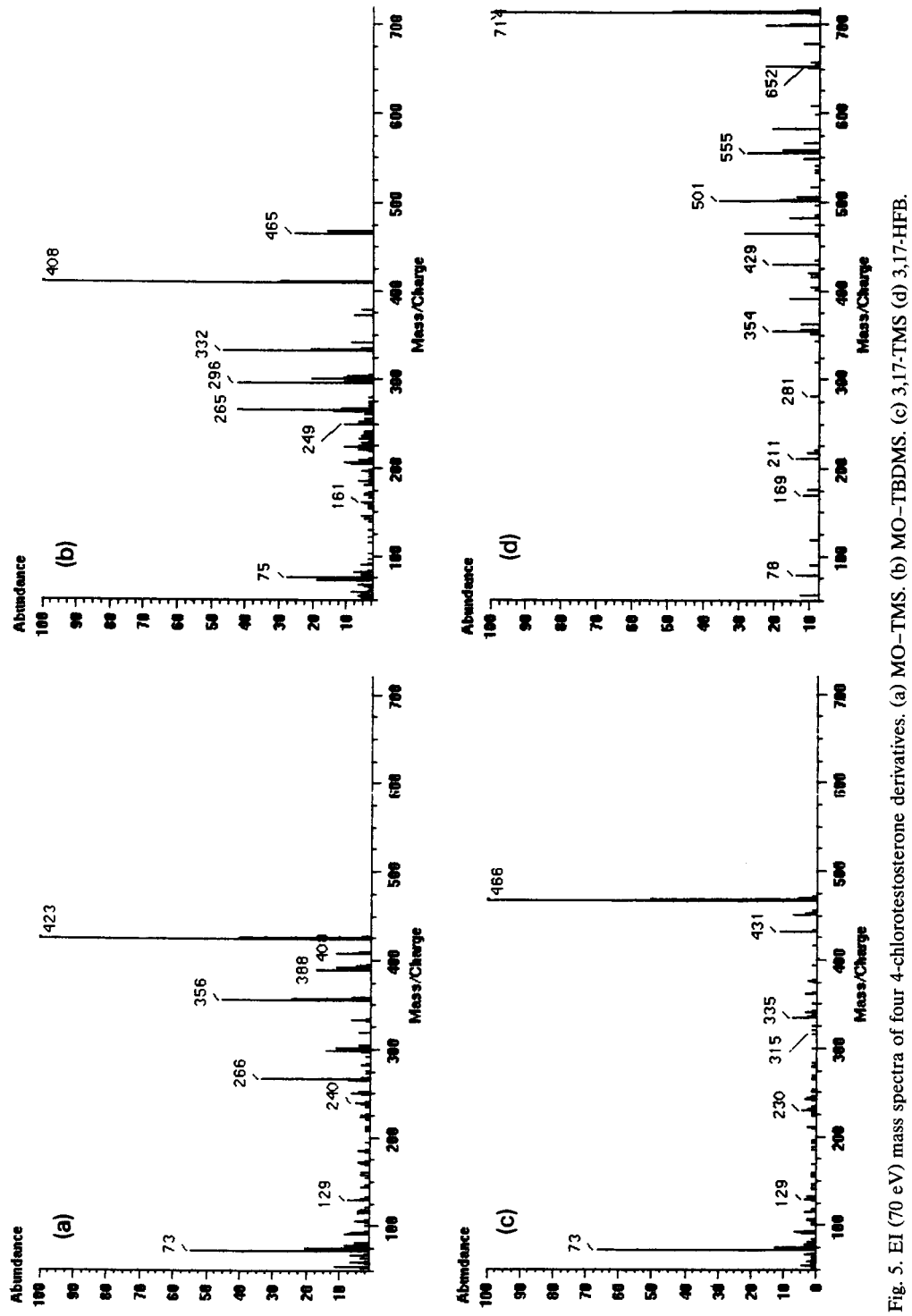

Fig. 5. EI (70 eV) mass spectra of four 4-chlorotestosterone derivatives. (a) MO–TMS. (b) MO–TBDMS. (c) 3,17-TMS (d) 3,17-HFB.

in water]. The organic phase was dried over anhydrous sodium sulphate and evaporated to dryness. The residue was then dissolved in 1 ml of toluene–ethyl acetate (EtOAc) (85:15, v/v) and passed through a silica gel 60 column previously conditioned in a glass column with the same solvent (gel bed height = 8 cm, i.d. = 1 cm).

The first 4 ml of toluene–EtOAc (85:15, v/v) were discarded and the following 10 ml eluted stilbenes and estradiol-like compounds (Fraction I). A 100-ng amount of dichlorophene was added as external standard.

A 12-ml volume of toluene–EtOAc (50:50, v/v) eluted androgens and gestagens (Fraction II). Each fraction was evaporated to dryness and the residue was dissolved in CH_2Cl_2 and transferred into a 1-ml conical vial. The solvent was removed under a stream of nitrogen at 40°C and the residue was derivatized.

Preparation of derivatives

Trimethylsilyl ether derivatives. These derivatives are suitable for the GC–MS screening of steroids containing only hydroxyl functional groups (e.g., stilbenes, estradiol-like compounds). The dry residue (Fraction I) was treated with 20 μl of MSTFA and heated at 60°C for 30 min [12,13].

Trimethylsilyl enol–trimethylsilyl ether derivatives. Androgens and gestagens (Fraction II), which bear enolizable keto and hydroxyl groups, were derivatized with 20 μl of MSTFA–TMIS (1000:5, v/v) for 30 min at 60°C.

MO–trimethylsilyl ether derivatives. A 30-μl volume of methoxylamine hydrochloride (2%) in dry pyridine was added to the androgen–gestagen compounds and heated for 30 min at 60°C. The residue was dried and 20 μl of MSTFA–TMIS (1000:5, v/v) were added.

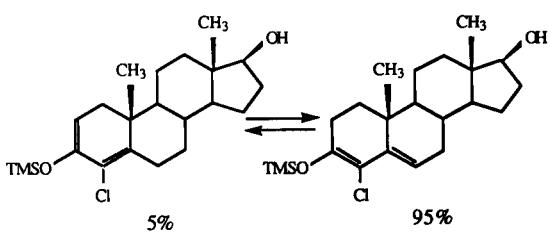

Fig. 6. Tautomeric forms of the TMS-enol–TMS-ether derivative of 4-chlorotestosterone.

TABLE 1

4-Chlorotestosterone derivatives compared in terms of sensitivity (signal-to-noise ratio for high-mass ions) and specificity (number of high-mass ions) [a]

Parameter	3,17-TMS	3-MO--17-TMS	3-MO--17-TBDMS	3,17-HFB
Sensitivity	++++	++	+	++
Specificity	++	++++	+++	++

[a] ++++ = Excellent; +++ = good; ++ = passable; + = bad.

TABLE 2

List of steroids screened with their respective diagnostic ions (in italics) and retention times

Molecule [a]	Diagnostic ions (*m*/*z*)	Retention time (min)
cis-DES	*412*/397/*383*/368	11.63
Dichlorophene (I.S.)	412/377	12.07
Hexestrol	414/*207*/206/191	12.33
Dienestrol	410/395/381/245	12.38
trans-DES	*412*/397/*383*/368	12.46
α-Nandrolone	*418*/403/287/*194*/182	14.75
β-Nandrolone	*418*/403/287/*194*/182	15.20
α-Trenbolone	*414*/399/192	15.37
Stanolone	*434*/419/*405*/202	15.39
Boldenone	*430*/415/325/299/*206*	15.49
β-Estradiol-d_2 (I.S.)	418/287	15.50
β-Estradiol	*416*/401/298/*285*	15.52
β-Testosterone	432/417/301/207	15.63
β-Trenbolone	*414*/399/192	15.82
Norethandrone	*432*/417/342/*287*	16.21
Dianabol	*444*/339/299/283/*206*	16.50
Methyltestosterone-d_3 (I.S.)	449/301	16.63
Methyltestosterone	*446*/431/356/*301*	16.66
Ethynylestradiol	*440*/425/300/*285*	16.67
Bolasterone	460/445/355/315	16.72
Ethynyltestosterone	*456*/441/316/*301*	16.90
Zeranol	*538*/523/*433*/335/307	16.97
Norgestrel (E.S.)	456/316	17.43
α-Chlorotestosterone	468/*466*/451/*431*	17.50
Norethandrolone	*446*/431/356/*287*	17.57
β-Chlorotestosterone	468/*466*/451/*431*	18.25
Fluoxymesterone	*552*/462/*407*/319	18.43
Bromoestradiol	*496*/494/481/*365*	18.90
Chloromethandienone	482/*480*/465/390/*335*	19.20
Medroxyprogesterone	560/330/315	19.41

[a] DES = diethylstilbestrol; I.S. = internal standard; E.S. = external standard.

tert-Butyldimethylsilyl ether derivatives. A 15-μl volume of MTBSTFA + 1% TBDMSCl and 15 μl of dry pyridine were added to the stilbenes and estradiol-like compounds. The solution was heated for 1 h at 70°C.

A 2-μl volume of each derivative was injected for GC–MS analysis.

Gas chromatography–mass spectrometry

Samples were analysed using an HP-5971 mass-selective detector (Hewlett-Packard, Palo Alto, CA) interfaced to an HP 5890A gas chromatograph equipped with an HP 7673 autosampler and an OV-1 fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). EI ionization was applied at 70 eV. Injections were made in the splitless mode (1-min delay) using helium as carrier gas (1 ml min^{-1}). The column temperature was maintained at 120°C for 1 min, then programmed to 250°C at 15°C min^{-1} and to 300°C at 5°C min^{-1} and maintained at 300°C for 4 min. The transfer line and injector were set at 280 and 250°C, respectively. The GC–MS system was controlled by a Hewlett-Packard 7.0 Unix station. Acquisition was performed in the selected ion monitoring (SIM) mode with 2–3 ions per molecule for screening purposes.

RESULTS AND DISCUSSION

Hydrolysis steps and purification

Most steroid metabolites in bovine urine are conjugated with either glucuronic or sulphuric acid but few steroids remain unchanged. This is the reason why the hydrolysis must be efficient in order to recover virtually all of the metabolites. The most commonly used method is enzymatic

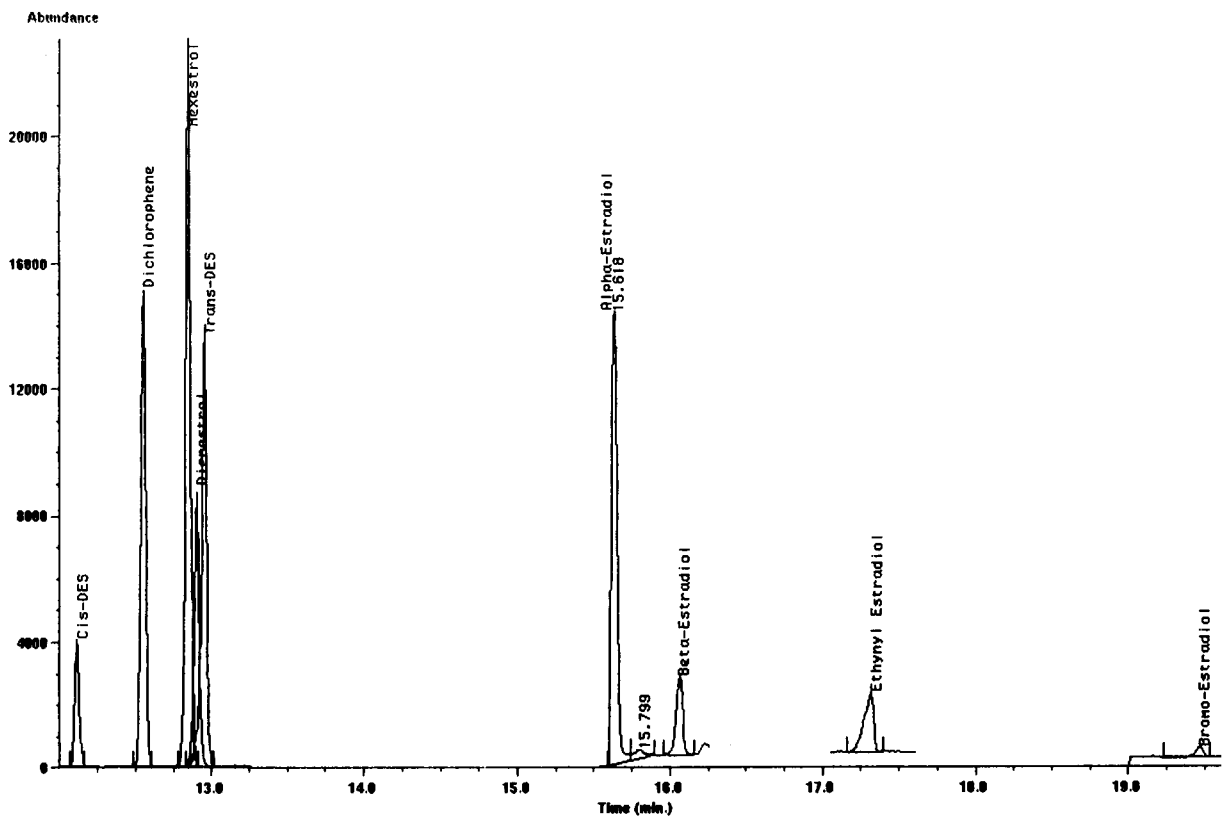

Fig. 7. Results for a 2 $\mu g\ l^{-1}$ urine extract showing stilbenes and estradiol-like compounds in their TMS ether forms.

hydrolysis with the digestive juice of *Helix pomatia*, which contains β-glucuronidase and arylsulphatase. The respective theoretical enzyme pH and temperature cannot be followed in practice. Figures 1 and 2 show the results of the study to establish the optimum pH and temperature for the deconjugation of testosterone 17β-sulphate and nandrolone 17β-glucuronate by *Helix pomatia*. It is clear that the two enzymes do not have the same efficiency.

For glucuronate forms, the glucuronidase is very active whatever the temperature (in the range 4–80°C) and the pH (from 4 to 7). The release of the free forms is complete and very rapid (a few minutes).

This experiment also shows the difficulty of hydrolysing the 17β-sulphate forms with sulphatase. The action of this enzyme is very sensitive to pH and temperature. The optimum values were pH 5.2 and 52°C. A difference of 10°C or of 0.5 pH unit can lead to a loss of 50% of deconjugation for the same hydrolysis time. Nevertheless, even when testosterone 17β-sulphate was hydrolysed under the optimum conditions, the recovery even after 15 h of hydrolysis did not give 100% of free testosterone. This is the reason why chemical hydrolysis appears to be necessary to release the remaining sulphate forms. However, the conjugated and unconjugated metabolites must first be extracted from urine using C_{18} Mega Bond Elut (2 g) cartridges. The modified silica is able to adsorb both free and conjugated forms from urine. According to Teale and Houghton [8], elution directly with ethyl acetate–methanol–sulphuric acid gains time and minimizes losses during evaporation and transfers.

Figure 3 shows the solvolysis kinetics of testosterone 17β-sulphate. We obtained almost the

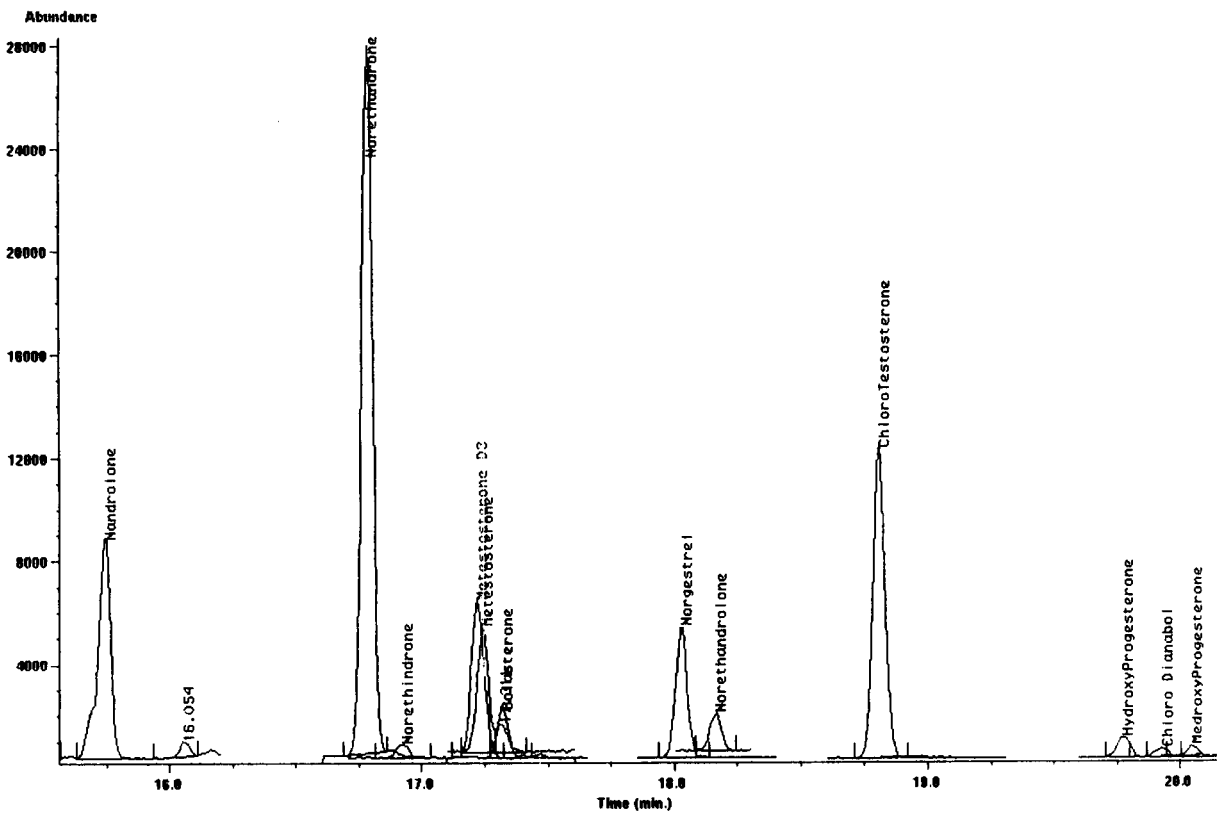

Fig. 8. Results for a 2 $\mu g\ l^{-1}$ urine extract showing androgen and gestagen compounds in their TMS-enol–TMS-ether forms.

same results as Tang and Crone [14]; in 30 min at 50°C, 80% of free testosterone was released but no free nandrolone. It seems that methanolysis [14,15] could provide a means of hydrolysing completely both steroid sulphate and glucuronide conjugates. This method appears to be inexpensive, rapid and efficient, but perhaps slightly drastic.

The combination of alkaline liquid–liquid extraction and silica gel 60 purification gave good results. A urine sample containing nandrolone at 0.5 $\mu g\ l^{-1}$ was extracted first by C_{18} solid-phase extraction (SPE) and second by C_{18} SPE combined with liquid–liquid extraction and silica purification. The comparison of the two molecular ion chromatograms shows the efficiency of and the necessity for the complete method (Fig. 4). Also, with "dirty" urine samples, we took advantage of the phenolic hydroxyl group of stilbenes and phenolic steroids and their pH-dependent partitioning between aqueous and organic phases.

Derivatization of the steroids

Derivatization reagents were chosen in order to enhance the formation of derivatives that would give high-mass ions of good intensity under EI ionization conditions. The reason is that screening methods are aimed at avoiding false-negative results although minimizing false-positive samples. 4-Chlorotestosterone derivatives were compared in terms of sensitivity and specificity (Fig. 5 and Table 1). For screening purposes, one high-mass-sensitive ion is ideal. The TMS-enol–TMS-ether of chlorotestosterone was the derivative that was least fragmented because of the stabilization of the molecular ion. Consequently, the total ion current is limited in some ions, necessarily very sensitive.

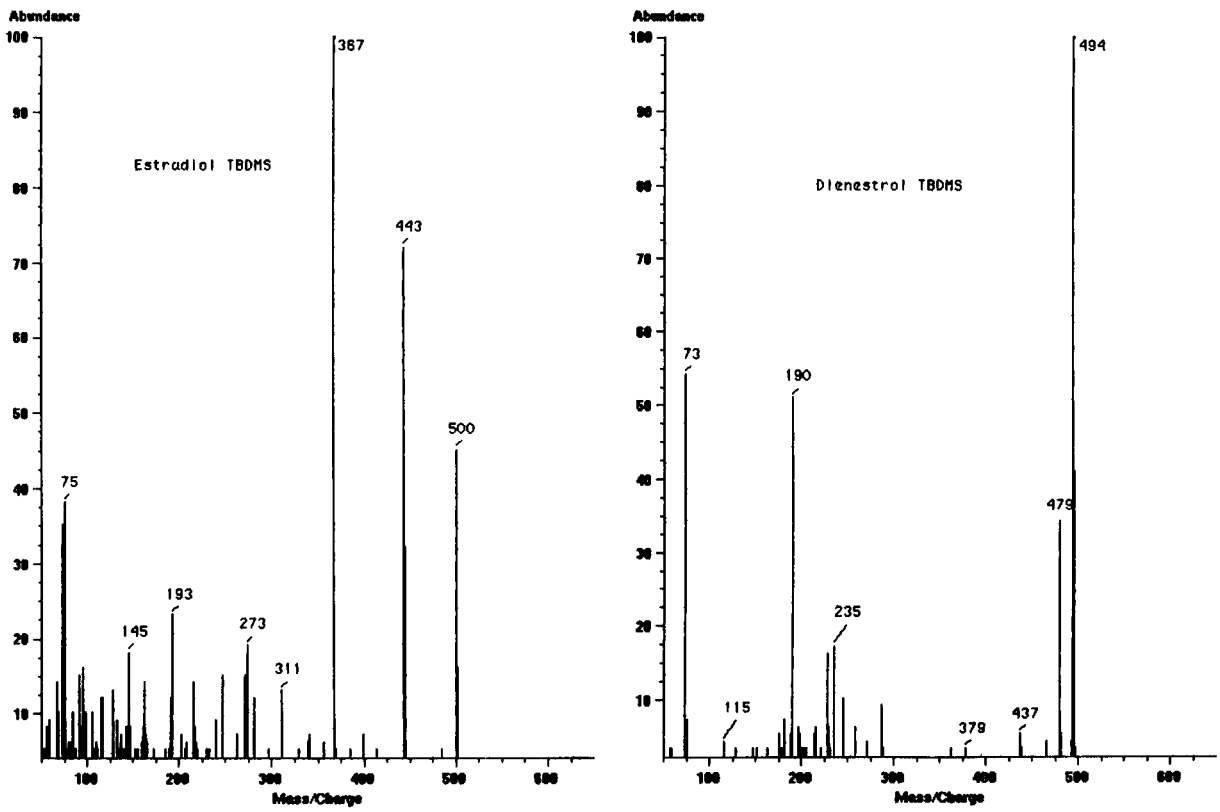

Fig. 9. EI (70 eV) mass spectra of TBDMS derivatives of (left) estradiol and (right) dienestrol.

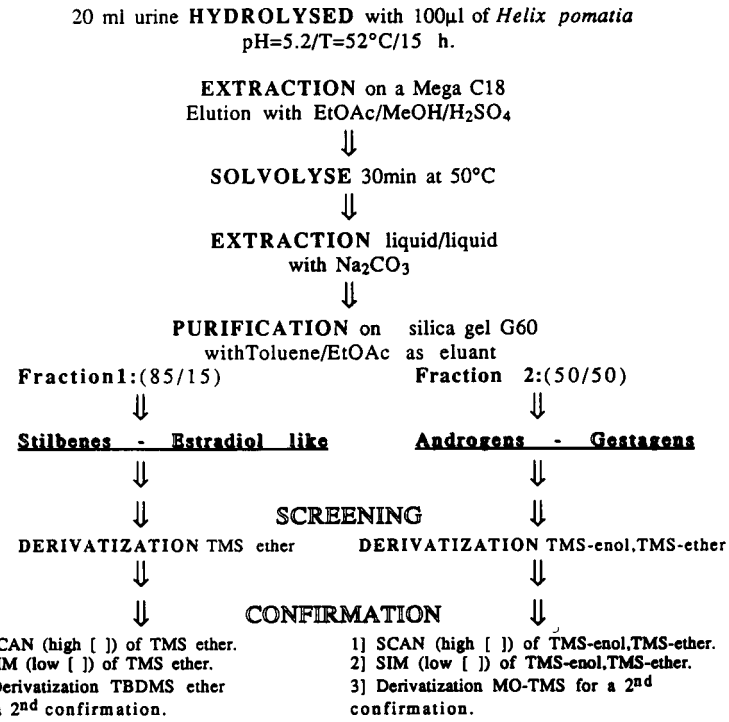

Fig. 10. General scheme of the method. [ ] = concentration.

Generally, the molecular ion is the base peak in the spectrum and the $[M - Me]^+$ and $[M - TMSOH]^{+\cdot}$ ions often give prominent ions in addition to other fragmentations concerning the B ring (fissions of both the 9,10- and 6,7-bonds or both the 9,10- and 7,8-bonds) of the D ring (fissions of both the 13,17- and 14,15-bonds or both the 13,17- and the 15,16-bonds).

To synthesize these derivatives, a mixture of MSTFA as silylating agent and TMIS as catalyst was used to promote the regioselective formation of the TMS-enol–TMS-ether of ketonic groups and the formation of the TMS ether of hydroxyl functions even at hindered positions. Massé et al. [7] described the mechanism of this reaction accurately.

Although different tautomeric forms are possible, only one is predominantly formed, except for rare anabolic steroids [16] (Fig. 6).

It was found that TMS-enol derivatives were sensitive to hydrolysis and several precautions must be taken. The derivatized extract must be kept at 4°C in a hermetically sealed vial before injection; under these conditions no degradation was observed during 1 week. Moreover, it was observed, as elsewhere [7], that repeated injections can lead to washing out of the column. Consequently, some active sites appeared and hence other anabolic compounds such as β-agonistic drugs, which bear a secondary amino group, can form bonds with these sites; the chromatogram shows extensive peak tailing. To resolve this problem, two solutions are available: either to cut off the first few centimetres of the column or to inject MSTFA in order to deactivate the adsorption sites.

Analysis of urine samples

In this laboratory, the method described is used for screening purposes. A list of some of the steroids screened together with the respective ions for multi-screening is given in Table 2. Examples of results for a 2 $\mu g\ l^{-1}$ urine extract are given in Figs. 7 and 8 for the stilbenes and

estradiol-like compounds and for the androgen and gestagen molecules, respectively.

If an anabolic steroid is detected by this procedure, its presence can be confirmed. The confirmation is based on the full mass spectrum of the suspected molecule when its concentration is sufficient. For low concentrations, injection in the SIM mode with at least four diagnostic ions with their respective $m/z-1$ and $m/z+1$ ions is performed while respecting the criteria of EC directive 87/410 [17]. When another confirmation is required or when not enough characteristic ions are obtained with the TMS derivatives, a new extraction is performed using another derivatization procedure (Fig. 9). For the stilbenes and estradiol-like compounds, a TBDMS derivative is formed. The EI mass spectrum shows several diagnostic ions, notably corresponding to $M^{+\cdot}$, $[M-Me]^+$ or $[M-tBu]^+$. For the androgen and gestagen molecules, MO–TMS derivatization provides good specificity with numerous characteristic ions at high mass even if the sensitivity is lower.

The general scheme of the method is shown in Fig. 10. The procedure has been tested in this laboratory for 1 year and has been found to be efficient in the control of anabolic steroids in meat-producing animals.

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Determination of 19-nortestosterone in finnish boar plasma and urine samples

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Abstract

Urine and plasma samples of non-castrated, cryptorchid and castrated boars were analysed in order to establish the endogenous concentration of 19-nortestosterone. A combination of three different analytical techniques was used in this work. ELISA and gas chromatography were used for quantitation and gas chromatography–mass spectrometry for confirmation of positive samples. The results obtained confirm the endogenous origin of 19-nortestosterone in boars and give a natural range of concentration of about 1–300 ng ml^{-1} for urine and 1–15 ng ml^{-1} for plasma samples.

Keywords: Gas chromatography; Mass spectrometry; Anabolic steroids; Nadrolone; 19-Nortestosterone;

The use of anabolic steroids in farm animals is forbidden in the EEC because of the adverse health effects they can have on the meat consumers. In Finland the use of anabolics in farm animals has been forbidden by the Ministry of Agriculture and Forestry with the circular letter No. 572/41-88. 19-Nortestosterone is perhaps the most common anabolic steroid illegally used. Some problems have arisen with the detection of 19-nortestosterone in different slaughter animals. The existence of endogenous 19-nortestosterone has been shown for example in stallions and bovines [1–3]. Several studies confirm the presence of endogenous 19-nortestosterone also in boars [4–7]. However, there is only little information about to which extent 19-nortestosterone may be present in biological fluids of boars. Furthermore, the methods used for quantitation were generally based on immunological techniques which may sometimes give false results.

In order to determine the endogenous level of 19-nortestosterone in finnish boars, cryptorchid and castrated boars, urine and plasma samples from non-treated animals were studied by using a combination of different analytical techniques. An enzyme immunoassay was used as a general screening method. The samples were also measured by gas chromatography (GC) with electron capture detection and confirmed by gas chromatography–mass spectrometry (GC–MS).

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EXPERIMENTAL

Chemicals

19-Nortestosterone and mesterolone were purchased from Steraloids (Wilton, NH). D-Norgestrel was supplied by Sigma (St. Louis, MO). β-Glucuronidase of *Escherichia coli* (1 000 000–5 000 000 U/g) was from Sigma. Deionized water was prepared with a Milli-Q system (Millipore, Bedford, MA). Heptafluorobutyric anhydride was obtained from Pierce (Rockford, IL). All other chemicals were purchased from Merck (Darmstadt) or Fluka (Buchs) and were of the highest purity grade available.

Samples

From a population of about 300 swines of the breed Land Race Large White, urine and plasma samples were collected from 20 boars, 10 cryptorchid boars, and 23 castrated boars, in two different slaughterhouses, under the control of a veterinarian. None of the animals was treated with 19-nortestosterone.

Instrumentation

Solid phase extraction for sample preparation was carried out using octadecyl disposable columns (BondElut, Analytichem, Sunnyvale, CA) in connection with a VacElut SPS 24 (Analytichem) vacuum manifold.

All enzyme immunoassay testing was performed using an ELISA kit from Geneco (Gorizia) according to the manufacturers instructions.

Gas chromatography was carried out by using a HP 5890A gas chromatograph equipped with data system and with electron capture detector (Hewlett-Packard, Palo Alto, CA) and with a HP-1 crosslinked dimethyl silicone fused silica capillary column (25 m × 0.32 mm i.d. × 0.17 μm film thickness). Helium (flow-rate, 1 ml/min at 60°C) was used as a carrier gas and nitrogen as a make-up gas. The injector temperature was set at 260°C, and detector at 300°C. The oven temperature program was as follows: After holding for 1 min at 60°C the temperature was raised up to 160°C at 45°C/min. After 2 min it was increased at 15°C/min to the final temperature of 260°C and maintained isothermal for 10 min. Two μl of sample were injected in the splitless mode.

Gas chromatography–mass spectrometry was carried out by using a Jeol DX-300 mass spectrometer equipped with data system and coupled with a HP 5890A gas chromatograph. The chromatographic parameters were the same as described above. The ion source was operated in the electron impact mode. The ionization voltage and the ionization current were 25 eV and 300 μA respectively. The acceleration voltage was 3 kV. Interface temperature was 265°C. The instrument was operated in the selected ion monitoring mode.

The ions 666, 667, 452 and 453 (m/z) were used to monitor for 19-nortestosterone. The detection limits for 19-nortestosterone in plasma and urine samples are 7 and 5 ng ml^{-1}, respectively using GC, with electron capture detection (ECD), and 10 and 7 ng ml^{-1}, respectively, when using GC–MS in the selected ion monitoring (SIM) mode.

Sample preparation for GC and GC–MS – urine samples

2.5 ml of 2 M sodium acetate buffer (pH 3), and 25 μl of internal standard (mesterolone 16 μg ml^{-1} in methanol) were added to 10 ml of urine. The sample was extracted twice (12.5 and 10 ml) with diethyl ether–ethanol (3 : 1) for 1 min on a tube shaker. After centrifugation (750 *g*, 5 min) the organic phase was separated and evaporated to dryness (temp. 30°C). The residue was dissolved in 2 ml of 0.5 M acetate buffer (pH 6.8) and after addition of 100 μl of β-glucuronidase (500 000 units/ml prepared in the same buffer), the mixture was incubated at 37°C overnight. The sample was supplemented with 2 ml of 2 M sodium acetate buffer (pH 3) and centrifuged at 410 *g* for 15 min. A Bond-Elut C18 cartridge was activated with 10 ml of methanol, 5 ml of water and 5 ml of 0.2 M sodium acetate buffer (pH 3) before transferring the sample to the cartridge. After washing twice with 4 ml of 0.2 M sodium acetate buffer (pH 3) the sample was eluted with 4 ml of methanol and the eluate evaporated to dryness under nitrogen stream below 60°C. The residue was dissolved in 6 ml of ethyl acetate–

cyclohexane (6:4, v/v). After adding 2 ml of 1 M NaOH saturated with NaCl the tubes were shaken for 30 s. After centrifugation the solvent phase was separated. The NaOH phase was further washed with 2 ml of solvent. The combined extracts were evaporated and the residues were stored at −20°C until derivatization. For quantitation, spiked urine samples (7.5, 15, 25, 40, 60 and 100 ng ml^{-1}) were used as standards.

Sample preparation for GC and GC–MS – plasma samples

25 μl of D-norgestrel (4 mM in methanol) and 25 μl of internal standard (mesterolone 16 μg ml^{-1}) were added to 5 ml of plasma. The D-norgestrel competes in the steroid–protein interaction, improving considerably the sample recovery [8]. After adding 3 ml of water, 2 ml of 2 M sodium acetate buffer (pH 5) and 10 ml of triethylammonium sulfate, the sample was extracted with 30 ml acetone for 1 min on a tube shaker. After centrifuging (1250 *g* for 10 min), the acetone was separated and evaporated to dryness. The residue was supplemented with 2 ml water, and transferred to a BondElut C18 cartridge activated as already described. After washing with 8 ml of 0.2 M sodium acetate buffer pH 5, the cartridge was eluted with 4 ml of methanol and the solvent evaporated to dryness under nitrogen. The residue was dissolved in 2 ml of 0.5 M sodium acetate buffer (pH 6.8) and, starting from the enzymatic hydrolysis with β-glucuronidase, the procedure was continued as described above for the urine samples. For quantitation, spiked plasma samples (1, 3, 6, 12 and 20 ng ml^{-1}) were used as standards.

Derivatization

The residues of urine and plasma extracts were supplemented with 100 μl of heptafluorobutyric anhydride, without any solvent, and reacted at 60°C for 45 min. After evaporating the excess reagent the sample residues were dissolved in 500–200 μl of hexane. Samples were analyzed by GC and GC–MS.

RESULTS AND DISCUSSION

All urine and plasma samples were screened by ELISA. The results are presented in Table 1, and all of them were found to be positive for 19-nortestosterone. Some of the samples were quantified by GC and confirmed by GC–MS. The urine and plasma samples collected from castrated boars were not analyzed by GC and GC–MS, because the level of nandrolone present was too low. The plasma, when analyzed by GC, gave results with no significant differences from those obtained by ELISA assay, except for three samples with negative GC results, as presented in Table 2. On the other hand, GC analysis of urine samples by GC gave much higher amounts than those obtained with ELISA, except for two of them which were found negative (see Table 3). All the samples found to be positive by GC were confirmed by GC–MS. The reason why some

TABLE 1

Results of ELISA assay for plasma and urine samples [a]

	Number of samples	Urine $\bar{x}$ (ng/ml)	Plasma $\bar{x}$ (ng/ml)
Boars	20	25 (10–55)	4.8 (0.6–17.5)
Cryptorchid boars	10	25 (10–47)	5.0 (1.1–15)
Castrated boars	23	1.0 (0.3–2.1) ($n = 19$)	0.5 (0.1–1.2)

[a] The numbers in parentheses represent the range of concentration.

TABLE 2

Results of plasma samples analyzed by gas chromatography with EC and MS detection
(Only boar or cryptorchid boar samples were analyzed) [a]

	Number of samples	$\bar{x}$ (ng/ml) (ECD)	MS
Boars	6	4 (2–8)	Positive
	1	N.D.	N.D.
Cryptorchid boars	3	6 (2–13)	Positive
	2	N.D.	N.D.

[a] N.D. = Not detected. The numbers in parentheses represent the range of concentration.

TABLE 3

Results of urine samples analyzed by gas chromatography with EC and MS detection
(Only boar or cryptorchid boar samples were analyzed) [a]

	Number of samples	$\bar{x}$ (ng/ml) (ECD)	MS
Boars	10	83 (1–277)	Positive
	1	N.D.	N.D.
Cryptorchid boars	6	93 (10–146)	Positive
	1	N.D.	N.D.

[a] N.D. = Not detected. The numbers in parentheses represent the range of concentration.

plasma samples resulted negative by GC could be found in the lower sensitivity of that technique when compared to ELISA. However in the case of urine samples, the only explanation for the two negative results obtained by GC is that ELISA may give false positives because of cross-reaction. The higher values for nandrolone in urine, obtained by GC, are most probably due to the lack of linearity of the calibration graph for the ELISA in the high range of concentration. The GC–ECD method seems to be, especially when used together with GC–MS, more reliable than ELISA for quantitation and screening of 19-nortestosterone in biological fluids of boars. The amount of nandrolone detected in boars and in cryptorchid boars is very similar in plasma as well as in urine, whereas the level of the castrated boars is clearly lower, as was expected. The concentration level in urine of boars and cryptorchid boars, when compared to plasma samples, resulted to be 20 and 16 times higher respectively. From a population of about 300 animals was selected a group sufficient to give enough information about the natural level of 19-nortestosterone in urine and plasma samples of boars, castrated and cryptorchid boars, and to confirm its endogenous origin. The research work will continue at this Institute in order to obtain more complete information on the presence of 19-nortestosterone and its metabolites in feeding pigs.

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Measuring the nandrolone threshold ratio by enzyme-linked immunosorbent assay for 5α-estrane-3β,17α-diol

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Abstract

The international threshold for nandrolone in equine urine is reached when the ratio of 5α-estrane-3β,17α-diol to 5(10)-estrene-3β,17α-diol exceeds one. When this ratio is exceeded, the sample is positive for administration of a nandrolone preparation. The present method for measuring the ratio requires hydrolysis of the urine sample followed by clean-up procedures, then analysis of the derivatized residue by gas chromatography–mass spectrometry (GC–MS). A quicker measurement of the ratio for screening purposes is desirable. A hybridoma that secretes monoclonal anti-5α-estrane-3β,17α-diol antibody has been developed. Using inhibition enzyme-linked immunosorbent assay (ELISA), this monoclonal antibody detects 5α-estrane-3β,17α-diol at 1 ng ml^{-1} and is far more sensitive than a rabbit polyclonal antiserum. The monoclonal antibody is specific to 5α-estrane-3β,17α-diol, 5α-estrane-3β-ol-17-one and 5α-estrane-3β-ol-17-carboxymethyloxime. Slight cross-reactivity with other structurally related steroids is observed at concentrations higher than 200 ng ml^{-1}. Work is in progress to raise a monoclonal antibody against 5(10)-estrene-3β,17α-diol. The two ELISAs should provide a rapid screening test to measure the nandrolone threshold ratio. Detailed GC–MS analysis would then only be required for confirmation.

Keywords: Immunoassay; Enzyme-linked immunosorbent assay; Anabolic steroids; Equine urine; Estranediol; Nandrolone threshold ratio; Urine

Conjugates of 5α-estranediols and nandrolone are the major metabolites of nandrolone in the horse [1–4]. The international threshold for nandrolone in equine urine is a 5α-estrane-3β,17α-diol to 5(10)-estrene-3β,17α-diol ratio of one [4]; when this ratio is exceeded, the sample is positive for administration of a nandrolone preparation.

The present method for measuring the ratio requires enzymatic hydrolysis [4] or methanolysis [5,6] of the urine samples, then a thin-layer chromatographic clean-up [7] or a C_{18} extraction procedure [8]. The residues are derivatized with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide to form the *tert*-butyldimethylsilyl derivatives for analysis by gas chromatography–mass spectrometry (GC–MS) [4]. These procedures are time consuming and, unless nandrolone administration is suspected, they are unsuitable for screening purposes. A quicker measurement of the ratio is desirable. This paper describes the production of a polyclonal and a more specific monoclonal anti-

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5α-estrane-3β,17α-diol antibody for screening and preliminary determination of 5α-estrane-3β,17α-diol in equine urine.

Work is in progress to raise a monoclonal anti-5(10)-estrene-3β,17α-diol antibody. Once completed, enzyme-linked immunosorbent assays (ELISAs) using the two monoclonal antibodies should provide a rapid screening test to measure the nandrolone threshold ratio.

EXPERIMENTAL

Chemicals

5α-Estrane-3β,17α-diol, 5(10)-estrene-3α,17β-diol and 5α-estrane-3β-ol-17-one were purchased from Steraloids (Wilton, NH), bovine serum albumin (fraction V), sheep serum albumin (fraction V), ovalbumin (grade V) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride from Sigma (St. Louis, MO), carboxymethoxylamine hemihydrochloride from Aldrich (Milwaukee, WI), MOX (2% methoxyamine hydrochloride in pyridine) from Pierce (Rockford, IL), Regisil [*N*,*O*-bis(trimethylsilyl)trifluoroacetamide + 10% trimethylsilyl (TMS) chloride] from Chrompack (Middelburg, Netherlands) and Sep-Pak C_{18} cartridges from Waters (Milford, MA).

Equipment

The TMS derivatives of 5α-estrane-3β,17α-diol and 5(10)-estrene-3α,17β-diol were determined using a Model 3400 gas chromatograph (Varian, Sunnyvale, CA) connected to an ITS40 ion-trap detector (Finnigan MAT, San Jose, CA). The column (a cross-linked dimethylsilicone capillary, 12.5 m × 0.2 mm i.d., Ultra 1) (Hewlett-Packard, Avondale, PA) was initially held at 150°C for 1 min, then heated at 15°C min^{-1} to a final temperature of 300°C, which was maintained for 5 min. Injections were splitless.

Administration of a proprietary preparation

Durabolin (16 ml containing 400 mg of nandrolone phenylpropionate in oily solution) (Organon, Oss, Netherlands) was administered intramuscularly to a thoroughbred gelding (ca. 450 kg; 11 years old). Urine samples were collected before the steroid preparation was administered, then daily for 22 days.

Immunization of rabbits with steroid–protein conjugates; ELISA for the IgG antibody activity

Male New Zealand white rabbits (1.5–2.0 kg) were immunized by monthly injections of Freund's adjuvant emulsified with 5α-estrane-3β-ol-17-carboxymethyloxime (5α-estrane-3β-ol-17-CMO) conjugated to bovine serum albumin (EA–BSA). This conjugate was synthesized by carboxymethyloximation of 5α-estrane-3β-ol-7-one with carboxymethylamine, then carbodiimide coupling with BSA [9]; the molar ratio of 5α-estrane-3β-17-CMO to BSA was found to be 26:1 by UV spectrophotometry [10]. Blood was obtained before immunization and 14 days after each injec-

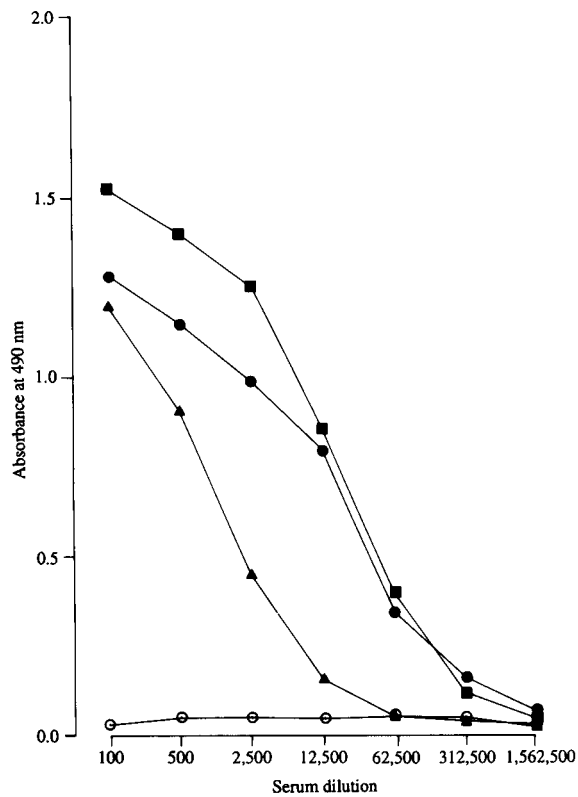

Fig. 1. Appearance of 5α-estrane-3β-ol-17-CMO specific antibody. ○ = Preimmunization; ▲ = 14 days after initial immunization; ■ = 14 days after first booster; ● = 14 days after second booster.

tion, then clotted overnight and centrifugated to obtain serum.

Antibody in the rabbits' serum specific to 5α-estrane-3β,17α-diol was monitored by ELISA. Microtitre plates were coated with 5α-estrane-3β-ol-17-CMO conjugated to ovalbumin [EA–OVA; 0.01 mg ml^{-1} in 0.05 M phosphate buffer (pH 7.2)]. Anti-estrane-3β,17α-diol antibody titres were calculated as the dilution of antiserum giving 0.5 absorbance (Fig. 1).

After a 1:100 dilution, inhibition ELISA was performed as shown in Fig. 2.

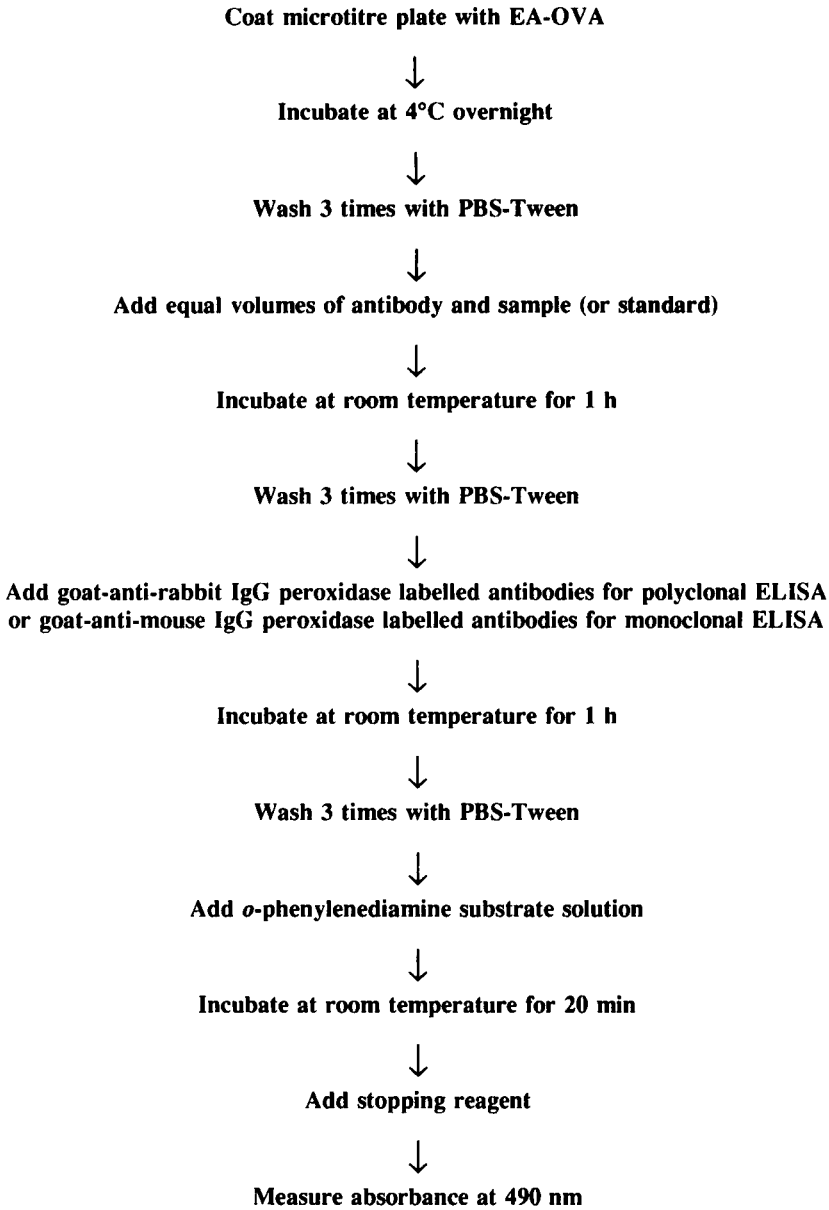

Fig. 2. Procedure for the inhibition ELISA.

Hybridoma preparation

Male C57 mice (10–15 g) were immunized monthly by intraperitoneal injection of 5α-estrane-3β-ol-17-CMO conjugated to sheep serum albumin (EA–SSA). The molar ratio of 5α-estrane-3β-ol-17-CMO to SSA was found by UV spectrophotometry [10] to be 29:1. Hybridoma secreting monoclonal anti-5α-estrane-3β,17α-diol antibody was produced as shown in Fig. 3.

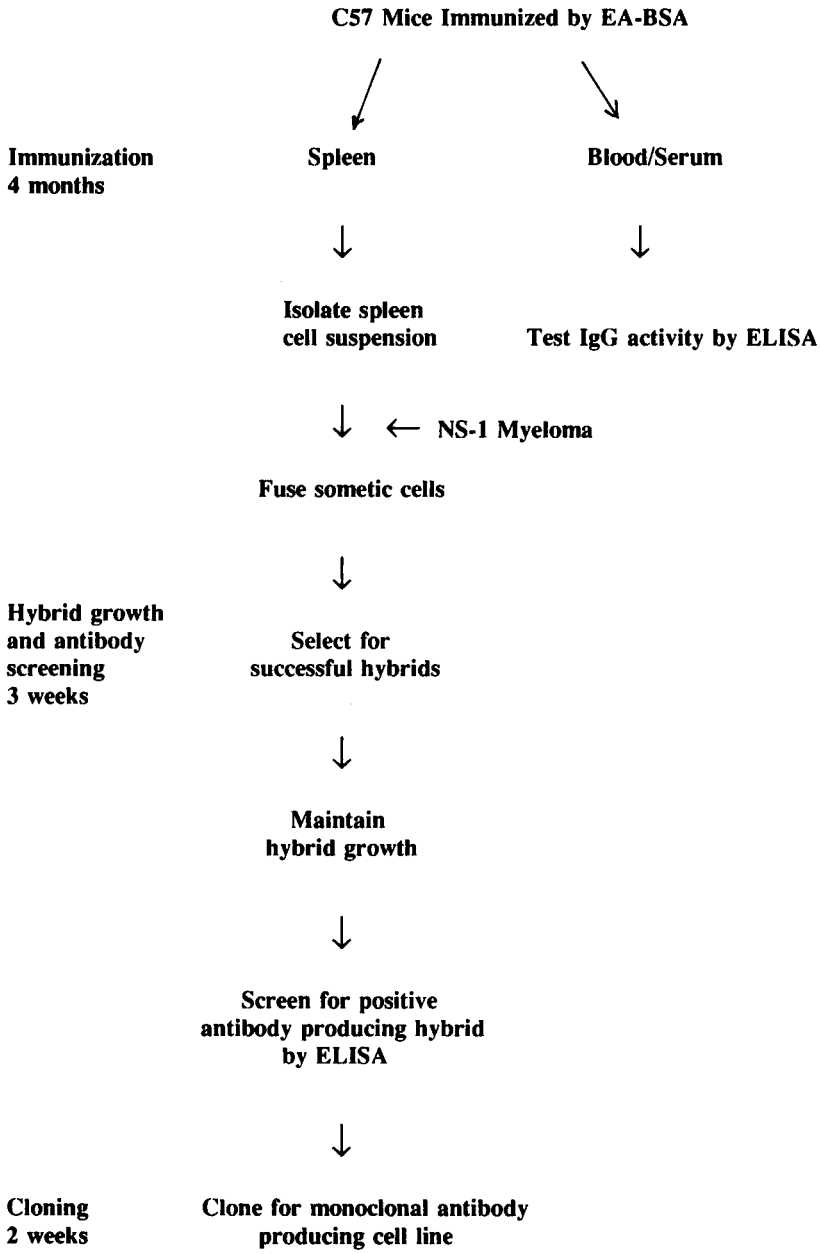

Fig. 3. Procedure for the development of hybridoma.

All serum from EA–SSA-immunized mice showed anti-estranediol IgG activity with titres about 1 in 20000 (Fig. 4).

All mice were killed and their splenocytes isolated and fused with NS-1 myeloma cells. Cells producing positive antibodies were cloned. The method of limiting dilution was employed and, after colonial growth, wells with more than one colony and those without any cell growth were discarded. The remainder were screened again for desirable antibody production with ELISA.

Inhibition ELISA with monoclonal anti-5α-estrane-3β,17α-diol antibody

Monoclonal antibody in the hybridoma supernatant specific to 5α-estrane-3β,17α-diol was determined by ELISA. Microtitre plates were coated with EA–BSA (0.01 mg ml^{-1}). Antibody titres were calculated as the supernatant dilution giving 0.5 absorbance (Fig. 5).

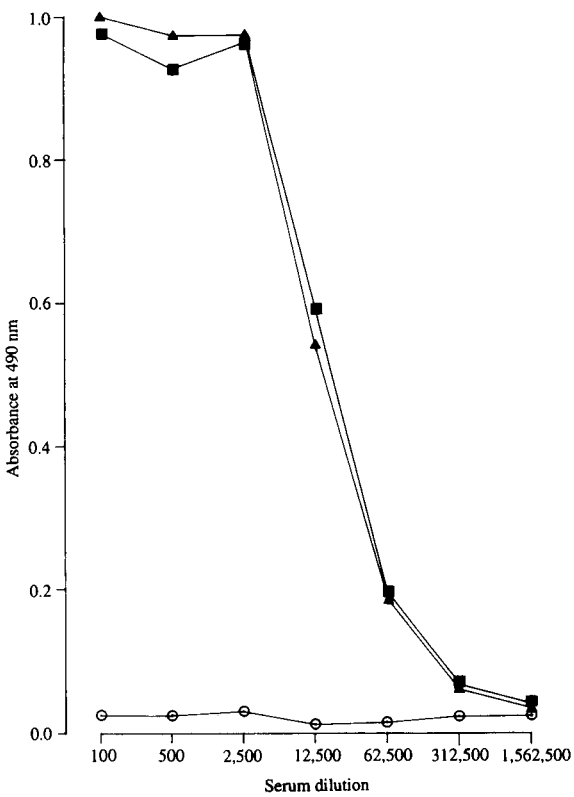

Fig. 4. Inhibition ELISA for anti-estranediol IgG activity in mouse antiserum after EA–SSA immunization. ■ = Mouse 1; ▲ = mouse 2; ○ = control serum.

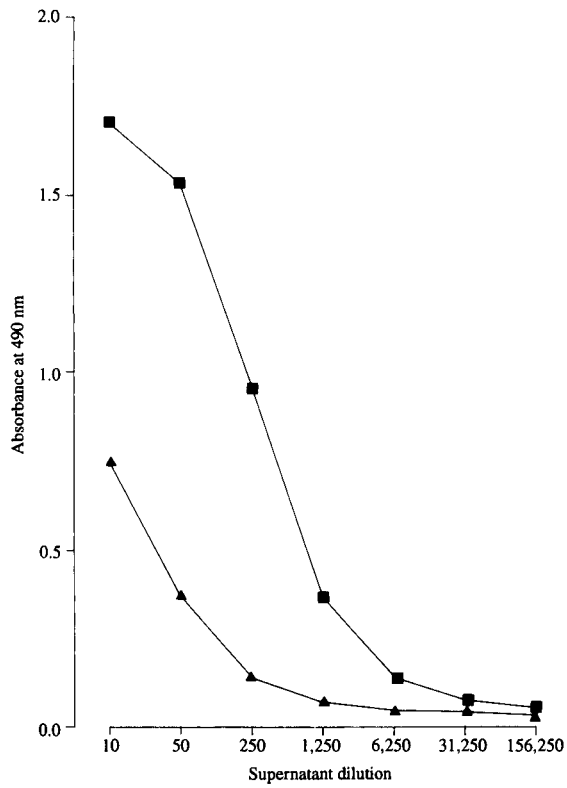

Fig. 5. Inhibition ELISA for anti-estranediol IgG activity in the supernatants of hybridoma clones (■) C1E7 and (▲) C3D10.

Hybridoma supernatant (1:200 dilution) was used for all inhibition ELISA screenings. Forty-six steroid inhibitors (Table 1) were used for cross-reactivity according to the procedure shown in Fig. 2.

GC–MS determination of 5α-estrane-3β,17α-diol in urine collected after administration of nandrolone

Duplicate urine samples (AD4 and AD12 collected 4 and 12 days, respectively after administration of nandrolone; 5 ml each) and duplicate spiked negative urine samples (containing 0, 50, 250, 500, 1000, 2000 or 3000 ng of 5α-estrane-3β,17α-diol; 5 ml each) were methanolysed and derivatized with MOX and BSTFA [5,6]. 5(10)-Estrene-3α,17β-diol (500 ng) was added as the

TABLE 1

Steroids used for cross-reactivity studies

5α-Androstane-3β,17α-diol	5α-Estrane-3α,17β-diol
5α-Androstane-3β,17β-diol	5α-Estrane-3β,17α-diol
Androstenediol	5α-Estrane-3β,17β-diol
Androstenedione	α-Estradiol
Androsterone	Estradiol
Betamethasone	Estratriol
Boldenone	4-Estrene-3β,17β-diol
Corticosterone	5(10)-Estrene-3,17-dione
Cortisone	5(10)-Estrene-3α,17β-diol
11-Dehydrocorticosterone	5(10)-Estrene-3β,17α-diol
Dehydroepiandrosterone	Estrone
Dexamethasone	Hydrocortisone
Dihydroandrosterone	Methandriol
5α-Dihydrocortisol	Methylandrostanolone
5β-Dihydrocortisol	Methylprednisolone
5α-Dihydrotestosterone	Methyltestosterone
Epiandrosterone	Nandrolone
Epitestosterone	Prednisolone
5α-Estrane-3α-ol-17-one	Prednisone
5α-Estrane-3β-ol-17-one	Progesterone
5α-Estrane-3β-ol-17-CMO	Stanozolol
5α-Estrane-17α-ol-3-one	Testosterone
5α-Estrane-17β-ol-3-one	Trenbolone

TABLE 2

Comparison between the cross-reactivities of polyclonal and monoclonal anti-estranediol antibodies

Steroid	Cross-reaction (%)	
	Rabbit polyclonal antiserum	Monoclonal antibody C1E7
Cross-reacting steroids		
5α-Estrane-3β,17α-diol	100	100
5α-Estrane-3β-ol-17-one	> 100	201
5α-Estrane-3β-ol-17-CMO	> 100	159
5α-Estrane-3α,17β-diol	42.6	0.66
Weakly cross-reacting steroids		
Epiandrosterone	14.3	0.2
5α-Estrane-3β,17β-diol	45.1	< 0.1
5α-Estrane-17α-ol-3-one	20.6	4.2
5α-Estrane-17β-ol-3-one	16.6	5.4
5α-Estrane-3α-ol-17-one	7.7	0.19
4-Estrene-3β,17β-diol	22.8	5.7
5(10)-Estrene-3,17-dione	2.0	5.3
5(10)-Estrene-3α,17β-diol	3.3	0.43
5(10)-Estrene-3β,17α-diol	5.8	3.8
Nandrolone	10.5	4.1
Testosterone	2.7	< 0.01

internal standard immediately after methanolysis. Portions (1 μl) were analysed by GC–MS and 5α-estrane-3β,17α-diol was determined by ratioing the peak area of the ion at m/z 242 to the peak area of the ion at m/z 330 (from the internal standard).

Monoclonal ELISA determination of 5α-estrane-3β,17α-diol in urine collected after administration of nandrolone

Duplicate urine samples (AD4 and AD12; 15 μl each) and duplicate spiked negative urine samples (containing 420, 210, 105, 52.5, 26.25 or 0 ng ml^{-1} of 5α-estrane-3β,17α-diol; 15 μl each) were assayed with monoclonal C1E7 antibody according to the procedure shown in Fig. 2.

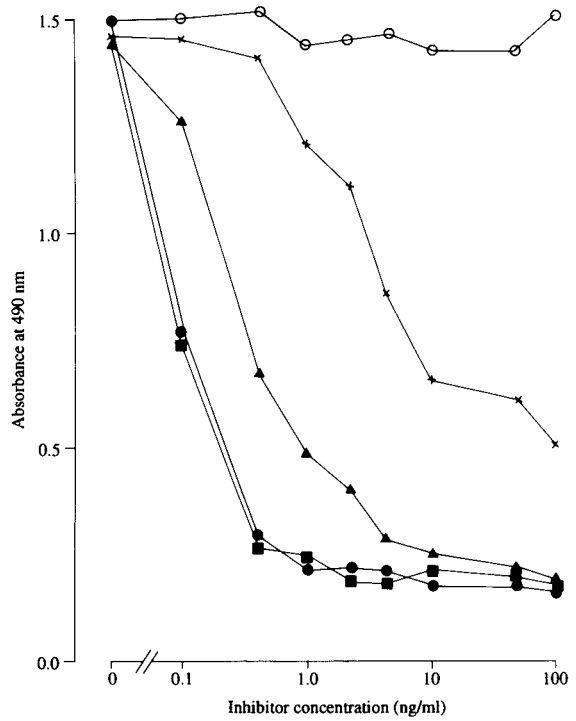

Fig. 6. Inhibition ELISA with polyclonal anti-estranediol antibody. ■ = 5α-Estrane-3β-ol-17-one; ▲ = 5α-estrane-3β,17α-diol; ● = 5α-estrane-3β-ol-17-CMO; × = other inhibiting steroids; ○ = methanol.

RESULTS

Inhibition ELISA with polyclonal anti-5α-estrane-3β,17α-diol antibodies

Inhibition ELISA studies were used to find the specificity of the IgG antibody for 5α-estrane-3β,17α-diol. Only steroids that are structurally similar to 5α-estrane-3β,17α-diol inhibit the antibody binding (Table 2 and Fig. 6).

Inhibition ELISA with monoclonal anti-5α-estrane-3β,17α-diol antibody

Immunized mice with positive estranediol IgG responses were chosen for the hybridization. After fusing three times with NS-1 myeloma cells, 68 wells were plated. Supernatants from three wells (A4, C1 and C3) were found to be anti-estranediol positive. These wells were isolated and cloned. The supernatant from one well (A4) lost its IgG activity after culturing for 10 days.

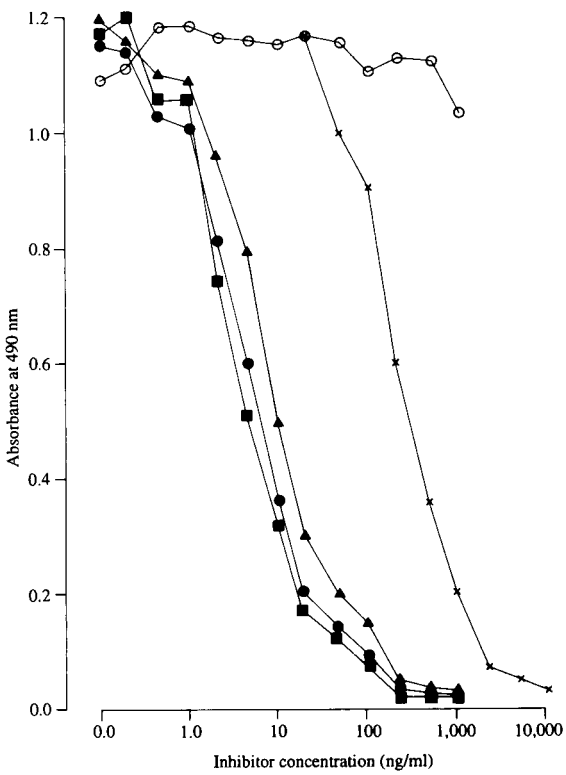

Fig. 7. Inhibition ELISA with monoclonal C1E7 anti-estranediol antibody. Symbols as in Fig. 6.

Two other clones (C1E7 and C3D10), however, continued to secrete monoclonal anti-5α-estrane-3β,17α-diol IgG (Fig. 5). The monoclonal C1E7 antibody had a higher titre and so was chosen for use.

The specificity of the monoclonal C1E7 antibody to 5α-estrane-3β,17α-diol was confirmed by inhibition ELISA. Of the 46 steroids studied, 15 showed inhibition at 10 μg ml^{-1} (Table 2), the strongest being 5α-estrane-3β,17α-diol, 5α-estrane-3β-ol-17-one and 5α-estrane-3β-ol-17-CMO (Fig. 7). 5α-Estrane-3β,17α-diol had a linear dose–response to the monoclonal antibody within the range 1–100 ng ml^{-1}. Except for the three strongest inhibiting steroids, the 50% inhibition concentration (IC_{50}) was at 200 ng ml^{-1} or higher and cross-reactivity was negligible after a tenfold dilution.

Comparison of monoclonal ELISA with GC–MS for the determination of 5α-estrane-3β,17α-diol in urine collected after administration of nandrolone

By GC–MS, the 5α-estrane-3β,17α-diol concentration in urine AD4 was 545 ± 10 ng ml^{-1} and in AD12 it was 135 ± 5 ng ml^{-1}. By ELISA, the concentrations were 559 ± 15 and 123 ± 10 ng ml^{-1} after methanolysis and 526 ± 13 and 143 ± 10 ng ml^{-1} without methanolysis.

DISCUSSION

The hybridoma developed secretes a monoclonal anti-5α-estrane-3β,17α-diol antibody which specifically recognizes 5α-estrane-3β,17α-diol, 5α-estrane-3β-ol-17-one and 5α-estrane-3β-ol-17-CMO. 5α-Estrane-3β,17α-diol is a urinary metabolite of nandrolone in the horse. Like 5α-estrane-3β,17α-diol, 5α-estrane-3β-ol-17-one is also a urinary metabolite of nandrolone in the horse [1], but at a lower concentration (only traces of 5α-estrane-3β-ol-17-one were detectable by GC–MS). Although its cross-reactivity is 200%, its contribution to the measured concentration of 5α-estrane-3β,17α-diol is insignificant. The other cross-reacting species, 5α-estrane-3β-ol-17-CMO, is not a metabolite of nandrolone or a naturally occurring steroid.

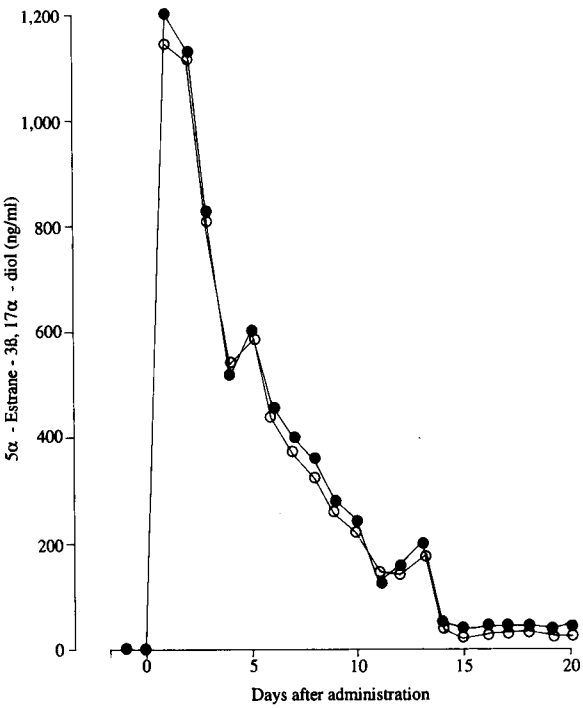

Fig. 8. Inhibition ELISA for 5α-estrane-3β,17α-diol in urine after administration of nandrolone. ○ = With methanolysis; ● = without methanolysis.

The ELISA using the monoclonal antibody could not differentiate 5α-estrane-3β,17α-diol from its conjugates (Fig. 8).

With urine AD4 and AD12 collected after administration of nandrolone, 5α-estrane-3β,17α-diol, as determined by the monoclonal ELISA, matched the concentrations determined by GC–MS to within 10%.

Work is in progress to raise a monoclonal anti-5(10)-estrene-3β,17α-diol antibody for determining 5(10)-estrene-3β,17α-diol, the denominator in the nandrolone threshold ratio.

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Influence of matrix and applied method on the detection of anabolic residues in biological samples

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Abstract

The influence of the matrix and the applied method on the results for the detection of anabolic residues is discussed. A comparative study was done by analysing different kinds of samples but from the same animal. Injection sites, fat and meat sampled from the same carcass were analysed. Different techniques (liquid chromatography with UV detection, high-performance thin-layer chromatography and gas chromatography–mass spectrometry) were used to detect and identify the residues and to confirm and compare the results.

Keywords: Gas chromatography–mass spectrometry; Liquid chromatography; Thin-layer chromatography; Anabolic steroids; Biological samples

The use of anabolic steroids as growth promoters in cattle fattening is well known. Despite the EEC prohibition of their use, there is still substantial illegal use of hormonal compounds, the list of which has to be updated constantly. When the number of combined substances in mixtures is increased [1], the concentration of each product can be decreased to obtain the same growth-promoting effect. The residue concentration for each substance will then be much lower, which may be a difficult problem for the analyst. Multi-residue analysis as a consequence becomes more difficult, and harmonization of the methodology, with well defined quality criteria, becomes essential [2,3].

Even in regulatory control it is not surprising that when doing a re-examination on a second sample from the same animal in another laboratory using a different method, contradictory results can arise. This problem motivated a comparative study of the analysis of different kinds of samples, but all from the same animal, with different methods.

Many techniques have been optimized for the selective detection of anabolic residues. Immunoassays and multi-immunoaffinity are highly selective methods [4,5]. On-line liquid chromatography (LC) with diode-array spectrophotometric detection has been applied to steroidal hor-

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mones in illegal preparations [6]. High-performance thin-layer chromatography (HPTLC) is a rapid screening technique for the identification of esters of anabolics in injection sites [1]. Not only for identification or confirmatory purposes but also for routine analysis there is an increasing use of coupled techniques such as gas chromatography–mass spectrometry (GC–MS) [7,8]. Combination of direct and indirect information obtained from the different techniques increases the specificity and reliability [9]. The aim of this work was to demonstrate that there is an important influence of the matrix and the applied method on the results obtained.

EXPERIMENTAL

Apparatus

A homogenizer (e.g., Ultra Turrax, 20 000 rpm), a water-bath, a centrifuge equipped with centrifugation tubes of 450 ml (e.g., Beckman), a mechanical extractor (e.g., Stomacher), a rotary vacuum evaporator (e.g., Rotavapor, Speed-Vac, Vortex), extraction flasks of 100 ml and 250 ml, a solid-phase extractor (e.g., Baker), chromatographic tanks and a UV transilluminator ($\lambda = 366$ nm) were used. The sample applicator used was a semi-automatic Camag Linomat IV. The LC system consisted of a Series 4 pump (Perkin-Elmer), an ISS-100 autoinjector (Perkin-Elmer), a VICI automatic switching valve (Valco), a Model 440 UV detector (Waters) and a Model 202 fraction collector (Gilson). The gas chromatograph–mass spectrometer was an ITS 40 ion trap (Finnigan MAT).

HPTLC plates were obtained from Merck (Darmstadt, Germany).

Reagents, reference compounds and solutions

Most of the reference compounds were obtained from Steraloids (Wilton, NY) or Sigma (St. Louis, MO). All solvents were of analytical-reagent grade or LC grade from J.T. Baker (Phillipsburg, NJ); diethyl ether was obtained from Gifrer & Barbezat (Decines, France). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was purchased from Machery–Nagel (Düren, Germany) and iodotrimethylsilane (ITMS) and dithioerythritol (DTE) from Pierce (Rockford, IL).

Solutions

Stock standard solutions were prepared in ethanol at a concentration of 1 mg ml^{-1}. For routine control purposes, working standard solutions were prepared by dilution of the stock standard solutions to 50 ng μl^{-1}. The same concentration was used for the following solutions of mixtures of the most frequently used anabolic compounds and corresponding to the different fractions collected from the LC column: mixture 1, diethylstilbestrol (DES), zeranol (Z), trenbolone (Tb), ethinylestradiol (EE_2), β-nortestosterone (βNT), methylboldenone (MeBol), and α-estradiol (αE_2); mixture 2, α-nortestosterone (αNT) and β-testosterone (βT); mixture 3, α-testosterone (αT), methyltestosterone (MT) and acetoxyprogesterone (AP); and mixture 4, chlormadinone acetate (CMA), medroxyprogesterone acetate (MPA) and chlortestosterone acetate (ClTA).

The following solvent systems were used to develop the HPTLC plates: 1 = *n*-hexane–diethyl ether–dichloromethane (25 + 45 + 30); 2 = chloroform–acetone (90 + 10); 3 = cyclohexane–ethyl acetate–ethanol (60 + 40 + 2.5); 4 = chloroform–*n*-hexane–acetone (50 + 40 + 10); 5 = tetrahydrofuran–*n*-hexane (35 + 65); 6 = methanol–toluene (95 + 5); 7 = *n*-hexane–dichloromethane–acetonitrile (80 + 10 + 5).

LC columns

A semi-preparative C_{18}, Ultraphere ODS (80 Å pore size, particle size 5 μm) column (250 mm × 10 mm i.d.) was obtained from Beckman Instruments, C_{18} pellicular ODS (particle size 37–53 μm) guard column (30 mm × 4.6 mm i.d.) from Whatman and a C_{18} MCH-10 cartridge (particle size 10 μm) precolumn (30 mm × 4.6 mm i.d.) from Varian.

Solid-phase extraction (SPE) columns

Bond Elut columns were obtained from Varian. Different sorbents were tried such as polar silica (Si) and non-polar octadecyl (C_{18}) and phenylsilane (PH) materials. Before use, all these

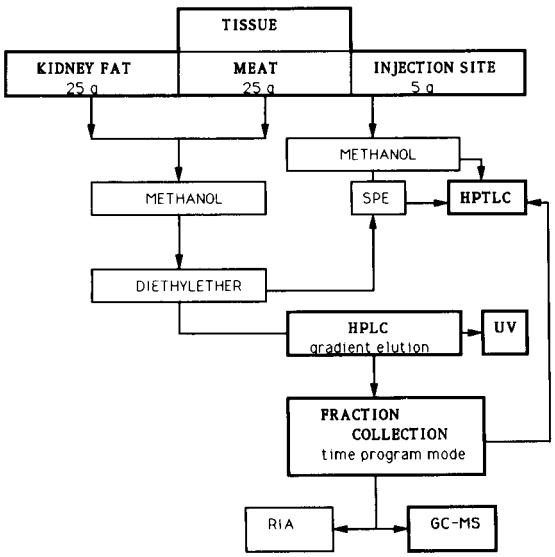

Fig. 1. Overall scheme of the extraction procedure.

columns were conditioned with the appropriate solvents, as will be described later.

Extraction

The overall scheme of the extraction procedure is given in Fig. 1. Meat or kidney fat is cut into small pieces, 25 g are weighed into a polypropylene flask and 50 ml of sodium acetate buffer (0.04 M, pH 5.2) are added. The fat samples are melted on a water-bath at 70°C for 20 min, the mixture is homogenized with a Ultra Turrax for 1 min, 50 ml of methanol are added and the mixture is homogenized again for 1 min. The flask is centrifuged at 5000 *g* for 20 min and the supernatant is filtered over silanized glass-wool in a separating funnel. The methanolic supernatant is extracted once with 25 ml of *n*-hexane and the hexane phase is discarded. The anabolics are then extracted into 1×100 ml and 1×50 ml of diethyl ether. The combined ether phases are washed with 10 ml of carbonate buffer (pH 10.25) and twice with 15 ml of water and then evaporated to dryness. The residue is transferred with 3×1 ml of methanol into a conical tube and concentrated again with a vacuum evaporator. Injection sites are extracted with methanol as described previously [1].

LC clean-up [10,11]

Gradient elution on a semi-preparative Ultrasphere ODS column was applied. A column-switching system was used to achieve a sufficient degree of purification and to prolong the lifetime of the column.

As shown in Fig. 2, an on-line combination of a guard column and a precolumn with a switching valve in connection with the analytical column was used.

The residue obtained after evaporation of the ether extract is dissolved in methanol and transferred into a 300-μl polypropylene vial for the autosampler. After a front-cut, the fraction of interest is chromatographed with gradient elution on the semi-preparative column. Preliminary injection of a mixture of standards gives the exact retention times of the different reference compounds. With these values the start and end of the windows needed to programme the fraction collector can then be determined. Figure 3 gives the gradient and fractionation conditions and Fig. 4 shows a chromatogram of some anabolic compounds eluted under the described conditions, obtained with UV detection.

Solid-phase extraction

Three different sorbents were tested for purifying the meat and fat extracts. The aim was to

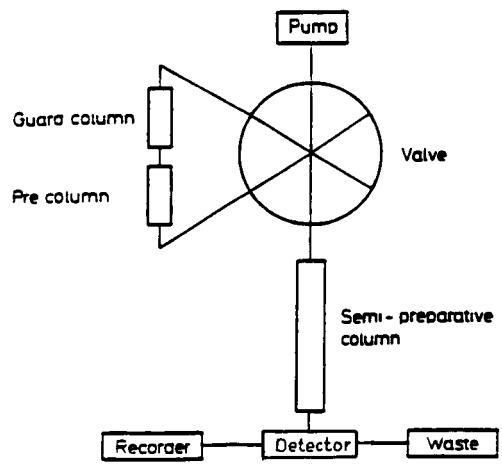

Fig. 2. System configuration of the column-switching technique.

evaluate the possibility of replacing one or more extraction or purification steps with an SPE. Bond Elut silica was used to replace the defatting extraction with hexane. The other two sorbents were tried for their potential to replace the LC purification step [12]. The conditions are described in Table 1.

The eluted fraction is evaporated to dryness and is then ready to be chromatographed by LC or immediately by HPTLC.

LC–UV detection

Before the fractions are collected the eluate passes a UV detector ($\lambda = 254$ nm), giving some information about the possible composition of the extract (see Fig. 5).

Two blocks of impurities are separated by a zone where it is possible, according to the retention times, to detect concentrations of 5 ng g^{-1} or less of most of the anabolics of interest.

High-performance thin-layer chromatography

For screening purposes the extract is chromatographed on a precoated silica gel plate (20 cm × 10 cm). The dry residue is dissolved in 20 μl of ethanol and 5 μl are spotted on the plate, 0.5 cm of the edge. Another 5 μl of the same fraction is spotted on the opposite site of the plate. The horizontal distance between the spots can be 0.5 cm so that up to 30 fractions can be analysed on one plate, every fourth spot being reserved for standards.

Chromatographic development is performed in the first direction with solvent system 3 over a distance of 5 cm. Then the plate is removed, air dried and eluted in the opposite direction with

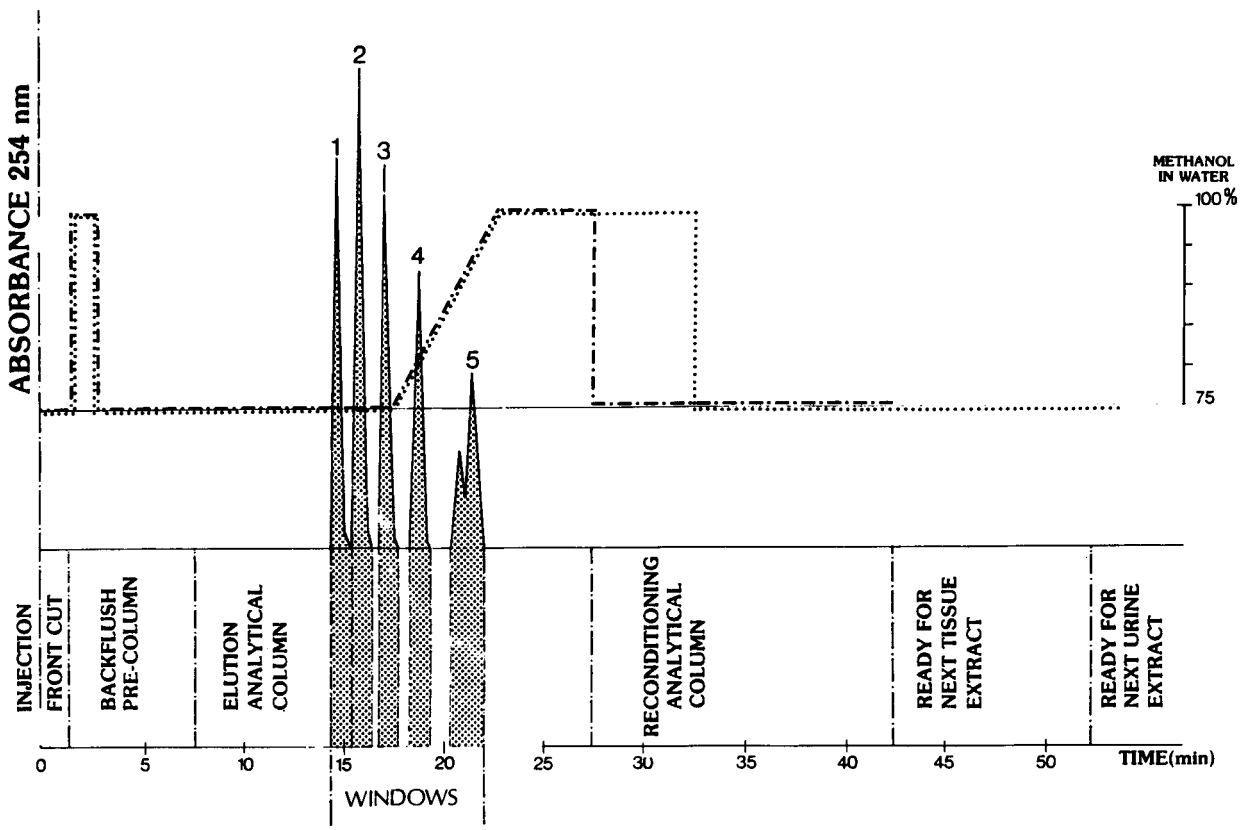

Fig. 3. According to the retention time of the standard products, a sequence of time-based collection and drain steps is installed. Each fraction, also called a window, contains a limited number of well defined anabolics.

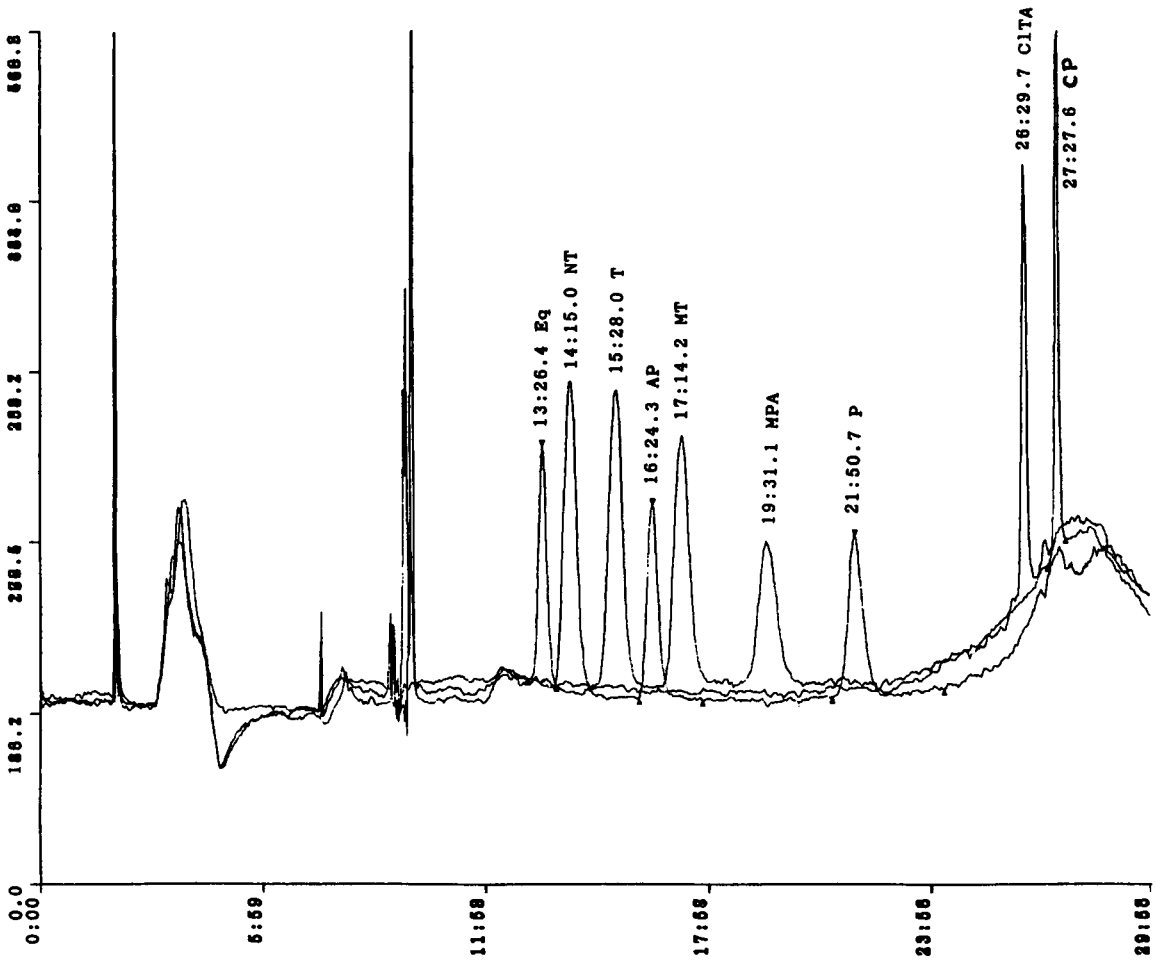

Fig. 4. Retention times of equilenine (Eq), nortestosterone (NT), testosterone (T), acetoxyprogesterone (AP), methyltestosterone (MT), medroxyprogesterone acetate (MPA), progesterone (P), chlortestosterone acetate (CITA) and caproxyprogesterone (CP).

TABLE 1

Chromatographic conditions for the different SPE columns

	Sorbent		
	Si	C_{18}	PH
Conditioning	$CHCl_3$ (5 ml)	CH_3OH (2 × 2 ml)	CH_3OH (10 ml)
	n-Hexane (5 ml)	H_2O (2 × 2 ml)	H_2O (20 ml)
Sample solution	$CHCl_3$–*n*-hexane	CH_3OH–H_2O	CH_3OH–H_2O
	(0.5 ml–5 ml)	(40 + 60) (2 ml)	(1 ml–10 ml)
Removal of impurities	*n*-Hexane (5 ml)	CH_3OH–H_2O	CH_3OH–H_2O
		(40 + 60) (2 × 2 ml)	(30 + 70) (10 ml)
Elution	$CHCl_3$–acetone	CH_3OH–H_2O	CH_3OH (10 ml)
	(90 + 10) (5 ml)	(80 + 20) (2 ml)	

solvent system 4. The plate is then dipped into a 5% ethanolic solution of sulphuric acid for 30 s; induction of fluorescence is accelerated by heating the plates in an oven at 95°C for 10 min.

The spots are identified by viewing by transillumination under UV radiation ($\lambda = 366$ nm) and confirmed by their specific colours under visible light [1]. The identity of the anabolics is evaluated by comparing the R_F values and the colours with those of reference substances.

For confirmatory purposes, the second half of the fractions is spotted on a 10 cm × 10 cm plate and chromatographed by two-dimensional co-chromatography [13].

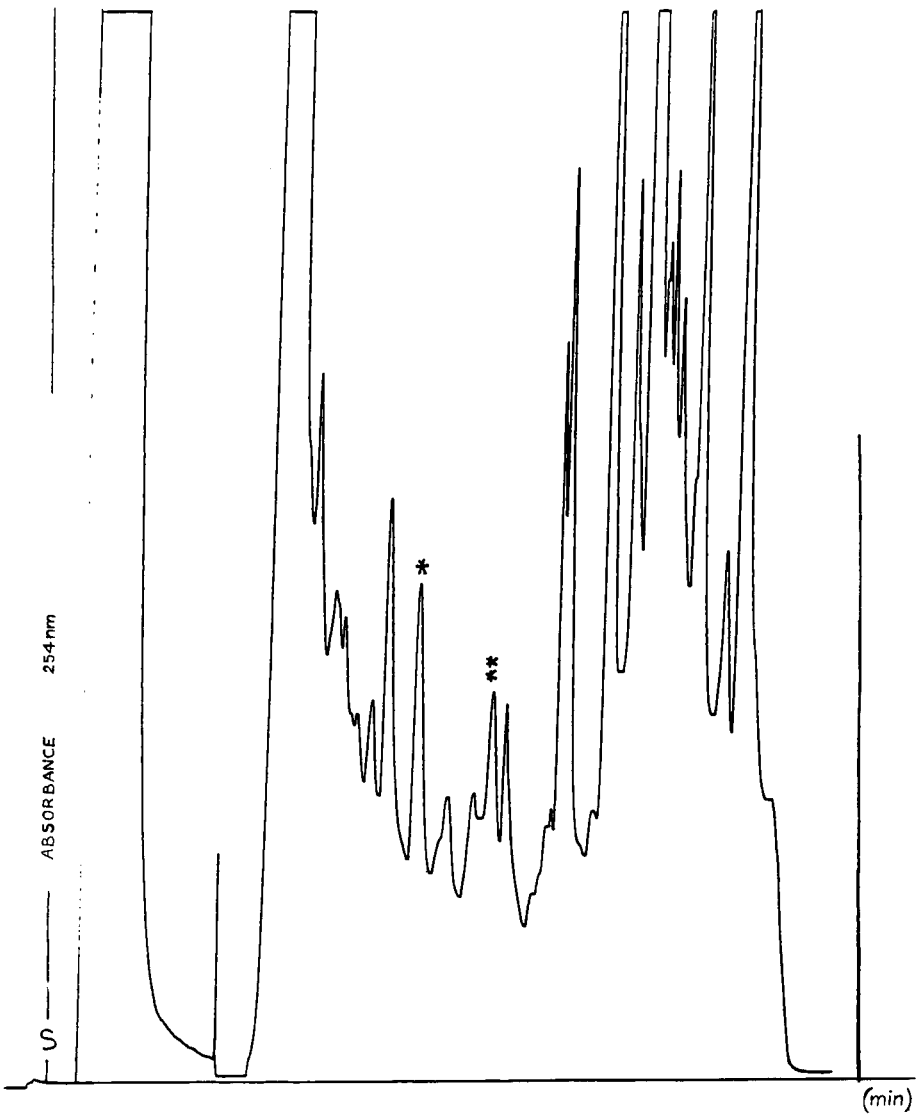

Fig. 5. Chromatogram with UV detection of a fat sample (25 g) spiked with 5 ng g^{-1} of β-nortestosterone (*) and 5 ng g^{-1} of methyltestosterone (**).

Gas chromatography – mass spectrometry

The derivatization reagent mixture is MSTFA–ITMS–DTE (1000 + 2 + 2). Extracts should be evaporated to dryness and then stored in a desiccator prior to derivatization.

To the tube containing the extract, 10 μl of the reagent mixture are added and heated at 60°C for 15 min. The tube is allowed to cool and then 1 μl is injected in the splitless mode into the GC–MS instrument.

The analyses were carried out on a Finnigan ITS 40 ion trap in the full-scan mode. The GC column used was DB-5 fused silica (30 m × 0.25 mm i.d.) with a 0.25-μm film thickness and a carrier gas (helium) flow-rate of 1 ml min^{-1}. The temperature settings were as follows: injector, 260°C; transfer line, 300°C; oven, programmed from 100 to 200°C at 16.7°C min^{-1} and from 250 to 300°C at 4°C min^{-1}, the final temperature of 300°C being maintained for 3.5 min.

RESULTS AND DISCUSSION

Comparative study

With the techniques described above, samples obtained from 55 carcasses were analysed. From each animal that was examined, three different kinds of samples were analysed: the injection site, the kidney fat and meat. Information about the sex and previous treatment of the animal and about the location of the sampled materials was not available. As we also had no idea of the period between treatment and slaughter, the result for an old injection site will give little indication of the initially injected concentration. The results of these analyses give only an indication of what kind of anabolic compounds could be expected in the meat and fat from the same animal. These sites sometimes contain a mixture of seven or more different compounds at more or less slightly different concentrations. The detected substances in the fat and meat were compared with the analytical results for the corresponding injection sites. All these results are summarized in Tables 2–5.

Table 2 summarizes the HPTLC results for sixteen fat and meat samples purified by SPE.

TABLE 2

HPTLC results for sixteen fat and meat samples purified by SPE

Sample No.	Injection site	Concentration (ng g^{-1}) [a]	
		Kidney fat	Meat
89/363	MPA CMA NT	MPA < 0.5 T < 0.5	NRD
89/364	MPA CMA NT	MPA > 3 CMA > 2 T > 2	MPA < 0.5 T > 2
89/368	MPA	MPA > 3 T < 0.5	MPA < 0.5
89/369	MPA MEGA	MPA > 3	MPA > 2
89/370	MPA	MPA > 10 CMA < 0.5	MPA < 0.5
89/371	MEGA NT βT + ester βE_2 + ester EE_2	MEGA > 10	MEGA > 6
89/372	MPA MEGA NT + ester βT ester βE_2 + ester	MPA > 3	MPA < 0.5
89/373	MPA MEGA NT + ester βT + ester βE_2 + ester EE_2	MPA > 3	NRD
89/386	MPA CMA	MPA > 6	MPA < 0.5
89/387	MPA CMA	MPA > 4	NRD
89/388	MPA CMA	MPA > 3	NRD
89/396	MPA	MPA > 3	NRD
89/399	MPA	MPA > 4	NRD
89/400	MPA MEGA	MPA > 10	MPA > 3
89/401	MPA NT + ester EE_2	MPA > 6	MPA < 0.5
89/402	MPA CMA	MPA < 0.5	NRD

[a] NRD = no residues detected.

Only gestagens were detected, namely MPA and CMA, at concentrations between 2 and 10 ng g^{-1} in the fat and between traces (< 0.5 ng g^{-1}) and

2–3 ng g^{-1} in the meat. Although these results are semi-quantitative evaluations by visual comparison of spot intensities, it can be concluded that there is a concentration difference of a factor of 4 or more. The lack of detection of androgens is related to the SPE. SPE gives only the collection of one fraction, resulting in a very interesting time-saving effect. However, when chromatographing a single fraction from the SPE column on an HPTLC plate, not all anabolics are completely separated (αNT, βNT, αT and βT have almost identical R_F values). Even the phenylsilane column, in our hands giving the best clean-up (C_{18}, Si and PH columns were tested), still gives confusing results in the NT zone.

LC purification on a semi-preparative column therefore has advantages over other clean-up techniques. First there is the greater capacity of this type of column, which is very convenient for biological extracts. The front cut switching technique combined with gradient elution results in such a good separation that different fractions can be collected containing a limited number of well defined anabolics without other interfering matrix components. The chromatograms obtained from these fractions by HPTLC are very clean, which facilitates the interpretation.

Figure 5 shows a liquid chromatogram obtained with UV detection which gives an idea of the separation and purification ability of this system.

Table 3 summarizes the HPTLC results for fifteen fat and meat samples purified by such LC. In addition to gestagens there are also residues of testosterone, methyltestosterone and nortestosterone detected. The difference in concentration can be estimated as a factor 5 or more, except for two samples where the same results were found in the fat and meat. These results show a better correlation between the three different kinds of samples. No estrogens are detected. This is probably due to the lower concentration of the estrogen components in the anabolic mixtures but also to the lower sensitivity of HPTLC for estrogens.

Table 4 demonstrates that the LC clean-up gives more complete results than the SPE purification. Nine fat samples were analysed twice, once with LC purification and a second time with SPE clean-up. The HPTLC results show a difference due to a different interpretation related to the clean-up of the extract. Another factor is that the recovery is slightly better with LC.

TABLE 3

HPTLC results for fifteen fat and meat samples purified by LC

Sample No.	Injection site	Concentration (ng g^{-1}) [a]	
		Kidney fat	Meat
89/364	MPA	MPA > 10	MPA > 3
	CMA		
	MEGA		
	NT	NT > 2	NT > 2
		βT < 0.5	βT < 0.5
		αT > 3	αT > 3
	MT		
89/370	MPA	MPA > 10	MPA < 0.5
89/372	MPA	MPA > 8	
	NT		
	βT + ester	αT > 2	αT < 0.5
	βE_2 + ester		
89/373	MPA	MPA > 4	
	MEGA		
	NT		
	MT		
	βT + ester	αT > 2	αT > 2
	βE_2 + ester		
	EE_2		
89/401	MPA	MPA > 6	NRD
	CMA		
	NT + ester		
	EE_2		
89/403	MPA	MPA > 2	NRD
	CMA		
	T	αT < 0.5	
	NT		
	MT	MT < 0.5	
89/410	MPA	MPA > 8	NRD
		αT < 0.5	
89/411	MPA	MPA > 6	NRD
89/412	MPA	MPA < 4	MPA < 0.5
	CMA	CMA < 0.5	
	NT + ester	NT > 2	NT < 0.5
	T + ester	αT > 2	αT < 0.5
89/420	MPA	MPA < 0.5	NRD
	CMA	CMA < 0.5	
		MT > 2	
		αT > 2	
		βT < 0.5	
89/421	MPA		NRD
	CMA		
	NT		
		αT < 0.5	

TABLE 3 (continued)

Sample No.	Injection site	Concentration (ng g^{-1}) [a]	
		Kidney fat	Meat
89/422	βE_2 + ester		NRD
	βT	$\alpha T < 0.5$	
90/26	NT + ester		
	T + ester	$\alpha T > 3$	$\alpha T < 0.5$
	MT		
90/27	MPA	MPA > 10	MPA > 4
	βT + ester	$\alpha T < 0.5$	
	βE_2 + ester		

[a] NRD = no residues detected.

Table 5 gives the results for samples from another 24 animals. On one half of each extract HPTLC was performed and the other half served for analysis by GC–MS. The conclusions drawn from the other tables are confirmed by the results in Table 5. The main difference is that with GC–MS low concentrations of estrogens are also detected. For the gestagens and ClTA, another derivatization technique is necessary, and these compounds were not confirmed by GC–MS.

These results indicate that with the combination of detection techniques a better qualitative correlation is obtained between the results for the different kinds of samples.

HPTLC

The main advantage of one-dimensional TLC is its applicability to the rapid screening of large numbers of fractions. It is possible to chromatograph five fractions of six samples on one plate when the spots are placed as described earlier. Plates 20 cm wide do not show any border effect, which facilitates the reading of the chromatograms.

Figure 6 shows the results of double one-dimensional elution on the same plate. As can be seen, interpretation is not hindered by interfering compounds. The difference in intensity of the spots corresponding to the same compound extracted from meat and fat samples originating from the same animal can be seen.

HPTLC gives the first reliable results of the analysis. The fraction containing the residues of progesterone was not analysed by HPTLC because of its too low sensitivity.

Figure 7 shows the results of two-dimensional co-chromatography.

LC–UV detection

The UV trace of the LC eluate shows that most anabolics are eluted in a relatively clean zone. This means that for concentrations even lower than 5 ng g^{-1} it is possible to have a first interpretation of whether a sample is suspect. It should be possible to improve the purification so that quantitative detection can be performed but in combination with a diode-array detector in order to have more specific information about the identity of the detected peak.

TABLE 4

Comparison of HPTLC results obtained after LC or SPE purification

Sample No.	Concentration (ng g^{-1})	
	LC	SPE
89/363	MPA > 2	MPA < 5
	NT < 2	
	$\alpha T < 0.5$	
89/364	MPA > 20	MPA > 10
	CMA > 5	CMA > 2
	$\beta NT > 2$	
	$\beta T < 0.5$	T > 2
	$\alpha T > 2$	
89/368	MPA > 5	MPA > 5
	$\beta E_2 > 2$	
	$\beta T < 0.5$	
89/369	MPA > 8	MPA > 5
	$\beta E_2 > 1$	
	$\beta NT > 1$	
	$\alpha NT < 0.5$	
89/370	MPA > 10	MPA > 10
	$\beta NT < 1$	CMA < 0.5
	$\alpha T < 0.5$	
	$\beta E_2 > 1$	
89/371	MEGA > 50	MEGA > 10
	$\beta E_2 > 1$	
	$\beta T < 0.5$	
89/372	MPA > 10	MPA > 8
	$\beta E_2 > 1$	
	$\beta T < 0.5$	
89/373	MPA > 5	MPA > 3
	$\beta E_2 > 1$	
	$\beta T < 0.5$	
89/401	MPA > 6	MPA < 5

TABLE 5

Comparison of results for HPTLC of injection site, fat and meat, sampled from the same animal, and HPTLC and GC–MS of the same extract

Sample No.	Injection site	Concentration (ng g^{-1}) [a]			
		Kidney fat		Meat	
		HPTLC	GC–MS (without gestagens)	HPTLC	GC–MS (without gestagens)
89/363	MPA	MPA > 3	–		
	CMA				
	MT	MT < 0.5			
	NT	NT > 2			NT < 0.5
		T < 0.5		αT < 0.5	αT
89/371	MEGA	MEGA > 10	–	MEGA > 5	
	NT + ester	NT < 0.5			
	βT + ester	βT < 0.5			
		αT > 2		αT > 2	αT
	βE_2 + ester				
	EE_2				
89/426	MPA		–		
	MEGA				
	NT + ester				
	βT + ester	βT < 0.5			
		αT > 2		αT > 2	αT
	βE_2 + ester				
90/25	NT + ester	NT > 2	–	NT > 2	NT
	MT				
	βT + ester	αT < 0.5		αT < 0.5	T < 0.5
	βE_2 + ester				
91/92	βE_2 + ester		βE_2 < 0.5	NRD	NRD
			αE_2 < 0.5		
	βT + ester	βT < 4	βT		
		αT < 2	αT		
			P		
	Stan				
	CITA				
91/93	βE_2 + ester		βE_2 < 0.5	NRD	βE_2 < 0.5
	βT + ester	βT < 2	βT		
		αT < 2	αT < 0.5		
			P		
	Stan				
	CITA				
91/94	βE_2 + ester		βE_2 < 0.5	NRD	–
	βT + ester		βT		
		αT < 0.5	αT		
			P		
	Stan				
	CITA				
91/99	MPA	MPA > 10		NRD	
	CMA	CMA < 4			
	βE_2 + ester				
	βE_2		βE_2 < 0.5		βE_2 < 0.5
	NT + ester		βNT		
	T + ester	βT < 0.5	βT		βT < 0.5
		αT < 0.5	αT		
			P < 0.5		

TABLE 5 (continued)

Sample No.	Injection site	Concentration (ng g^{-1}) [a]			
		Kidney fat		Meat	
		HPTLC	GC–MS (without gestagens)	HPTLC	GC–MS (without gestagens)
91/100	MPA	MPA > 10 CMA < 3	βE_2	MPA < 2	
	βE_2 + ester				$\beta E_2 < 0.5$
	βT + ester	$\beta T < 3$	βT	$\beta T < 0.5$	$\beta T < 0.5$
		$\alpha T < 2$	αT		$\alpha T < 0.5$
		$\beta NT < 2$	βNT	$\beta NT < 0.5$	$\beta NT < 0.5$
	Stan		P		P
91/108	MPA	MPA < 4		MPA < 2	
	βE_2 + ester				
	NT + ester		βNT $\alpha NT < 0.5$		$\beta NT < 0.5$
	T + ester	βT	βT		$\beta T < 0.5$
			αT		$\alpha T < 0.5$
91/109	βE_2		$\beta E_2 < 0.5$		
	βE_2 + ester				
	MT				
	CITA				
	AP	AP < 3		AP < 0.5	
			$\beta T < 0.5$		
		$\alpha T < 0.5$	$\alpha T < 0.5$	$\alpha T < 0.5$	$\alpha T < 0.5$
91/110	βE_2		βE_2		βE_2
	βE_2 + ester				
	MT				
	CITA				
	AP	AP < 4		AP < 1	
		$\beta T < 0.5$	$\beta T < 0.5$		$\beta T < 0.5$
		$\alpha T < 2$	αT	$\alpha T < 1$	αT
91/111	βE_2		βE_2		$\beta E_2 < 0.5$
	βE_2 + ester				
	CITA				
		$\beta T < 2$	βT		$\beta T < 0.5$
		$\alpha T > 2$	αT	$\alpha T < 1$	αT
	AP	AP < 4		AP < 1	
91/115	MPA	MPA < 10		MPA < 2	
	βE_2				
	βE_2 + ester				
	NT + ester	$\beta NT < 2$	βNT	$\beta NT < 0.5$	$\beta NT < 0.5$
		$\beta T < 2$	βT	$\beta T < 0.5$	βT
		$\alpha T < 2$	αT	$\alpha T < 1$	αT
	MT	MT > 2	MT P	MT < 1	MT < 0.5
91/120	MPA	MPA > 3		MPA < 2	
	MEGA	MEGA > 3			
	AP	AP < 2		AP < 0.5	
			$\beta E_2 < 0.5$		$\beta E_2 < 0.5$
			$\beta NT < 0.5$		$\beta NT < 0.5$
			$\beta T < 0.5$		
		$\alpha T < 2$	$\alpha T < 0.5$ P	$\alpha T < 0.5$	$\alpha T < 0.5$ P

(Continued on p. 158)

TABLE 5 (continued)

Sample No.	Injection site	Concentration (ng g^{-1}) [a]			
		Kidney fat		Meat	
		HPTLC	GC–MS (without gestagens)	HPTLC	GC–MS (without gestagens)
91/121	MEGA	MEGA > 3			
	MPA	MPA > 3		MPA > 2	
	AP	AP < 4		AP < 0.5	
			βNT		
			βE_2		$\beta E_2 < 0.5$
			βT		βT < 0.5
		αT < 4	αT	αT < 0.5	αT
			P		P
91/123	βE_2 + ester		$\beta E_2 < 0.5$	NRD	$\beta E_2 < 0.5$
			βT < 0.5		βT < 0.5
		αT < 2	αT		αT < 0.5
			P < 0.5		P
91/124			βT	NRD	
		αT < 0.5	αT		αT < 0.5
	CIT				
	CITA		P		P < 0.5
91/128	MPA	MPA < 8		MPA < 2	
	CMA	CMA < 2			
		βNT < 0.5	βNT		βNT < 0.5
		αT > 1	αT	αT < 1	αT < 0.5
	MT	MT > 1	MT		
	CITA				
91/129	MPA	MPA < 8			
			βNT < 0.5		
			βT		
		αT < 2	αT	αT < 1	αT < 0.5
			P		
91/136		MPA < 6		NRD	NRD
	βE_2 + ester				
	βT + ester		βT < 0.5		
		αT < 0.5	αT < 0.5		
	NT + ester		βNT		
			P < 0.5		
91/164	βE_2 + ester			NRD	$\beta E_2 < 0.5$
	CITA				
	AP	AP < 6			
		αT < 2	αT < 0.5		
91/165	βE_2 + ester		βE_2	NRD	$\beta E_2 < 0.5$
	AP	AP < 6			
			βT		
		αT < 2	αT		
	CITA				
			P		
92/23	βE_2 + ester	$\beta E_2 < 0.5$	βE_2		$\beta E_2 < 0.5$
	βT + ester	βT > 1	βT		
		αT > 2	αT	αT < 1	αT < 0.5
	Stan				
	CITA				
	CP				
			P		P

[a] NRD = no residues detected.

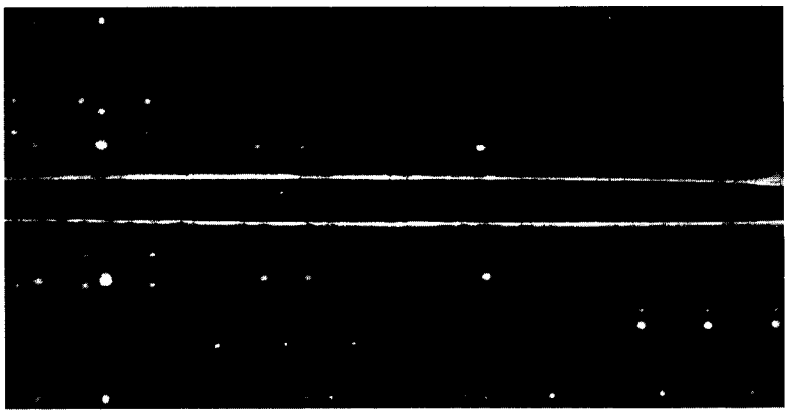

Fig. 6. Double one-dimensional elution of different fractions on the same 20 cm × 20 cm plate.

Figure 8 shows that one can have an idea of the difference in concentration of the same substance (MPA) in the meat and fat samples. Figure 9 shows the influence of the method used on the purification of the extract, the detected substances and their concentration. Figure 10 shows

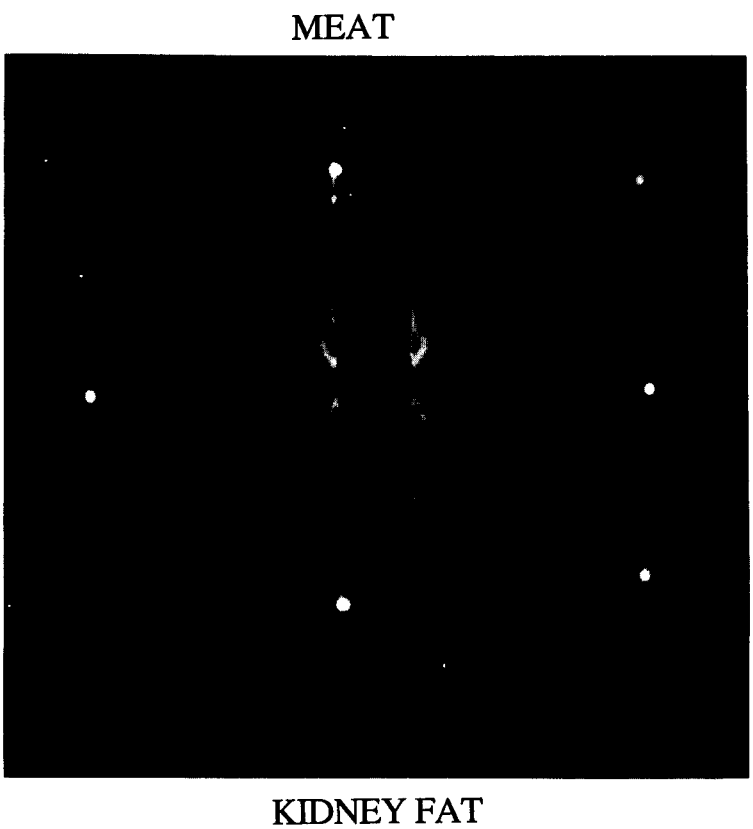

Fig. 7. Two-dimensional co-chromatography of the fraction containing MT and αT. Lower part of the plate, kidney fat extract; upper part, meat extract.

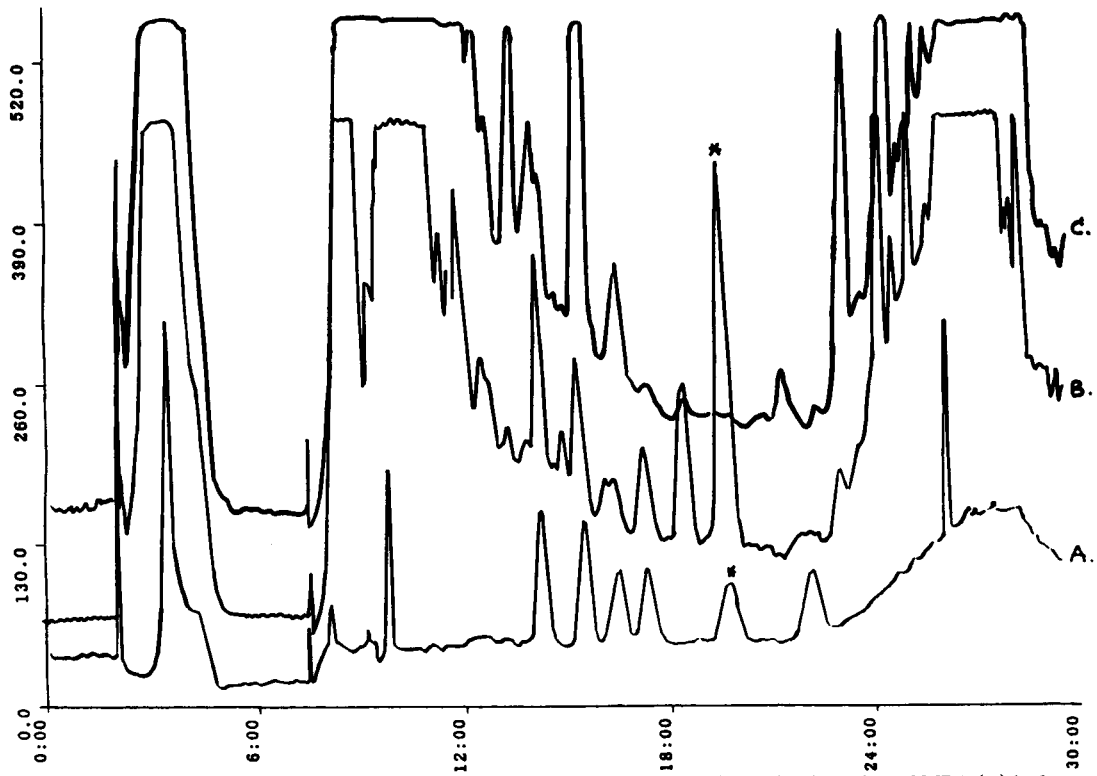

Fig. 8. Chromatogram with UV detection showing the influence of the matrix on the detection of MPA (*) in fat and meat samples originating from the same carcass. (A) Standard mixture; (B) kidney fat extract; (C) meat extract.

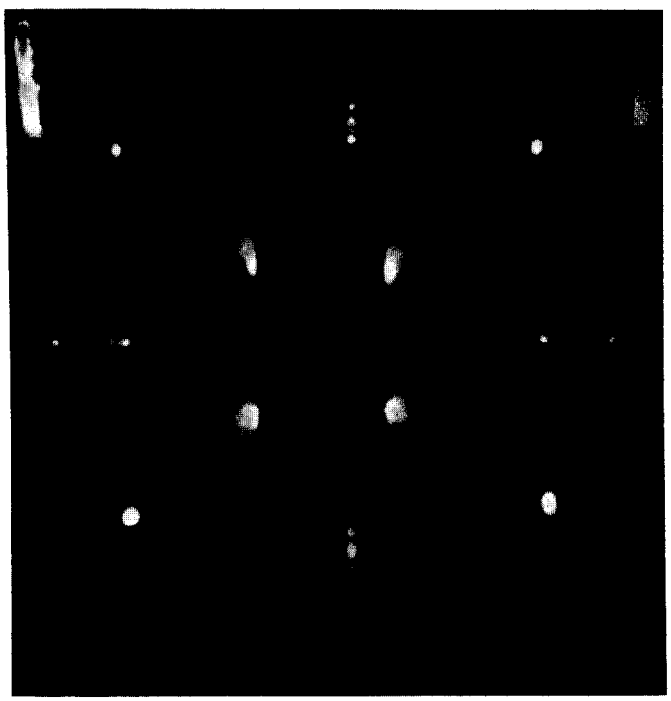

Fig. 9. HPTLC showing the influence of the method used on the purification of the extract, the detected substances and their concentration.

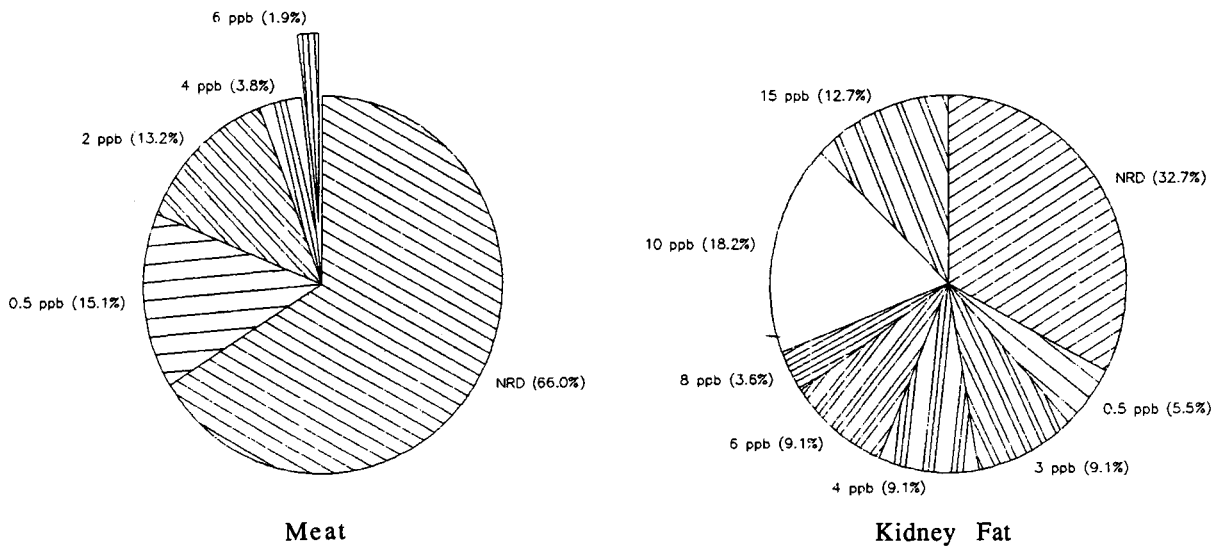

Fig. 10. Concentrations of MPA detected in 55 kidney fat and meat samples (ppb = ng g^{-1}).

the proportional occurrence of the different ranges of MPA in fat and meat.

GC–MS

As this technique is more sensitive and selective than LC–UV detection and HPTLC, more complete results on the composition of the extracts were obtained. This is confirmed by the results summarized in Table 5.

Conclusions

The influence on the results of which matrix is analysed has been clearly demonstrated. Injection sites contain residues of anabolics in their non-metabolized form, as they are injected, and in a concentration where there normally are no detection problems. It appears that there is a considerable difference in concentration of the detected substances depending on the kind of tissue analysed. As the concentrations in the fat and even more in the meat samples are much lower than those in the injection sites, the results are also dependent on the sensitivity and selectivity of the method used.

The main conclusion is that it should be stressed that no one analytical technique possesses sufficient specificity for a positive identification. Evidence from many separate sources of data (e.g. UV detection, HPTLC, GC–MS) is required in order to increase the probability of a correct interpretation.

Finally, the importance of uniformity in the choice of the method when a particular kind of sample is to be analysed by different analysts in different laboratories is stressed. Strict quality criteria should assist the maximum correlation between results.

Thanks are due to the laboratory team for technical assistance, especially Mrs. Martine Delval-Deridder, Mrs. Christiane Pâques-Sauveur and Mrs. Annick Debbaut for their cooperation in the development and application of the chromatographic and application techniques.

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Metabolism of anabolic agents in the racing greyhound

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Abstract

As in other sports, the use of anabolic agents is not permitted in Greyhound Racing. Currently, there is little data available on the metabolism of steroids in the racing greyhound. In the present study, the normal urinary steroid profile has been established in greyhounds which are actively being trained. The excretion of radio labelled and stable isotopically labelled testosterone was examined to provide a greater understanding of the production, turnover and excretion in the animals. The pharmacokinetics of nandrolone (19-nortestosterone) was also examined. It was noted that the levels of naturally occurring steroids in greyhound urine are far lower than in humans and it was difficult to demonstrate the presence of any steroid in urine except by GC–MS in the selected ion recorded mode. There also exists a sex difference between dogs and bitches with corticosteroid levels being higher in the bitch than the dog. In parallel to approaches now being suggested for other sports, the levels of specific endogenous steroids were examined. It was noted that although the levels of endogenous steroids were depressed following the dosing of an anabolic agent, no significant changes in the ratios of endogenous steroids were noted.

Keywords: Gas chromatography; Mass spectrometry; Anabolic steroids; Animal samples; 19-Nortestosterone; Greyhound racing

As with other sporting bodies, the National Greyhound Racing Club (NGRC) has declared the use of chemical substances to enhance or diminish the performance of a racing animal to be contrary to the rules of the sport. To achieve this aim, urine samples from racing animals are screened for the presence of illegal doping substances. In a further attempt to eliminate any chemical manipulation of the greyhounds, racing animals which perform outside certain limits, based on their established form, are subject to a second screen based on a post race sample of blood or urine. The latter samples are sent to the reference laboratory in Glasgow for further analysis by radioimmunoassay (RIA), liquid chromatography (LC), gas chromatography (GC), and when necessary gas chromatography–mass spectrometry (GC–MS). To extend and improve the detection and identification of illegal doping substances in the racing greyhound, an active research program is in progress dealing with the metabolism and excretion of contraceptive and anabolic steroids in the greyhound.

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EXPERIMENTAL

Materials and equipment

Steroid standards, deuterium-labelled testosterone standard, *Helix pomatia* digestive juice, creatinine assay kits and heptafluorobutyric acid anhydride were purchased from Sigma (Poole). [^{3}H]-Testosterone (specific activity 319 mCi/mg) was purchased from Amersham. [^{3}H]-Corticosterone, [^{3}H]-dehydroepiandrosterone sulphate and [^{3}H]-estradiol glucuronide were purchased

from New England Nuclear. Methoxyamine hydrochloride, trimethylsilylimidazole (TMSI), *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) and hexamethyldisilazane (HMDS) were purchased from Pierce and Warrender (Chester). Insta-gel scintillation fluid and Lipidex-5000 were purchased from Canberra Packard. Laurabolin (nandrolone laurate) was obtained from Intervet (Cambridge). All other solvents and reagents employed were of analytical grade or higher.

Gas chromatography–mass spectrometry

All GC–MS work was carried out using the following equipment and conditions.

The gas chromatograph was a Hewlett-Packard Model HP5890 fitted with a fused silica column (CP-Sil 5, 25 m × 0.32 mm i.d., d_f 0.4 μm). Samples were injected using a Hewlett-Packard Model 7673 autoinjector and a split/splitless injector fitted with a goosenecked liner. The latter was operated in the splitless mode for 0.7 min after injection. The mass spectrometer used was a VG Analytical Model 70-250S double focusing instrument. The delivery line from the chromatograph to the mass spectrometer was held at 280°C.

Scintillation counting

All scintillation counting used a Canberra Packard 2200CA Tri-Carb liquid scintillation analyser.

Animals

All animals employed in this study were fit, healthy greyhounds. All experimental procedures employed were subject to ethical and regulatory approval.

Normal urinary steroid profile of the racing greyhound

The following method was used, based on procedures published by Shackleton and co-workers [1–3].

Extraction and hydrolysis. Urine (10 ml) was centrifuged at 2500 rpm (1200 *g*) for 10 min to remove any particulate material. The urine was applied to a C18 Sep-Pak® cartridge which had been conditioned with methanol (10 ml) and distilled water (10 ml). The cartridge was washed with a further portion of distilled water (10 ml) and eluted with methanol (5 ml). The extract was taken to dryness under a stream of nitrogen at 60°C. Once dry, the residue was suspended in sodium acetate buffer (pH 4.6, 0.2 M, 20 ml) and *Helix pomatia* digestive juice extract containing approximately 100 000 units of glucuronidase activity and 8000 units of sulphatase activity was added. This was incubated in a water bath at 55°C for 3 h or at 37°C for 24 h.

Following incubation, the sample was re-extracted through the same C18 Sep-Pak® cartridge previously used following the same procedure described above. The extract was taken to dryness under a stream of nitrogen at 60°C and the residue was dissolved in 5 ml cyclohexane–ethanol (4:1, v/v).

Sample purification. Sephadex LH-20 (1 g) was equilibrated in an excess of cyclohexane–ethanol (4:1, v/v). Once equilibrated, the gel was transferred to a chromatography column (20 cm × 1 cm i.d.) with a reservoir (50 ml) at the top and a sintered glass plate above a stopcock at the lower end. The sample was loaded onto the Sephadex column, taking care not to allow the Sephadex to dry out. The column was eluted with a further 45-ml portion of cyclohexane–methanol (4:1, v/v). This fraction contained the steroids while the more polar components of the matrix were retained on the column. The column was regenerated by washing with methanol (75 ml) before re-conditioning it with cyclohexane–ethanol (4:1, v/v) prior to loading the next sample.

The fractions containing the eluted steroids were concentrated by rotary evaporation, transferred to vials and stored at −20°C until required for analysis.

Sample derivatisation. Each extract was dried under a stream of nitrogen and a solution of methoxyamine hydrochloride (2%, w/v in pyridine, 50 μl) was added. After incubation at 60°C for 60 min, TMSI (50 μl) was added and the mixture heated for a further 16 h at 100°C. To remove the excess derivatisation reagents the mixture was passed through a short column of Lipidex-5000 equilibrated with cyclohexane–pyridine–HMDS (98:1:1, v/v/v). Once the sample was applied, the column was eluted with a

further portion of cyclohexane–pyridine–HMDS (2 ml). This fraction was collected and evaporated under a stream of nitrogen at 60°C. The residue was redissolved in hexane (100 μl) and aliquots (1 μl) analysed by GC–MS.

GC–MS conditions. The injector temperature was 280°C and the column oven was programmed from 60°C (held for 0.5 min) at 20°C/min to 200°C (held for 0.5 min) then at 5°C/min to 300°C (held for 15 min). MS was operated in full scan mode (1 second per decade, interscan time 0.3 s) using electron impact ionisation at 30 eV.

Metabolic studies

[^{3}H]-Testosterone

Dose preparation. Radioactive testosterone (ca. 150 μCi) was dissolved in 60% aqueous ethanol. The dose homogeneity was checked by taking small known aliquots of the dose and assessing the radioactivity present. The coefficient of variation (C.V.) was determined and found to be acceptable (C.V. = 0.87%, $n = 6$). The dose was sterilised by passing it through a Millix-OR 0.2-μm filter obtained from Millipore, and sealed in a sterile vial with a rubber septum.

Dose administration. One greyhound was dosed intravenously with 1 ml of the prepared solution, injected into the jugular vein.

Housing. The greyhound was housed in a steel metabolic cage to allow complete collections of urine and faeces to be made, and to prevent the radioactive contamination of the kennel environment.

Radioactive sample collection. To facilitate the collection of blood samples, a cannula was inserted into the jugular vein of the animal under local anaesthetic prior to dosing. Blood samples were collected by allowing blood to pass through the cannula into the heparinised sample tubes and were separated into red blood cells and plasma by centrifugation. The plasma was removed for analysis. Separate urine collections were made at 8, 24, 48, 72 and 120 h post dose into pre-weighed containers.

Analysis of radioactive samples. Portions of the plasma were taken and made up to 1 ml where necessary with water. Scintillant (Insta-gel, 10 ml) was added and the mixture shaken. The radioactivity present was determined by scintillation counting for 5 min.

The total weight of urine excreted was determined. A portion of the sample (ca. 1 g) was weighed into a scintillation vial and scintillant (Insta-gel, 10 ml) was added. The radioactivity present was determined by scintillation counting for 5 min.

[$^2H_3$]-Testosterone

Dose preparation. [$^2H_3$]-testosterone (8 mg) was dissolved in 60% aqueous ethanol. The solution was filtered through a Millix-OR 0.2-μm filter, and transferred to a sterile vial which was then sealed with a sterile rubber septum.

Dose administration. One greyhound was dosed intravenously with 1 ml of the prepared solution, injected into the jugular vein.

Sample collection. Blood samples were taken from the jugular vein by syringe and transferred to heparinised blood tubes for separation into red blood cells and plasma. Urine samples were collected by a kennel nurse in a bowl during exercise periods.

Sample extraction. This extraction method is based on a procedure by Belanger et al. [4]. Ethanol (5 ml) was added to the plasma sample (1 ml) and gently mixed for 5 min. The sample was centrifuged [2500 rpm (1200 *g*), 5 min] and the supernatant removed. The pellet was washed with a further portion of ethanol (5 ml), centrifuged and the supernatant was removed and combined with the supernatant already obtained. The combined extracts were taken to dryness under a stream of nitrogen at 60°C. The dry residue was suspended in 2 ml methanol–water (5:95, v/v; solution A). A C18 Sep-Pak cartridge was conditioned with methanol (10 ml), distilled water (10 ml) and solution A (10 ml). The sample was loaded onto the column and washed with solution A (10 ml). The steroids were eluted from the column with 3.5 ml methanol–water (85:15, v/v; solution B).

The extract from the Sep-Pak cartridge was taken to dryness under a stream of nitrogen at 60°C. To each sample, internal standard, 17β-trenbolone was added. Trimethylsilyl derivatives

TABLE 1

Selected ion recording parameters used for the analysis of selected steroids as their trimethylsilyl derivatives

Compound	Ions (m/z)	Dwell time (ms)
17β-Trenbolone	327.1780	5
	342.2015	5
Testosterone	345.2250	50
	360.2485	50
[$^2H_3$]-Testosterone	348.2438	50
	363.2673	50

were formed by adding MSTFA (50 μl) and incubating at 60°C for 15 min.

Gas chromatography parameters. The injector temperature was 280°C. The column oven was programmed from 100°C (held for 0.5 min) at 10°C/min to 300°C (held for 15 min). Aliquots (1 ml) were injected using a Hewlett-Packard Model 7673 autoinjector.

Mass spectrometry parameters. The ions monitored and the dwell times are listed in Table 1. The ratio of [$^2H_3$]-testosterone to [$^2H_0$]-testosterone was calculated from the relative areas under selected ion chromatograms for their molecular ions of [$^2H_3$]-testosterone and [$^2H_0$]-testosterone.

Nandrolone (19-nortestosterone)

Dosing. Two dogs were dosed intramuscularly with 1 ml of Laurabolin, a commercial preparation containing 50 mg of nandrolone laurate per ml of oily solution. Sample collection was as for the [$^2H_3$]-testosterone experiment except that the samples were taken from the saphenous vein.

Sample extraction. Plasma samples were extracted as for the [$^2H_3$]-testosterone samples.

Urine samples were extracted using Bond-Elut® C18 cartridges containing 500 mg of sorbent. The urine sample (10 ml) was loaded on a cartridge which had been conditioned with methanol (5 ml), water (5 ml) and 5 ml methanol–water (5:95, v/v). After loading the sample, the cartridge was washed with 5 ml methanol–water (5:95, v/v) and eluted with 5 ml methanol–water (80:20, v/v). This extract was taken to dryness under a stream of nitrogen at 60°C and redissolved in sodium acetate buffer (pH 4.6, 0.2 M, 20 ml). *Helix pomatia* digestive juice extract containing approximately 100 000 units of glucuronidase activity and 8000 units of sulphatase activity was added and the mixture was incubated in a water bath at 37°C for 16 h. The samples were then re-extracted through the C18 cartridges used previously using the same methodology.

Sample derivatisation. Sample derivatisation was based on a method published by Ehrsson et al. [5]. The extracts obtained were taken to dryness under a stream of nitrogen at 60°C and dissolved in toluene (0.5 ml). Triethylamine (0.05 M in toluene, 100 μl) and heptafluorobutyric acid anhydride (100 μl) were added and the resulting mixture heated at 100°C for 15 min. Following incubation, the solvent was removed under a stream of nitrogen at 60°C and the residue redissolved in toluene (100 μl), and analysed by GC–MS.

GC–MS parameters. The GC system used was the same as for [$^2H_3$]-testosterone. The mass spectrometer was employed in the selected ion recording mode, monitoring the ions listed in Table 2.

Creatinine measurements. The levels of creatinine in each urine sample were determined with a commercial kit according to the manufacturer's instructions, using a suitable dilution of the urine.

TABLE 2

Selected ion recording parameters used for the analysis of selected steroids as their heptafluorobutyrate derivatives

Compound	Ions (m/z)	Dwell time (ms)
Testosterone/ epitestosterone	665.1373	50
	680.1608	50
Nandrolone	666.1451	50
[$^2H_3$]-Testosterone	668.1561	10
	683.1795	10
Etiocholanolone/ androsterone	471.1770	50
	486.2005	50

RESULTS AND DISCUSSION

Normal urinary steroid profile

It was initially difficult to establish the normal urinary steroid background profile in the racing greyhound because of the low levels of steroids normally present. However, following the development of the extraction procedure described, significant differences between dogs and bitches were noted. There were no steroids detectable in the urinary extract of the male animals by repetitive full scan GC–MS, but the presence of estradiol, cortisol, β-cortol, tetrahydrocortisol (two peaks), and $3\alpha,11\beta,17\alpha,20\alpha$-tetrahydroxy-$5\beta$-pregnane was noted in the urine from bitches (Fig. 1). The two peaks present for tetrahydrocortisol were due to the formation of diastereoisomeric oxime derivatives which were separated on the GC column.

[^{3}H]-Testosterone

High specific activity [^{3}H]-testosterone was used to avoid enlarging the endogenous pool size of testosterone in the greyhound. The weight of injected testosterone was around 6 ng. The apparent volume of distribution at t_0 was 5.8 l as calculated from the initial exponential part of the curve. The approximate blood volume of the greyhound in question is 3 l. This result shows

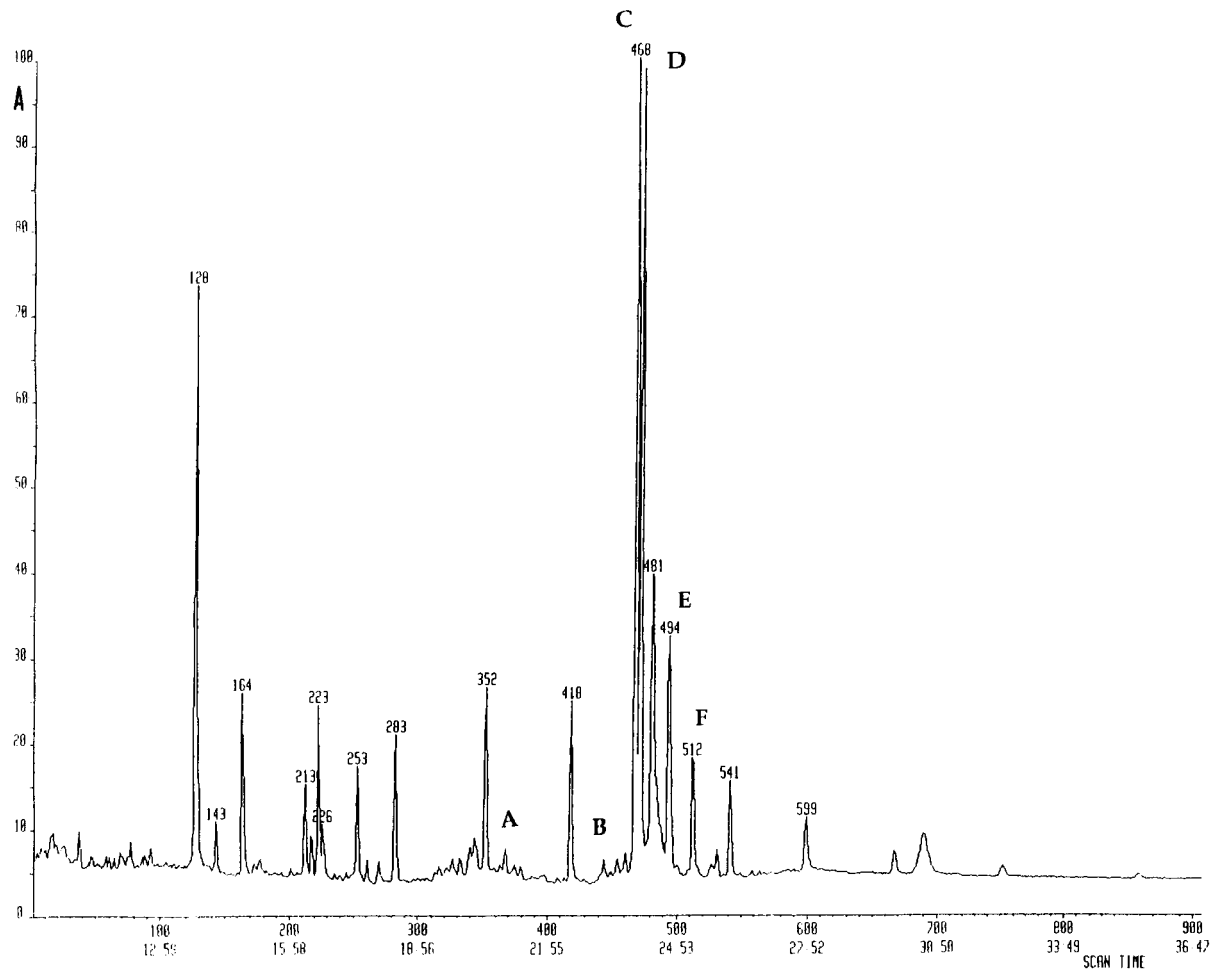

Fig. 1. Total ion current trace for greyhound bitch normal urinary steroid profile. A = Estradiol; B = cortisol; C and D = tetrahydrocortisol; E = β-cortol; F = $3\alpha,11\beta,17\alpha,20\alpha$-tetrahydroxy-$5\beta$-pregnane.

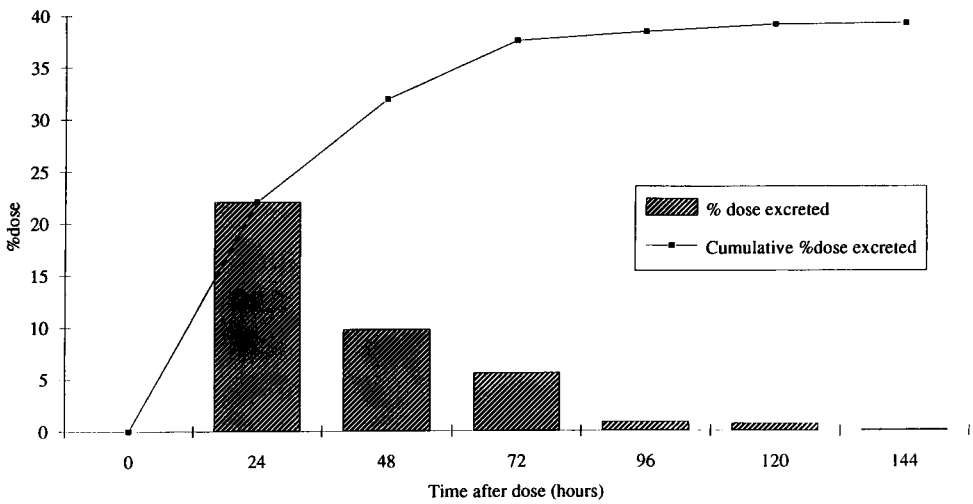

Fig. 2. Dose recovered in urine (%) versus time after dose following intravenous injection of a greyhound with [^{3}H]-testosterone.

that the testosterone was rapidly distributed into the tissues of the animal from the bloodstream with approximately 50% of the dose entering the tissues.

The radioactive testosterone and metabolites were excreted partially in the urine with 22% excreted within in the first 8 h, 32% being excreted in 24 h and a total of 39% of the dose excreted in the urine in 144 h (Fig. 2). This long period of excretion suggests that the dose was being retained significantly within the animal. If the plasma curve is examined (Fig. 3), there is the suggestion that after the initial rapid distribution phase, the level of radioactivity in the plasma oscillated over time, probably as a result of enterohepatic circulation. This recirculation also

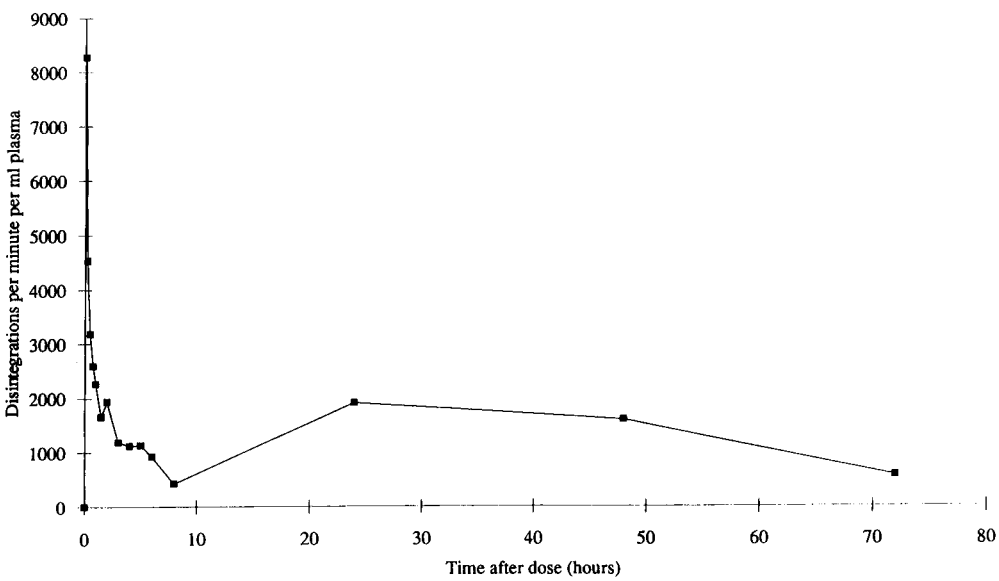

Fig. 3. Disintegrations per minute per ml of plasma versus time after dose following intravenous injection of a greyhound with [^{3}H]-testosterone.

provides an explanation for the prolonged presence of radioactivity in the urine.

[$^2H_3$]-Testosterone

The dose size was designed to enlarge significantly the endogenous pool of testosterone in the dog and to provide information on the pool size and the production rate of testosterone. Figure 4 shows the change in the ratio between [$^2H_3$]-testosterone and [$^2H_0$]-testosterone against time. The overall pattern of the curve is similar to that of Fig. 3, again with an increase in the concentration between 8 and 24 h post dose. As with the radio labelled dose this suggests the recirculation of the testosterone within the animal.

The ratio at 15 min post dose was 10.5 and this gave a value for the pool size of 186 μg compared to 35 μg in the human male. The ratio between labelled and unlabelled material was still significant after 24 h (0.65), suggesting a slow testosterone turn over rate for the system once equilibrium has been established.

Nandrolone (19-nortestosterone)

Due to the low levels of steroids in the plasma and urine all the results obtained from dosing with nandrolone laurate were obtained using selected ion recording. On initial analysis runs with standards, it was noted that nandrolone and epitestosterone had very similar retention times and baseline separation was not always possible when utilising trimethylsilyl, *O*-methyl oxime/trimethylsilyl, trifluoroacetyl or pentafluoropropionyl derivatives. This would normally not be a problem when employing a selected ion recording system where it is possible to distinguish between two compounds by looking at their separate ions. In this case, however, there was a possibility of interference due to the presence of isotopic peaks. The molecular ion of nandrolone is 1 a.m.u. heavier than the M-15 fragment of epitestosterone and the chromatographic overlap with epitestosterone could affect the apparent levels of nandrolone present. The use of heptafluorobutyryl derivatives allowed baseline separation of epitestosterone and nandrolone and so removed the source of error. It was also noted that 3-*keto*-Δ-4-steroids formed bis-heptafluorobutyryl derivatives as a result of *keto–enol* tautomerism. If no double bond was present in the A ring, as for example in etiocholanolone, only a mono derivative was formed.

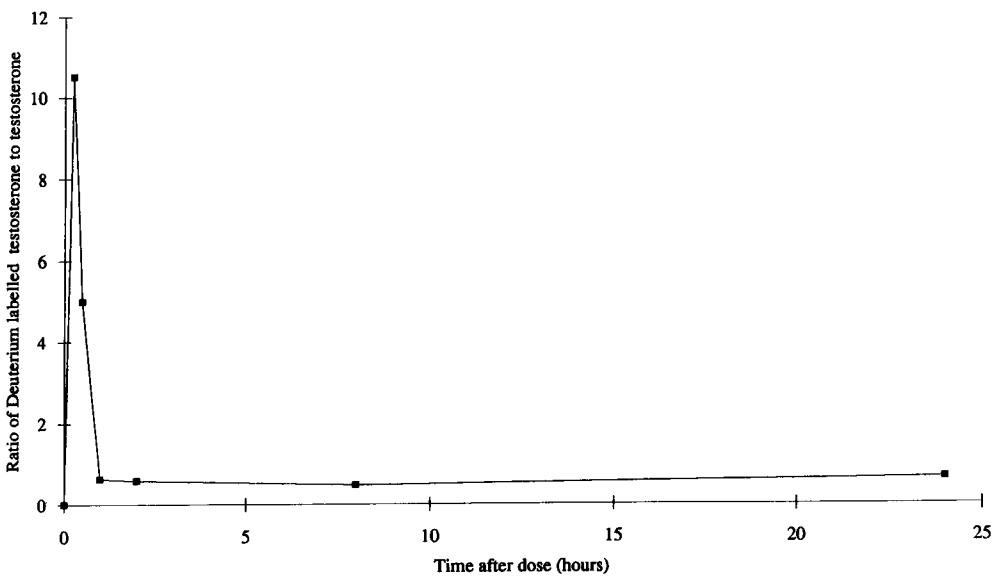

Fig. 4. Ratio of [$^2H_3$]-testosterone to testosterone in plasma versus time after dose following intravenous injection of 2 mg of [$^2H_3$]-testosterone in a racing greyhound.

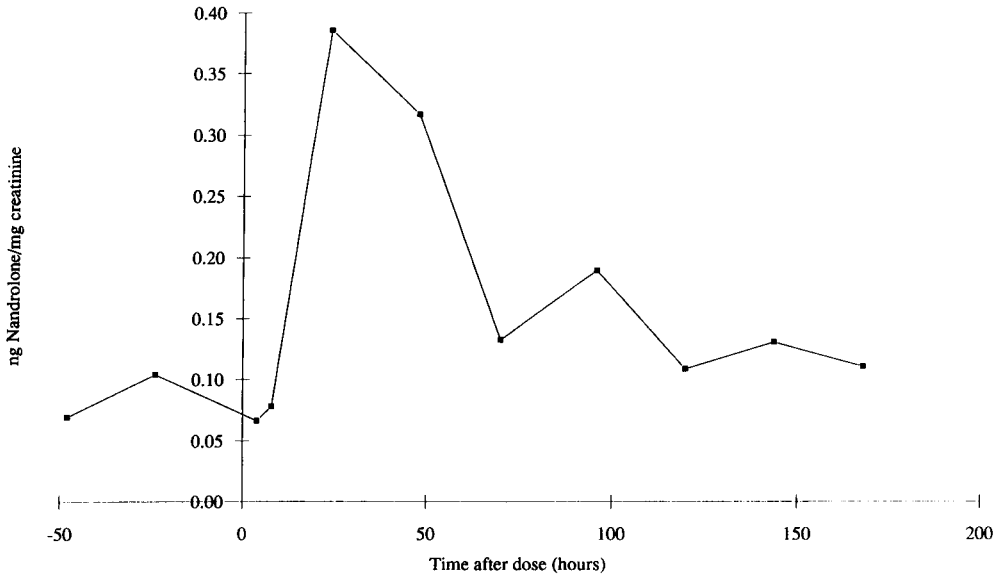

Fig. 5. Levels of nandrolone per mg creatinine in the urine of a greyhound versus time after dose following the intramuscular injection of 50 mg of nandrolone laurate.

As the animals involved in the experiment were under training, it was not possible to obtain complete urine collections daily. To overcome this problem of varying dilutions of the urine being analysed, all results were corrected with respect to the levels of creatinine present in the sample. The levels of nandrolone detected in the urine of the dogs are shown in Fig. 5. There was

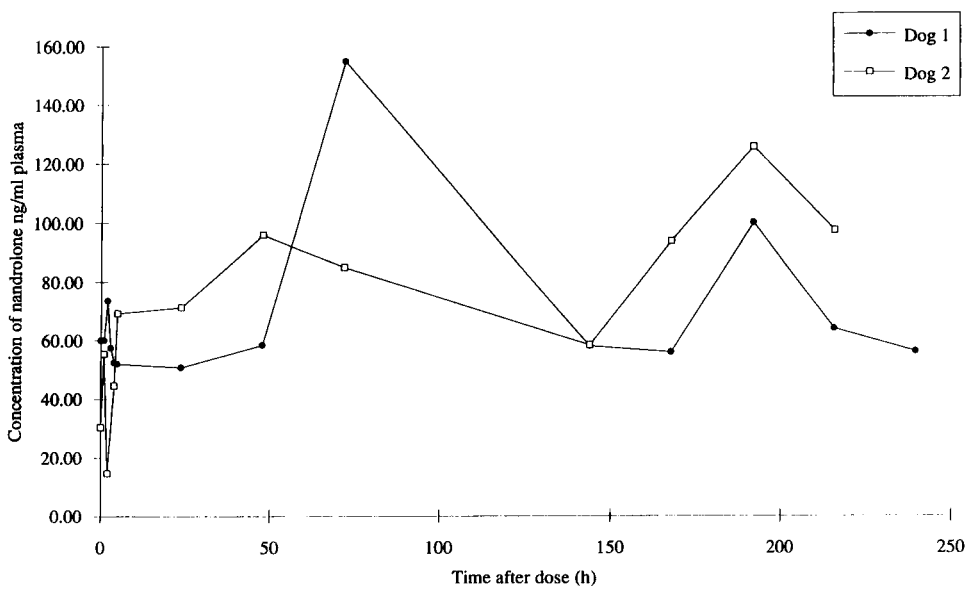

Fig. 6. Concentration of nandrolone in the plasma of greyhounds versus time after dose following intramuscular injection of 50 mg of nandrolone laurate.

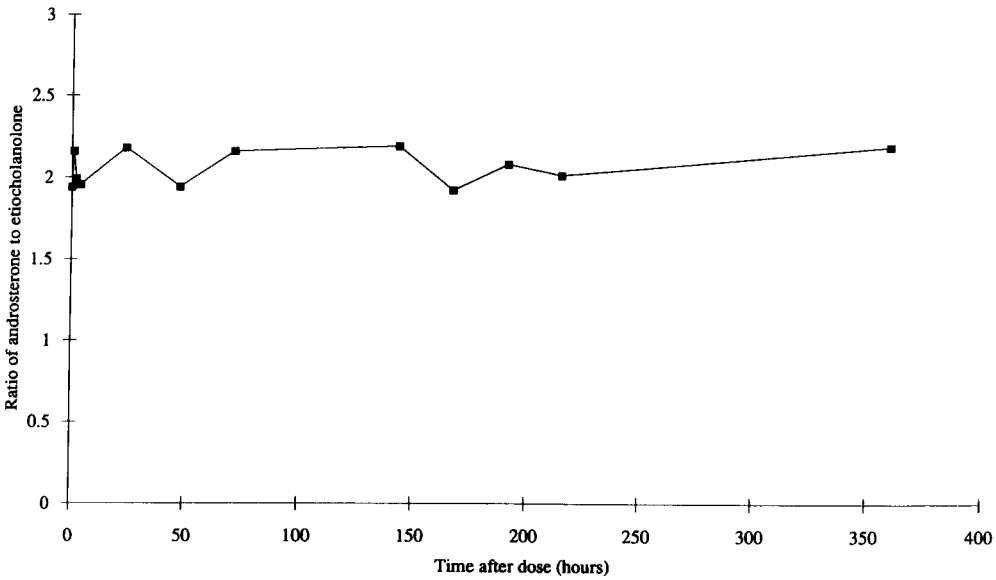

Fig. 7. Ratio of androsterone/etiocholanolone in greyhound plasma versus time after dose following intramuscular injection of nandrolone laurate.

an increase in the level of nandrolone detected after 24 h but this rapidly fell below the limit of detection by 72 h. This suggests that by far the largest portion of the drug is excreted in a metabolised form.

In plasma, endogenous levels of nandrolone of around 30 ng/ml in one dog and 60 ng/ml in the other dog were detected. Following injection of the anabolic agent, there is, in both animals, an initial rise in the concentration of nandrolone followed by a drop to below the initial sample level (Fig. 6). The release of nandrolone into plasma from the injection depot appears to be episodic as no steady level was attained in the plasma. The maximum concentrations of nandrolone in plasma are 125 ng/ml and 154 ng/ml for each dog and raised levels of nandrolone are detectable in plasma for at least 10 days following a single intramuscular dose.

It has been suggested [6] that when an anabolic steroid is introduced to a system in equilibrium, the biosynthesis and metabolism of endogenous steroids would be affected and as a result the ratios of excreted metabolites would be altered, in particular the ratio androsterone: etiocholanolone, which are the main androgen metabolites. While the levels of epitestosterone, testosterone, androsterone and etiocholanolone were all reduced in plasma and in one dog the levels of androsterone and etiocholanolone fell below the levels of detectabilty for several days, the ratio between androsterone and etiocholanolone did not vary significantly (Fig. 7). In urine, as in plasma, the levels of the steroids mentioned fell, but were still detectable throughout the period examined. Again there appeared to be no significant change in the androsterone to etiocholanolone ratio.

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Detection of growth hormones in the plasma of cows treated with recombinant bovine growth hormone

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Abstract

The levels of bovine growth hormone (bGH) in the plasma of cows treated with recombinant bGH (r-bGH) were measured in order to discriminate between treated and untreated animals. bGH concentrations were detected with an enzyme immunoassay (EIA) that uses polyclonal antibodies raised against pituitary bGH. The method can detect levels of bGH as low as 0.25 ng ml^{-1}. The r-bGH used for the animal treatment showed, in EIA, a cross-reactivity with the pituitary bGH of 77.23%. The animals were injected monthly with 640 mg of r-bGH in slow-release form, with a total of four injections. The experimental procedure showed, in the pattern of bGH concentrations of the treated cows, a peak on the seventh day after each injection. The increase was statistically significant in comparison with the basal level (56% increase; $P < 0.01$). The control animals did not show any significant variation in the plasma concentration of bGH throughout the experiment. The results indicate the usefulness of the EIA in discriminating r-bGH-treated from untreated animals.

Keywords: Enzymatic methods; Immunoassay; Cows; Hormones; Plasma; Recombinant bovine growth hormone

The administration of bovine growth hormone (bGH) to dairy cattle, in the recombinant form (r-bGH), has so far been authorized in India, South Africa, Czechoslovakia and the Community of Independent Republics (CSI) [1]. As precaution, in EEC countries its use is banned until December 1993 because, according to Directive 82/851/EEC, the recombinant forms have to be assessed as free from counter side effects. These are mainly related to consumer safety, animal health and production, and genetic selection.

In view of the above, a control strategy has been developed aimed at detecting, in real time, the administration of r-bGH to dairy cattle. The method, which takes into account the naturally occurring levels of bGH in the plasma of lactating cows, is also able to reveal the different levels of the hormone following the administration of r-bGH in the sustained-release form.

The availability of an enzyme immunoassay (EIA) procedure that detects both the natural and the recombinant form of bGH in plasma is the first step in a method for the routine screening of dairy cattle under treatment.

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EXPERIMENTAL

EIA procedure

The concentration of bGH in plasma was measured with a previously described EIA [2].

Polyclonal antibodies, raised against pituitary bGH, were used both immobilized in the solid phase to capture the antigen and as a second biotin-labelled antibody. Highly purified pituitary bGH was used as a standard. The method can detect concentrations of bGH as low as 0.25 ng ml^{-1}.

The cross-reactivity between the pituitary and the recombinant bGH was assessed by testing, in the EIA, serial dilutions of the r-bGH. From the curves obtained the ED_{50} was measured, defined as the amount of hormone (ng ml^{-1}) required for 50% of the total transmittance scale change, observed under the conditions used for the test. The percentage of cross-reactivity was given by the ratio (ED_{50} for bGH/ED_{50} for r-bGH) × 100.

r-bGH treatment

The experimental procedure has been described previously [3]. Forty Fresian cows (about 70 days post-partum; milk yield 23 kg day^{-1}) were divided into four groups. Two groups (Treated 1 and Treated 2) received a subcutaneous injection of 640 mg per cow of r-bGH slow release (Somidobove, Eli Lilly) every 28 days with a total of four injections. The other two groups (Control 1 and Control 2) received a placebo. The groups differed also in the dietary energy supplied: Control 1 and Treated 1 (low-energy diet) were fed 0.35 kg of concentrate No. 2 per kg of milk yield more than 18 kg day^{-1} and Control 2 and Treated 2 (high-energy diet) were given 0.5 kg of concentrate No. 2 per kg of milk exceeding 18 kg day^{-1}. Milk yield and composition were monitored once a week. Blood samples were collected from the jugular vein on days 0, 7 and 21 of each cycle and immediately centrifuged [2500 rpm (850 *g*), 10 min] and the plasma was stored at −40°C until used. Each sample was assayed in duplicate.

Statistical analysis

The GH secretory patterns measured in the four groups of cows were analysed with the Student *t*-test by comparing, in the individual groups, the baseline concentration with the level measured during the whole treatment period and with the concentration detected on the seventh day after injection. The baseline GH concentration was calculated according to Brinkley et al. [4], and was defined as those values lying within a normal distribution.

RESULTS AND DISCUSSION

The calibration graph used in the EIA for bGH was obtained by plotting transmittance (T) versus log[bGH concentration (ng ml^{-1})]. The response was linear over the range 0.25–10 ng ml^{-1} and the linear regression corresponded to the equation $\log(\text{ng ml}^{-1}\ \text{bGH}) = -0.02T + 1.15$. The ED_{50} for bGH, calculated from the regression parameters, was 1.41 ± 0.07 (S.D.) ng ml^{-1} ($n = 9$) [2]. The exogenous r-bGH showed, in the EIA for bGH, an ED_{50} value of 1.83 ± 0.10 ng ml^{-1} ($n = 6$), which, compared with the ED_{50} of bGH, gave a cross-reactivity of 77.23%. This value was not unexpected, as the recombinant hormone used was not identical with the pituitary hormone. In fact, the r-bGH contains eight amino acid residues added to the *N*-terminus of the sequence (M–F–P–L–D–D–D–D), and the first amino acid of the pituitary bGH, Ala, is replaced with Lys [5]. Because of this, the sequence homology between the two hormones is 95.5%.

The modifications introduced in the primary structure of the r-bGH molecule are responsible for the change in its molecular weight: the pituitary hormone shows an average molecular weight of 21 800, whereas the r-bGH has a molecular weight, calculated on the basis of the amino acid sequence, of 22 825. These modifications also influence the isoelectric point, which is 5.9 for the r-bGH and 7.9 for the hypophyseal hormone, as computed using the PC/GENE program (IntelliGenetic, Mountain View, CA). Moreover, the hydropathic profile, calculated according to Hopp and Woods [6], in the *N*-terminal part of the r-bGH molecule becomes more hydrophilic, suggesting that it could be a highly immunogenic area [6].

The injectable r-bGH used to treat the animals was in the slow-release form to overcome the problem of daily injections. In this form it was not detectable by EIA, because of the interfer-

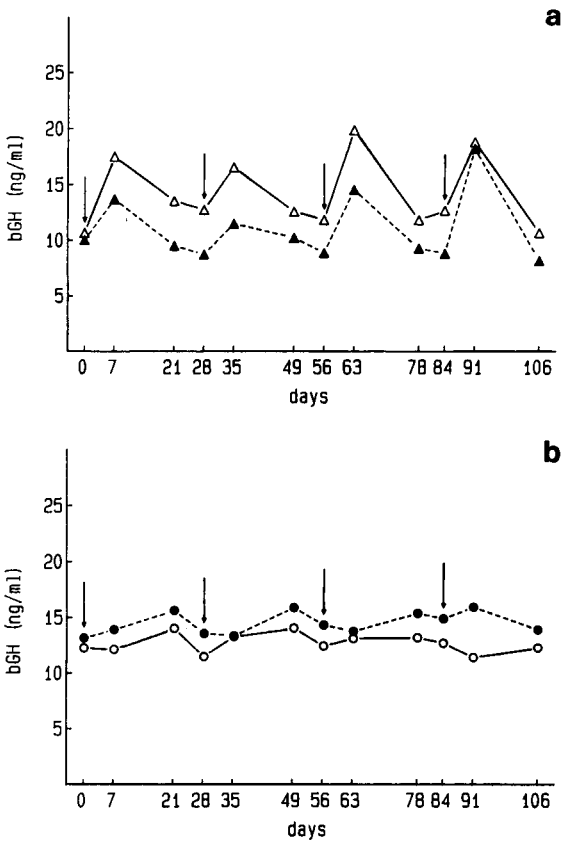

Fig. 1. GH secretory patterns measured in the plasma of the four groups of cows used (ten cows per group). (a) △ = Treated 1 and ▲ = Treated 2; each cow received an injection of 640 mg of r-bGH in the slow-release form on the days indicated by the arrows. (b) ○ = Control 1 and ● = Control 2; each cow received a placebo on the days indicated by the arrows. Each point represents the mean of the ten values determined in each group (ten cows per group).

ence of the drug medium. Nevertheless, the r-bGH was available to be assayed once released into the blood.

In Fig. 1 the bGH concentration profiles measured in the four groups of cows during the experimental period are shown. Each point is the mean of the ten values determined in each group (ten animals per group).

In Table 1 the plasma concentrations of bGH in the control and treated groups during the r-bGH treatment are reported. The baseline concentrations were slightly lower in the treated than in the control groups (11.99 ± 0.99 and 9.11 ± 0.60 ng ml^{-1} in comparison with 12.22 ± 0.52 and 13.98 ± 0.77 ng ml^{-1}).

The experimental procedure used revealed, in the patterns of bGH concentrations of the treated groups (Fig. 1a), a peak on the seventh day after each injection of r-bGH. Three weeks after injection, the levels fell to the baseline. Because of the hormone fluctuations, the bGH levels throughout the treatment were not significantly higher than the basal concentrations (Table 1). However, the increase in the plasma concentration of bGH detected on the seventh day after each injection of r-bGH was statistically significant in comparison with the basal level (52% and 59%, respectively, for the two groups; $P < 0.01$) (Table 1). As the experiment went on, this increment became particularly notable, reaching a maximum of a 100% increase for the treated group No. 2 on day 91. The control animals did not show any significant variation in the plasma concentration of

TABLE 1

Plasma levels of bGH during the treatment with r-bGH

Cows	Baseline [a] (ng ml^{-1}) [b]	Level during treatment (ng ml^{-1}) [b]	t	P [c]	Level on the 7th day after injection (ng ml^{-1}) [b]	t	P [c]
Control 1	12.22 ± 0.52	12.69 ± 0.87	1.03	n.s.	12.49 ± 0.89	0.53	n.s.
Control 2	13.98 ± 0.77	14.47 ± 1.02	0.88	n.s.	14.24 ± 1.15	0.38	n.s.
Treated 1	11.99 ± 0.99	14.12 ± 3.21	1.28	n.s.	18.20 ± 1.46	7.03	< 0.01
Treated 2	9.11 ± 0.60	10.97 ± 3.04	1.19	n.s.	14.50 ± 2.80	3.76	< 0.01

[a] The baseline was defined as those values lying within a normal distribution [4]. [b] Mean ± S.D. ($n = 10$). [c] n.s. = Not significant.

bGH (Fig. 1b and Table 1) throughout the experiment.

The results indicate that with the EIA used, it was possible to follow the release of bGH in the plasma of cows treated with r-bGH and to discriminate them from the untreated animals.

Regarding health and animal production aspects, counter side effects may arise from the release of r-bGH administered in the slow-release form, which may interfere with the natural secretion mechanism of bGH. Further, the structural differences between the recombinant and natural forms may lead to the formation of anti-bGH antibodies in dairy cattle treated over more than one lactation. Finally, the chemical matrices containing the r-bGH polypeptide may introduce a risk factor for consumer health, such as outbreaks of allergic phenomena [7].

The availability of a specific and rapid diagnostic assay such as the bGH EIA is useful for monitoring the administration of hormones licensed for veterinary use.

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Development of a high-performance thin-layer chromatographic method for the multi-screening analysis of corticosteroids

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Abstract

In order to screen injection sites for the presence of corticosteroids, a high-performance thin-layer chromatographic (HPTLC) system was developed for the determination of 26 corticosteroid standards. Different mobile phases were tested and chromatography with chloroform–methanol was thoroughly evaluated. The development on preloaded Kieselgel 60 HPTLC plates with chloroform–methanol (92 + 8, v/v) yielded the best separation. After examining different detection methods, a resorcylaldehyde spray was selected, yielding the best colour differentiation and fluorescence at 366 nm for several components.

Keywords: Thin-layer chromatography; Corticosteroids; Meat; Steroids

When suspected injection sites, collected from cattle in Belgian slaughterhouses, were routinely analysed for the presence of anabolic steroids, a decreasing percentage of positive results was observed (62% in 1990, 42% in 1991, 33% in the first half of 1992). One explanation for this decrease could be the misuse of "new" unknown anabolic steroids or of other xenobiotic products. Corticosteroids, for example, were suspected to be used in cattle production because of their ability to promote water retention in the body. In the literature, few data are available on the multi-screening analysis of corticosteroids.

As high-performance thin-layer chromatography (HPTLC) has been applied with success by several routine laboratories in the multi-residue analysis of anabolic steroids in injection sites, in this work attempts were made to develop a similar system for the determination of corticosteroids. Special attention was paid to the separation of dexamethasone and betamethasone and their esters, as commonly used veterinary pharmaceutical corticosteroid preparations on the Belgian market contain dexamethasone and its esters.

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EXPERIMENTAL

Chemicals

All reagents were of analytical-reagent grade and were used as received. Twenty-six corticosteroid standard materials, all of pharmacopoeial grade, were obtained from different sources.

The corticosteroid standards were divided into five standard mixtures (as listed in Table 1), based on the hR_F values ($hR_F = R_F \times 100$) in the different solvent mixtures tested. Stock standard solu-

TABLE 1

Corticosteroids, used as standards, divided into five standard mixtures with their mean hR_F values in the solvent system chloroform–methanol (92 + 8, v/v)

Mixture	Compound	hR_F	n	R.S.D. (%)
1	Deoxycorticosterone acetate	96	14	1.8
	Deoxycorticosterone	84	14	4.2
	Corticosterone acetate	75	14	5.3
	Betamethasone acetate	58	14	5.2
	Corticosterone	51	14	4.9
	Betamethasone	33	14	5.2
2	Cortisone acetate	68	14	6.2
	Prednisolone acetate	55	14	5.8
	Cortisone	47	14	5.1
	Allotetrahydrocorticosterone	46	6	2.2
	6α-Methylprednisolone	31	14	6.5
3	Dexamethasone acetate	62	14	6.5
	Betamethasone valerate	59	6	3.9
	Triamcinolone acetonide	48	14	6.3
	Dexamethasone	35	14	7.4
	Prednisolone	33	6	8.8
	Triamcinolone	23	14	12.2
4	Beclomethasone dipropionate	92	6	1.7
	Hydrocortisone acetate	60	14	7.0
	Dehydrocortisone	45	14	6.7
	Hydrocortisone	34	14	7.9
	Tetrahydrocortisol	24	6	3.8
5	Flumethasone pivalate	84	6	3.6
	Fludrocortisone acetate	67	6	5.2
	Fluorometholone	50	6	5.0
	Tetrahydrocortisone	34	6	4.7

tions contained 1 mg ml^{-1} in methanol and were stored at −20°C.

HPTLC conditions

HPTLC separations were carried out on preloaded Kieselgel 60 HPTLC plates (10 × 10 cm) (Merck, Darmstadt, Germany). A 0.75-μl volume of each standard solution was applied to the plate, 1 cm from the lower edge. The plates were preloaded for 20 min. Development was carried out in a Camag twin-through chamber at room temperature with 5 ml of mobile phase over a distance of 5 cm. The mobile phase was chloroform–methanol (92 + 8, v/v).

Detection

After elution, the plate was dried under a cool air stream and sprayed with the appropriate detection reagent. This spray reagent was prepared by dissolving 1% resorcylaldehyde (Janssen Chimica, Geel, Belgium) in acetic acid (solution A) and 10% sulphuric acid in acetic acid (solution B). Before use, equal amounts of solutions A and B were mixed. After spraying, the plate was heated for 10 min at 95°C in an oven and examined in daylight and under UV radiation at 366 nm.

RESULTS AND DISCUSSION

Solvent optimization

Solvent optimization is often based on the solvent selectivity triangle of Snyder [1]. Snyder classified solvents into eight groups, depending on their relative ability to function as a proton acceptor (X_e), a proton donor (X_d) or a strong dipole interactor (X_n). These three coordinates of a solvent determine its position in a triangle. The groups that are obtained in that way each contain solvents of more or less the same selectivity. By using a mixture of solvents with the greatest difference in selectivity, which means from the three apices of the selectivity triangle or in other words from group I (e.g., diethyl ether), group V (e.g., dichloromethane) and group VIII (e.g., chloroform), it is possible to achieve all possible solvent selectivities. Usually, a non-polar diluent (polarity < 2), e.g., hexane, is used to adjust to the ideal solvent strength.

In Table 2 the composition and polarity index (p') [1] of the tested solvent systems are listed. None of them was usable. Solvents of group A gave problems of insufficient separation, tailing and diffuse spots. In some solvents all components moved with the front (group B) or did not migrate at all (group C).

It was difficult to define the ideal solvent strength (p') of the mobile phase. As is shown in Table 2, solvent mixtures with comparable p' values, e.g., benzene–isopropanol (80 + 20, v/v) ($p' = 2.9$), diethyl ether–methanol (90 + 10, v/v) ($p' = 3$) and dichloromethane ($p' = 3.1$) gave completely different chromatographic behaviours of the standards. Therefore, solvent selection was based more on a trial-and-error approach and on data published by Szepesi and Gazdag [2].

Finally a chloroform–methanol mixture, which has often been reported [2–4], was tested as the mobile phase, yielding a good separation of all standards. The best chloroform to methanol ratio was determined by plotting the hR_F values versus the methanol concentration for the different standard solutions and by evaluation of the separation by the "equal spread criterion" of De Spiegeleer et al. [5]. This criterion is indicative of the distribution of the spots on the plate: when all components are equally spaced from each other and from the chosen boundaries, the function has the maximum value of 100% response. This criterion is given by the following function:

$$\left\{[hR_F(\max) - hR_F(n)][hR_F(l) - hR_F(\min)] \times \prod_{i=1}^{n-1}[hR_F(i+1) - hR_F(i)]\right\} \times \left\{\{[hR_F(\max) - hR_F(\min)]/(n+1)\}^{n+1}\right\}^{-1} \times 100\%$$

where hR_F(max) and hR_F(min) are the boundaries within which the spots must lie; there are n components in the mixture with l being the component of lowest hR_F and n being the component of highest hR_F.

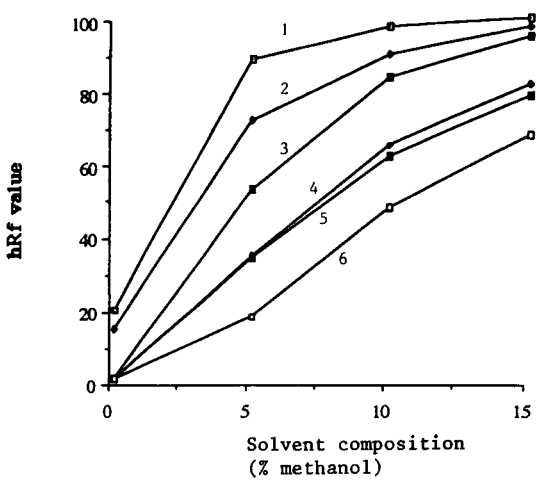

Fig. 1. Plot of hR_F values of standard mixture 1 versus the solvent composition (% methanol in chloroform). 1 = Deoxycorticosterone acetate; 2 = deoxycorticosterone; 3 = corticosterone acetate; 4 = betamethasone acetate; 5 = corticosterone; 6 = betamethasone.

TABLE 2

Solvent mixtures tested for solvent optimization

Mobile phase	Composition (v/v)	Polarity index, p'
Group A		
Diethyl ether	100	2.8
CH_2Cl_2–CH_3OH	90+10	3.3
Ethyl acetate	100	4.4
CH_2Cl_2–hexane–CH_3OH	50+48+2	1.7
$CHCl_3$–light petroleum–isobutanol–CCl_4	40+20+20+20	2.8
Light petroleum–isobutanol	52+50	2.0
Light petroleum–isobutanol–CCl_4	50+30+40	1.9
Benzene–CH_3OH	90+10	2.9
Benzene–isopropanol	80+20	2.9
Group B		
Diethyl ether–CH_3OH	90+10	3.0
Group C		
CH_2Cl_2	100	3.1
$CHCl_3$	100	4.1
Diethyl ether–hexane	70+30	2.0
Diethyl ether–hexane	88+12	2.5
CH_2Cl_2–hexane	90+10	2.8
$CHCl_3$–diethyl ether	90+10	4.0
$CHCl_3$–ethyl acetate	90+10	4.1
$CHCl_3$–CH_3OH–H_2O	90+10+1	4.3

A plot for standard mixture 1 is shown in Fig. 1. Chloroform–methanol (92 + 8, v/v) was found to be optimum, as is also illustrated by the response percentage values for the five standard mixtures for the different chloroform to methanol ratios as presented in Fig. 2.

TABLE 3

Response percentage values (boundaries 0–100) for the different standard mixtures under different conditions: normal saturation (NS), chamber saturation (CS) and preloading

Mixture	NS	CS	Preloading	n
1	21.2	24.8	25.1	6
2	5.9	6.9	7.2	5
3	6.0	4.6	6.2	6
4	31.4	39.9	47.3	5
5	46.6	51.0	78.6	4

Using this chloroform–methanol mixture (p' = 4.18) in a normal saturation (NS) chamber, "edge effects" occurred. Two possible solutions to this problem were tested: chamber saturation (CS) (30 min) and preloading of the plate (20 min). Table 3 shows the response percentages for the different standard mixtures using NS, CS and preloading conditions.

On preloading the plate for 20 min, the quality of the chromatogram was clearly superior. Under these conditions, the mean hR_F values (n) and relative standard deviation (R.S.D.) for the standards were determined and are given in Table 1. Slightly different hR_F values for dexamethasone and betamethasone and their respective esters were obtained with this chromatographic system.

Detection

A sulphuric acid solution in an alcohol is a generally used spray reagent for corticosteroids [2–4,6]. A 10% solution of sulphuric acid in methanol was tested. After heating the plate for 10 min at 95°C, all standards were detectable at the amount used (750 ng per spot). However, this spray reagent was found not to be selective enough, as components with comparable hR_F values (e.g., dexamethasone and betamethasone) were identically coloured (Table 4). Other reported detection reagents [2,6,7] were tested, but were not used for the reasons indicated in Table 5. Finally, a resorcylaldehyde spray reagent [6] was applied, yielding good selectivity. The composition of this reagent was slightly modified from that published [7]. As shown in Table 4, the two isomers dexamethasone and betamethasone were differently coloured, which resulted in an certain identification.

The detection limit was determined by serially diluting the stock standard solutions in methanol (1:2). For a clear detectable spot, standards should be spotted in an amount of at least 380 ng per spot.

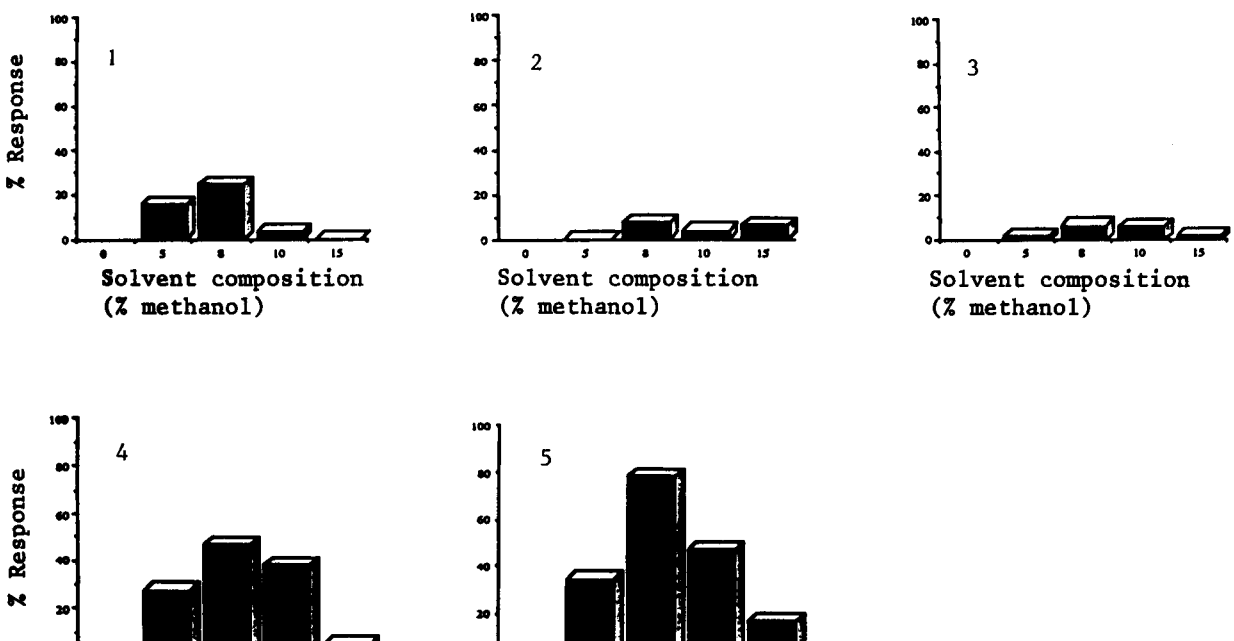

Fig. 2. Response percentage values (boundaries 0–100) of the five standard mixtures for the different solvent compositions, showing that the composition chloroform–methanol (92 + 8, v/v) yields the best chromatographic separation.

TABLE 4

Appearance of the corticosteroid spots after spraying with methanolic sulphuric acid solution and resorcylaldehyde reagent

Compound	Colour		Fluorescence at 366 nm	
	H_2SO_4	Resorcylaldehyde	H_2SO_4	Resorcylaldehyde
Deoxycorticosterone acetate	Purple	Purple		
Deoxycorticosterone	Purple	Purple		
Corticosterone acetate	Blue-purple	Brown	Yellow	Yellow
Betamethasone acetate	Blue-grey	Blue		
Corticosterone	Blue-purple	Brown	Yellow	Yellow
Betamethasone	Blue-grey	Blue		
Cortisone acetate	Light yellow	Yellow		
Prednisolone acetate	Burgundy-brown	Burgundy-brown		
Cortisone	Light yellow	Yellow		
Allotetrahydrocorticosterone		Purple		
6α-Methylprednisolone	Burgundy-brown	Burgundy-brown		
Dexamethasone acetate	Blue-grey	Green-grey		
Betamethasone valerate		Blue		
Triamcinolone acetonide	Light yellow	Purple		
Dexamethasone	Blue-grey	Green-grey		
Prednisolone		Burgundy-purple		
Triamcinolone	Light yellow	Purple		
Beclomethasone dipropionate		Blue		
Hydrocortisone acetate	Yellow-brown	Brown	Yellow	Yellow
Dehydrocortisone	Orange-yellow	Orange-yellow		
Hydrocortisone	Yellow-brown	Brown	Yellow	Yellow
Tetrahydrocortisol		Purple		
Flumethasone pivalate		Yellow-green		
Fludrocortisone acetate		Orange-brown		
Fluorometholone		Yellow-green		Yellow
Tetrahydrocortisone		Brown		

TABLE 5

Detection reagents tested and their shortcomings

Detection reagent	Shortcoming
Phosphoric acid in methanol	Detection limit > 750 ng per spot
Tetrazolium blue	All standards similarly coloured
p-Toluenesulphonic acid	Not specific
2,3,5-Triphenyl-2H-tetrazolium chloride (TTC)	All standards similarly coloured

In future work this method will be applied to the analysis of injection sites for the presence of corticosteroids.

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Development of an enzyme immunoassay for the determination of porcine growth hormone in plasma

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Abstract

A competitive enzyme immunoassay for the determination of porcine growth hormone (pGH) was developed. The assay is based on (anti-rabbit IgG) sheep IgG-coated microtitre plates, anti-pGH from rabbits, biotinyl-pGH and streptavidin–horseradish peroxidase. The assay is done directly with 40 μl of plasma and the calibration graph (90% relative binding at 0.36 ng ml^{-1} and 50% relative binding at 4.4 ng ml^{-1}) is prepared in plasma without measurable endogenous pGH. Recovery of pGH added to different plasma samples amounted to 102–119%, and all variabilities were < 8.6%. The assay shows 50% cross-reaction with recombinant pGH, but no significant binding of porcine prolactin, follicle stimulating hormone and luteinizing hormone. The assay detects high and low plasma pGH levels within the physiological variation as well as changes in plasma pGH after stimulation with growth hormone releasing factor or treatment with recombinant pGH. Hence it offers a reliable alternative to radioimmunoassay. However, a reference method providing evidence on the identity of exogenous pGH will be required for proving illegal treatment with pGH and for forensic purposes.

Keywords: Enzymatic methods; Immunoassay; Plasma; Porcine growth hormone

In addition to the classical sex hormones (estrogens and androgens) and the β-agonists, growth hormone has also gained scientific and practical importance for growth promotion [1,2]. Porcine and bovine growth hormone (pGH, bGH) are 191 or 190 amino acid proteins, they share a high degree of homology (90%) and they are produced in the anterior pituitary. Today both proteins can be synthesized by transformed bacteria [3]. Liver, skeleton, adipose tissue and muscle are the major target tissues. There are clear metabolic effects of GH and the reduced glucose utilization by adipose tissue seems to be most important. In addition, amino acid degradation in the liver is reduced. In consequence, more glucose and amino acids are available for muscle and skeleton growth [4,5]. Induced hepatic IGF-1 may also be of importance for increased growth rates [6].

To study the control of GH secretion or to monitor the application of GH, a sensitive assay is required. Recently a competitive enzyme immunoassay (EIA) for luteinizing hormone was described that uses the double antibody technique and biotin–streptavidin–horseradish peroxidase amplification [7]. In order to avoid any radiolabel a similar assay was developed for pGH.

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EXPERIMENTAL

Preparation of biotinyl-rpGH label

A 200-mg amount of biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimideester (Biotin-X-NHS; Sigma, Deisenhofen, Germany) dissolved in 20 ml of *N*,*N*-dimethylformamide (Aldrich, Steinheim, Germany) was used for coupling with 500 μg of recombinant porcine growth hormone (rpGH) (kindly provided by Pitman-Moore, Mundelein, IL, lot number 148–215) dissolved in 500 μl of 50 mM carbonate buffer (pH 9.98). The reagents were mixed and incubated at room temperature with gentle stirring for 4 h. The reaction was stopped by adding 0.4 mg of glycine (Serva, Heidelberg, Germany) in 1 ml of carbonate buffer. After overnight incubation at 4°C, 2 mg of bovine serum albumin (BSA) (lot number 12031, Serva) in 1 ml of carbonate buffer were added to the mixture and dialysed three times against carbonate buffer at 0°C overnight. After dialysis the conjugate was diluted with assay buffer to achieve a final concentration of 50 μg ml^{-1}, pipetted into aliquots and stored at −20°C until used.

pGH antibody

A GH-specific antibody raised in rabbits (code K-14) was kindly supplied by Dr. Schams (Institute of Physiology, Technical University of Munich, Weihenstephan, Germany). The cross-reactivities of rpGH (Pitman-Moore, lot number 148–215), prolactin (porcine PRL, USDA pPRL I-2, AFP-500), porcine luteinizing hormone (LH) (Bioproducts, Brussels, lot number 004/1) and porcine follicle stimulating hormone (FSH) (Bioproducts, Brussels, lot number 008/2) were determined at 50% binding of pituitary GH.

Preparation of affinity-purified sheep IgG antirabbit IgG

A 15–20-ml volume of plasma from a sheep immunized against rabbit IgG, containing 6 mM EDTA (Merck, Darmstadt, Germany) was applied to a small column prepared with 5 g of rabbit IgG agarose gel (Sigma). Low affinity bound proteins were eluted with 10 ml of 0.5 M NaSCN (pH 8.0) followed by 10 ml of 0.1 M glycine–HCl (pH 3.5). Proteins bound specifically were eluted with 15 ml of 0.1 M glycine–HCl (pH 2.0). All steps were performed at room temperature. To achieve an immediate neutralization of the eluted product, the collecting vials were prefilled with 2 ml of 1 M Tris–HCl (pH 8.0). The eluted IgG was dialysed against 66 mM NaH_2PO_4–Na_2HPO_4, (pH 7.2) and determined by the biuret procedure.

Enzyme immunoassay procedure

First coating. The first coating was achieved by adding 1 μg of sheep–anti-rabbit IgG dissolved in 100 μl of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) per well of the microtitre plate (Nunc, Roskilde, Denmark, No. 439454). The plates were subsequently incubated for either 2 h at room temperature or overnight at 0°C with slight shaking. After the incubation the plates were decanted.

Second coating. To achieve saturation of the remaining binding sites, 350 μl of assay buffer (40 mM Na_2HPO_4, 150 mM NaCl, pH 7.2, containing 0.1% BSA) were added per well and incubated for 15–45 min at room temperature before subsequent decantation. Non-specific binding (NSB) in the absence of antibody was checked in duplicate on each plate. Optical density values for NSB ranged between 0.045 and 0.135. Coated plates can be stored at −20°C for up to 6 months.

Washing. Prior to use in the assay, the coated plates were washed twice with 375 μl of 0.05% Tween 80 per well.

Assay protocol. Lyophilized pituitary porcine GH (pit. pGH) (Bioproducts, lot number 009/1) was dissolved in 50 mM carbonate buffer (pH 9.98) at a concentration of 100 ng ml^{-1}. Standard solutions (0.306–40 ng ml^{-1} pit. pGH) were prepared by serial dilution (steps of 1:2) in plasma with undetectable levels of endogenous porcine GH ($\leqslant$ 0.3 ng ml^{-1}). Volumes of 40 μl of standards and unknown plasma samples were diluted in duplicate into 100 μl per well of antibody-containing (1:40 000) assay buffer using a diluter–dispenser (Hamilton Microlab 1000). The plates were then incubated under constant gentle shaking for 48 h at 4°C and subsequently decanted. After decantation, 2 ng per well of biotinylated rpGH in 100 μl of assay buffer were added and

incubated for 2 h at 4°C. Following another decantation, 20 ng per well of streptavidin peroxidase (Boehringer, Mannheim, Germany) in 100 μl of assay buffer were added and incubated for 15 min at 4°C until decantation.

Substrate reaction. The plates were washed four times with 375 μl of 0.05% Tween 80, after which 150 μl of substrates A and B (1 : 1) were added per well [substrate A, 1.0 g l^{-1} hydrogen peroxide–urea, 18 g l^{-1} $Na_2HPO_4 \cdot 2H_2O$, 10.3 g l^{-1} citric acid monohydrate, 0.1 ml l^{-1} Kathon (Rohm and Haas, Frankfurt, Germany), pH 5.0; substrate B, 500 mg l^{-1} tetramethylbenzidine, 40 ml l^{-1} dimethyl sulphoxide, 10.3 g l^{-1} citric acid monohydrate, pH 2.4]. The reaction was stopped with 50 μl of 2 M H_2SO_4 per well and the extinction was measured at 450 nm using an eight-channel microtitration plate photometer (Titertek Multiscan MC, Flow Lab., Meckemheim, Germany).

RESULTS

Titration of porcine biotinyl-rpGH and anti-pGH serum

In order to determine the optimum concentration of antibody K-14 and biotinyl-rpGH for the assay a two-dimensional titre determination was performed. Antibody dilutions of 1 : 10 000–1 : 1 280 000 and biotinyl-rpGH concentrations of 0.125–4 ng per well were tested. The minimum concentrations of antibody and biotinyl-rpGH accepted as suitable for the assay were taken as those sufficient to achieve an optical density (OD_{450}) of ca. 1. For the actual assay with an incubation period of 2 days an antibody dilution of 1 : 40 000 and a biotinyl-rpGH concentration of 2 ng per well were chosen.

Assay validation

Influence of incubation temperature on assay kinetics. To determine the optimum incubation time and temperature, microtitre plates were incubated for either 4 or 24 h at 22°C or for either 24 or 48 h at 4°C (Fig. 1). The relative binding was less with incubation for 4 h at 22°C and 24 h at 4°C compared with 24 h at 22°C and 48 h at 4°C. Despite the good relative binding with incubation for 24 h at 22°C the slope of the calibration graph was inferior with a low repeatability and higher standard deviations among duplicate determinations. For this reason an incubation period of 48 h at 4°C was chosen as being optimum for the assay.

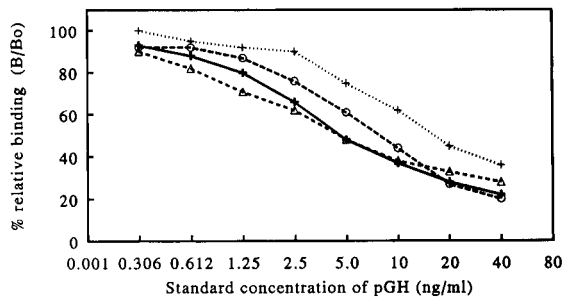

Fig. 1. Influence of incubation period and temperature on pGH binding. + · · · + = 4 h at 22°C; △ - - - △ = 24 h at 22°C; ○ - - - ○ = 24 h at 4°C; + ——— + = 48 h at 4°C.

Assay sensitivity. To determine the possible interference of plasma with the assay sensitivity, various amounts of plasma (5, 10, 20 and 30 μl) were added to the pit. pGH standard dilutions. An interference of added amounts of plasma with the calibration graph was observed. Because of this, all standard dilutions were subsequently prepared in plasma, which had unmeasurable amounts of endogenous pGH ($\leqslant$ 0.3 ng ml^{-1}). Samples and standards were standardized to a volume of 40 μl per well to guarantee sufficient assay sensitivity (Fig. 2). Using standards prepared in plasma, recoveries rates of various con-

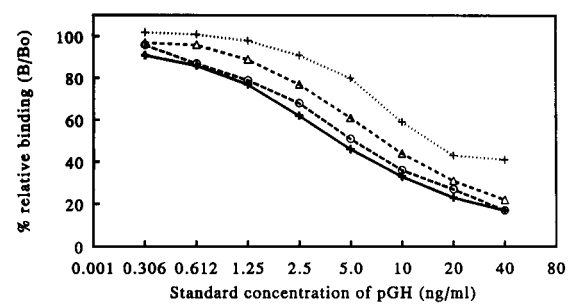

Fig. 2. Influence of volume of standard on pGH binding. Volume per well: + · · · + = 10; △ - - - △ = 20; ○ - - - ○ = 30; + ——— + = 40 μl.

centrations of pit. pGH (0.625, 1.25, 2.5, 5.0 and 10.0 ng per ml) added to unknown samples were determined and acceptable results were obtained (Table 1).

At a volume of 40 μl of standard per well the detection limit of the assay (optical density values significantly different from those at 100% B/B_0) was found to be 0.361 ± 0.11 ng per ml of pit. pGH (mean $\pm$ S.D., $n = 14$, R.S.D. = 21%, 90% B/B_0) and the 50% relative binding (B/B_0) was at 4.41 ± 0.43 ng ml^{-1} of pit. pGH (mean $\pm$ S.D., $n = 14$, R.S.D. = 9.8%).

Intra- and inter-assay variation

Intra- and inter-assay variation were determined using plasma samples with various pit. pGH concentrations. Repeated measurements of plasma concentrations (mean $\pm$ S.D.) of 4.79 ± 0.28 ($n = 14$), 11.04 ± 0.43 ($n = 5$), and 17.22 ± 0.93 ng ml^{-1} ($n = 5$) of pit. pGH resulted in R.S.D.s of 5.75, 3.87 and 5.4%, respectively. The inter-assay variation was 8.6% and 4.3% using plasma samples with concentrations of 1.20 ± 0.19 ($n = 14$) and 4.44 ± 0.19 ($n = 14$) ng ml^{-1} of pit. pGH, respectively.

Cross-reactivity with other hormones

The antibody K-14 that was used in this assay showed 50% cross-reaction with rpGH, but no cross-reactivity ($\leqslant 1\%$) was observed with either porcine LH, FSH and prolactin.

Clinical validation

Plasma samples obtained from a German Landrace sow (DL 685) and a Göttingen miniature pig (MINI 667) at 15 min intervals via an indwelling jugular catheter were analysed using the standard EIA procedure described above. Both animals had received a single i.v. stimulation with human growth hormone-releasing factor (hGRF 1–29, 7 μg kg^{-1} body weight) (Saxon Biochemicals, Hannover, Germany) at 0 h to induce marked changes of plasma pGH concentrations (Fig. 3). In the German Landrace pig a first increase in pGH in plasma could be observed within 5 min after the injection, reaching a peak concentration at 30 min, and followed by a steady decline until basal levels were reached at 90 min after the injection. In the Göttingen miniature pig the pGH response to hGRF was delayed; the peak concentration of pGH was found at 75 min after hGRF. The response curves were similar in increase and timing to those in other experiments performed previously.

TABLE 1

Recoveries of pituitary-derived GH added to different plasma samples at various concentrations

Pit pGH added (ng ml^{-1})	Repititions (n samples)	Recovery (%)	Variation (%)
0.612	6	119	3.87
1.250	12	107	4.16
2.500	18	108	3.54
5.000	18	104	4.90
10.000	12	102	14.14

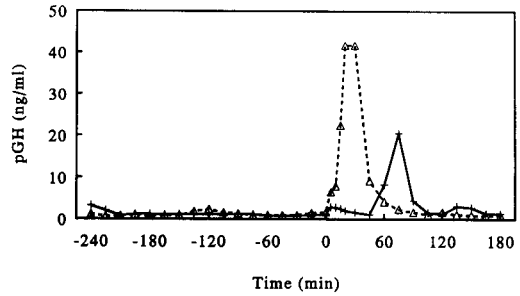

Fig. 3. Plasma pGH levels in two individual pigs (+ = MINI 667; Δ = DL 685) before and after stimulation with GRF (7 μg kg^{-1} body weight i.v.).

DISCUSSION

The method described here is the first report of a pGH EIA. The use of the second antibody for coating the wells instead of hormone-specific antibody is preferred as it reduces assay variabilities associated with uneven binding of the latter antibody to the wells and further reduces the amount of hormone-specific antibody needed in the EIA [8]. The amount of hormone-specific antibody required is ten times less than in a radioimmunoassay and 1000 times less than in a sandwich ELISA.

In our EIA an acceptable decrease in optical density was observed when increasing plasma volumes were used. Nevertheless, it is essential to compensate for this effect and to use the same plasma volumes for standards and unknowns. Suitable sensitivity and low non-specific binding were obtained over a range of up to 40 μl of plasma, sufficient to measure low baseline and high pGH levels in the pig. The sequential saturation of the antibody with cold and labelled hormone further improved the sensitivity of the EIA. However, this required optimization of the tracer concentration and incubation period so as to produce the desirable saturation without displacement of the cold hormone. Lower tracer concentrations than those employed in both EIAs could be used but were less preferred as longer incubation periods were needed to attain saturation.

The validation data presented above indicate that the performance characteristics of the EIA are acceptable with respect to sensitivity, variability, specificity and recovery of pGH in different samples. The stimulation of pGH by GRF was clearly detectable. The results of a current experiment in which pigs were treated with rpGH show a 5–10-fold elevation of pGH levels in porcine plasma.

In conclusion, the assay detects high and low plasma pGH levels within the physiological range and changes in plasma pGH after stimulation with GRF or treatment with recombinant pGH. Hence it offers a reliable alternative to radioimmunoassay. However, a reference method providing evidence on the identity of exogenous pGH will be required for proving illegal treatment with pGH and for forensic purposes.

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In vitro investigations of β-agonist accumulation in the eye

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Abstract

The eye is said to be the most accumulating tissue for β_2-adrenergic agonists in bovines. To avoid animal experiments, a perfusion system was developed for the eye. Therefore the eyes were perfused via arteria ophtalmica with defined amounts of Ringer solution containing 1 ng ml^{-1} of the respective β-agonist [clenbuterol, mabuterol, 2-amino-3-chloro-5-(1′-hydroxy-2′-amino-*tert*-butylethyl)pyridine (ACP), salbutamol and terbutaline]. β-Agonists were extracted, cleaned-up with cartridges containing LiChrospher 60 RP select-B and determined by enzyme immunoassay. The tests prove accumulation for other β-agonists also besides clenbuterol and the accumulation shows good correspondence to the metabolic stability in the organism [clenbuterol > mabuterol > 2-amino-3-chloro-5-(1′-hydroxy-2′-amino-*tert*-butyl-ethyl) pyridine > salbutamol > terbutaline]. The accumulation in retina + uvea was about 35 times higher than in vitreous humour + aqueous humour. The amount of accumulated clenbuterol corresponds with the volume of perfused Ringer solution containing the β-agonist. Preliminary data investigating the visual system suggest a very weak, if any, interaction of β-agonists with opsin.

Keywords: Immunoassay; ACP; β-Agonists; Clenbuterol; Eye tissue; Mabuterol; Perfusion system; Salbutamol; Terbutaline

Most β-agonists are derivatives of epinephrine and are normally used for the treatment of pulmonary diseases or for tocolysis in humans and animals. However, they are also used illegally in meat production, because of their repartitioning effect, which means that they 'improve' carcass composition by decreasing fat for the benefit of muscle mass [1–3]. To guarantee the safety of the consumer a multiresidue enzyme immunoassay was developed [4] for the detection of nine β-agonists in urine. This system is useful during the treatment period. Because of a half-life time of 13–14 h the detection of β-agonists in urine is possible only 3–4 days after withdrawal even with a sensitive assay [5]. Therefore we looked for an organ for residue monitoring even after withdrawal. Meyer and Rinke [5] showed highest accumulation of clenbuterol in the eye with very slow elimination. To investigate whether these results can be transferred to other β-agonists by avoidance of animal experiments we wanted to develop an in vitro system which could simulate in vivo conditions. By the use of this system we also wanted to know more about the localization and the kinetics of the β-agonists in the eye. Furthermore we examined possible effects of β-agonist accumulation on the visual system.

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EXPERIMENTAL

Perfusion system

The eyes of slaughtered animals were perfused by truncated microlances via arteria ophtalmica. First they were rinsed with 5 ml of water containing 200 U of heparin and with 10 ml containing 20 U of heparin. Then they were coupled to the perfusion system, which consists of a container with perfusion solution, a peristaltic pump plus flexible tubes. Eyes were perfused with Ringer solution containing 1 ng ml^{-1} of the respective β-agonist. This concentration is similar to the plasma concentrations achieved in animal experiments with anabolic dosages of clenbuterol [1], salbutamol and terbutaline [6]. To standardize the system defined amounts of perfusion solution were used.

Extraction and clean-up of eye fluids and tissues

Vitreous humour and aqueous humour

To 3 g of sample homogenized with an ultraturrax (Janke and Kunkel, Staufen i. Br., Germany) 10 ml of 500 mM KH_2PO_4 (pH 3.0) were added and incubated at 4°C overnight. After centrifugation (1250 *g*, 4°C, 30 min) 10 ml of the supernatant were cleaned up with cartridges containing 400 mg of LiChrospher RP-select B (Merck, Darmstadt). After the cartridges had been rinsed with 8 ml of methanol and 6 ml of water, respectively, they were equilibrated with 4 ml of 50 mM KH_2PO_4 (pH 3.0). Then 10 ml of supernatant were applied to the columns and washed with 4 ml of 50 mM KH_2PO_4 (pH 3.0). The β-agonists were eluted with 2 ml of methanol–water (50:50, v/v). All solvents were passed through the cartridges by suction (vacuum approx. 30–35 kPa). The eluates were evaporated to dryness and the residues dissolved in 500 μl assay buffer [7.12 g l^{-1} $Na_2HPO_4 \cdot 2H_2O$ (Merck), 8.50 g l^{-1} NaCl (Merck) pH 7.2, 1.00 g l^{-1} bovine serum albumin (No. 11930, Serva, Heidelberg)]. Duplicates of 20 μl were used for the enzyme immunoassay (EIA), which was described in detail earlier [4].

Retina and uvea, consisting of iris, choroid plus ciliary muscle

Clenbuterol. To defined amounts of tissue (up to 2 g) water was added up to 8 g . After addition of 3 ml of methanol, 500 μl of 5 M HCl and 5 ml of chloroform the samples were incubated for 3 h at 40°C with gentle shaking. Then the samples were centrifuged at 1200 *g*, the upper aqueous phase was removed and 600 μl of 5 M NaOH plus 6 ml of *tert*-butyl methyl ether were added. The samples were shaken for 10 min, centrifuged like before and the ether phase was decanted. The aqueous phase was extracted again with 6 ml of *tert*-butyl methyl ether, the ether was combined and evaporated in a water bath at 60°C. The residue was redissolved in 200 μl of assay buffer and duplicates of 20 μl were used for analysis by EIA.

Mabuterol, ACP, salbutamol or terbutaline. The tissues were homogenized 1 + 9 with water and 10 g of the homogenate (1 g of the respective tissue) were used for extraction. 300 μl of 5 M HCl were added to reach pH 2 and the homogenates were shaken for 2 h at room temperature and incubated overnight at 4°C. After incubation the homogenates were centrifuged at 1250 *g* at 4°C for 30 min . To the supernatants 250 μl of 5 M NaOH were added and incubated for 1 h. After a similar centrifugation (if necessary) the supernatant was incubated with 10 ml of 500 mM KH_2PO_4 (pH 3.0) overnight at 4°C. The samples were centrifuged once more and the supernatants were cleaned up with cartridges containing 400 mg LiChrospher RP-select B and measured by EIA as described above.

Accumulation and localization of the β-agonists in the eye

The eyes were perfused with 500 ml of Ringer solution containing 1 ng ml^{-1} of the respective β-agonist (flow-rate, 1–1.5 ml min^{-1}). Then the eyeball was divided into fluid parts (vitreous humour plus aqueous humour) and tissues (retina and uvea). The lens was not examined. These parts were extracted, cleaned up and analysed as mentioned before. We also separated retina without pigmented epithelium and uvea including pig-

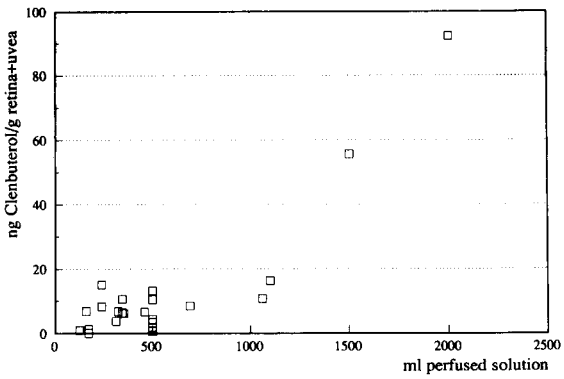

Fig. 1. Correlation between perfused clenbuterol and its accumulation.

mented epithelium, because this tenth layer of the retina could not be separated from the choroid. These two parts were extracted separately as mentioned (extraction of retina and uvea).

Kinetics of clenbuterol accumulation in the eye

The eyes were perfused with different amounts (Fig. 1) of Ringer solution containing 1 ng of clenbuterol ml^{-1}. The concentration of clenbuterol was determined in retina + uvea.

Effects of β-agonists on the visual system

Three methods were available to investigate the influence of β-agonists on the visual system. Detailed information on experimental methods and the evaluation of results is described by Hofmann [7], but the analytical strategy is given in brief.

(1) Meta II measurement

The absorbance maximum of the active conformation of rhodopsin (Meta II form) is at 380 nm whereas that of the inactive form (Meta I) is at 480 nm. Starting the measurement with a flash, rhodopsin rapidly forms an equilibrium between Meta I and Meta II. Transducin stabilizes the Meta II form that was quantified at 380 nm. Possible influences of β-agonists on the formation of Meta II were investigated: the sample, which consists of 1.5 μM rhodopsin, 0.5 μM transducin and 0.5 μM β-agonist was compared to the positive control without β-agonist and the negative control which contains only rhodopsin.

(2) Light-scattering

In the dark all transducin is bound to the membrane. After activating the membrane receptor rhodopsin by a flash, transducin forms a complex with Meta II and is activated by guanosine triphosphate (GTP), which leads to a dissociation of activated transducin from the membrane. This dissociation causes a loss of mass that is measured via the decrease of light scattering at 800 nm. Possible effects of β-agonists on the dissociation of transducin could be measured. For the experiment 3 μM rhodopsin in washed membranes, 0.5 μM transducin, 500 μM GTP in 10 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 2.5 mM NH_2OH (pH 8) served as a control. The positive sample included 0.5 μM of the respective β-agonist.

(3) Fluorescence measurement

The Gα-subunit of transducin contains two tryptophan moieties, which contribute to the fluorescence intensity at 340 nm when illuminated at 280–300 nm. After activation the fluorescence intensity of the Gα-subunit is increased. Starting the measurement with GTP, best activation is achieved in the presence of light-activated rhodopsin, but also opsin, which regenerates to a pseudo-active form [8], is able to activate transducin. For this experiment 0.5 μM rhodopsin, 0.5 μM transducin and 100 μM GTP were used for positive controls, the negative control included 0.5 μM opsin instead of rhodopsin and the sample contained 0.5 μM opsin or rhodopsin, 0.5 μM transducin, 100 μM GTP, 100 μM of the respective β-agonist.

RESULTS AND DISCUSSION

The different procedures provided the following recoveries.

(i) Extraction and clean-up of vitreous and aqueous humour: 65% for clenbuterol, 62% for mabuterol and 90–100% for ACP, salbutamol and terbutaline.

TABLE 1

Accumulation of clenbuterol and other β-agonists in the retina + uvea after perfusion with 500 ml Ringer solution containing 1 ng ml^{-1} of the respective β-agonist

	μ (ng g^{-1}) ± S.D. (ng g^{-1}) (n)	C.V.(%)
Clenbuterol	5.4 ± 4.5(5)	83
Mabuterol	5.3 ± 3.1(7)	58
ACP	1.4 ± 0.9(3)	62
Salbutamol	1.0 ± 0.4(9)	39
Terbutaline	1.3 ± 0.6(8)	47

(ii) extraction and clean-up of tissues: recoveries of 60–70% for clenbuterol (ether extraction), 40% for mabuterol and ACP, 70% for salbutamol and 90% for terbutaline.

All results were corrected in view of the recoveries of the different extractions. Table 1 presents the results concerning the question whether other β-agonists are also accumulated in the eye. Clenbuterol and mabuterol show the highest accumulation with mean values of 5–5.5 ng g^{-1} in whole retina and uvea. Salbutamol, terbutaline and ACP give only mean accumulations up to 1.4 ng g^{-1}. The accumulation shows a good correspondence to the metabolic stability of the compound. The high variability points to the individual perfusabilities of the eyes and the individual release of the retina (nine layers, without the pigmented epithelium) from the choroid.

The results with regard to the localization of the β-agonists in the eye are shown in Table 2. The accumulation in retina + uvea are up to 35 times higher than in vitreous and aqueous humour. Table 3 shows the results achieved after separation of retina without pigmented epithelium and uvea with pigmented epithelium. There is up to 14 times higher accumulation of clenbuterol in uvea plus pigmented epithelium than in the nine layers of the retina. This indicates an accumulation either in the pigments or in the vessels, but it can not be excluded that there is also an accumulation in the rods and the cones, because the rods and the cones are buried in the pigmented epithelium by influence of light.

TABLE 2

Accumulation of β-agonists in corpus vitreum + aqueous humor (c + h) and retina + uvea (r + u)

	c + h	r + u
Negative controls (ng Clenbuterol g^{-1})	< 0.01	0.1
Clenbuterol (ng g^{-1})(n)	0.03 ± 0.03(3)	11.0 ± 3.6(3)
Mabuterol (ng g^{-1})(n)	0.02 ± 0.01(4)	6.1 ± 3.6(4)
ACP (ng g^{-1})(n)	0.04 ± 0.02(2)	1.44 ± 1.22(2)
Salbutamol (ng g^{-1})(n)	0.11 ± 0.05(4)	0.9 ± 0.3(4)
Terbutaline (ng g^{-1})(n)	0.13 ± 0.02(3)	1.2 ± 0.6(3)

TABLE 3

Clenbuterol concentrations in retina and uvea with pigmented epithelium

	Uvea + pigmented epithelium	Retina
Samples μ(ng $g-1$) ± S.D. (ng g^{-1})(n)	14.6 ± 8.9(4)	1.1 ± 0.8(4)
Negative controls (ng g^{-1})	< 0.03	< 0.05

The perfusions of the eyes with different amounts of clenbuterol showed a slight parallelism between the amount of clenbuterol and its accumulation and a coefficient of correlation of $r = 0.87$ was calculated. Up to 2 l of perfusion solution no saturation was seen (Fig. 1).

The investigation of effects on the visual system gave the following results. (i) The addition of clenbuterol and salbutamol does not show any significant influence on the production of meta-II–GTP complexes. (ii) The light-scattering method did not indicate significant influences of clenbuterol, pirbuterol and BRL 31344. (iii) In the presence of rhodopsin the signals were unaltered. The addition of salbutamol, terbutaline, orciprenaline, BRL 31344, BRL 28410 and mabuterol in the presence of opsin led to a shift of the signal from the negative to the positive control, whereas clenbuterol, isoetharine, carbuterol and pirbuterol had no effect. In view of these different results no definitive conclusions are possible. The fact that there are no obvious influences on the normal enzyme cascade does not show whether there is any binding of β-agonists to membranes or proteins, but it is shown that β-agonists do not effect the on-reaction of the enzyme cascade.

Conclusions

It could be demonstrated that there is also an accumulation of several β-agonists in the eye, that means that the eye would be an ideal organ for detection of β-agonists even after withdrawal of the drug. The developed in vitro system can only simulate the contact of the eye with β-agonists, but is not identical to an intact in vivo system because of the partial detachment of the retina from the choroid after death. In addition, the different individual perfusabilities of the eyes cause high standard deviations. Negative effects of β-agonists on the visual system could not be identified, but can also not be excluded.

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Determination of clenbuterol residues in bovine liver, urine, and eye by enzyme immunoassay

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Abstract

Simple enzyme immunoassays are described which enable clenbuterol to be determined in bovine liver homogenate and eye (aqueous humour and choroid/pigmented retinal epithelium) by direct analysis, and in urine following solid-phase extraction and concentration. The sensitivity, analytical recovery and linearity of response of the assays proved appropriate to the determination of clenbuterol in cattle seven days after cessation of a programme of treatment (0.8 μg clenbuterol per kg bodyweight, twice daily for four days). Concentrations determined in liver (range < 0.25 ng g^{-1} to 0.35 ng g^{-1}) were approximately ten times higher than in urine (range 0.015–0.030 ng ml^{-1}). Although clenbuterol could not be detected in aqueous humour, the choroid/pigmented retinal epithelium contained concentrations ranging between 12 and 14 ng g^{-1}. These preliminary findings clearly indicate that choroid may be the tissue of choice to enable use or abuse of clenbuterol to be established following slaughter.

Keywords: Enzymatic methods; Immunoassay; β-Agonists; Bovine; Clenbuterol; Eye; Liver; Urine

There is currently appreciable concern about the potential consumer health hazards associated with the illegal use of clenbuterol as a growth promoting agent. The β_2-adrenoceptor agonist (β-agonist), clenbuterol, is licensed in certain EC states for use in the relief of bovine and equine respiratory diseases and as a tocolytic. Following administration (rat, dog, rabbit and cow), it is rapidly eliminated, being largely excreted in urine as the parent drug [1–4].

In cattle, the 12-day withdrawal period specified for clenbuterol in the UK is aimed at ensuring that residue concentrations in edible tissues will not exceed the maximum residue limit (MRL) of 0.5 ng g^{-1} tissue and thus that consumer exposure to the drug is minimal. When used at the therapeutic dose (0.8 μg kg^{-1} body weight), the high β_2-receptor selectivity of clenbuterol ensures that important β_1-adrenoceptor related side-effects (e.g., tachycardia) in the target species, are kept to a minimum.

When presented at high concentrations (around 10 times the therapeutic dose) clenbuterol gives rise to, amongst other effects, mobilisation of fat depots and protein accretion ("repartitioning"), providing a more lean carcase. Although such treatment may markedly improve profitability for the producers, through better carcase prices at intervention, such an application is not licensed in the EC and many other countries. Most importantly, since this use is illegal, uncontrolled and dose levels are so high, there is an appreciable potential health risk to both the unscrupulous operator (at dosing) and to consumers (particularly those with a heart condition), since the drug may be administered for a prolonged period to a point near to slaughter. The

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real dangers of such abuse are underscored by recent reports of human food poisoning in Spain and France [5,6] in which consumption of liver containing clenbuterol residues was implicated: these cases are amongst the very few published records where clinical signs observed in man have been associated with veterinary drug residues contained in food.

Body fluids or edible tissues randomly sampled from food-producing animals (cattle, pigs, sheep) at the slaughterhouse, are analysed for clenbuterol or other β-agonists, under the provisions of the Residues Directive (86/469/EEC), to monitor for misuse or illegal use. The particular challenge in developing analytical methods for clenbuterol screening derives from the rapid rate of elimination from the body and the low MRL. Physico-chemical screening methods currently described for clenbuterol, such as liquid chromatography (LC) [7,8] cannot readily achieve limits of detection down to the MRL. Immunoassays, in particular, offer the requisite degree of sensitivity, although samples may need to be extracted and concentrated prior to analysis [4,9,10]: such procedures may be used in conjunction with LC to provide some degree of confirmation [11]. In determining illegal use there are obvious advantages in selecting tissues which accumulate residues: of the edible tissues, liver appears to contain the highest concentrations throughout the withdrawal period [10]. Of the samples which may be taken from the live animal (e.g., blood, urine and faeces) it would be anticipated that urine should contain the highest clenbuterol concentrations post-withdrawal and may therefore be the sample of choice. A pharmacokinetic study recently reported by Meyer and Rinke [10] clearly indicated that the eye may achieve concentrations an order of magnitude higher than in liver, providing evidence for the value of this organ in screening for abuse in the slaughter population.

In the present study we investigated the use of enzyme immunoassay for the determination of clenbuterol residues in bovine liver, urine, and eye (aqueous humour and choroid/pigmented retinal epithelium) and the value of such determinations in establishing use and abuse of clenbuterol in cattle.

EXPERIMENTAL

Reagents

Clenbuterol hydrochloride, ^{3}H-clenbuterol (11.21 Ci mmol^{-1}), hydroxyclenbuterol [4-amino-3,5-dichloro-α-(2-hydroxy-1,1-dimethyl)ethylamino methylbenzyl alcohol], 4-amino-3,5-dichlorobenzoic acid and 4-amino-3,5-dichlorohippuric acid were obtained from Boehringer (Ingelheim), gelatin from Difco (West Molesey), Subtilisin Carlsberg protease (type VIII) from Sigma. (Poole), Ultrafluor scintillator from National Diagnostics (Aylesbury), Freund's complete adjuvant from ICN (High Wycombe) and Morris' non-ulcerative incomplete adjuvant from Guildhay Antisera (Guildford). Alkaline phosphatase (calf intenstinal, code ALPI12G) was purchased from Biozyme (Blaenavon). Microtitre plates (NUNC-Maxisorp F96-cert) and plate adhesive sealing tape were from Gibco (Uxbridge).

Buffers

Phosphate buffers (0.1 M; pH 7.0) were prepared with sodium dihydrogenorthophosphate dihydrate and disodium hydrogenorthophosphate dihydrate, with sodium chloride (0.9%, w/v; PS buffer), sodium azide (0.02%, w/v; PAS buffer), and gelatin (0.1%, w/v; PAS–gelatin buffer) added.

Animals and samples

Friesian cross heifers (around 80 kg) were treated orally (by drench) with clenbuterol ($n = 4$, 0.8 μg kg^{-1} body weight) or with salbutamol ($n = 4$, 2 μg kg^{-1}) by intramuscular injection, twice daily for four days in order to incur residues. Animals were slaughtered seven days after the last treatment, at which time samples were collected and stored at -20°C until required. Urine and aqueous humour were aspirated from the bladder and anterior chamber of the eye, respectively, using a syringe and needle.

Control tissues and aqueous humour were obtained from cattle of known history, after slaughter, and stored at -20°C until used. Control urine samples were collected either following natural evacuation of the bladder, or at slaughter, using a syringe and needle. Other urine samples were acquired routinely as part of the UK Na-

tional Residues Surveillance Scheme and were selected because they showed clear signs of degradation: although of no defined age, these dark-brown pigmented samples are described here as "aged".

Preparation of choroid / PRE samples

Following excision of eyes from slaughtered animals, the aqueous humour, cornea, lens, vitreous humour and retinal tissue were removed and the choroid teased from the everted eye ball with a scalpel. Tissues and fluids were stored at −20°C until required. Choroid prepared in this manner will have associated with it the pigmented retinal epithelium (PRE) layer.

Choroid/PRE digest was prepared using protease from Substilisin Carlsberg protease (20 mg ml^{-1}, 10 μl; stored at −20°C) which was added to a portion of choroid/PRE (0.1 g) in 0.4 ml of phosphate buffer (0.1 M; pH 8.0) in a capped polythene microtube (Sarstedt). The preparation was incubated at 56°C for 1 h (with vortex mixing every 20 min), then for 15 min at 98–100°C. The resulting inky suspension was centrifuged (13 000 *g*) for 15 min and the clear amber supernatant (choroid/PRE digest supernatant) was stored at −20°C until required.

Enzyme immunoassay for clenbuterol

Preparation of immunogen. Immunogen was prepared by coupling diazotised clenbuterol to carrier protein, essentially as described by Yamamoto and Iwata [9]. Ovalbumin, rather than horse serum albumin, was used as carrier protein. A 40:1 molar ratio of diazotised clenbuterol: ovalbumin was used in the reaction mixture (pH 9.5) at coupling.

Antibody production. The immunogen was prepared for inoculation in an emulsion of sterile saline (0.9%, w/v) with Freund's complete adjuvant (primary inoculation) or Morris' incomplete adjuvant (subsequent "boost" inoculations) in a ratio of 1:1.3. Sheep (Suffolk Cross wethers, about 2 years old) were inoculated with 3 mg (in 1.3 ml emulsion, primary) or 1 mg (in 2.3 ml, boost) of immunogen per animal. Each dose was distributed equally between four injection sites (three subcutaneous and one intramuscular). Boost injections were given at intervals of three months after the primary inoculation and blood samples taken by jugular venepuncture nine days later. The γ-globulin fraction was prepared by ammonium sulphate (final concentration 33% saturated) precipitation, essentially by the method of Garvey et al. [12] and stored in aliquots at −20°C until required.

Preparation of antibody-coated microtitre plates. The γ-globulin fraction of antiserum SC248/5, reconstituted to the original serum volume in deionised water, was diluted 1:1 (v/v) in glycine buffer (0.3 M, pH 2.2; [13]) and incubated at ambient temperature (22–25°C) for 35 min. The preparation was then diluted as appropriate (1:16 000) in tris(hydroxymethyl) methylamine (Tris) buffer (0.05 M; pH 8.5) and incubated for 1 h (22–25°C) before addition (0.2 ml) to microtitre plate wells. Plates were then sealed with adhesive film and incubated for 3 h (22–25°C), before emptying and washing with PAS–gelatin buffer (0.3 ml). After emptying the wells, further PAS–gelatin buffer was added (0.3 ml) before finally sealing with adhesive film and storing at 4°C until required. Binding activity of adsorbed antibodies was retained for several months under these conditions.

Preparation of alkaline phosphatase–clenbuterol conjugate. Hydroxyclenbuterol was coupled using essentially the procedure described by Yamamoto and Iwata [9]. Alkaline phosphatase, rather than β-galactosidase, was used as enzyme label, however: this was exhaustively dialysed against PS buffer prior to conjugation using a 20:1 molar ratio of diazotised hydroxyclenbuterol: alkaline phosphatase. Following coupling, the labelled clenbuterol was purified and stored in the manner described elsewhere for progesterone-alkaline phosphatase conjugate [14].

Using the microtitre plate procedures described below, titration of antibody dilution against conjugate dilution in the presence of a range of clenbuterol concentrations enabled the optimal EIA conditions to be determined.

Enzyme immunoassay for residues in urine, aqueous humour and choroid / PRE

The enzyme immunoassay was performed essentially as described by Sauer et al. [14] for

progesterone EIA, but substituting appropriate clenbuterol standards (10 μl, in quadruplicate, unless otherwise stated; range 0.1–50 ng ml^{-1}) and hydroxyclenbuterol–alkaline phosphatase conjugate (CL-AP24, 80 ng ml^{-1}). Adhesive film was used to seal plates during incubations. The absorbance (405 nm) was determined using a MR710 automatic plate reader (Dynatech, Billinghurst).

Enzyme immunoassay for residues in liver homogenate

This procedure has been described in full elsewhere [15] and enables clenbuterol residues in liver homogenate (20%) to be analysed by direct EIA. A combination of clenbuterol–ovalbumin antiserum and salbutamol–horseradish peroxidase as enzyme label was used to introduce a degree of heterology; this enabled improved assay sensitivity to be achieved.

Urine extraction and concentration using octadecyl silica (C_{18}) columns

The method used was essentially that described by Meyer et al. [11]: 200 mg, rather than 100 mg columns, were used (Analytichem C_{18}, 3 ml per 200 mg; Jones Chromatography, Gwent) to facilitate extraction of larger volumes of urine. The C_{18} columns were conditioned by elution with methanol (2 × 2 ml) then NaOH solution (1 mM, 2 × 2 ml) and urine samples (1–5 ml) extracted by passage through the column under gravity, or using minimal pressure applied over the column. Columns were then washed with NaOH solution (1 mM, 2 × 2 ml) then 15% methanol in water (2 × 4 ml) before elution of clenbuterol with methanol (2 ml) into conical polypropylene test tubes (Sarstedt). The eluate was concentrated to dryness under nitrogen and finally dissolved in an appropriate volume of PAS–gelatin buffer (100–500 μl), with the aid of sonication (10 min) in a water bath at ambient temperature (18–23°C).

Extracts were stored at 4°C for up to two days, or at −20°C, until analysed by EIA.

Aqueous humour (direct analysis)

After thawing, aqueous humour samples from the right and left eyes were blended for each animal. Aliquots from each untreated control animal were fortified with clenbuterol solution (20 ng ml^{-1}, prepared in aqueous humour pooled from all control animals) so as to contain 0.33 ng ml^{-1} per ml aqueous humour. Aliquots (10 μl) of fortified samples, or those from untreated control and clenbuterol- and salbutamol-treated animals were assayed in duplicate by EIA. Standards (10 μl) prepared in PAS–gelatin buffer were used to provide assay calibration.

TABLE 1

Sensitivity of EIA procedures using standards prepared in various matrices

Matrix	Sample size (ml)	Limit of detection (pg per well) [a]	Calibration graph mid-point (pg per well) [b]	Limit of determination (ng ml^{-1} or ng g^{-1})
PAS–gelatin buffer				
Urine (extract) [c]	0.20	2	20	0.01
Chroid/PRE digest	0.01	2	20	0.2
Aqueous humour	0.02	2	20	0.1
Urine (pooled) [d]	0.01	2	20	0.2 [e]
Liver homogenate (20%) [f]	0.20	20	40	< 0.5

[a] Confidence limit 99%. [b] Mass of clenbuterol required to reduce label binding by 50%. [c] Extract concentrated five-fold compared with original urine volume. [d] This direct assay was not used to determine residues in treated animals since it was insufficiently sensitive. [e] Highly pigmented samples may give rise to high blank values, increasing the limit of determination. [f] Data derived from the method of Bucknall et al. [15].

Choroid / PRE digest supernatant (direct analysis

Samples of choroid/PRE digest supernatant (CDS) from clenbuterol treated animal ($n = 4$) were serially diluted in CDS from untreated control animals ($n = 5$) to enable linearity of the assay response to be determined under constant conditions. Aliquots (10 μl) of diluted samples and of CDS from clenbuterol- and salbutamol-treated animals were assayed in duplicate by EIA using standards prepared in PAS–gelatin buffer for assay calibration.

RESULTS

Immunoassay characteristics

The sensitivity of each EIA procedure is shown in Table 1, as defined both by the limit of detection [16] and the calibration graph mid-point [17]. Values shown were determined as appropriate from calibration curves, using standards prepared in PAS–gelatin buffer, aqueous humour, pooled urine ($n = 18$) or liver homogenate (20%, w/v).

Specificity of EIA. The cross-reactivities of various β-agonists and metabolites (dissolved in PAS–gelatin buffer) with the antiserum (SC245/5, 1:16000 dilution) were determined as described by Abraham [16]. Hydroxyclenbuterol, salbutamol, terbutaline, isoproterenol and fenoterol cross-reacted 5.7, 3, 2.7, 0.07 and 0.001% respectively; all others (isoxsuprine, adrenalin and noradrenalin) including the metablites 4-amino-3,5-dichlorobenzoic acid and 4-amino-3,5-dichlorohippuric acid, giving rise to minimal ($< 0.001\%$) interaction.

Analytical recovery of clenbuterol added to samples

Liver (direct analysis). In the case of liver homogenate analysis, to date, attention has been paid to the ability to discriminate between samples from untreated animals and those containing residue concentrations around the MRL: validation data for this procedure have been reported elsewhere [15].

TABLE 2

Analytical recovery of clenbuterol added to urine samples ($n = 5$) collected from calves following natural evacuation of the bladder and determined by direct EIA

Amount Added (ng ml^{-1})	Mean concentration determined (ng ml^{-1})	C.V. (%)	Recovery (%)
0	< 0.1	–	–
0.2	0.19	7.7	96
0.5	0.45	17.0	90
2.0	1.84	8.2	92

Urine (direct analysis). Urine from eighteen calves, of known history, were sampled from the bladder by syringe and needle after slaughter. Aliquots of five samples (1 ml), were spiked with clenbuterol standard (20 or 200 ng ml^{-1}, prepared in PS buffer), to provide final concentrations of 0, 0.2, 0.5 and 2 ng clenbuterol per ml. Clenbuterol standards (0.1 –50 pg ml^{-1}) were prepared in urine, pooled from the eighteen calves. Analysis of samples by EIA enabled mean concentrations and percentage recoveries of clenbuterol to be determined (Table 2). Linear regression of mean concentrations measured (y) on the values expected (x) gave $y = 0.9577x - 0.0176$ and a correlation coefficient $r = 0.9999$ ($n = 20$).

Urine recovery following C_{18}-column extraction

Aliquots (50 ml) of control pooled urine (prepared as above, for direct analysis) were spiked with an appropriate volume of clenbuterol solution (100 ng ml^{-1} deionised water), a proportion consisting of ^{3}H-clenbuterol (final concentration 20 pg ml^{-1}, 11.1 Bq ml^{-1}) to provide final concentrations of 20, 50, 100 and 200 pg clenbuterol per ml urine. Spiked urine (1, 2 or 5 ml) was extracted in duplicate, the methanolic eluate (1 ml) was evaporated to dryness under nitrogen and the residue dissolved in PAS–gelatin buffer (0.1, 0.2 or 0.5 ml). An aliquot of the spiked urine (0.1 ml), the methanol eluate (0.2 ml) and the final solution of extract in PAS–gelatin buffer (20% of the final volume) were mixed with Ultrafluor (4 ml) and analysed by liquid scintillation spectrometry to enable clenbuterol recovery to be

determined. Consistent tracer recoveries of 98.4 ± 6.9% (mean ± S.D., $n = 72$) for methanol eluates and 89.6 ± 5.8% (mean ± S.D., $n = 72$) for PAS–gelatin solutions were found, irrespective of the volume of urine extracted, the clenbuterol concentration or the volume in which the extract was finally dissolved.

Recovery of clenbuterol in ODS extracts, determined by EIA

Recovery of clenbuterol from spiked urine samples, as determined by EIA, was consistent with that found in the tracer studies. However, where samples showed clear signs of deterioration as indicated by dark brown pigmentation (aged), recovery was found to be dependent on the concentration of the final extract and the size of the sample taken for analysis. When two aged control urine samples (containing 0.1, 0.2, 0.5 or 1 ng clenbuterol ml^{-1}) were extracted in duplicate, concentrated (two-fold) and assayed (10 μl by EIA, linear regression analysis of mean concentrations determined (y) against those expected (x) gave $y = 1.195x - 0.061$ ($r = 0.9999$, $n = 16$), demonstrating good recovery and linearity of response. Further aged urine samples ($n = 10$), spiked at 0.2 and 0.8 ng clenbuterol per ml urine gave consistent recoveries (mean ± S.D.) of 140 ± 14% and 122 ± 11%, respectively. This degree of sample concentration, however, provides little prospect of determining concentrations below 0.2 ng ml^{-1}.

Higher concentration factors (10 to 50-fold, EIA of 10–50 μl of sample extract) enabled lower concentrations to be determined, but introduced a sample blank equivalent to about 0.02 ng ml^{-1}. In this study randomly selected urine samples, supplemented to contain 0.02, 0.05, 0.1 or 0.2 ng clenbuterol per ml were extracted, analysed by EIA and data for each original concentration (at each fold concentration and for each extract volume analysed) were pooled ($n = 16$–26). Linear regression analysis of concentrations determined (y) against those expected (x) gave $y = 0.7078x$

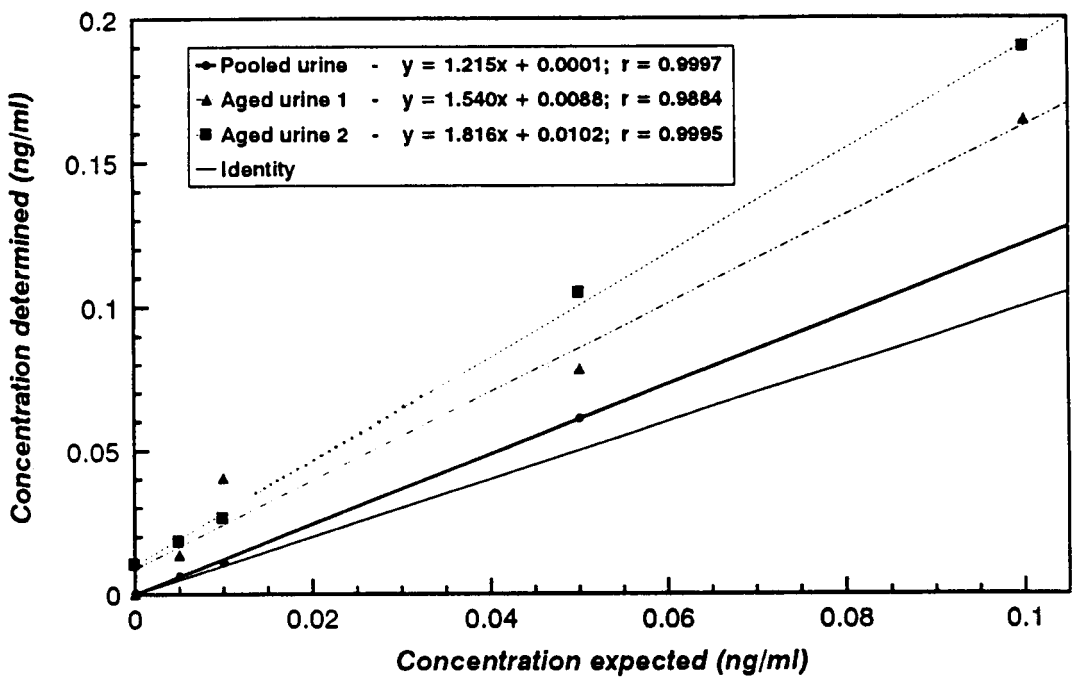

Fig. 1. Analytical recovery of clenbuterol from spiked, aged urine samples compared with pooled urine following extraction, concentration and determination by EIA. Final extracts in PAS–gelatin buffer were equivalent to a five-fold concentration of urine; a 0.2-ml aliquot was taken for analysis.

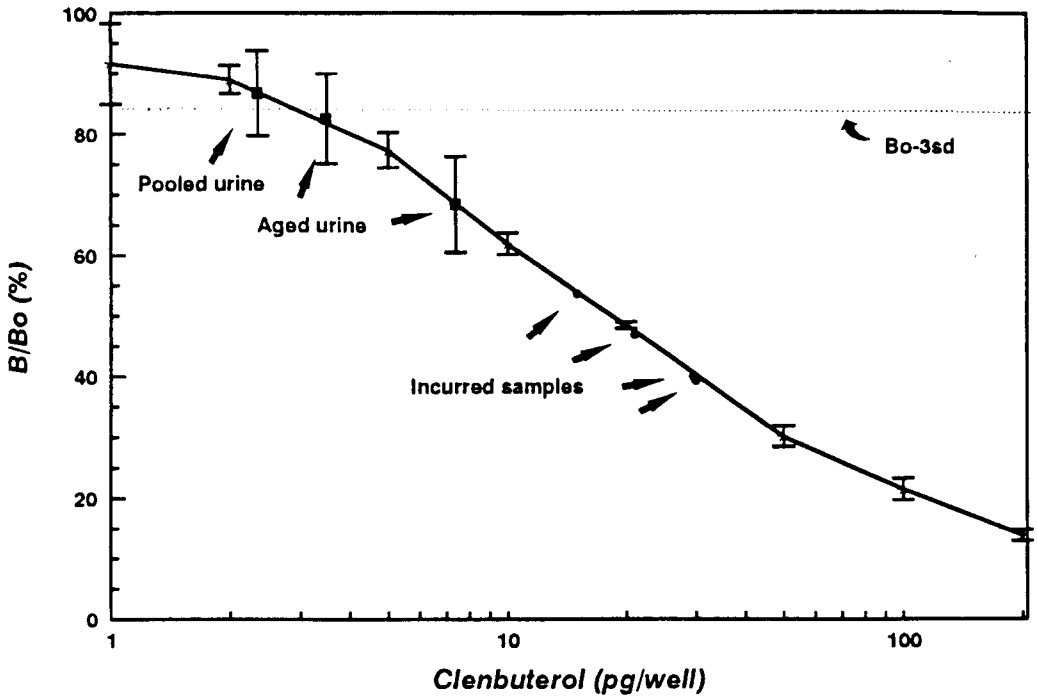

Fig. 2. EIA calibration graph. Analysis of extracts of negative control urine samples (pooled control urine and aged urine samples, determined in quadruplicate; mean ± S.D.) and urine samples from animals in which clenbuterol residues had been incurred. Extracts in PAS–gelatin buffer represented a five-fold concentration; a 0.2-ml aliquot was taken for analysis. B_0 = The quantity of enzyme-labelled clenbuterol bound to antibody in the absence of competing clenbuterol. B/B_0 = The quantity of labelled clenbuterol bound to antibody in the presence of competing analyte (B) relative to B_0.

+ 0.0228 and $r = 0.9999$. When two highly pigmented samples (5-fold concentration, EIA of 200 μl sample extract, were analysed a pronounced positive bias was apparent when compared with pooled control urine (Fig. 1): in this "worst-case" situation it was still possible to distinguish these samples from those in which residues had been incurred (Fig. 2), however.

TABLE 3

Clenbuterol residues in liver, urine, aqueous humour and choroid/PRE seven days after cessation of clenbuterol treatment

Animal No.	Tissue/fluid concentration			
	Liver (ng g^{-1})	Urine (ng ml^{-1})	Aqueous humour (ng ml^{-1})	Choroid/PRE (ng g^{-1})
CF176	0.35	0.03	< 0.2	12.0
CF177	0.28	0.03	< 0.2	12.1
CF178	< 0.25	0.015	< 0.2	12.0
CF179	0.29	0.021	< 0.2	14.1
CF180–183 [a]	below limit of detection			

[a] Animals CF180–CF183 were salbutamol-treated controls.

Recovery of clenbuterol in choroid / PRE digest supernatants

Pooled choroid/PRE digest supernatant from five control animals was used to dilute samples from four animals in which clenbuterol had been incurred. Samples analysed contained 25, 50, 75 or 100% of incurred supernatant. To normalise data for all four animals, recovery for each dilution was expressed as a per cent of the concentration determined in the undiluted (100%) sample. Regression analysis of %recovery (y) against % of original sample (x) gave $y = 1.08x - 8.103$ and $r = 0.9899$: the degree of linearity provided a good indication that clenbuterol, specifically, was being measured. This is further supported by

data from salbutamol-treated animals, in which no clenbuterol was detected (Table 3).

Determination of clenbuterol in aqueous humour

Residues of clenbuterol in aqueous humour from animals in which clenbuterol had been incurred could not be distinguished from the reference (0 ng ml^{-1}) calibration standard or from values obtained from untreated or salbutamol treated animals.

Clenbuterol concentrations in liver, urine, aqueous humour and choroid / PRE of treated animals

The limits of detection of the assays used to determined clenbuterol concentrations in the animals in which clenbuterol had been incurred are shown in Table 1.

Following a four-day period of clenbuterol administration at the therapeutic dose and seven days withdrawal, concentrations in liver were 0.35 ng g^{-1} or below (Table 3). Concentrations in urine were approximately one-tenth of those in liver, clearly indicating the need for appreciable sample concentration to enable reliable estimation. In aqueous humour, concentrations were below the detection limit of the direct EIA.

Choroid/PRE showed clear indication of clenbuterol accumulation since concentrations exceeded those in liver by about forty-fold (Table 3). It is tempting to speculate that accumulation of clenbuterol is associated with melanocytes contained within the choroid. It should be borne in mind, however, that choroid derived by the simple process described will have associated with it pigmented epithelial cells derived from the retina; such cells also contain large quantities of melanin. Histological and cell separation techniques may enable determination of whether retina, choroid/PRE tissue or both are involved. Such accumulation is not unusual and has been reported for drugs such as phenothiazine [18], chloroquine [19] and rifampicin [20] as well as adrenaline and noradrenaline [21] and in these cases has been associated with an affinity for melanin-containing tissues. The potential importance of accumulation in choroid/PRE tissue to programmes in which the slaughter population is monitored to establish use, or illegal use, of veterinary drugs in food-producing animals has not previously been appreciated, however.

Conclusions

It is apparent that concentrations of clenbuterol in liver fall below the MRL (0.5 ng g^{-1}) well within the twelve day withdrawal period when animals are dosed according to recommended practice. Although concentrations in urine were appreciably lower they could nevertheless be reliably determined following ODS extraction and concentration. Serious doubts are raised about the accuracy of such determinations, however, under circumstances in which urine samples are allowed to deteriorate and become highly pigmented: the use of alternative clean-up procedures such as immunoaffinity adsorption may then prove valuable [22]. It is clear from these studies that the high concentration of clenbuterol in the eye, as reported by Meyer and Rinke [10], may in large part be due to accumulation by the choroid/pigmented retinal epithelium. Choroid/PRE may thus be the tissue of choice to enable use or abuse to be established at slaughter. If a clear relationship could be established between depletion rates in liver and choroid/PRE then this may be of value in determining withdrawal period compliance. Clearly, if a similar pattern of accumulation for other β-agonists were established this would greatly facilitate detection of illegal use long after drug withdrawal.

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Cryotrapping gas chromatography–fourier transform infrared spectrometry: a new technique to confirm the presence of β-agonists in animal material

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Abstract

The usefulness of cryotrapping gas chromatography–Fourier transform infrared spectrometry for the analysis of the repartitioning agents clenbuterol, mabuterol and salbutamol in cattle has been evaluated. It is demonstrated that the technique is able to detect and identify the trimethylsilyl derivatives of these compounds in samples extracted from urine and liver of veal calves and cattle with a deteciton limit of 1 ng on column, equivalent to 2.5 $\mu g\ l^{-1}$ or $\mu g\ kg^{-1}$ of the original sample. It is anticipated to become a valuable method for confirmation in residue analysis, in addition to e.g. gas chromatography–mass spectrometry.

Keywords: Gas chromatography; Infrared spectrometry; β-Agonists; Clenbuterol; Mabuterol; Salbutamol

Structural elucidation of analytes in complex matrices is an important aspect of residue analysis. Particularly in the analysis of materials related to human food, unambiguous identification of substances with suspected toxic properties is required to fulfill stringent forensic and regulatory demands.

The ultimate identification of an analyte is preferably based on specific structural information. In residue analysis, gas chromatography (GC) combined to mass spectrometry (MS) with e.g. electron impact ionization (EI) is widely accepted as the method of choice, because of the high sensitivity and selectivity of the detecting technique. However, sometimes the spectral information obtained with this mass spectrometry, is insufficiently diagnostic for univocal identification of the compound of interest. In such cases additional structural analysis is necessary, e.g. by soft chemical ionization (CI) techniques or by using another specific detecting principle.

An example of the shortcomings of GC–EI-MS occurred in the analysis of β-agonists, a large group of *N*-phenylethanolamines illegally used as repartitioning agents in veal calve and cattle production. EI spectra as a rule only show one or two abundant diagnostic fragment ions with sufficient intensity for identification at residue levels

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[1]. However, European Community directives [2] state that for reference purposes at least four ions have to be monitored. As a consequence additional CI-MS measurements are needed for the determination of the molecular ion and related fragments as only the combination of EI and CI-MS can give sufficient information.

The present methodology to determine the presence of these compounds in animal material includes a three-step procedure [1]: (i) extraction and subsequent purification from samples of urine or liver by means of immunoaffinity chromatography; (ii) derivatization of the hydroxyl groups by means of trimethysilylation and (iii) GC–MS (EI) measurement of the derivatized extracts for identification and quantitation. Occasionally additional CI measurements are necessary [3].

In principle, GC combined to Fourier transform infrared (FT-IR) spectrometry is an attractive alternative for identification purposes because of the high discriminating properties of IR spectrometry. Moreover, the complementary character of the information obtained might fill gaps left by MS. However, thus far GC–FT-IR has hardly been applied to residue analysis because of the limited sensitivity of the conventional lightpipe-based GC–FT-IR systems.

Recently a new type of interfacing capillary GC and FT-IR has been developed with a sensitivity which is 1–2 orders of magnitude better than the lightpipe detection. The principle of this so-called cryotrapping technique is condensation of the GC eluates at 77 K on a moving IR transparant window with subsequent scanning of the trapped compounds by means of FT-IR microscopy [4]. A unique feature of the system is the possibility of performing extended post-run scanning of the previously condensated compounds. Thus considerable improvement of the signal-to-noise ratio of the infrared spectra is obtained facilitating identification and increasing the limit of detection. The technique has been succesfully applied to the structural elucidation and isomer differentiation of pesticides [5], beer constituents [6], phenolic photodegradation products [7] and polyaromatic hydrocarbons [8]. Detection limits into the picogram range have been reported.

In view of this sensitivity, cryotrapping GC–FT-IR might be a useful method to confirm the presence of clenbuterol and related β-agonists in biological samples. To get insight into this the applicability to the analysis of the trimethylsilyl-derivatized agents clenbuterol, mabuterol and salbutamol has been evaluated.

EXPERIMENTAL

Sample preparation

Samples and standards were prepared as solutions in toluene according to the procedure previously described [1,3].

For clenbuterol, eight standard solutions, two samples of blank urine, two samples of blank urine spiked with clenbuterol, and two samples of urine from a veal calve treated with clenbuterol, have been examined. The concentration of the derivatized standards matched with respectively 100, 50, 25, 10, 5, 2.5, 1 and 0.5 ng μl^{-1} toluene. The concentration of the spiked samples corresponded to 1 ng μl^{-1} and real samples to approximately 1 ng μl^{-1}.

For mabuterol, four standard solutions, one sample of blank urine and one sample of urine obtained from a veal calve treated with mabuterol, have been studied. The concentration of the derivatized standards matched with 50, 25, 5 and 1 ng mabuterol μl^{-1} and the concentration of the urine sample with approximately 1.6 ng μl^{-1}.

For salbutamol, four standard solutions, one sample of blank liver and two samples of liver obtained from a treated animal, have been studied. The salbutamol concentration of the derivatized standards matched with respectively 50, 25, 5 and 1 ng μl^{-1} toluene. The 1 ng μl^{-1} standard was additionally diluted to a concentration of 0.25 ng μl^{-1}. The concentration of the liver extracts matched with approximately 0.3 ng μl^{-1}.

Gas chromatography

GC separations were carried out on a Carlo-Erba MEGA 5160 gas chromatograph with split/splitless injector. The GC apparatus was equipped with a CP-Sil-5 CB capillary column (Chrompack)

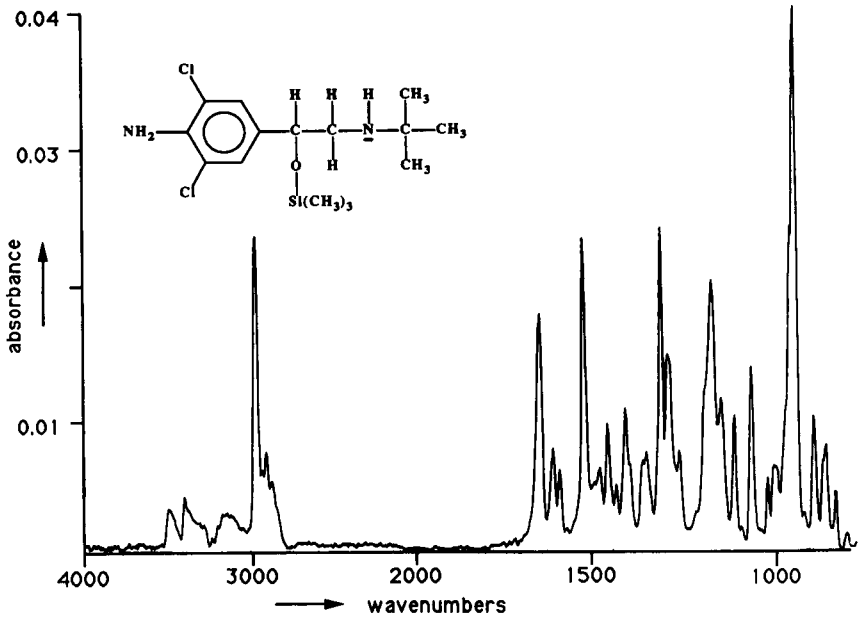

Fig. 1. Cryotrapping GC–FT-IR spectrum and molecular structure of trimethylsilylclenbuterol. Injected amount 25 ng, optical resolution 8 cm^{-1}, 4 scans coadded.

of 25 m × 0.25 mm i.d. and 0.25 μm film thickness. The injection volume was 1 μl. Carrier gas was helium with a calculated flow-rate of 0.9 ml min^{-1} at 120°C. Temperature programming for all separations was 80°C hold for 1 min followed by a temperature increase of 25°C min^{-1} to 180°C

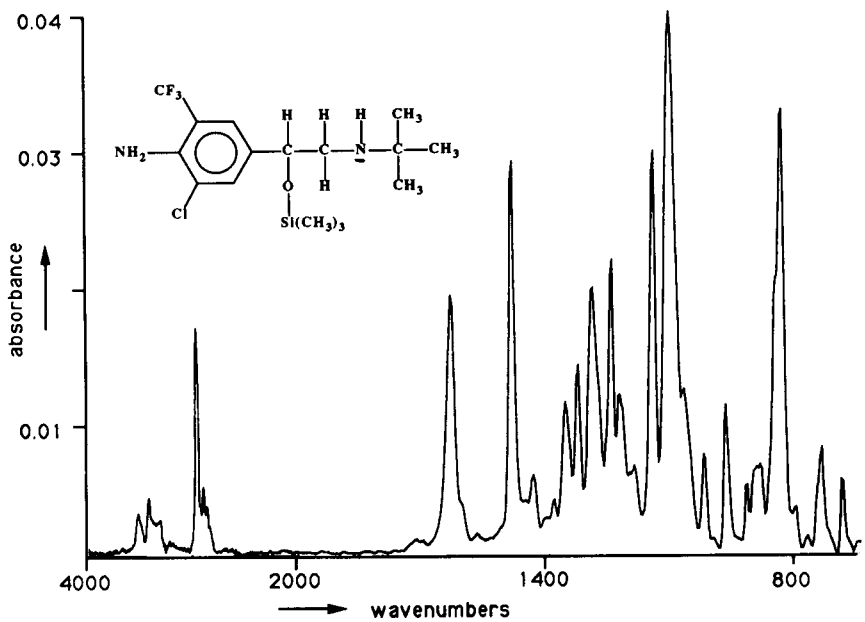

Fig. 2. Cryotrapping GC–FT-IR spectrum and molecular structure of trimethylsilylmabuterol. Injected amount 25 ng, optical resolution 8 cm^{-1}, 4 scans coadded.

and of 10°C min^{-1} to 290°C where it was hold isothermal for 5 min. The column end was connected to a fused-silica transfer line with an internal diameter of 150 μm, by means of glass press fit connector (Supelco). The transferline was guided into the FT-IR spectrometer interface by means of a stainless-steel tube heated to 250°C.

Spectrometry

Infrared spectrometric detection was performed with a Digilab FTS-40 Fourier transform instrument equipped with a Digilab Tracer GC interface and an SPC 3200 computer for data processing. Chromatograms were processed as the Gram–Schmidt chromatogram, representing changes in the infrared signal as a function of time in the range 4000–660 cm^{-1}, and as functional group chromatograms representing changes in a preselected wavenumber interval. Spectra were recorded during the GC run, immediately after deposition (on-the-fly detection) with 2 scans s^{-1}/4 scans coadded, and after completion of the GC run (post-run scanning) with 512 scans coadded. Scanning was carried out at optical resolutions of 8 cm^{-1} (data point resolution 4 cm^{-1}) and 2 cm^{-1} (data point resolution 1 cm^{-1}).

RESULTS AND DISCUSSION

In order to identify a compound by means of infrared spectroscopy, the IR reference spectrum has to be available. To our knowledge, condensed-phase IR spectra of trimethylsilylclenbuterol, trimethylsilylmabuterol and tris(trimethylsilyl)salbutamol have not been published before and for this reason these were recorded first. For this purpose, standard solutions containing 25 ng μl^{-1} were injected and scanned. Elution of each derivatized compound was clearly visible in the corresponding Gram–Schmidt reconstructed chromatogram and the obtained on-the-fly spectra showed good signal-to-noise ratios. These spectra are presented in Figs. 1, 2 and 3 together with the molecular structures. Although the molecular structures are quite alike, the spectra are evidently different which illustrates the discriminating power of infrared spectrometry. Dif-

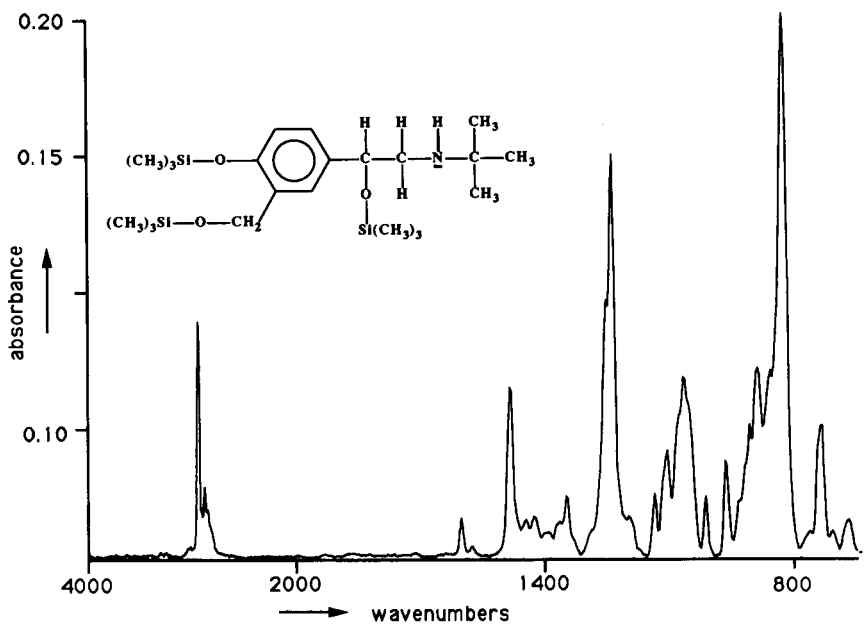

Fig. 3. Cryotrapping GC–FT-IR spectrum and molecular structue of tris(trimethylsilyl)salbutamol. Injected amount 25 ng, optical resolution 8 cm^{-1}, 4 scans coadded.

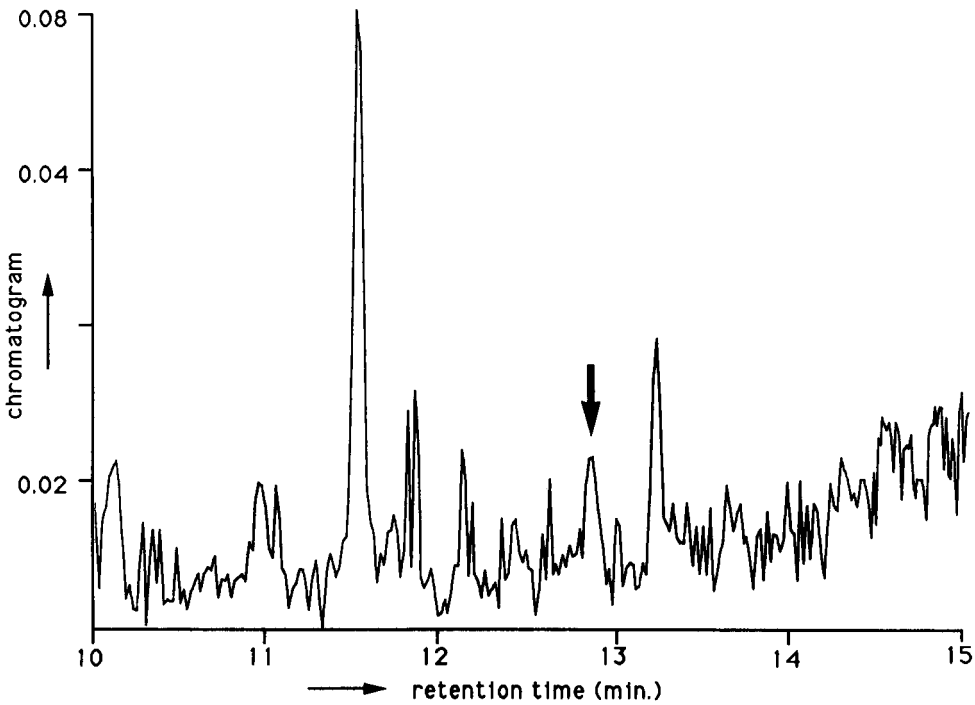

Fig. 4. Functional group GC–FT-IR chromatogram (interval 846–838 cm^{-1}) of a standard solution trimethylsilylclenbuterol in toluene. Concentration 2.5 ng μl^{-1}. Elution of trimethylsilylclenbuterol is indicated with an arrow.

ferences mainly occur in the region 1750–660 cm^{-1} and for that reason this region was used for purposes of identification in samples.

After a GC run, measurement of the trapped samples was carried out at optical resolutions of 2 and 8 cm^{-1} to further improve the spectral quality. As it appeared, the intensity and shape of the absorption bands were only slightly affected by scanning at higher resolution. In view of the much better signal-to-noise ratio of the spectra recorded at 8 cm^{-1}, this resolution has been used in further experiments.

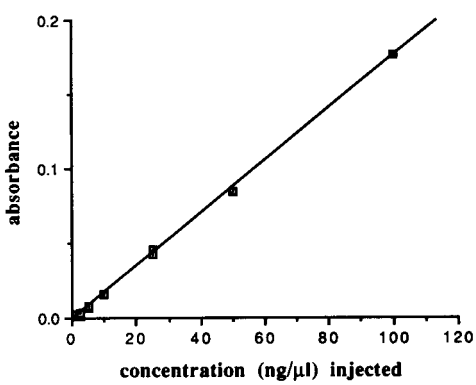

Fig. 5. Absorbance of the 842 cm^{-1} absorption band of trimethylsilylclenbuterol as a function of the concentration.

Next to the Gram–Schmidt chromatogram, so-called functional group (FG) chromatograms can be used for the detection of eluting compounds. FG-chromatograms represent the detected absorption of a preselected wavenumber interval. The main adavantage is an increased detectability of compounds absorbing in that particular interval. All three derivatives have a strong absorption band at 842 cm^{-1} which originates from a vibration of the oxygen-trimethylsilyl group. This band is the most suited one to detect the elution of the trimethylsilyl derivatives and therefore an FG-chromatogram with a window of 846–838 cm^{-1} (i.e. 842 $cm^{-1} \pm$ the data point resolution) was used.

Clenbuterol

The retention time t_R of trimethylsilylclenbuterol was determined from the injected standards as 12.80 ± 0.02 min. As illustrated in Fig. 4, detection was still possible in the FG-chromatogram of the 2.5 ng μl^{-1} standard but not for the 1- and 0.5-ng injections. It follows that the FG-chromatogram is not suited to draw quantitative conclusions at low concentrations.

However, an identifiable spectrum was produced by means of post-run scanning of the 1-ng standard over a short retention time interval (12.78–12.82 min). According to Beer's law, spectra can be used for quantitative analysis as well,

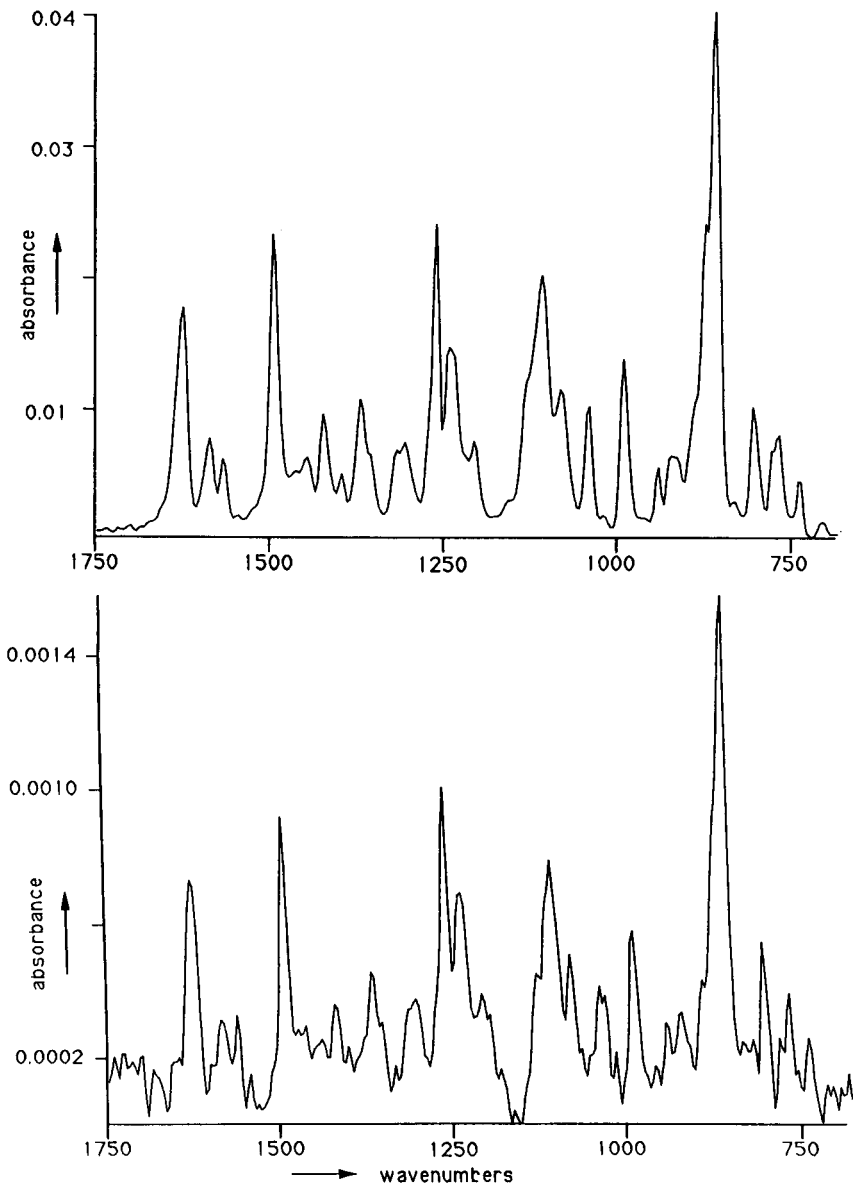

Fig. 6. Cryotrapping spectra of trimethylsilylclenbuterol in the range 1750–660 cm^{-1}; 25 ng standard (top) and urine extracted sample of approx. 1 ng (bottom).

and therefore the absorbance of the 842 cm^{-1} band of the post-run recorded standards was plotted as a function of the concentration. As can be seen from Fig. 5 a straight calibration was obtained which implies that this band can be used to determine the injected amount of clenbuterol.

The viability of these results was tested by the analysis of extracts of blank urine, the spiked urine samples and the urine samples of treated animals. As expected the obtained FG-chromatograms were very similar but not showing trimethylsilylclenbuterol. Therefore post-run scanning was carried out over an extended retention time interval (i.e. 12.76–12.84 min) in order to cover

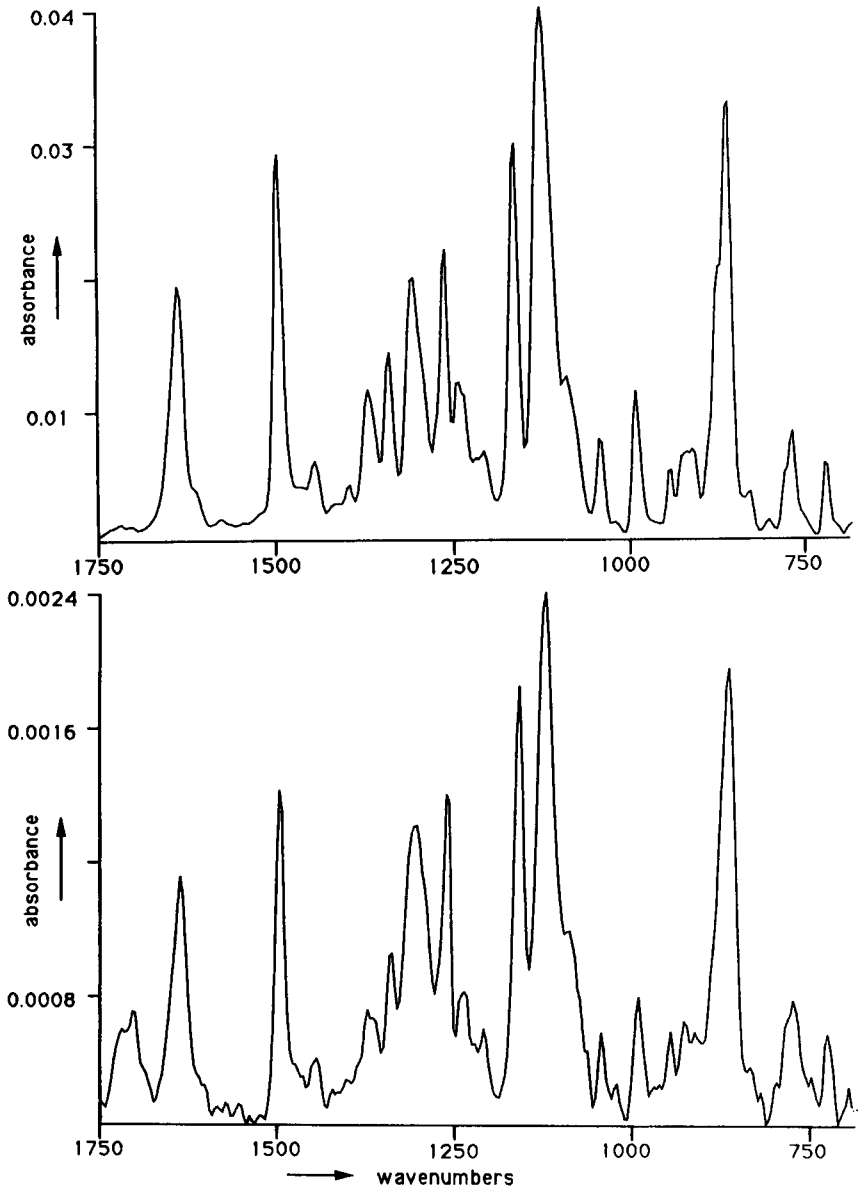

Fig. 7. Cryotrapping spectra of trimethylsilylmabuterol in the range 1750–660 cm^{-1}; 25 ng standard (top) and urine extracted sample of approx. 1 ng (bottom).

minor changes of t_R. For the extracts of blank urine, absorption was found not to occur in this interval whereas for the spiked and real samples identifiable spectra were obtained. This is illustrated by comparison of the spectra of the 25-ng standard and one of the real samples as shown in Fig. 6. The corresponding absorption frequencies are given in Table 1 and as can be seen practically all relevant bandmaxima are found within the data point resolution of 4 cm^{-1}.

The agreement in the absorption frequencies combined to the visual similarity of the spectra largely reflects the ability to identify trimethylsilylclenbuterol from the cryotrapped IR spectrum.

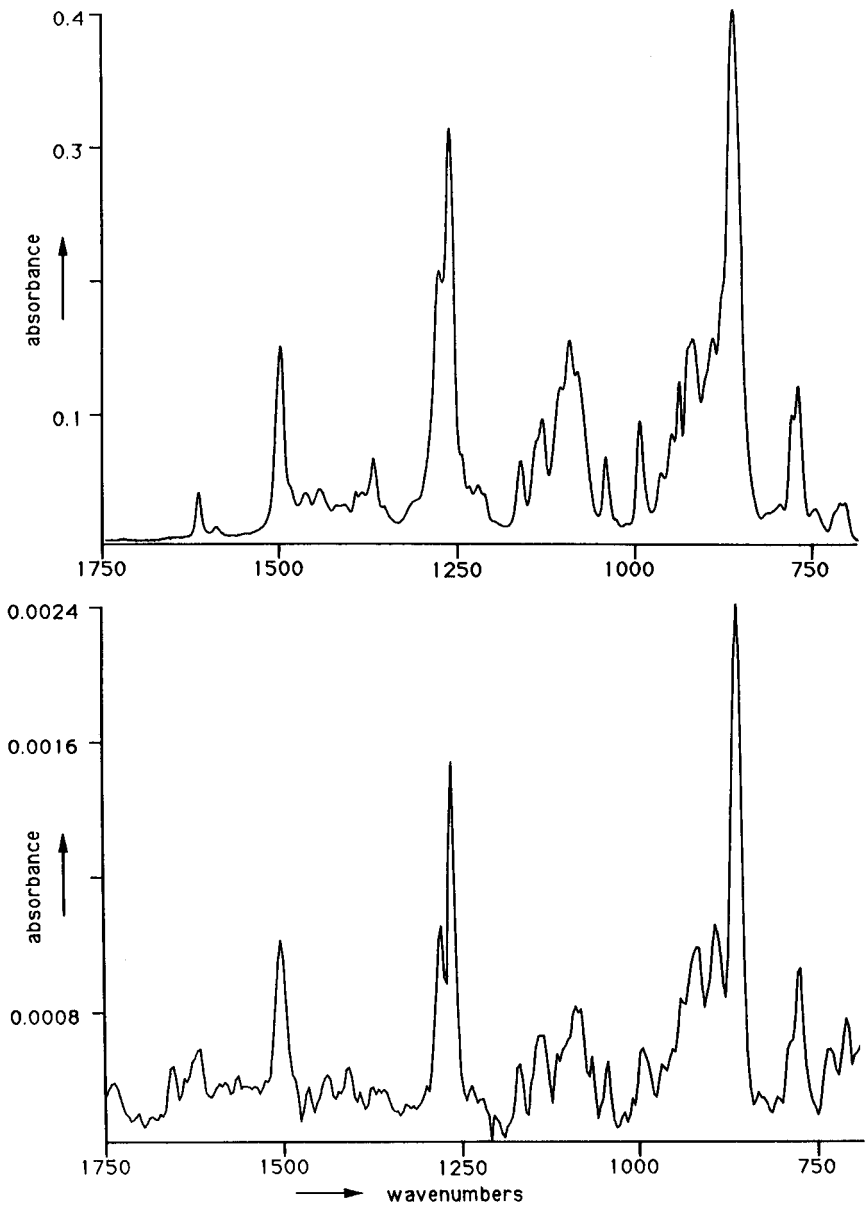

Fig. 8. Cryotrapping spectra of tris(trimethylsilyl)salbutamol in the range 1750–660 cm^{-1}; 50 ng standard (top) and liver extracted sample of approx. 0.3 ng (bottom).

TABLE 1

Infrared absorption maxima of trimethylsilyl-derivatized clenbuterol in the frequency range 1750–660 cm^{-1}

Reference (25 ng)	Standard (1 ng)	Urine extract (±1 ng)
719.5	719.4	721.0
749.9	749.8	749.3
786.2	786.5	786.4
842.0	842.0	842.2
855.1	855.6	–
905.5	906.6	907.9
926.6	926.9	928.2
976.0	975.9	977.0
1027.5	1027.2	1026.0
1067.7	1068.2	1068.6
1095.5	1094.8	1095.6
1195.3	1198.1	1199.1
1229.9	1225.7	1228.1
1250.5	1251.5	1250.5
1296.6	1298.8	1297.0
1360.8	1360.8	1360.3
1415.2	1413.2	1414.4
1448.1	1487.8	1488.6
1561.4	1555.4	1557.9
1581.6	1582.9	1580.4
1621.3	1621.2	1622.9

TABLE 2

Infrared absorption maxima of trimethylsilyl-derivatized mabuterol in the frequency range 1750–660 cm^{-1}

Reference (25 ng)	Standard (1 ng)	Urine extract (±1.6 ng)
700.4	702.2	702.5
749.4	751.8	750.4
842.7	845.7	845.3
902.3	908.7	907.3
927.6	929.3	929.5
976.4	976.4	975.4
1028.4	1030.7	1031.1
1077.3	1078.2	–
1110.9	1111.9	1115.8
1150.9	1150.1	1151.5
1196.4	1199.3	–
1232.0	1227.5	1226.5
1251.2	1252.1	1247.7
1297.8	1297.8	1299.2
1331.8	1332.6	1332.8
1361.5	1365.1	1364.1
1439.5	1438.8	–
1490.4	1491.6	1491.9
1634.7	1635.2	1634.5

Analogous to the criteria for the identification of an analyte by means of infrared spectrometry as proposed by De Ruig et al. [9], the presence of a minimum number of absorption frequencies that agree in relative absorbance, seems to be useful to confirm the presence of this β-agonist in a biological sample.

Mabuterol

The analysis of samples containing mabuterol was accordingly performed. The retention time of the trimethylsilyl derivative in the range 50–2.5 ng μl^{-1} was established at 10.60 ± 0.01 min but elution of the 1 ng μl^{-1} standard was not observed in the FG-chromatogram. Therefore post-run scanning was applied and a good quality spectrum was obtained within the interval t_R ± 0.01 min. The presence of mabuterol in the sample of urine was confirmed by post-run scanning of the t_R interval 10.54–10.64 min. As can be seen from Fig. 7, the obtained spectrum compares very well with the one of the 25 ng μl^{-1} standard. The corresponding absorption frequencies are given in Table 2. All observed band maxima are found within the data point resolution.

Salbutamol

Trimethylsilyl-derivatized salbutamol differs from trimethylsilylclenbuterol and trimethylsilylmabuterol by the fact that it contains two more trimethylsilyl-oxygen groups. As a consequence, the trimethylsilyl-oxygen absorption band around 842 cm^{-1} is even more dominating in the spectrum of derivatized salbutamol then it is in the spectra of trimethylsilylclenbuterol and trimethylsilylmabuterol. Apparently the extinction of this band is increased and thus the limit of detection is expected to be improved. This was confirmed by the measurement of the tris(trimethylsilyl) salbutamol standards. The FG-chromatogram showed a clearcut peak down to the 1 ng μl^{-1} concentration while in the post-run mode a spectrum matching with 250 pg salbutamol on column could be obtained. The retention time was determined at 13.19 ± 0.02 min.

Post-run measurement of the interval 13.15–13.23 min was applied to detect the presence of

TABLE 3

Infrared absorption maxima of trimethylsilyl-derivatized salbutamol in the frequency range 1750–660 cm^{-1}

Reference (50 ng)	Standard (1 ng)	Liver extract (±0.3 ng)
687.9	689.6	684.1
750.3	751.3	750.1
841.0	841.2	841.0
871.6	–	871.0
903.2	901.5	901.1
921.4	920.9	–
978.3	988.9	976.6
1026.9	1027.1	1026.3
1078.6	1076.9	1072.2
1119.4	1118.0	1120.6
1149.5	1149.8	1154.6
1210.7	–	–
1250.7	1250.6	1250.1
1264.0	1263.6	1266.5
1360.5	1363.1	–
1495.1	1495.6	1496.7
1612.0	1612.3	1613.8

tris(trimethylsilyl)salbutamol in the extracts of the liver samples. In the extract of the blank sample, no significant interfering absorptions were observed. In the extracts of the real samples, the obtained spectra were clearly recognized as originating from tris(trimethylsily)salbutamol at a level of approximately 0.3 ng μl^{-1}. However, as can be seen from the spectra in Fig. 7, the number of absorption bands with sufficiently high intensity to be used for identification is small. It implies that the spectrum is less discriminative than that of trimethylsilylclenbuterol and trimethylsilylmabuterol. Yet, as can be seen from Table 3, even for weak bands the agreement in the absorption frequencies is fairly good and therefore it is concluded that the presence of tris(trimethylsilyl)salbutamol can be confirmed at this low level as well.

Conclusions

In summary, it is concluded that cryotrapping GC–FT-IR can be used to detect and identify trimethylsilyl-derivatized clenbuterol, mabuterol and salbutamol in extracts of biological samples. The limit of detection is established in the range 0.3–1 ng μl^{-1} on column, corresponding to 1–2.5 ng l^{-1} or $\mu g\ kg^{-1}$ analyte in the original material.

The similarity of the analyte spectra and the reference spectra, expressed as the agreement in frequency and relative absorbance of a minimum number of absorption bands, can be used as a criterion for identification.

In view of the present results, it is concluded that cryotrapping GC–FT-IR is a valuable technique for purposes of confirmation, particularly when it is used in conjunction with GC–MS.

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Application of an enzyme-linked immunosorbent assay kit for β-agonist screening of bovine urines in north-eastern Italy

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Abstract

The application and the results of a commercially available enzyme-linked immunosorbent assay (ELISA) kit for the screening of β-agonists are presented. The kit was adopted as a screening method because it allows very rapid multi-analyte detection without extraction. β-Agonists such as clenbuterol, mabuterol, salbutamol and terbutaline can be detected in bovine urine with a decision limit of 0.6, 0.9, 1.6 and 2.5 ng ml^{-1}, respectively. Confirmation of positive ELISA results concerning the presence of clenbuterol and salbutamol was made by liquid chromatography and by gas chromatography–mass spectrometry, with immunoaffinity clean-up. By employing the described strategy, in a 5-month period, 59 out of 815 samples were found to be positive. Positive groups of samples were from both therapeutic and anabolic use of β-agonist drugs. Adopting 1 ng ml^{-1} clenbuterol as a cut-off value, the percentage of unconfirmed positive ELISA herds was 3.6%. The reliability of the overall control strategy and of the kit in terms of the percentage of false-positive results is evaluated.

Keywords: Enzymatic methods; Immunoassay; β-Agonists; Bovine urine; Enzyme-linked immunosorbent assay; Urine

During the last 2 years the use of β-agonist drugs (in particular clenbuterol) in livestock breeding has became widespread in Italy. In order to prevent the presence of residues due to incorrect therapeutic doses or to anabolic use, veterinary drug control laboratories require extensive monitoring of such compounds. Hence, screening techniques that should not be subject to false-negative results should be adopted. For this reason a multi-analyte and extraction-free method, that overcomes the low recoveries of some compounds during normal liquid–liquid or solid-phase extractions, is to be preferred. Up to now, enzyme-linked immunosorbent assays (ELISAs) for β-agonists have required time-consuming sample clean-up and they sometimes showed insufficient sensitivity for salbutamol [1,2]. The ELISA kit employed in this study is a no-extraction assay for *tert*-butyric β-agonists, which in comparison with gas chromatography–mass spectrometry (GC–MS) showed no occurrence of

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false-negative results [3]. In order to verify the applicability of the kit to a high sample throughput routine, we considered the results obtained during a period of 5 months in a government laboratory in charge of monitoring the use of anabolic compounds and veterinary drugs in north-eastern Italy, devoting particular attention to the occurrence of false-positive results.

EXPERIMENTAL

Materials

Clenbuterol, salbutamol and β-glucuronidase were obtained from Sigma. Methanol acetonitrile, water, toluene, triethylamine (TEA) were of liquid chromatographic (LC) grade. All other solvents were of analytical-reagent grade. C_{18} cartridges were obtained from Baker (Schilling Italia, Milan). The β-agonist ELISA kit and Multi-prep III immunoaffinity column were supplied by Genego (Gorizia, Italy).

Analytical strategy

Urine samples were submitted to ELISA. Any sample whose concentration was above the cut-off limit chosen (0.6 or 1 ng ml^{-1}) was considered to be positive. Any group in which at least one positive animal was found was considered to be positive. For identification and confirmation, positive samples (> 0.6 ng ml^{-1}) were cleaned up and then submitted to LC and GC–MS for clenbuterol detection. If the clenbuterol analysis was negative, the sample was submitted to the salbutamol detection procedure (LC and GC–MS).

Samples

Urine samples (40–50 ml) were collected and sent refrigerated to the laboratory within a few hours. The number of samples from each breeding or slaughter house ranged from 2 to 20.

ELISA kit

Screening was performed by using Genego quantitative β-agonist ELISA kit, which contains the following components: a 96-well microtitre plate (split into six strips of sixteen wells each), precoated with anti-rabbit immunoglobulin (IgG); anti-β-agonist antibody; salbutamol–horseradish peroxidase enzyme conjugate; six clenbuterol standard solutions (0.3–10 ng ml^{-1}); chromogen [2,2′-azinobis-3-ethylbenzothiazoline sulphonate (ABTS)]; developing buffer (citrate buffer, hydrogen peroxide); stop solution; dilution buffer; and washing buffer.

The assay is a simple and rapid competitive ELISA with time requirements of 0.5 h for the preparation of 30 samples and 1 h for implementation of the test. Detection limits (1 + 1 sample dilution) are 0.6 ng ml^{-1} for clenbuterol and mabuterol, 1.5 ng ml^{-1} for salbutamol and 2.5 ng ml^{-1} for terbutaline. Recoveries from urine and serum samples were 97–110%.

ELISA procedure

Urine samples were diluted 1 + 1 with dilution buffer, centrifuged for 5 min at 1000 *g* and directly tested in the ELISA: 50 μl of standard or sample with 50 μl of salbutamol–enzyme conjugate were added to each well, followed by the addition of 100 μl of diluted antiserum. Maximum binding was assessed by adding no inhibitor (zero standard) to relevant wells and the background (blank) was assessed by adding no antibody to the relevant wells. The plate was then incubated at room temperature for 35 min. After washing five times, the enzymatic activity was determined by the addition of 200 μl per well of developing solution [chromogen–citrate buffer (1 + 24, v/v)]. After a 30-min incubation, 50 μl of stop solution were added to each well. The absorbance was determined at 414 nm by an automatic microtitre reader (Multiskan Titertek MCC; Flow Italia, Milan). Samples were always assayed in duplicate; by using the whole plate three calibration graphs were obtained whereas two were obtained by using half the plate or less.

ELISA data handling

ELISA raw data from the microtitre absorbance reader were processed by RIASMART software linked to an Autogamma Cobra 5002 gamma counter (Canberra Packard Italia, Milan).

SAMPLE PREPARATION

Solid-phase extractions

Clenbuterol. A 10-ml volume of urine was made alkaline (pH 9) with 5 M NaOH and processed as

described by Beernaert and Degroodt [4]: C_{18} cartridges (500 mg of gel) were rinsed with 10 ml of methanol and 10 ml of water. The sample was applied to the cartridge, which was then washed twice with 5 ml of water, once with 5 ml of methanol–water (25 + 75) and once with 5 ml of 0.05 M NaOH. After drying the gel under vacuum for 15 min, clenbuterol was eluted with 2 ml of methanol. The eluate was evaporated to dryness and the residue was dissolved in 3 ml of methanol—Multi-prep III buffer (10 + 90, v/v).

Salbutamol. A 10-ml volume of urine was hydrolysed at pH 5.2 with β-glucuronidase (overnight at 37°C) and processed as described by De Groof [5]: C_{18} cartridges (500 mg of gel) were rinsed with 5 ml of methanol and 5 ml of water. The pH of the digested sample was adjusted to 8 with 5 M NaOH, then the sample was applied to the cartridge, which was washed twice with 5 ml of water. After drying the gel under vacuum for 15 min, salbutamol was eluted with 2 ml of methanol–acetic acid (95 : 5, v/v). The eluate was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 3 ml of methanol–multi-prep III buffer (10 + 90, v/v).

Immunoaffinity clean-up

Immunoaffinity clean-up of C_{18} urine extracts was performed by employing a Genego Multi-prep III kit (20 ng of β-agonists total capacity) and following the manufacturer's instructions. Briefly, the procedure was as follows. Dissolved extracts were loaded on the columns and gently mixed with the gel for 1 min, then the columns were left to elute by gravity and washed with extraction buffer (10 ml). β-Agonists were recovered by elution with ethanol–10 mM acetic acid (96 + 4). The eluates were evaporated to dryness under a stream of nitrogen at 60°C and the residue was dissolved in 100 μl of 0.01 M HCl (LC confirmation) or 50 μl of hexamethyldisilazane (HMDS) (GC–MS confirmation).

Liquid chromatography

The LC system consisted of a Varian Model 2510 pump, a Waters Model 484 UV–visible detector, a Jasco Model 820 FP spectrofluorimetric detector, a Shimadzu CR4 integrator and a Spherisorb C_8 (5 μm) column (25 cm × 4.5 mm i.d.) (Labservice, Bologna).

Clenbuterol. The UV detector was set to 245 nm; elution was done with NaH_2PO_4–acetonitrile (70 + 30), adjusting the 'pH' to 4 with H_3PO_4; the flow-rate was 1 ml min^{-1}.

Salbutamol. The spectrofluorimetric detector was set at 275 nm (excitation) and 305 nm (emission); elution was done with a 0.1% solution of TEA, adjusting the 'pH' to 4 by adding H_3PO_4–acetonitrile (90 + 10); the flow-rate was 0.9 ml min^{-1}.

GC–MS

GC–MS analysis was performed according to Van Ginkel [6]. Briefly, the dried residue was dissolved in 50 μl of HMDS and the mixture was heated at 60°C for 30 min. The reagent was evaporated and the dried derivatized residue was dissolved in 50 μl of toluene. GC–MS was done with a Hewlett-Packard HP 5890 gas chromatograph equipped with an HP1 fused-silica capillary column (12 m × 0.2 mm i.d., film thickness 0.33 μm) coupled to an HP 5970 mass-selective detector. Injections were made in the splitless mode with an injection volume of 2 μl. The injector temperature was 250°C. The column temperature was maintained for 1 min at 80°C, then programmed at 20°C min^{-1} to 280°C and maintained there for 4 min. Analysis of urine extracts was performed in the single-ion monitoring (SIM) mode by monitoring specific ions of the trimethylsilyl (TMS) derivatives at m/z 86, 243, 262, 277, 369 and 440.

RESULTS

Following the above strategy during a 5-month period, 815 bovine urine samples (from 180 herds) from breeding and slaughter houses were analysed. Slaughter houses represented about 30% of the total number of cases. A number of samples were from certified therapeutical treatments.

Figure 1 shows an example of a calibration graph obtained by using the ELISA kit. Internal quality control was performed (doses: 1.25, 2.5 and 5 ng ml^{-1}) and the intra-assay relative standard deviation (R.S.D.) was found to be below

6% ($n = 6$) and the inter-assay R.S.D. below 9% ($n = 5$). The accuracy of analysis, measured by spiking urine blanks with 1 ng ml^{-1} of clenbuterol or 2 ng ml^{-1} of salbutamol, showed a tendency for slight overestimation of the analyte concentration (10–15% in the low ng ml^{-1} range). The confirmation procedure (in which a C_{18}-immunoaffinity clean-up step was introduced) gave satisfactory results in terms of background, recoveries rates (75% for clenbuterol- and 65% for salbutamol-spiked samples) and sensitivity (0.6 ng ml^{-1} for clenbuterol and 1 ng ml^{-1} for salbutamol). Figure 2 shows an example of a liquid chromatogram obtained for a clenbuterol-positive sample.

A total of eighteen positive herds were found; of these eighteen, seventeen cases were due to clenbuterol (49 samples) and one to salbutamol (ten samples).

In order to decide which cut-off limit was to be preferred for application in the future, it was decided to calculate the number of ELISA positive samples over 1 ng ml^{-1} and in the range 0.6–1 ng ml^{-1}. The number of samples analysed was 815, the numbers of ELISA positives were 141 (17%) > 0.6 ng ml^{-1} and 89 (10.9%) > 1.0 ng ml^{-1} and the number of confirmed positives was 59 (7.2%). The number of herds analysed was 180, the numbers of ELISA positives were 35 (19.4%) > 0.6 ng ml^{-1} and 25 (13.8%) > 1.0 ng ml^{-1} and the number of confirmed positives was 18 (10.0%).

Applying the two different cut-offs, different numbers of positive herds were detected (eighteen using 0.6 ng ml^{-1} and seventeen using 1 ng ml^{-1}) and different numbers of unconfirmed samples were obtained. The ELISA positives unconfirmed by chromatography were 9.4% of cases and 10.0% of samples for the 0.6 ng ml^{-1} cut-off

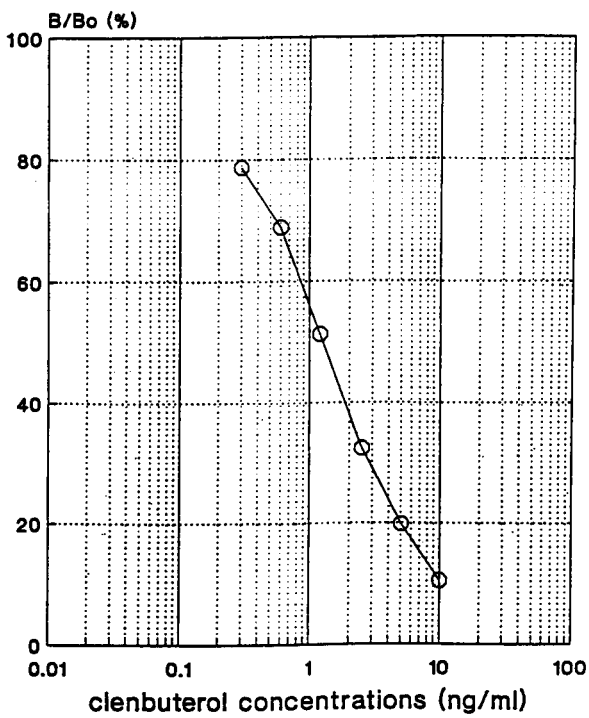

Fig. 1. Example of an ELISA calibration graph. B = absorbance of each standard or sample; B_0 = absorbance of standard = control.

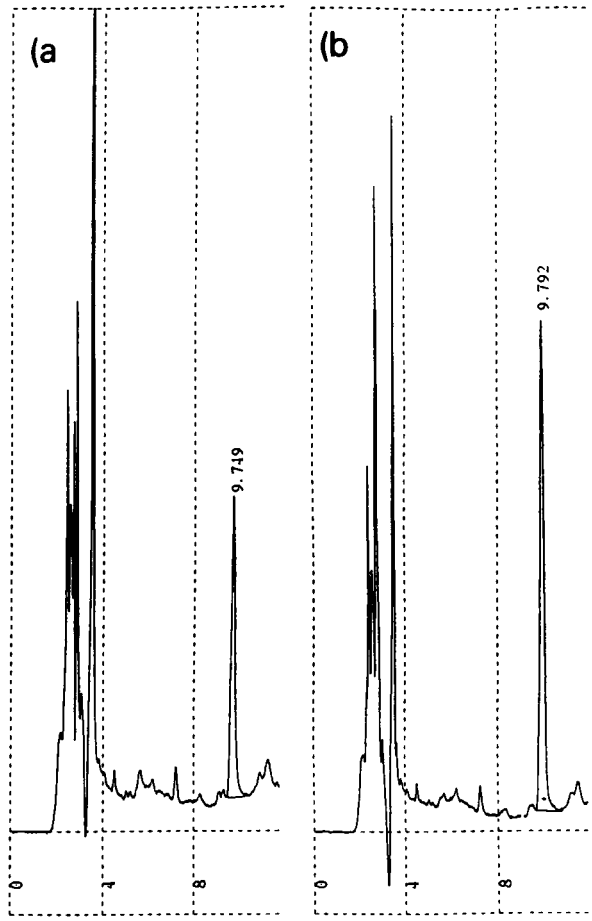

Fig. 2. Liquid chromatogram of a positive urine sample: (a) sample 234; (b) sample 234 spiked with 2 ng ml^{-1} clenbuterol.

and 3.6% of cases and 3.8% of samples for the 1.0 ng ml^{-1} cut-off. Of eighteen confirmed positive herds, six were identified in slaughter houses; the concentrations of clenbuterol found in slaughter houses were in a few instances fairly high (> 20 ng ml^{-1} in ELISA). Chromatographic analysis was not performed for quantitative purposes, so that a precise correlation between ELISA and GC–MS or LC results is not possible. Moreover, the standard clean-up procedure prior to chromatographic analysis did not allow quantitative recoveries from samples containing more than a few ng ml^{-1}, owing to the limited capacity of the immunoaffinity column. Anyway, in the low ng ml^{-1} range good agreement was observed between screening and confirmation steps.

DISCUSSION

During the past few years, the detection of veterinary drugs in urine samples by ELISA has been published, but this appears to be the first paper reporting data on the routine application of an ELISA kit for the detection of β-agonists. Out of 180 herds, the ELISA kit allowed the detection of 18 positive herds. With the exception of one case (due to salbutamol), the positive results were due to clenbuterol. The concentration values found covered the whole range of the kit calibration graph (0.6–20 ng ml^{-1}). In spite of available data on the bovine clenbuterol elimination rate [7] the concentrations found in samples coming from slaughter houses were high.

Concerning the cut-off values to be adopted, the results showed that the percentage of unconfirmed samples was acceptable in the case of the 0.6 ng ml^{-1} limit (10%) and very good in the case of the 1 ng ml^{-1} limit (3.8%). As the positive samples were analysed by chromatography for the presence of clenbuterol and salbutamol only, in unconfirmed ELISA positives (apparent false-positives) the presence of other β-agonists (i.e. terbutaline or mabuterol) could not be excluded. At present the monitoring laboratory is devoting its efforts to setting up a full multi-residue analytical procedure to confirm several other β-agonist molecules.

By employing the highest limit (1 ng ml^{-1}), one positive herd was not detected, but in these samples the clenbuterol concentrations were below 1 ng ml^{-1}. Considering that the results did not indicate the risk of underestimation, it is concluded that, by employing the Genego ELISA kit, a 1 ng ml^{-1} cut-off could be a reasonable value to obtain a very low false-positive percentage, probably avoiding any false-negative results.

By ELISA screening the number of samples submitted to time-consuming sample clean-up and chromatographic analysis was considerably reduced. The performance of the assay was very simple and the results of internal quality control were in agreement with the manufacturer's information.

In conclusion, ELISA is very useful for the screening of large groups of samples and the kit employed has good reliability even in routine application.

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Application of matrix solid phase dispersion for the determination of clenbuterol in liver samples

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Abstract

A rapid method for determination of clenbuterol in liver samples has been developed. It involves combining matrix solid phase dispersion with radioimmunoassay as an efficient technique for residue determination at the sub ng g^{-1} level. The technique was optimised for extract clean-up and recovery of residue using radiolabelled [^{3}H]-clenbuterol. Inter- and intra-assay validation, carried out on fortified liver samples, showed high recoveries over the range 1–5 ng clenbuterol g^{-1}. The determination of incurred residues in liver samples has been investigated. The developed procedure allows for determination of residues of clenbuterol in tissues at levels of less than 0.5 ng g^{-1}.

Keywords: β-Agonists; Clenbuterol; Matrix solid phase dispersion

Clenbuterol {4-amino-α-[(*tert*-butylamino) methyl]-3,5-dichlorobenzyl alcohol hydrochloride}, a β-adrenergic agonist drug, has been used illegally as a growth promoter for beef animals. When administered as an additive in feed, clenbuterol exhibits a repartitioning effect, causing increased deposition of lean meat and reduction in fat [1]. To control the possible misuse of the drug and to meet public demands for residue-free edible tissues, a wide range of analytical techniques have been developed. Because the drug is cleared rapidly from the body, plasma and urine clenbuterol levels are often not detectable by many of the analytical techniques. Clenbuterol persists in liver at levels much higher than in other edible tissues [2]. Rapid methods for determination of clenbuterol in liver samples are required.

Liquid chromatography (LC) has been used for the determination of clenbuterol in biological matrices [3–8] and pharmaceutical preparations [9] with electrochemical detection proving to be most sensitive (low ng g^{-1} levels). LC using post-column derivatisation has allowed detection at levels below 1 ng g^{-1} [10,11]. A recent development is the use of immunoaffinity chromatography as a preconcentration step prior to analysis by LC with UV detection [12], enzyme immunoassay [13] and gas chromatography–mass spectrometry (GC–MS) [14]. Radioimmunoassay (RIA) [15] and enzyme immunoassays [16–19] can detect clenbuterol at the sub ng g^{-1} level. GC–MS methods have been developed [20–23] which provide very

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specific and sensitive assays for clenbuterol but require extensive clean-up procedures.

The extraction procedure described in this paper, matrix solid phase dispersion (MSPD), has found wide applicability in residue analysis of pesticides, antibiotics and other veterinary drugs [24,25]. It has been found that blending tissues with lipophilic C_{18} packing material disperses the sample on the solid support and, when the mixture is formed into a column, residues can be isolated by solvent elution. Applications of the MSPD technique to extraction of residues from tissues have utilized LC for determination of the extracted residues. The limits of determination of these methods have been above 10 ng g^{-1}. For assay of clenbuterol in liver, a limit of determination of less than 1 ng g^{-1} is required. This study reports the application of the MSPD technique to such low levels of residue in tissues and the coupling of the MSPD technique with RIA for determination of the extracted residue.

EXPERIMENTAL

Reagents and equipment

Hexane (extra pure grade) and ethanol (absolute) from Merck (Darmstadt), dichloromethane and methanol (Hypersolv grade) from BDH (Poole) and doubly distilled water were used. Other chemicals used were AnalaR grade from BDH, or equivalent. Clenbuterol hydrochloride from Sigma (St. Louis, MO) was used for fortification of samples. A radioimmunoassay kit, supplied by Laboratoire d'Hormonologie (Marloie, Belgium) was used for determination of clenbuterol. The [^{3}H]-clenbuterol in this kit, with a specific activity of 13 Ci $mmol^{-1}$ (481 GBq $mmol^{-1}$), was used for method optimisation. The clenbuterol antiserum in the kit has principal cross-reactivity (relative to 100% for clenbuterol) of 9% (terbutaline), 7.7% (salbutamol, free base), 6.8% (salbutamol, sulphate salt), 2.75% (cimaterol) and 0.11% (pirbuterol).

Preparative grade Bondesil C_{18} (40 μm) packing material, supplied by Analytichem (Harbor City, CA), was used for the extraction procedure. This material was prepared by placing it in a plastic syringe barrel (50 ml) and washing sequentially with two column volumes each of hexane, dichloromethane and methanol and drying by vacuum aspiration [25]. Extraction columns were prepared in prewashed plastic syringe barrels (10 ml) and qualitative filter paper discs (No. 1, Whatman, Maidstone), were used as frits.

The RIA buffer was 0.01 M phosphate, pH 7.0, containing 1 g l^{-1} gelatin and 0.1 g l^{-1} thiomersal. Dextran-coated charcoal was prepared by adding 2 g of activated charcoal (Sigma) and 0.25 g of dextran T70 (Pharmacia, Uppsala), to 500 ml doubly distilled water. The scintillation cocktail was Cocktail T (BDH).

Methods

Samples and sample preparation. All samples of bovine liver were stored frozen, until assay. Liver samples of known history and certified as free of clenbuterol were homogenised for use in fortification studies and for calibration extracts.

Fortification. The method was optimised by extraction of samples fortified with 193 Bq [^{3}H]-clenbuterol per 0.5 g of tissue. The method was validated by analysis of samples fortified with clenbuterol; 0.5, 1.0 and 2.5 ng of clenbuterol, in 10 μl ethanol, were added to 0.5 g of tissue prior to extraction. Fortification was carried out by pipetting the material on to the surface of the sample and leaving for 10 min prior to extraction.

Extraction. 0.5 g of liver was weighed into a glass mortar and 10 μl of ethanol or an appropriate clenbuterol solution added. 2 g of the C_{18} packing material was added to the mortar and blended with the tissue by mixing with a pestle for 40 s. The mixture was removed from the mortar and transferred to a 10-ml syringe barrel containing two filter paper discs and with a 100-μl pipette tip attached to its outlet. The syringe barrel was tapped to settle the mixture and two filter paper discs were placed on top of the mixture. The mixture was compressed to a volume of 4.5 ml with a syringe plunger (from which the rubber seal and pointed plastic retainer had been removed). The column was washed with 8 ml of hexane and 8 ml of doubly distilled water. After each solvent had flowed through the column,

positive pressure was applied to the top of the syringe barrel with a pipette bulb to remove surplus solvent. 8 ml of methanol was added to the column, the first 1 ml of eluate (mainly water) was discarded and the remaining eluate collected. This eluate was evaporated to dryness under a stream of nitrogen, at 55°C on a sample concentrator, and redissolved in 0.5 ml of ethanol.

Radioimmunoassay. Standard curves (10–1000 pg clenbuterol per tube) were prepared both with and without tissue extract. The curve for the extract was prepared by the addition of 0.1 ml of a pooled extract of control tissue samples to each standard tube. 0.1 ml of tissue extracts were assayed, in duplicate. The contents of all tubes were evaporated to dryness under a stream of nitrogen at 40°C and 0.5 ml of phosphate gelatin buffer was added. After vortexing and incubation for 15 min at 37°C, 0.1 ml of [^{3}H]-clenbuterol and 0.1 ml of antiserum were added. The tubes were incubated at 37°C for 15 min and overnight at 4°C. 0.5 ml of dextran-coated charcoal suspension was added to separate bound from free radioactive clenbuterol and the tubes were centrifuged at 1200 *g* for 10 min. The supernatant was decanted into scintillation vials, 10 ml of scintillation cocktail added and the radioactivity counted.

RESULTS AND DISCUSSION

Method optimisation

A range of solvents of differing polarities were evaluated for their capacity to elute [^{3}H]-clenbuterol from the MSPD column; 8 ml of each solvent were used as such a volume has been found suitable in previous studies with the MSPD technique [24,25]. The [^{3}H]-clenbuterol was not removed from the column by non-polar solvents, such as hexane, and was only partly removed by solvents of intermediate polarity such as dichloromethane and diethyl ether. Polar solvents, such as ethyl acetate and methanol, were found to give almost complete recovery of added [^{3}H]-clenbuterol. The [^{3}H]-clenbuterol was not removed from the column with water. Figure 1 shows elution profiles for [^{3}H]-clenbuterol from the columns, obtained by counting 0.5-ml fractions of the methanol eluate. Most of the [^{3}H]-clenbuterol is recovered in the second to fourth

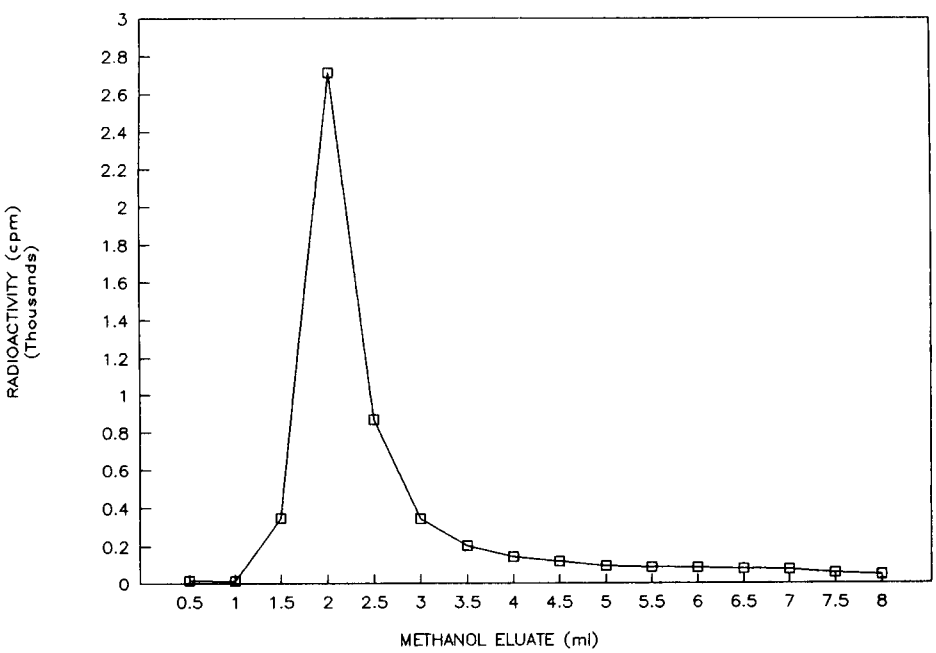

Fig. 1. Elution of clenbuterol from MSPD column.

ml of the methanol eluate and the first ml, which is mostly water retained from the aqueous wash step, can be discarded. A suitable clean-up and extraction procedure was the following: the column was washed with 8 ml hexane (to remove non-polar interferences) and 8 ml water (to remove polar interferences) prior to elution with 8 ml methanol. Using these conditions, recovery of [^{3}H]-clenbuterol in the methanol fraction was 101.9% (±6.0%, $n = 6$).

Radioimmunoassay

Standard curves for RIA were prepared both with and without tissue extract. For the extract curves, sufficient 0.5-g aliquots of a control liver sample were extracted by the MSPD procedure to give a pool of liver extract for the curve. 0.1 g equivalent of control sample extract was added to each standard tube. Figure 2 shows the standard curves (calculated from 8 separate assays) prepared with and without tissue extract. Good parallelism between the curves is found, indicating that a curve without tissue extract may be used. The value for the control liver extract given in the standard curves without extract was 23.6 pg per tube (±3.4 pg, $n = 4$).

TABLE 1

Inter-assay variation for MSPD–RIA determination of clenbuterol in liver; results are calculated on standard curves with (a) and without (b) tissue extract.

Clenbuterol added (ng g^{-1})	n	Clenbuterol determined (ng g^{-1})			
		a		b	
		Mean ± S.D.	C.V. (%)	Mean ± S.D.	C.V. (%)
1	6	0.98 ± 0.12	12.3	0.93 ± 0.18	19.3
2	6	1.94 ± 0.26	13.2	1.71 ± 0.32	18.8
5	6	4.99 ± 0.84	16.9	3.70 ± 0.60	16.1

Method validation

The inter- and intra-assay variations of the method are shown in Tables 1 and 2. Results are presented for RIA standard curves both with and without tissue extract. Satisfactory recovery of clenbuterol added at levels of 1, 2 and 5 ng g^{-1} is obtained (Table 1). The intra-assay results (Table 2) show acceptable variation within a single assay for samples fortified at 1 and 5 ng g^{-1}.

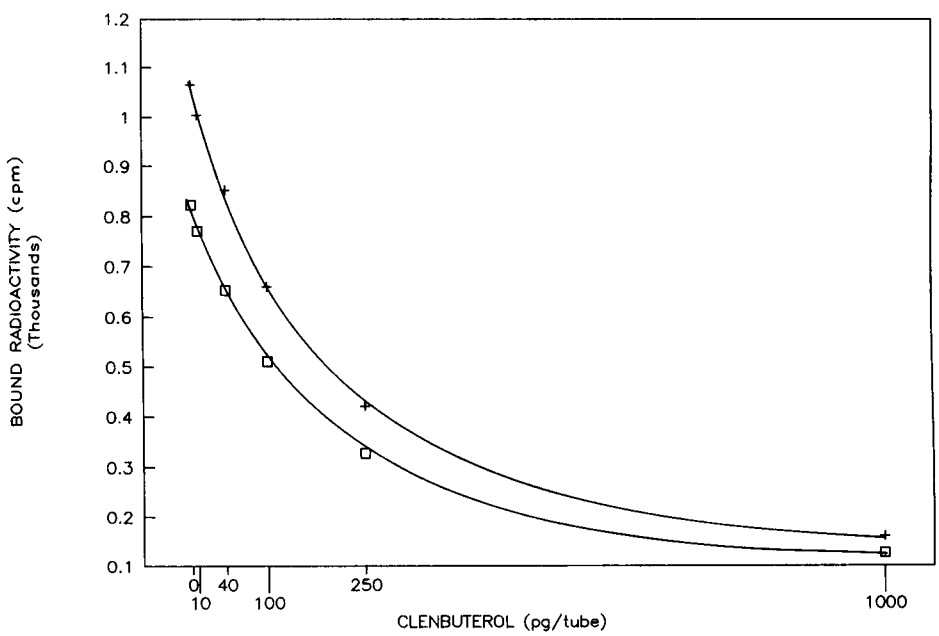

Fig. 2. Radioimmunoassay standard curves. □ = With extract; + = without extract.

TABLE 2

Intra-assay variation for MSPD–RIA determination of clenbuterol in liver; results are calculated on standard curves with (a) and without (b) tissue extract

Clenbuterol added (ng g^{-1})	n	Clenbuterol determined (ng g^{-1})			
		a		b	
		Mean ± S.D.	C.V.(%)	Mean ± S.D.	C.V. (%)
1	6	1.16 ± 0.07	5.9	0.91 ± 0.05	4.9
5	6	4.69 ± 0.60	12.7	3.88 ± 0.35	8.9

Sample analyses

Table 3 shows the results for analysis of samples containing incurred clenbuterol. The levels determined by the MSPD procedure, using standard curves with and without tissue extract, are compared with results obtained by the standard method used in the laboratory [26]. This method consists of protease digestion of the sample, multiple extraction with diethyl ether and determination by radioimmunoassay. Good comparison between the two methods has been found. The coefficient of variation (C.V.) for analysis of residue-positive samples by MSPD–RIA is 10–11% (Table 3); in the case of the standard method, a C.V. of 17.4% was obtained for a quality control sample ($\bar{x}$ = 0.33 ng g^{-1}, S.D. = 0.058 ng g^{-1}, n = 6).

A tentative limit of detection for the MSPD procedure was determined by analysis of liver samples free of clenbuterol residue. The mean value for these samples was 0.18 ng g^{-1} (±0.04 ng g^{-1}, n = 8), determined in a standard curve without tissue extract; the limit of detection, calculated as the mean +3 times the standard deviation, was 0.30 ng g^{-1}. Assay of a larger number of clenbuterol-free samples would be required to give a robust limit of detection for the method.

TABLE 3

Clenbuterol levels determined in residue-positive liver samples by MSPD–RIA, compared with a standard technique; results are calculated on standard curves with (a) and without (b) tissue extract

Sample	Clenbuterol determined (ng g^{-1})		
	Standard method	MSPD–RIA method (n = 6)	
		a	b
		Mean ± S.D.	Mean ± S.D.
A	3.60	3.61 ± 0.35	3.07 ± 0.34
B	1.62	1.84 ± 0.21	1.84 ± 0.19

Conclusion

The MSPD technique has been developed successfully for application to the analysis of clenbuterol in liver samples. Satisfactory results are obtained when the determination of residue levels is by RIA either with or without tissue extract. Use of a standard curve without tissue extract is preferred because of the decreased time and cost of the assay. Recovery of clenbuterol from fortified samples is greater than 70% and results for samples containing incurred residues compare well with the standard method used in the laboratory. The developed technique offers a simple, rapid procedure for assay of clenbuterol in tissue samples. The MSPD technique can be applied to assay of residues at levels of 0.5 ng g^{-1}, or lower, and suitable extracts are produced from the technique for determination by RIA.

For a typical analytical run of 10–20 samples, the MSPD procedure takes slightly more than half the operator time required for the standard procedure. The advantages of the MSPD procedure are low solvent usage and a reduced number of manipulative steps. Preliminary studies with other β-agonist drugs, such as salbutamol, suggest that it may be an appropriate procedure for multi-residue analysis.

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Determination of clenbuterol in bovine liver by enzyme immunoassay

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Abstract

Procedures are described for optimising and validating an ELISA method for measuring clenbuterol residues in bovine liver without prior sample enrichment. Optimal assay conditions are obtained by preincubating liver supernatant with an immobilised antibody raised to clenbuterol–ovalbumen. After further incubation with a salbutamol–peroxidase conjugate, colour is developed using a tetramethylbenzidene substrate. The assay will permit measurement of clenbuterol residues in liver at the maximum residue level of 0.5 ng g^{-1} with a confidence of > 99%.

Keywords: Enzymatic methods; Immunoassay; β-Agonists; Bovine liver; Clenbuterol; ELISA method

Clenbuterol is a β-agonist which is licensed only for the treatment of respiratory disorders in cattle and horses and as a tocolytic. At high dose it can be used as a repartitioning agent promoting the conversion of fat to muscle and therefore increasing carcase value and in this respect its use has been banned. As clenbuterol is toxic it is necessary to monitor levels of the residue in animal tissue destined for human consumption to ensure the maximum residue limit (MRL) for this compound is not exceeded. To support this laboratory's veterinary drug residue monitoring programme an ELISA method was developed for clenbuterol in urine [1]. It was necessary to optimise and validate this assay for rapidly and reliably measuring residues of clenbuterol in liver.

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EXPERIMENTAL

An ELISA method for clenbuterol in urine using an alkaline phosphatase enzyme system was performed in the presence of either urine or 10% bovine liver homogenate to assess the matrix effect of the liver (Fig. 1). In an attempt to decrease assay time a horseradish peroxidase (HRP) enzyme label was substituted for the alkaline phosphatase label (Fig. 2). The antisera obtained from an animal before (248/5) and after (248/6) rechallenging with clenbuterol–ovalbumen immunogen were compared (Fig. 3). Utilising the 4% cross-reactivity of the clenbuterol antibody with salbutamol, salbutamol–HRP was prepared (by the conjugation of salbutamol hemiscuccinate [2] to aminated HRP using the mixed anhydride method based on [3]) and substituted for the clenbuterol–HRP conjugate (Fig. 4). Sample volume was then increased from 10 to 200 μl and sample concentration doubled from 10 to 20%

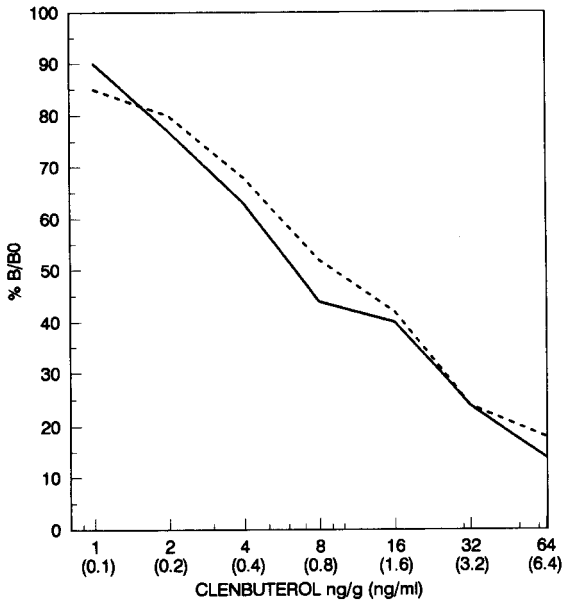

Fig. 1. Alkaline phosphatase clenbuterol ELISA in urine (———) vs. 10% liver (- - - - - -) homogenate.

(w/v) (Fig. 5). Figure 6 shows the effect of preincubation of the test samples and standards in the wells at approximately 10-min intervals up to 1 h, prior to addition of salbutamol–HRP.

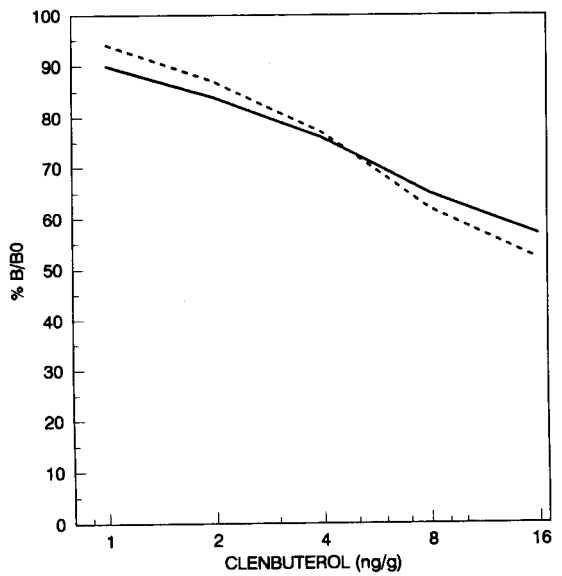

Fig. 2. Clenbuterol peroxidase (———)/alkaline phosphatase (- - - - - -) conjugates. Assay times are 0.5 and 4.5 h, respectively.

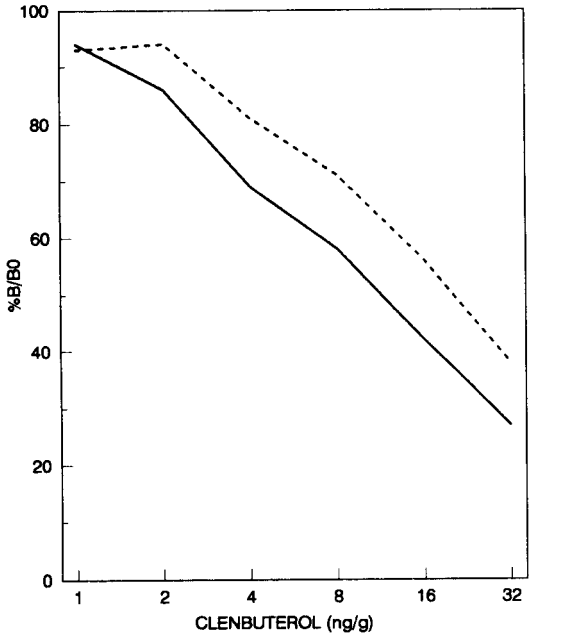

Fig. 3. Antisera bleed 248/5 (- - - - - -) vs. 248/6 (———).

The following ELISA procedure evolved from these investigations. Microtitre plates were coated with clenbuterol-specific antisera using partial denaturation of the antibody with glycine to in-

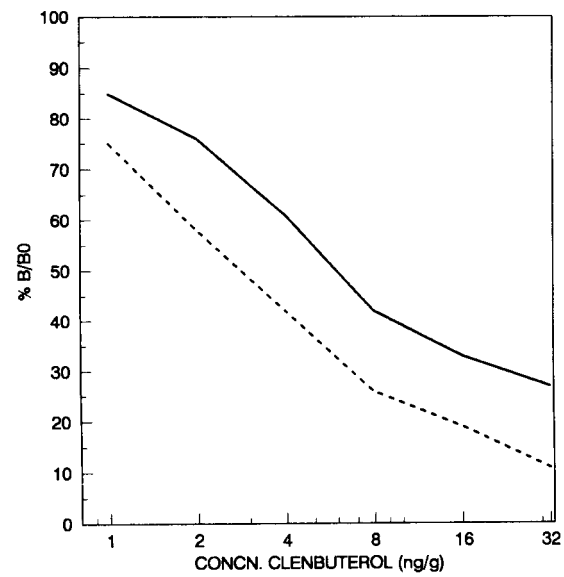

Fig. 4. Salbutamol peroxidase (- - - - - -) vs. clenbuterol peroxidase (———) conjugate.

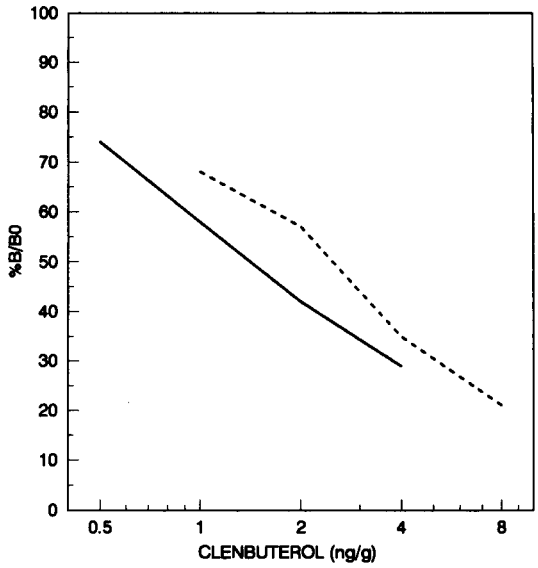

Fig. 5. Clenbuterol standard curves in 10% (- - - - -) and 20% (———) liver homogenate. Sample volume, 200 μl.

crease binding [4]. The plates were then washed, 6 × with phosphate buffered saline (PBS, 0.1 M, pH 7.0) containing Tween 20 (0.05%), sucrose (5%), bovine serum albumin (BSA, 1%), and dried prior to storage at 4°C. Bovine liver test samples and negative control liver were prepared by homogenization in PBS (0.05 M, pH 7.0) to give 20% (w/v) suspensions which were centrifuged (2500 *g*, 4°C, 15 min). The supernatant was retained for assay. Standards were prepared as above using control negative bovine liver fortified with clenbuterol HCl to provide a range between 0.25–8 ng g^{-1}.

TABLE 1

Assays of control liver fortified with clenbuterol HCl

	Standard concentration (ng g^{-1})			
	0	0.5	1.0	2.0
Inter				
Mean ($\%B/B_0$)	96	65	47	34
S.D.	12.5	1.8	1.9	1.6
C.V.	13	2.7	4.2	4.6
n	87	32	32	32
Intra				
S.D.	3.7	2.1	2.4	1.5
C.V.	3.8	3.3	5.1	4.4
n	8	8	8	8

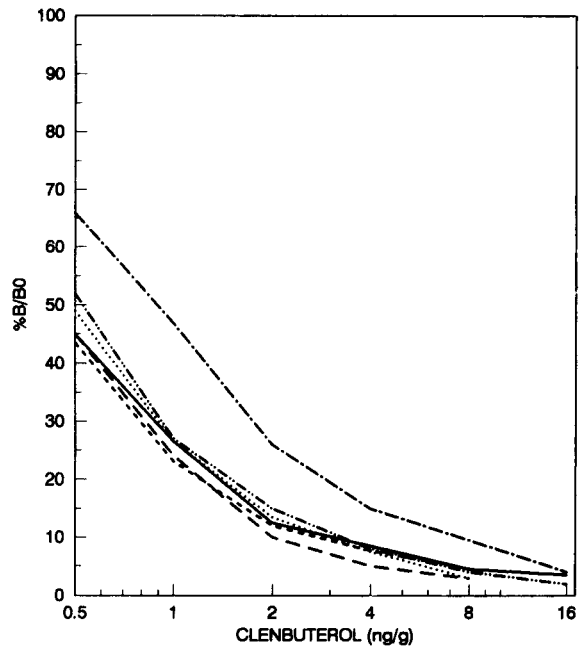

Fig. 6. Six clenbuterol standard curves with preincubation times: ———, 60; - - - - - -, 45; ·····, 30; — — —, 20; —·····—, 10; ·–·–·, 0 min.

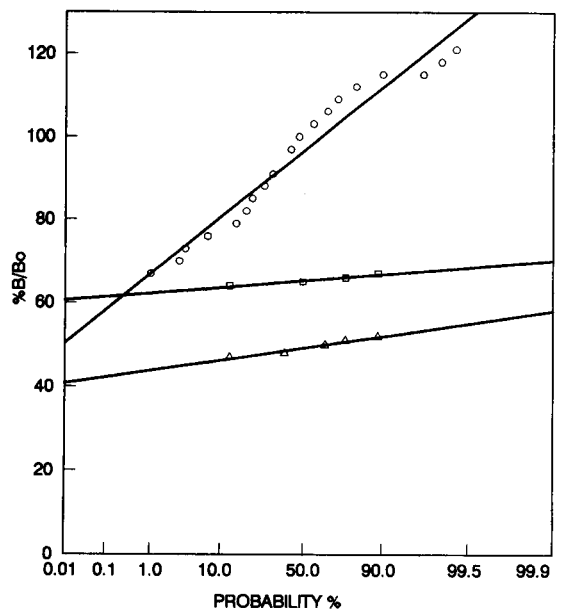

Fig. 7. Clenbuterol probability curves. ○, Negative samples; □, 0.5 ng g^{-1} standard; Δ, 1.0 ng g^{-1} standard.

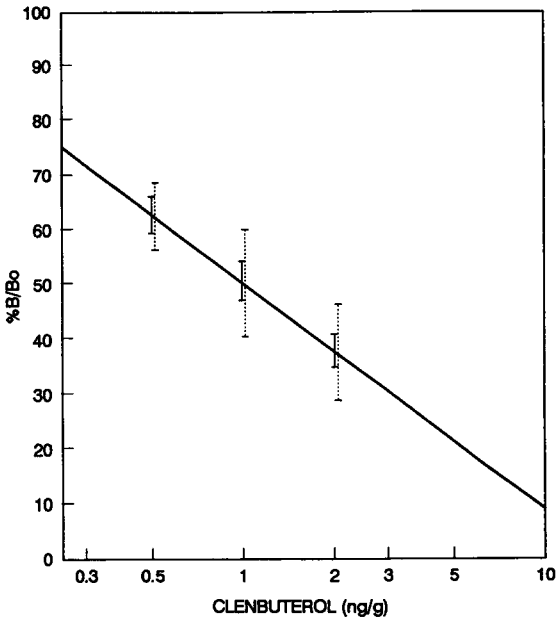

Fig. 8. Composite clenbuterol standard curve ±2S.D. ———, Intra-; - - - - - -, Inter-assay. $r^2 = 0.98$.

200 μl aliquots of samples, controls and standards were added to each well and the plate preincubated (40 min, room temperature); 50 μl of salbutamol–HRP was added and the plate was shaken for 15 min.

After incubation the plate was washed with Tween 20 (0.05%) and 200 μl of TMB–peroxidase substrate added to each well. After shaking the plate for 15 min, colour development was stopped by the addition of 50 μl of 10% H_2SO_4. Absorbance was read at 450 nm on a Dynatech MR700 ELISA plate reader.

Using this procedure a number of assays were performed using negative control liver fortified with clenbuterol HCl at 0, 0.5, 1.0 and 2.0 ng g^{-1}, to assess both inter- and intra-assay variation (Table 1). A probability curve was constructed by plotting percentage cumulative frequency of B/B_0 (probability) against B/B_0 for 176 negative tissue samples and homogenates spiked at 0.5 and 1.0 ng g^{-1} (Fig. 7).

RESULTS AND DISCUSSION

The following conclusions were drawn from the optimisation procedures described. Although liver matrix had a negligible effect on linear response the assay was not sensitive enough to detect the MRL of 0.5 ng g^{-1} liver. The introduction of clenbuterol–HRP conjugate gave a marginal increase in sensitivity and reduced assay time from 4.5 to 0.5 h. Rechallenging the immunised sheep with clenbuterol–ovalbumen resulted in a doubled sensitivity. Assay sensitivity was further improved by the substitution of a salbutamol–HRP conjugate owing to the antibodies lower affinity for salbutamol than for the clenbuterol in the test sample. By increasing sample volume and sample concentration, sensitivity was doubled without significantly increasing background colour. After preincubation of the sample with immobilised antibody the B/B_0 at 0.5 ng g^{-1} was lowered from 62 to 44%. At the MRL of 0.5 ng g^{-1} the inter-assay coefficient of variation (C.V.) was 2.7% when the number of samples tested was 32 and intra-assay C.V. was 3.3% when the number of batches tested was 8. At a B/B_0 value of approximately 65% there is a 1% probability of producing a false positive result and less than 0.5% probability of producing of false negative result at the 0.5 ng g^{-1} detection level. Cross-reactivity with other β-agonists was found to be salbutamol 4%, terbutaline 2%, isoproterenol, ractopamine, DL-4-hydroxy-3-methoxy mandelic acid, dihydroxymadaleic acid and noradrenaline less than 1%.

The optimised assay provides a robust, sensitive and specific analytical procedure with a linear range between 0.25 and 8 ng g^{-1} (Fig. 8) suitable for the screening of liver samples.

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Determination of clenbuterol residues by liquid chromatography–electrospray mass spectrometry

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Abstract

The application of electrospray ionization to the mass spectrometric determination of clenbuterol was investigated. Using electrospray, the energy of the ions extracted into the vacuum system can be controlled and, therefore, collisionally activated dissociations (with residual gas) can be induced in the high-pressure region of the electrospray ionization source. According to the energy thus conferred to the ions, the fragmentation of the molecule can be readily controlled. The influence of instrumental parameters on the fragmentation of clenbuterol was studied. Some results are presented that illustrate the potential of electrospray ionization for the mass spectrometric determination of clenbuterol. The sensitivity of the method is discussed, and preliminary results on a biomedical application are presented.

Keywords: Liquid chromatography; Mass spectrometry; β-Agonists; Clenbuterol; Pharmaceuticals; Urine

Clenbuterol is a β-agonistic drug used for the treatment of pulmonary diseases in humans and animals. Its chemical structure is closely related to that of catecholamines, and clenbuterol can interact with adrenergic receptors. In addition to their broncholytic properties, β-agonists possess strong repartitioning effects in meat-producing animals, owing to reduced protein catabolism and enhanced lipolysis, when adminitered at higher dosages. The use of β-agonists as animal feed additives has not yet been authorized. However, clenbuterol is the β-agonist most commonly and illegally used as a growth-promoting agent for animals.

Clenbuterol determinations have already been achieved either by immunochemical methods [1,2] or physico-chemical methods. Several procedures based on gas chromatography–mass spectrometry (GC–MS) have been described, using different derivatives (trimethylsilyl [3–5], pentafluoroacyl [6], cyclic boronates [5], etc.) and different ionization modes [electron impact (EI), positive ion (PICI) or negative ion (NICI) chemical ionization]. It has been shown that under EI conditions, the mass spectrum of the TMS derivative of clenbuterol presented abundant ions at low masses, while the most specific ions were characterized by low abundances [5]. Consequently, CI was preferably used to acheive good detection limits with sufficient specificity. The most sensitive method for the determination of clenbuterol was developed by Girault et al. [6], using GC of its pentafluoroacyl derivatives and NICI under selected-ion monitoring (SIM) recording conditions. With isotopic dilution, femtomole determinations have been achieved by this method. Gas chromatography–tandem mass spectrometry (GC–MS–MS) [4] and liquid chromatography–thermospray mass spectrometry (LC–TSP-MS) [7] have

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also been proposed for the determination of clenbuterol in bovine urine, and ng ml^{-1} level sensitivities were obtained using SIM with these methods.

Full-scan analyses have been performed on TMS derivatives, permitting the simultaneous determination of several different β-agonistic drugs with detection limits in the ng ml^{-1} range [3].

In recent years, electrospray mass spectrometry [8–10] has become a very powerful tool for the determination of polar and ionic compounds. As multiply charged ions can be formed, most of the applications of electrospray mass spectrometry have been devoted to high-mass compounds such as proteins or large peptides [9,11,12], while smaller molecules have been less extensively studied [13].

In this paper, some preliminary results of a study of the application of electrospray ionization mass spectrometry to the determination of clenbuterol are presented. Using electrospray, the energy of the ions extracted into the vacuum system can be controlled by changing the voltage difference between the exit of the glass capillary transfer and the skimmer, and therefore, collisionally activated dissociations (CADs) can be induced in the high-pressure region (skimmer region) of the atmospheric pressure ionization source [14,15]. The behaviour of clenbuterol towards electrospray ionization was investigated from standards solutions, in particular the ability for obtaining CAD mass spectra on a single quadrupole mass spectrometer. The sensitivity of the method for the acquisition of full-scan spectra of clenbuterol was investigated, based on standard solutions. A biomedical application is presented in which bovine urine spiked at 25 ng ml^{-1} with clenbuterol was extracted, chromatographed on a reversed-phase LC system and then analysed (off-line) by electrospray mass spectrometry.

EXPERIMENTAL

Chemicals and standards

All reagents and solvents were of analytical-reagent grade. Methanol and acetonitrile (LC grade) were purchased from Carlo Erba (Milan), *tert*-butyl methyl ether, trifluoroacetic acid and *n*-butanol from Aldrich (Strasbourg), and ammonium acetate from Prolabo (Paris). An authentic standard of clenbuterol was obtained from Boehringer (Engelheim).

Liquid chromatography

LC analyses were performed using a Spectra-Physics (Les Ulis) SP8100 pump, a Spectra-Physics SP8400 UV detector set at 270 nm and a Shimadzu (Touzart et Matignon, Vitry sur Seine) CR-3A integrator.

An Ultrabase C_{18} column (250 mm × 4 mm I.D.) from SFCC-Shandon (Eragny, France) was used for the separation. The mobile phase was 50 mM ammonium acetate–8 mM trifluoroacetic acid (TFA) (pH 5.4)–85% (v/v) acetonitrile, filtered through Millipore (Saint Quentin en Yvelines) 0.45-μm filter, helium degassed, and was used at a flow-rate of 1.2 ml min^{-1}, without recycling. The separations were performed at room temperature.

Sample preparation for mass spectrometry

Bovine urine (3 ml) was spiked with 75 ng of standard clenbuterol. The extraction was carried out according to the procedure proposed by Polettini et al. [5], based on the extraction of the alkalinized urine with *tert*-butyl methyl ether–*n*-butanol (9 + 1). After evaporation of the organic layer to dryness, the residue was dissolved in 50 μl of the LC mobile phase and then injected directly into the liquid chromatograph. The peak corresponding to clenbuterol was carefully collected. The mobile phase containing clenbuterol (2.5 ml) was then alkalinized with 250 μl of 4 M NaOH and extracted twice with 2 ml of *tert*-butyl methyl ether. The organic layer was separated and evaporated to dryness under a stream of nitrogen and the residue was dissolved in the appropriate mobile phase for electrospray mass spectrometric analysis.

Electrospray mass spectrometry

A Nermag R10-10H quadrupole mass spectrometer (Delsi-Nermag, Argenteuil, France) with

an API data system (P2A Systems, Vincennes, France) was used for data acquisition and processing. The mass spectrometer was fitted with an Analytica (Brandford, CT) electrospray source equipment. The sample solutions were infused with a Harvard Apparatus (South Natick, MA) Model 22 syringe pump into the electrospray interface at a flow-rate of 0.5 or 1 μl min^{-1}, according to the sample being analysed. For the CAD experiments, clenbuterol standard was dissolved (100 ng μl^{-1}) in acetonitrile–water (80 + 20, v/v). The potentials applied to the various components of the electrospray source were as follows: needle, ground; surrounding electrode, −3000 V; end-plate/nozzle, −4700 V; and metallized inlet end of the glass capillary, −5000 V. The nitrogen counter-current pressure was 5 psi (35 kPa). The spectra were acquired by scanning a mass range from m/z 100 to 300 in 10 s. All spectra presented in this paper are an average of five profiles acquired.

RESULTS AND DISCUSSION

Influence of experimental parameters on the fragmentation pattern

As has been pointed out by Loo et al. [14], ions produced by electrospray ionization can be submitted to excitation processes induced by collisions (with residual gas) in front of the skimmer. This feature enables collisonally activated dissociations on single quadrupole mass spectrometers, which is very useful for structural elucidation purposes [15]. Collisional activation can be controlled by changing the electrostatic field imposed in the skimmer region of the electrospray source, thus allowing a true energy-resolved breakdown to be performed [16]. With the Analytica electrospray source [17], this electrostatic field is directly related to the voltage difference (ΔV) between the metallized outlet of the glass capillary transfer and the skimmer.

In this work the skimmer voltage was kept constant at 20 V, whereas the voltage applied to the metallized outlet of the glass capillary transfer was changed from 80 to 240 V. The ions' energy corresponding to this voltage drop can thus be considered as the energy in the laboratory frame of reference for CAD processes.

Electrospray mass spectra of standard clenbuterol solutions are presented in Fig. 1, for various values of ΔV. The spectrum in Fig. 1a was obtained with $\Delta V = 60$ V. For this low voltage difference (i.e., ion energy value), the spectrum consists almost only of the protonated molecular ion, located at m/z 277 (279 and 281 for the chlorine isotopic contributions). When the energy of the ions is increased (e.g., $\Delta V = 120$ V, Fig. 1b), some fragmentations appear, giving rise to ions of m/z 259/261/263 and 203/205/207. With high energy values ($\Delta V = 180$ V, Fig. 1c), some other fragmentations (which seem more complex) occur, and the intensity of the molecular ion is dramatically reduced.

The relative intensities of the five most important ions arising from the collisionally activated dissociations of clenbuterol in the electrospray source are plotted in Fig. 2 versus the voltage difference between the glass capillary and the skimmer. The use of low ΔV values favours the production of the $[M + H]^+$ ion, while characteristic structural information can be obtained for ΔV values around 120 V. The first fragmentation observed consists in a loss of water (giving rise to m/z 259/261/263 ions) occurring from the lowest proton affinity site, followed by the consecutive loss of the *tert*-butyl group of the side-chain (m/z 203/205/207 ions). The subsequent fragmentations occur at higher values of ΔV, meaning that they require more energy. The isotopic profile of the m/z 168 ion indicates that an atom of chlorine has been lost in the formation of this fragment ion. The m/z value of this fragment is consistent with the elimination of a neutral *tert*-butylammonium chloride from the protonated molecular ion. However, this process (which has not been demonstrated) would imply the formation of a radical ion, which is unlikely. In a similar way, it can be deduced that the m/z 132 ion arises from the m/z 168 ion via the loss of HCl. From the above observations, an electrospray CAD-MS fragmentation pattern of clenbuterol is proposed as in Scheme 1.

Nevertheless, it appears clearly that a specific fragmentation can be produced by changing the

glass capillary voltage. Similar fragmentation pathways have been observed for other β-agonists [18], providing an interesting means of characterization for this class of compounds.

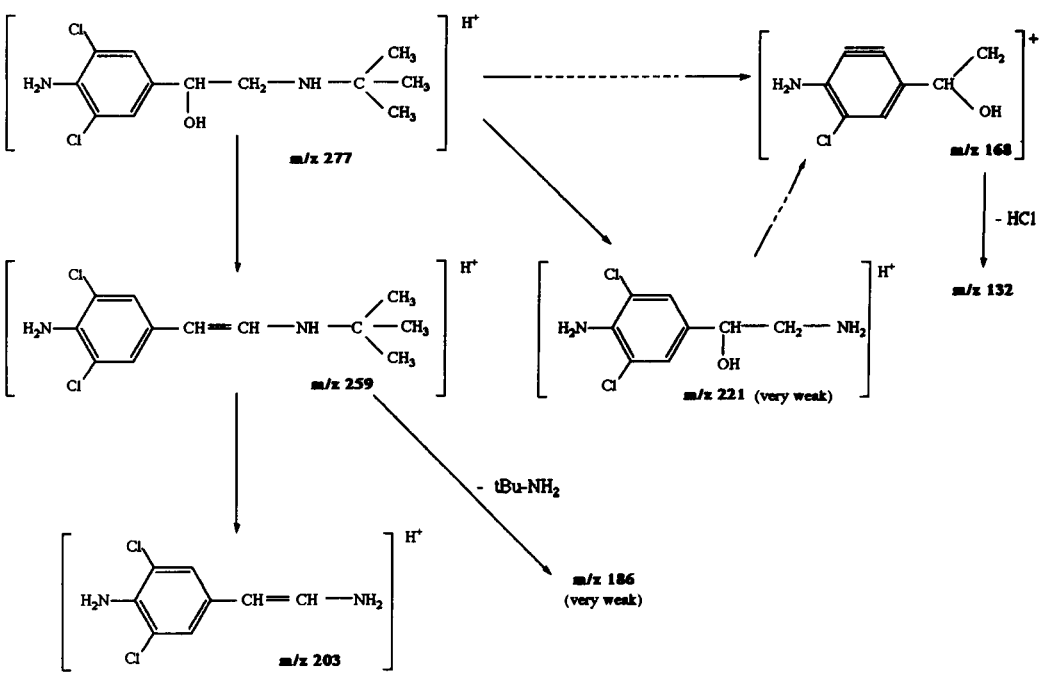
Influence of mobile phase composition

In LC, the retention of clenbuterol (and also other β-agonistic drugs) on reversed-phase systems is usually ensured by the use of ion-pairing reagents and buffered solutions [19,20], but the use of ion-pairing reagents constitutes a serious drawback for the direct LC–MS determination of such compounds. However, some volatile ion-pairing agents such as TFA have been proposed for the LC–TSP-MS determination of ionic compounds, together with volatile buffer solutions [21].

For the LC–TSP-MS determination of clenbuterol, Blanchflower and Kennedy [7] used an LC mobile phase consisting of only an ammonium acetate buffer. Under such conditions, the retention of clenbuterol on RP-18 stationary phases is poor, and the selectivity of the system was provided by the SIM acquisition mode. When dealing with full-scan analysis, the retention of clenbuterol should be greater in order to minimize the interferences due to overlapping peaks.

In order to assess the feasibility of direct LC–MS coupling for the analysis of clenbuterol with electrospray ionization, some assays were carried out on the effect of the LC mobile phase on the electrospray mass spectrometric response. A mobile phase system consisting of 50 mM ammonium acetate–8 mM TFA (pH 5.4)–15% (v/v) acetonitrile [21] was satisfactory for the retention of clenbuterol on an RP-18 stationary phase. Hence, the performance of such a solvent system as the electrospray ionization mobile phase was tested, in addition to simpler water–methanol or water–acetonitrile binary systems (for 10 ng μl^{-1} solutions).

Figure 3 shows the variation of the mass spectrometric response of clenbuterol as a function of the mobile phase composition. It appears that the response (expressed as the ion intensity corresponding to the main ions of the CAD spectrum of clenbuterol) is greatly disturbed when using a buffered system as the mobile phase. Indeed, the



Scheme 1. Fragmentation pathway proposed for the electrospray CAD of clenbuterol.

signal is decreased by a factor of about 7 when ammonium acetate buffer is added. In a recent study on organophosphorus pesticides using at-

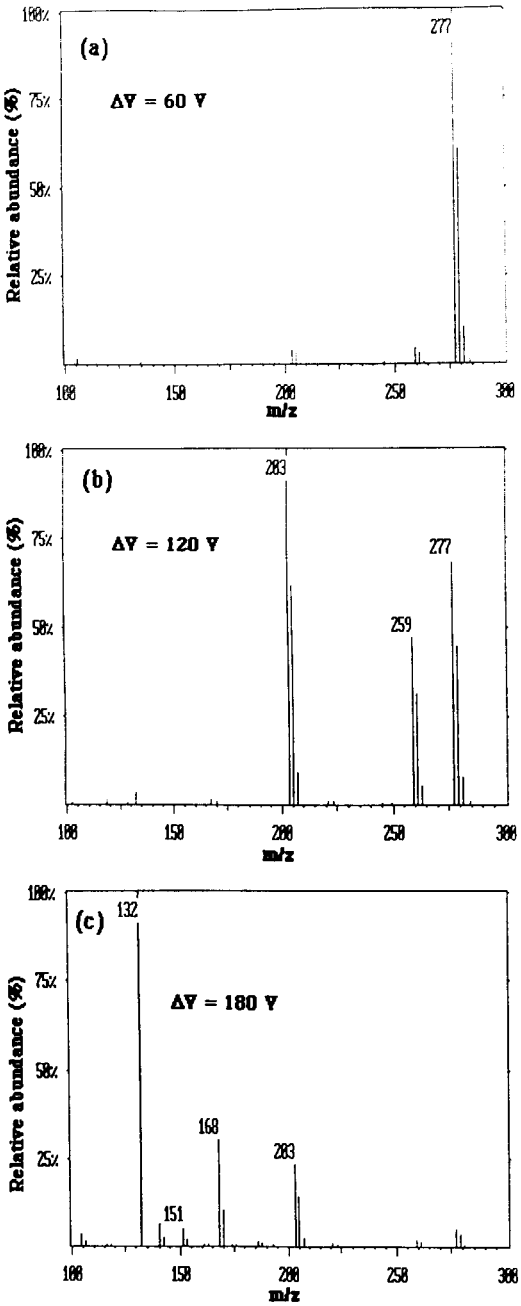


Fig. 1. Effect of change of the capillary transfer voltage on the spectra of clenbuterol.

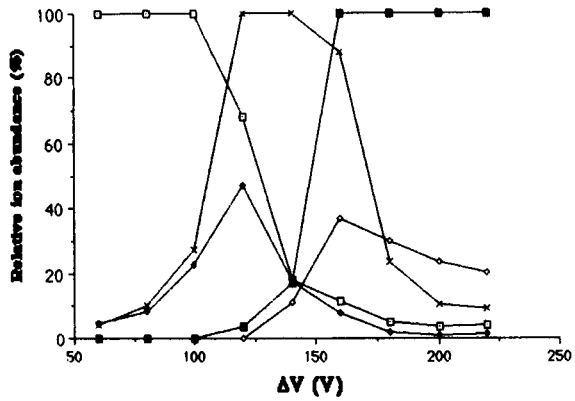


Fig. 2. Influence of ΔV on the relative intensity of the five most abundant ions of clenbuterol. □ = m/z 277; ♦ = m/z 259; × = m/z 203; ◇ = m/z 168; ■ = m/z 132.

mospheric pressure ionization, Kawasaki et al. [22] observed a similar trend on adding ammonium acetate to the mobile phase, and this feature has already been pointed out by other workers [23,24]. Although a more complete study should be made in order to provide more precise conclusions concerning the influence of the buffer (pH, concentration) on the mass spectrometric sensitivity, it can be concluded that the mobile phase composition is a critical parameter for the

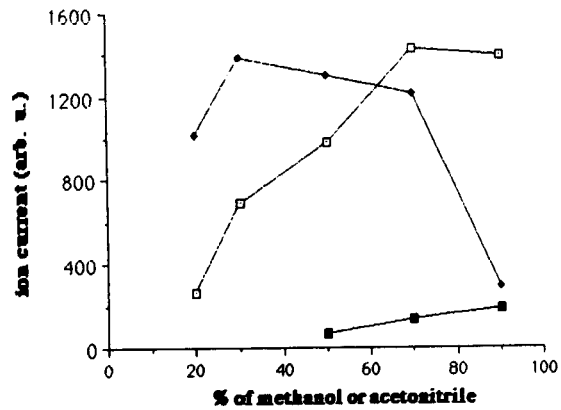


Fig. 3. Effect of the mobile phase composition on the mass spectrometric response. □ = Acetonitrile–water; ♦ = methanol–water; ■ = acetonitrile–ammonium acetate. The ordinate is the sum of the ion intensities corresponding to ions of m/z 207, 259, 261, 263, 277, 279 and 281 ($\Delta V = 120$ V).

determination of clenbuterol by electrospray ionization mass spectrometry (see Fig. 3).

Sensitivity

Full-scan analysis. The sensitivity of the system was assessed using standard solutions of clenbuterol, infused through the electrospray ionization source at a flow-rate of 1 μl min^{-1} for 40 s (i.e., five scans from m/z 100 to 300 at 8 s per scan). The voltage drop between the glass capillary metallized outlet and the skimmer was set to 120 V for this experiment, providing a good mass spectrometric characterization of clenbuterol (Fig. 1b). It should be noted that for this value of ΔV, no fragment ion is observed below m/z 200. However, scanning a mass range from m/z 100 to 300 was chosen because other β-agonistic agents (e.g., cimaterol or salbutamol) exhibit characteristic fragment ions between m/z 100 and 200 for $\Delta V = 120$ V [18]. In all instances, higher ΔV values can be used to enhance the structural information available for the mass spectrometric characterization.

Using the above conditions, Fig. 1b corresponds to the full-scan spectrum obtained with 66 ng of clenbuterol consumed. The standard clenbuterol solutions were then diluted until the signal-to-noise ratio for the ions considered became lower than 3. The absolute limit of detection in the full-scan mode was reached with a 1 ng μl^{-1} solution (i.e., 2.5 pmol of clenbuterol consumed for the acquisition). It should be noted that the spectrum obtained in this instance (not reported here) included some interfering peaks, probably due to the solvent contribution, which becomes more significant when the samples are very diluted. For the same consumption of the compound, these interfering peaks can be reduced by analysing a 2.5 ng μl^{-1} clenbuterol solution at a flow-rate of 0.5 μl min^{-1} [25].

Considering eventual direct LC–electrospray MS coupling for the determination of clenbuterol, the same level of sensitivity should be expected as the acquisition is achieved in 40 s, which is in agreement with the LC peak width.

SIM acquisition. An interesting feature of the electrospray mass spectrometry of clenbuterol is the ability to control its fragmentation by means of collisional activation. Thus, the selection of the ions to be monitored can be made merely by setting the ion acceleration using the capillary voltage.

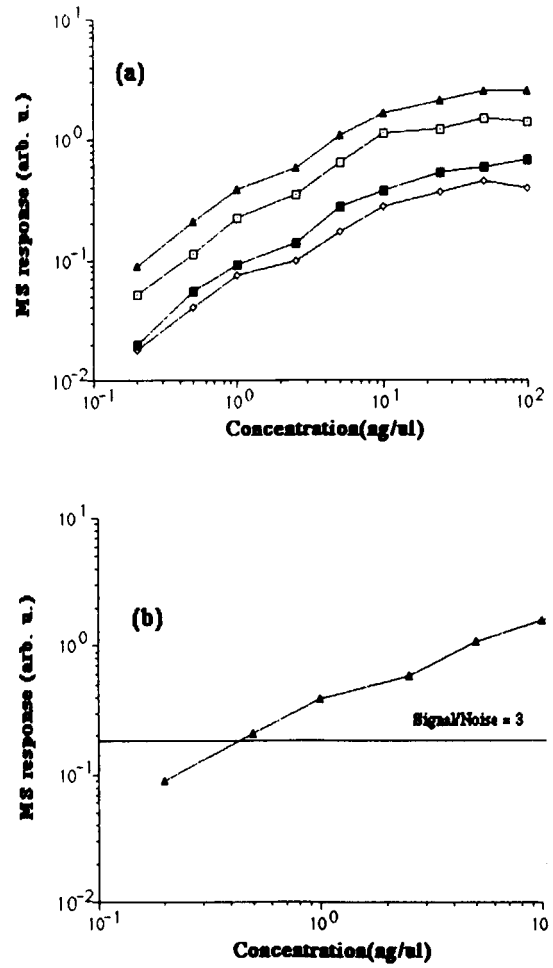
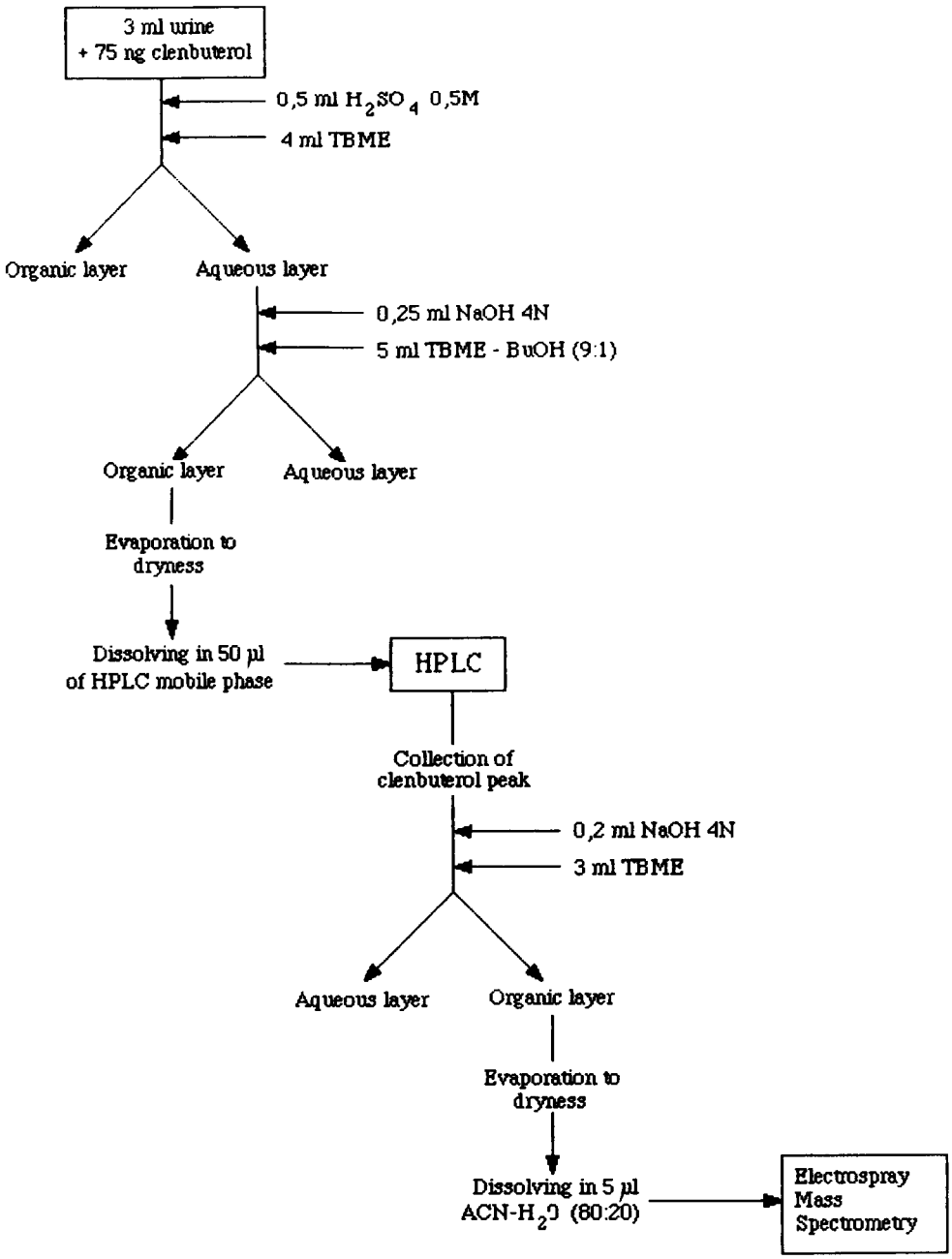


Fig. 4. Variation of the signal of clenbuterol with concentration: (a) between 0.2 and 100 ng μl^{-1} and (b) between 0.2 and 10 ng μl^{-1}. □ = m/z 203; ◇ = m/z 259; ■ = m/z 277; ▲ = total.

Using SIM conditions, the limit of detection of the system was tested by monitoring the m/z 277, 259 and 203 ions under the same mass spectrometric conditions as those used in the full-scan experiments. As no direct LC–MS coupling was available with this system, the study was carried out considering that the background noise corresponded to the ion current registered for an

acetonitrile–water (70 + 30, v/v) mixture containing no clenbuterol. Figure 4a shows the mass spectrometric response versus the sample concentration, obtained for the m/z 277, 259 and 203 ions. The total ion current corresponding to the sum of the ion currents considered is also shown.

Under these conditions, the limit of detection (signal-to-noise ratio = 3) with regard to the total



Scheme 2. Treatment of urine samples for the electrospray mass spectrometry of clenbuterol.

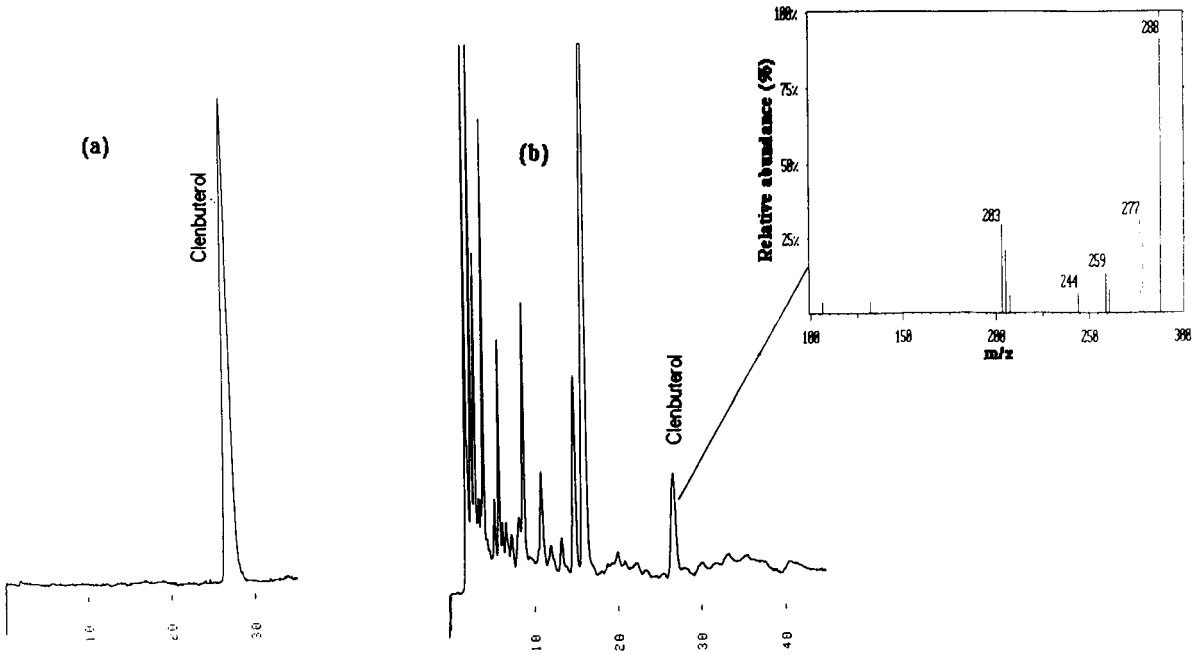


Fig. 5. Chromatograms of (a) clenbuterol standard and (b) an extract of bovine urine spiked with clenbuterol (50 ng ml^{-1}), with the electrospray mass spectrum associated with the peak of clenbuterol.

ion current (Fig. 4b) is obtained for a 0.5 ng μl^{-1} sample of clenbuterol infused in the mass spectrometric system at a flow-rate of 1 μl min^{-1} for 10 s. This corresponds to 80 pg (i.e., 300 fmol) of clenbuterol consumed.

Biomedical application: preliminary results

The application of electrospray ionization mass spectrometry was assessed on the measurement of clenbuterol in urine. Bovine urine (3 ml) was spiked with standard clenbuterol (25 ng ml^{-1}). The experimental procedure followed for the treatment of the urine sample is shown in Scheme 2. The chromatograms obtained for a standard solution of clenbuterol and for a spiked sample (50 ng ml^{-1}) of urine are shown in Fig. 5a and b, respectively.

As no direct LC–MS coupling was carried out in this work, the LC peak corresponding to clenbuterol had to be collected. After collection, the clenbuterol was extracted from the mobile phase with *tert*-butyl methyl ether in a basic medium, in order to eliminate the buffer, which is unsuitable for the electrospray ionization of clenbuterol (see Fig. 3). The ether extract was then evaporated to dryness and the residue was dissolved in 5 μl of acetonitrile–water (70:30, v/v) for mass spectrometric analysis. The electrospray mass spectrum recorded in the full-scan mode from 1 μl of such a sample is shown in Fig. 5b. In addition to the peaks of clenbuterol, some additional peaks appear (in particular at m/z 244 and 288) due to interfering compounds arising from the LC background present under the peak of clenbuterol. This (or these) interfering compound(s) probably possess the same basic character as clenbuterol, as they were not eliminated by the subsequent clean-up of the LC peak collected.

The minimum volume of the final extract was limited to 5 μl for practical reasons, mainly owing to the dead volume of the liquid introduction system. Nevertheless, only one fifth of this final extract was consumed in the mass spectrometric analysis and consequently the limit of detection

should reasonably be lowered merely by coupling a micro-LC system directly to the electrospray ionization source [26].

Conclusions

This study shows the potential of electrospray ionization mass spectrometry for the determination of clenbuterol. Fragmentations of the compound can be induced by means of collisional activation occurring in the high-pressure region (i.e., the skimmer region) of the electrospray source. Indeed, fragmentations can be readily controlled by changing the energy of the ions in this region. The structural information obtained in this way is very useful as characteristic cleavages are observed for clenbuterol and other β-agonists following the same fragmentation pathways [18].

The current sensitivity of the method allows the determination of clenbuterol in urine at the ppb level in the full-scan acquisition mode, without requiring any derivatization of the molecule. Under SIM conditions, the absolute limit of detection was reached at 300 fmol of standard clenbuterol. These preliminary results were obtained with an off-line system, and they should be improved by using direct LC–electrospray MS coupling. Its current limitations mainly lie in the LC mobile phase composition (and sample clean-up), which must be optimized in order to allow on-line LC–MS analysis without any loss of sensitivity.

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Determination of β-agonists in urine by an enzyme immunoassay based on the use of an anti-salbutamol antiserum

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Abstract

An enzyme immunoassay (EIA) method for β-agonists has been developed using an antiserum raised in rabbits by immunization against 3-*O*-salbutamol succinate coupled to bovine serum albumin. Horse radish peroxidase coupled to the same salbutamol derivative was selected as tracer. The dose of salbutamol which caused 50% binding inhibition was 16.8 pg per well and the limit of detection of the EIA directly performed on diluted urine was 0.14 ng ml^{-1} in urine (when the variability of blank values in samples from untreated animals was taken into account). Owing to the large cross reactivities of the antibodies with a number of β-agonists, the present assay is promising for the simultaneous determination of salbutamol, clenbuterol, mabuterol, terbutaline, cimaterol and probably also other β-agonists with a *tert*-butyl or isopropyl group at the extremity of the aliphatic side chain of the molecule.

Keywords: Enzymatic methods; Immunoassay; β-Agonists; Cattle; Clenbuterol; Urine;

It is well known that a number of compounds from the chemical class commonly referred to as β-adrenergic agonists are efficient enhancers of meat production. Following oral administration these substances have been shown to result in muscle hypertrophy, a reduction in carcass fat content and improved production efficiency in several food producing species [1,2]. The use of β-agonists as feed additives is, however, not yet permitted in the EEC since no compound from this class has, at this time, been listed in any of the Annexes to Directive 70/524/EEC. Nevertheless, recent reports of intoxication of consumers in Spain and France [3–6], caused by the presence of high concentrations of clenbuterol residues in edible tissues, confirm that these compounds are illegally used by unprincipled animal

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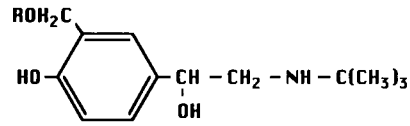
producers. The substances most often mentioned in connection with the illegal use of β-agonists as feed additives are clenbuterol and salbutamol. However, the enforcement agencies in a number of European countries pursing illegal producers, to protect the interests of both the agricultural community and consumer, have discovered the presence of not only a number of other β-agonists but also of new structural derivatives of some well-known compounds [7].

The availability of simple and sensitive analytical procedures is an important prerequisite for the combat of illegal use of β-agonists. In this context an enzyme immunoassay (EIA) method for the specific determination of clenbuterol in urine, blood and animal tissue has been developed in our laboratory [8]. In the present situation, however, where the possibility exists for the misuse of so many β-agonists, the application of EIA to the analysis of urine samples from animals suspected of being treated to allow the determination of several compounds in a single analysis would be of considerable advantage. In this paper we report on the development of such an assay. This EIA is based on the use of an anti-salbutamol antibody and a salbutamol succinate–enzyme conjugate as tracer which offers high cross reactivity with a number of benzyl ethanol derivatives.

MATERIAL AND METHODS

Reagents and equipment

Clenbuterol and cimaterol were generously provided by Boehringer (Ingelheim). Terbutaline, salbutamol, isoproterenol, pirbuterol, adrenaline, noradrenaline and fenoterol were obtained from Sigma (St. Louis, MO). Bovine serum albumin (BSA) was from Sigma, horse radish peroxidase (HRP) from Boehringer (Mannheim), *o*-phenylene diamine from Sigma, Tween-20 and thimerosal from Merck (Darmstadt) and Freund's complete adjuvant from Difco (Brussels). All other chemicals were of analytical-reagent grade or better and were used as obtained. Deionized water was purified on Milli-Q system (Waters). Buffers



salbutamol: R = H
succinyl derivative: R = – CO CH_2 – CH_2 – CO_2H

Fig. 1. Formula of salbutamol and its succinyl derivative.

were prepared as described by Degand et al. [8] in a previous publication.

All stock and standard solutions were stored at 4°C. ELISA plates were obtained from NUNC (NUNC-Immuno Plate Maxisorp F96-, Roskilde, Denmark).

Absorbance of plate wells was measured with a Multiscan MCC/340 microplate reader from Flow Laboratories.

Thin-layer chromatography (TLC) was performed on silica gel 60 HPTLC pre-coated plates (Merck) and eluted with the solvent mixture ethyl acetate–methanol–25% ammonium hydroxide (85 + 10 + 5, v/v/v). Spots were visualized after dipping in a solution of 2,7-dichlorofluoresceine (100 mg) in 100 ml ethanol.

Dialysis was performed in tubes of Spectra Por Membranes MWCO 2000 (Polylab, Antwerpen).

Immunogen preparation

(a) Preparation of salbutamol succinate. The salbutamol derivative (Fig. 1) was prepared as described by Beaulieu et al. [9] with minor modifications. Briefly, 80 mg of salbutamol free base (0.33 mmol) were dissolved in methanol (10 ml). The solvent was removed under vacuum using a rotatory evaporator (Büchi) and the oily residue obtained was redissolved in absolute ethanol (16 ml) using a magnetic stirrer. While stirring, 36 mg (0.36 mmol) of succinic anhydride were added. A cloudy white suspension appeared and the formation of the salbutamol succinate was monitored by thin layer chromatography (R_f salbutamol = 0.51; R_f succinyl derivative = 0.21). After 3 h at room temperature, the reaction appeared completed, the stirring was stopped and the suspension was centrifuged at 2750 *g* for 15 min. The solid phase was washed three times with absolute

ethanol and dried. The melting point of the cristals so obtained was 144°C which fits well with that reported by Beaulieu et al., viz. 143–144°C.

(b) Coupling of salbutamol succinate to BSA. The coupling was performed using the mixed anhydride method [10]. After dissolution of 40 mg of salbutamol succinate (0.12 mmol) in a mixture of dioxane–water–triethylamine (25 + 3 + 0.3, v/v/v), the solution was stirred during 30 min at room temperature. Isobutyl chloroformate (30 μl, 0.23 mmol) was added dropwise to a BSA solution (86 mg, 0.0012 mmol in 25 ml distilled water). The resultant solution was stirred overnight at 4°C and the mixture was then dialyzed against 3 l of 0.01 M phosphate buffer solution containing 0.15 M NaCl for 3 days with 6 changes of buffer. The trinitrobenzene sulphonic acid method of Habbeb [11] was used to estimate the free amino groups present in modified BSA. The dialyzed solution of immunogen was frozen at −20°C until use.

(c) Antibody preparation. Five rabbits (New Zealand breed) were immunized following the protocol of Vaitukaitis et al. [12] with an emulsion of 1 mg of salbutamol succinate conjugated to BSA in complete Freund's adjuvent. The animals received five injections with an interval of one month between each of them. After the third injection, a blood sample was taken from the ear vein on ten-day intervals to follow the apparition of the antibodies. This antiserum is now commercially available (Laboratoire d'Hormonologie Animale, Centre d'Economie Rurale, Marloie, Belgium).

Enzyme conjugate preparation

Salbutamol succinate used for the preparation of the immunogen served also as starting reagent for the synthesis of the salbutamol–enzyme conjugate by the mixed-anhydride procedure according to the modification by Dawson et al. [13] of the procedure of Erlanger et al. [10]. Briefly, 12.5 mg of salbutamol succinate (37.5 μmol) were suspended in dimethylformamide (2 ml) and triethylamine (30 μl). The reaction flask was placed in an ultrasonic bath (Branson 1200) during 15 min and two aliquots of distilled water (200 μl) were added. This step was essential for solubilizing the derivative. The solution containing the salbutamol succinate was cooled at −15°C in a cryostatic bath and mixed with 4 μl (37.5 μmol) of *N*-methylmorpholine. An amount of 37.5 μmol isobutyl chloroformate was then added and the mixture was stirred at −15°C during 5 min. This cooled mixture was added dropwise at −15°C to a horse radish peroxidase (HRP) solution in 0.01 M phosphate buffer, pH 7.5 (500 μl) to reach a molar ratio of 25 μmol derivative for 1 μmol HRP. After stirring for 1 h at −15°C and then for 2 h at 0°C, 10 mg of sodium bicarbonate were added and the mixture was dialyzed against 3 l of 0.01 M phosphate buffer. Unreacted material was then eliminated by gel filtration on Sephadex G-25.

Plate-coating procedure

The crude antiserum was aliquoted and stored at −20°C after dilution in 0.1 M phosphate-buffered saline (PBS)–glycerol mixture (serum–PBS–glycerol; 1 + 9 + 10, v/v/v) to give a final dilution of 1:20. A working solution of the antiserum was prepared by diluting 7 μl of the above stock solution (1:20) in 7 ml of carbonate coating buffer pH 9.6 (final dilution: 1:20 000). A volume of 100 μl of this antibody solution was added to the inner 60 wells of the polystyrene microtiter plate (the values for well to well variation are greatly reduced by omitting results from the perimeter wells). The plate was covered with a plastic film (TechGen, Zellik, Belgium) and stored at 4°C for 16 h or at 37°C for 2 h. The plate was washed four times with the washing solution to remove unbound antibodies.

Pretreatment of the urine samples

After centrifugation (2750 *g*, 15 min) to discard suspended material, urine samples were diluted ten times in 0.01 M PBS–0.1% BSA buffer (pH 7.4). A volume of 50 μl of diluted urine, corresponding to 5 μl of urine, was used in the assay.

Enzyme immunoassay

Volumes of 50 μl of standard solutions of salbutamol (range: 0.04–10 μg l^{-1}) or 10 times diluted urine were dispensed, in duplicate, into

individual wells of the microtiter plate already coated with the specific antibody. Then, 100 μl of the salbutamol–enzyme conjugate solution (8000-fold dilution in PBS–BSA buffer) were added. The plate was covered with a plate sealer and, after shaking, incubated overnight at 4°C. The wells were washed four times as described above in the coating procedure. Buffered enzyme substrate solution (150 μl) was then added to each well and the plate was incubated at room temperature (18–20°C) in the dark. After 30 min, the reaction was stopped by adding 50 μl of 6 M sulphuric acid to each well, in the same order and at the same rate as the substrate solution was added in order to keep the reaction time constant for all the samples. The plate was gently shaken before measuring absorbances at 492 nm with the microtiter plate reader.

Calculation

Results were calculated according to the method of Rodbard and Frazier [14] by interpolation from a calibration curve where the bound enzyme activity, expressed as the logit of the ratio (in percent) between absorbance increase per 30 min, at each concentration of salbutamol (B) and the bound activity in the absence of unlabeled salbutamol (B_0), was plotted versus log of salbutamol concentration. The Rodbard procedure of calculation was adapted (Logivet, Wodecq, Belgium) to enzyme immunoassay using EXCEL (Microsoft) on a Macintosh computer.

RESULTS

This competitive enzyme immunoassay is similar to those previously described for the analysis of artificial anabolics [15] and of clenbuterol [8] in urine: the same derivative of the hapten was used to prepare the immunogen by coupling to serum albumin and to horse radish peroxidase to synthesize the enzymatic tracer.

The specificity of the antiserum was established by EIA and is compared in Table 1 (column I) to that previously reported for the anti-clenbuterol antiserum (column II) [8]. Cross reactivities previously reported by other authors for anti-β-agonist antibodies are given for comparison in columns III–VI. It clearly appears that the anti-salbutamol antiserum (column I) is well suited for the simultaneous determination of at least clenbuterol, salbutamol, mabuterol, terbutaline and cimaterol.

TABLE 1

Comparison of the specificity of EIA of salbutamol to that previously described for other antibodies against β-agonists (Calculated according to Abraham [29].)

Compound	Antibody [a]					
	I	II	III	IV	V	VI
Salbutamol	100	2.7	3.3	100	12	11
Clenbuterol	115	100	100	75	100	100
Mabuterol	65					71
Terbutaline	31	5.6	2.8	7	13	10
Cimaterol	13	1.4	3.3		10	5.5
Isoproterenol	0.02	0.04		< 1		4
Pirbuterol	0.02	0.02				
Adrenaline	< 0.01	< 0.01		< 1	< 0.01	< 0.01
Noradrenaline	< 0.01	< 0.01			< 0.01	
Fenoterol	< 0.01	< 0.01	0.01	< 1		

[a] I, This work; II, [8]; III, [22]; IV, [18]; V, [19]; VI, [24].

The EIA calibration curve of the assay for salbutamol is shown in Fig. 2. The day to day variability of the standard curve was determined from 10 calibration curves. The mean slope ± standard deviation and coefficient of variation were: $-(0.82 \pm 0.01)$ (1%). These values are comparable to that determined for the clenbuterol-specific EIA: $-(0.67 \pm 0.02)$ (3%). The mid-point

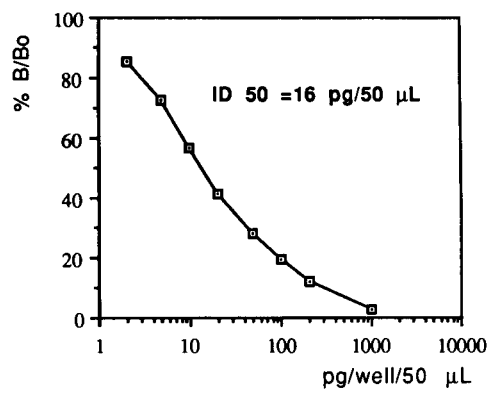


Fig. 2. Calibration curve for EIA of salbutamol. (ID_{50} = amount of unlabelled salbutamol required to inhibit 50% of the salbutamol–enzyme conjugate bound to the antiserum.)

TABLE 2

Accuracy and reproducibility of the assay in urine samples

Accuracy [a]			Reproducibility [b]		
Added ($\mu g\,l^{-1}$)	Measured ($\mu g\,l^{-1}$)	Recovery (%)	Concentration of salbutamol ($\mu g\,l^{-1}$)	Coefficient of variation (%)	
				Intra-assay ($n = 8$)	Inter-assay ($n = 8$)
1.0	1.1 ± 0.2	112	2.0	5.9	8.8
2.0	2.2 ± 0.3	113	5.0	7.2	9.7
5.0	4.8 ± 0.4	97			

[a] Salbutamol concentrations and recoveries were determined by enzyme immunoassay after addition of known amounts of salbutamol to a pool of urine samples from untreated veal calves. Values are means of eight determinations (blank values subtracted) with standard deviations. [b] Urine samples containing known concentrations of salbutamol.

of the calibration curve of the salbutamol EIA (intercept at 50% or ID_{50}) was 16.8 ± 2.2 pg (6.7%) ($n = 10$), similar to that of the specific clenbuterol EIA: 18.3 ± 1.4 pg (7.8%) [8].

When used to analyze urine samples, the limit of detection of this assay was estimated to be 0.14 $\mu g\ l^{-1}$ (mean determined concentration of 20 blank urine samples collected from untreated veal calves + 3 times standard deviation) and the limit of determination (mean of 20 blank urine samples + 6 times standard deviation) was 0.18 $\mu g\ l^{-1}$ (Commission Decision 89/610/EEC) [16].

The accuracy was estimated using urine samples enriched with known amounts of salbutamol (Table 2). The recoveries amounted to about 100% and the differences between the true value and the mean of the determinations are well below the limits −50% to +20% for real concentrations $\leq 1\ \mu g\ kg^{-1}$, recommended by the EEC Commission Decision (87/410) [17]. The intra- and inter-assay reproducibilities are also given in Table 2. They also fit with the recommendations of the EEC (for real concentrations $\leq 1\ \mu g\ kg^{-1}$: C.V. < 0.30). All these characteristics are comparable to those recorded for other immunoassays [8,15].

DISCUSSION

Owing to the suspected situation about the illegal use of β-agonists in animal husbandry [7], a rapid and simple method for the multicomponent analysis of residues of these drugs is needed. The present assay is promising for the simultaneous determination of salbutamol, clenbuterol, mabuterol, terbutaline, cimaterol and probably also other β-agonists such as cimbuterol (Aerts, 1991, personal communication). All these molecules contain a *tert*-butyl or isopropyl group at the right extremity of the structure (Fig. 1). The cross reactivity of our anti-salbutamol indicated that the state of substitution of the aromatic ring has little influence on the recognition of the β-agonist molecule by the antibodies.

Several immunoassays have been described for the detection of salbutamol simultaneously with other β-agonists [18–20]. Van Ginkel [21] and Schilt et al. [22] have used unspecific antisera for the determination of β-agonists by an analytical procedure involving a clean-up with immunoaffinity chromatography associated to GC–MS. In Table 1, we compare the reported cross reactivity of the antisera used by the authors of these works concerning either immunoassays or affinity chromatography. It is clear that our anti-salbutamol antiserum presented a larger specificity and higher cross reactivity values for several β-agonists suspected to be in circulation on the black market than the other antibodies of Table 1. The radioimmunoassay for albuterol (salbutamol) reported by Adam et al. [18] is based on a monoclonal anti-salbutamol antibody and tritiated salbutamol. According to the authors, it can be used for direct quantitation of salbutamol in horse urine without any clean-up or extraction step. However, their reported ID_{50} is 12 pmol ml^{-1} (2871.6 pg ml^{-1}) which is about nine times

higher than the corresponding value in our assay. This probably corresponds to a limit of detection that is one order of magnitude higher than that reported for our assay. The characteristics of the antisera described by van Ginkel [21] and by Schilt et al. [22] indicate that the affinities of these antibodies for β-agonists other than clenbuterol were too low for their application in a multianalyte enzyme immunoassay. Meyer et al. [19] described an enzyme immunoassay that can be applied to the multicomponent analysis of urine suspected to contain β-agonist residues. It involves a polyclonal antibody raised against clenbuterol diazo-BSA and carboxymethyl ether–biocytin as a label with the biotin–avidin amplification system (Meyer et al. [23]). The authors consider that this complex system, involving a bridge heterogeneity within the antigen and marker was required to obtain a good sensitivity (Meyer et al. [19]). This assay is manufactured by R-Biopharm (Darmstadt, Germany) as RIDASCREEN Clenbuterol; its cross reactivities are also given in Table 1. The association of LC to this immunoassay allows the differentiation of the different compounds (Meyer et al. [19]) and this LC–EIA method was used by these authors for the confirmation of positive samples detected during the screening using EIA alone. Their assay presents a good binding for clenbuterol, the other cross-reacting substances having about 10 times less affinity for salbutamol, cimaterol and terbutalin compared to clenbuterol. Furthermore, without clean-up, the blank values recorded for negative samples of urine were highly variable and could be as high as 1.9 ng. Consequently, these authors applied a pretreatment to the urine samples involving solid phase extraction and purification on C_{18} silica gel cartridges. This clean-up step takes about 1 h. Our system, when applied to salbutamol determination in urine required a simple 10-fold dilution of samples before performing EIA itself. Using this procedure we have observed low blank values for negative urine and the estimated limit of detection, calculated according to the EEC Commission Decision (89/610), was 0.14 ng ml^{-1}. In opposition to clenbuterol, which is present in urine in a free form, a part of salbutamol residues is present in urine in conjugated forms (mainly sulphate conjugates). Information about this subject is still scarce in literature [25–28]. The presence of conjugated forms of salbutamol in the sample does not impede its qualitative determination with our EIA with a good sensitivity, probably because the glucuronide or sulphate groups are present in the conjugated forms of salbutamol as ester derivatives of the CH_2OH group at position 3 of the aromatic ring. The derivatization of salbutamol in this position most probably does not inhibit antibody binding. However, the application of the EIA described in the present paper to the quantitative analysis of salbutamol will thus probably require a preliminary hydrolysis with Helix Pomatia juice.

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Spectrophotometric assay for β-lactam residues in kidney tissue

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Abstract

A total of 1617 samples has been analysed by a rapid screening technique for β-lactam antibiotics using reagents from a commercially available kit, adapted for use in tissues. Cross-reactivity data has been obtained and sensitivities compared using two signal systems.

Keywords: Spectrophotometry; β-Agonists; Antibiotics; Kidney tissue; β-Lactams;

β-Lactams are a group of compounds comprising both penicillins and cephalosporins. They are widely used in veterinary medicine, benzyl penicillin G (PenG) being the most notable. As this practice may result in a build-up of residues, Directive 86/469/EEC requires that monitoring of antimicrobial residues such as PenG takes place.

At the time of study, recommmended maximum limits were 100 ng g^{-1} for β-lactams. Current legislation laid down in January 1992 [The Animals, Meat and Meat Products (Examination for Residues and Maximum Residue Limits) Regulations, 1991, SI 1991, No. 2843, made under the Food Safety Act] now requires that named β-lactams such as PenG (50 ng g^{-1}), ampicillin (50 ng g^{-1}), amoxicillin (50 ng g^{-1}), oxacillin and cloxacillin (both 300 ng g^{-1}) may not be present in edible animal tissues in the parent drug form at levels exceeding those stated above.

A screening procedure has been developed and validated for β-lactam residues in kidney to an equivalent of 50 ng PenG per g tissue. The assay utilises inhibition of a commercially available bacterial D,D-serine carboxypeptidase (CPase) (see Experimental section) by a range of β-lactam antibiotics. Untreated tissue homogenates are incompatible with the CPase detection system, but a simple membrane filtration step has been shown to eliminate this interference and no other extraction or preparation step is required. This minimal sample treatment thus considerably reduces the cost of staff time.

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MATERIAL AND METHODS

Materials

Penzym™ kits were obtained from UCB-Bioproducts (Belgium), Centrifree™ columns from

Amicon (Stonehouse), and benzyl penicillin G from Glaxo (Greenford). ELISA plate strips (Nunc F8) and frames were obtained from Gibco (Paisley).

Reagents

Phosphate buffered saline (PBS) 0.01 M (pH 7.0) containing 0.9% sodium chloride. Spiked samples were made at 50 and 100 ng g^{-1} levels by adding penicillin G (PenG) standard solutions in water to kidney homogenate, previously analysed as negative for β-lactams and taken through the membrane filtration step.

Method

Kidney cortex (1 g) was homogenised with 9 ml PBS. A 1-ml aliquot was taken and spun in a plastic stoppered vial and centrifuged at 10000 g for 10 min. The supernatant was transferred in turn to a Centrifree column, capped and centrifuged at 2500 g for 15 min. The resultant filtrates from samples, spikes and negative controls were pipetted (50 μl) into microtitration plate wells. Substrate (100 μl) and reconstituted enzyme (50 μl) from the Penzym kit were added in turn, mixed briefly, covered and incubated in a water bath at 45°C for 30 min. The plate was then read on a microtitration plate reader at 510 nm. B/B_0 values for test and standard spike samples were then calculated.

Cross-reactivities were determined at 50 ng g^{-1} against PenG, normalised to 100% (Table 1). Two methods of signal generation were employed, using initially the Penzym kit reagents and latterly the Diaphorase system (Fig. 1).

(1) Penzym kit. The CPase hydrolyses the terminal D-alanine from the synthetic peptide of formula R-D-Ala-D-Ala. The D-alanine is oxidised by D-amino acid oxidase to liberate hydrogen peroxide. This in turn oxidises a colourless redox indicator to a pink-orange compound.

(2) 2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)-5-

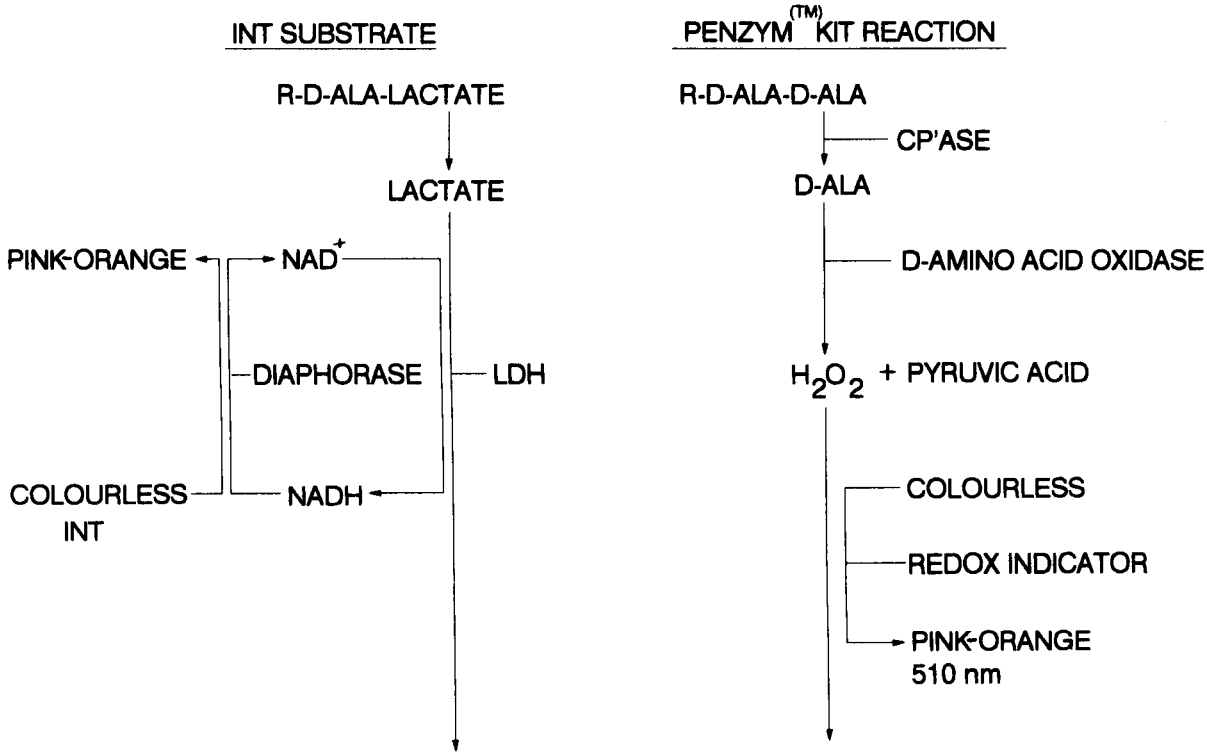

Fig. 1. Comparison of signal generation systems.

TABLE 1

β-Lactam cross reactivities (at 50 ng g^{-1}) (Penicillin = 100%)

Cephoxitin	217%	Oxacillin	6%
Penicillin V	182%	Nafcillin	5%
Cephalonium	143%	Cephuroxime	2.5%
Cephapirin	132%	Cephradine	2%
Cephalothin	70%	Cephalexin	2%
Ampicillin	70%	Cloxacillin	2%
Cephalosporin C	61%	Cephataxime	1%
Amoxycillin	40%	Methicillin	0.5%
Cephaloglycin	30%	Cefoperazone	0%
Phenethicillin	21%	Cefsulodin	0%

phenyltetrazolium chloride hydrate (INT) substrate. In this system, the CPase cleaves lactic acid from the synthetic peptide (Nα,NE-diacetyl-*l*-lysyl-*d*-alanyl-*d*-lactic acid). In turn, lactate dehydrogenase oxidises the lactate with the formation of NADH. Oxidised NAD^+ is regenerated by diaphorase with the simultaneous reduction of the colourless INT to produce the pink orange signal, which is proportional to CPase activity.

RESULTS

β-Lactam cross-reactivities for the D,D-serine CPase are given in Table 1 relative to PenG (50 ng g^{-1}) normalised to 100%. The two substrate systems employed (Fig. 1) were compared over a range of 12.5 to 800 ng g^{-1} of tissue (1.25–80 ng ml^{-1}) PenG (Fig. 2).

Probability curves were generated using the Penzym kit reagents for 50 and 100 ng g^{-1} PenG spiked tissue and 1617 negative samples, to demonstrate the performance of the assay in routine use (Fig. 3). The inter- and intra-assay variations were calculated on the data produced for 50

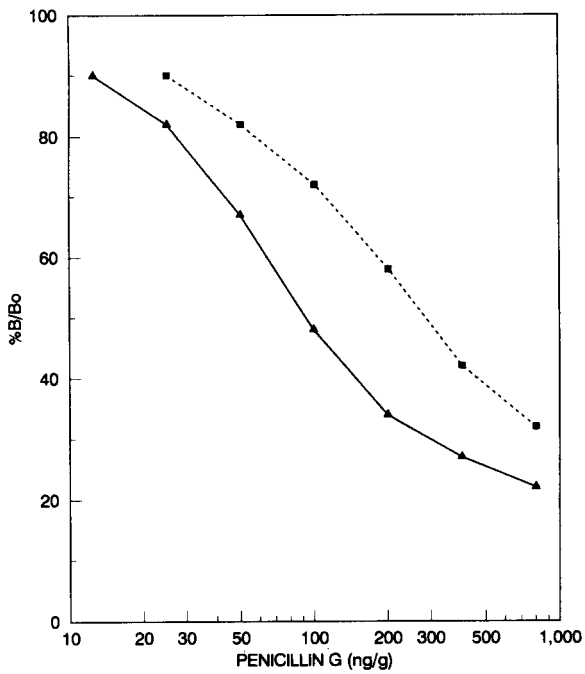

Fig. 2. Comparison of two CPase substrate systems: (▲) INT substrate; (■) Penzym hit substrate.

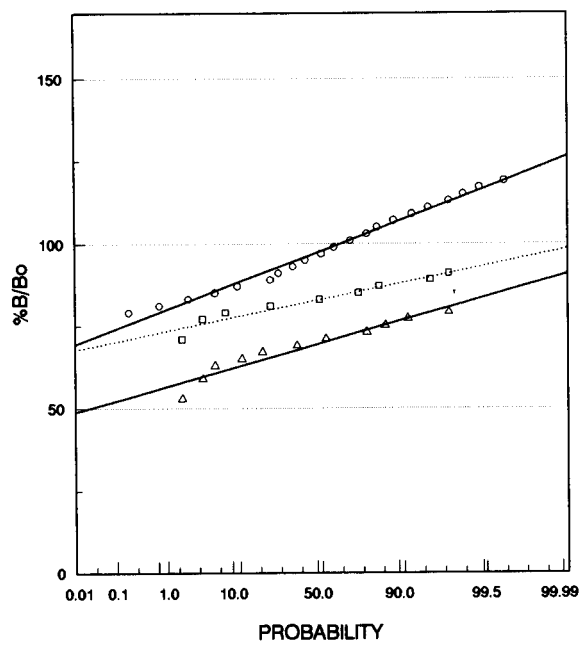

Fig. 3. Penicillin G: probability curves ($n = 1617$) using Penzyme reagents: (○) negative samples; samples spiked at: (□) 50 ng g^{-1}; (△) 100 ng g^{-1}. Horizontal lines are: (———) 5% probability of a false positive and 17% false negative at the 50 ng g^{-1} level; (- - - - -) b2) 1% false positive and 3.2% false negative at the 100 ng g^{-1} level.

TABLE 2

Inter/intra penicillin assay C.V.s ($n = 50$)

	Inter-assay (%)		Intra-assay (%)		
	50 ng g^{-1}	100 ng g^{-1}	50 ng g^{-1}	B_0	100 ng g^{-1}
mean	83.60	70.60			
S.D.	3.99	5.04			
C.V.	4.80	7.10			
mean C.V.			2.38	2.89	2.14
mean S.D.			1.30	1.85	1.00

separate batches of analyses and are shown in Table 2.

DISCUSSION

Microbiological tests have been used for antibiotic assays for a number of years [1,2]. Greater specificity has been obtained using isolated bacterial receptors to antimicrobial compounds, while still allowing detection of individual classes of antibiotics [3], especially for screening of milk samples.

The present system has been modified for use with tissue samples and to allow spectrophotometric measurement for quantitation. It was necessary, however, to filter the tissue homogenates to remove inherent factors causing elevation of the background signal. Centrifree micropartition columns (No. 4104) were found to be quick and easy to use for this purpose.

The assay was initially developed for use at 100 ng g^{-1}.

Figure 3 shows a plot of the % cumulative frequency of B/B_0 results obtained for a series of negative samples assayed directly and spiked at 50 and 100 ng g^{-1}. This is useful in obtaining an indication of the predictive performance of the assay with regards to the number of expected false positives and false negatives. At the 50 ng g^{-1} level for instance, a line drawn parallel to the axis at a B/B_0 of 89% gives a 5% probability of a false positive at this level and 17% false negative. At the 100 ng g^{-1} level a line drawn at 82% B/B_0 gives a probability of 1% false positive and 3.2% false negative. These figures show an acceptable level of false results (positive or negative) at 100 ng g^{-1}, but unacceptable at 50 ng g^{-1}.

The subsequent requirement for detection levels of 50 ng g^{-1} for PenG necessitated a more sensitive detection system with lower background to be developed. Use of the INT system effectively halved the detection limit (Fig. 2) and thus should allow a considerable decrease in the numbers of false positives and false negatives obtained.

The current method is sensitive to a wide range of β-lactams, both penicillins and cephalosporins (Table 2). The majority of commonly used β-lactams may therefore be detected using this system. Identification of individual compounds in any positive samples are carried out by either specific ELISAs [4] or liquid chromatographic assays. Alternative sources of CPase are currently under investigation.

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Multi-residue analysis for β-agonistic drugs in urine of meat-producing animals by gas chromatography–mass spectrometry

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Abstract

Two reliable gas chromatographic–mass spectrometric (GC–MS) procedures for the unequivocal determination of β-agonists in urine of meat-producing animals are described. The β-agonistic drugs are extracted using disposable mixed solid-phase extraction columns. The urine sample (10 ml) is buffered (pH 6.0) and applied to a Clean Screen DAU cartridge. The analytes are eluted with ethyl acetate containing 3% (v/v) of concentrated (32%) ammonia solution. The extracted analytes are derivatized to either their trimethylsilyl (TMS) or cyclic 2-(dimethyl)silamorpholine (DMS) derivatives and analysed by GC–MS under electron impact (EI) and positive-ion chemical ionization (PCI) conditions. Cyclic DMS derivatives proved to be useful for screening for or confirming the presence of clenbuterol analogues in the EI mode. If necessary, they could also be confirmed in the PCI mode using ammonia as reagent gas. With regard to TMS derivatives, their use provided a rapid and efficient method for screening for (EI) and confirming (PCI) the presence of at least thirteen β-agonists at the low ng ml^{-1} level.

Keywords: Gas chromatography; Mass spectrometry; β-Agonists; Meat; Pharmaceuticals

In addition to their well known therapeutic use as bronchodilators or tocolytics, some β-agonistic drugs, and especially those which have a higher affinity for β_2-adrenoreceptors, increase the protein to fat ratio in the carcasses of meat-producing animals [1,2], although this "repartitioning" mechanism is not fully understood [1,3]. Nevertheless, for ca. 4 years, β_2-agonists, particularly clenbuterol, which is the best known representative of this family of drugs and probably one of the most efficient, have been found to be illegally used to improve muscle mass production in farm animals during the fattening period, in spite of a European Community ban [4]. Further, over the past 2 years, several other analogues have appeared as "good candidates" for this kind of pharmacological manipulation. Among these, mabuterol and one of its methylated analogues (mapenterol) have been used in order to improve meat production efficiency in livestock [5]. These chlorotrifluoromethyl-substituted compounds may almost reach the anabolic effect of the corresponding dichloro-substituted β-mimetic clenbuterol [6]. Other β-agonists such as salbutamol, cimaterol and methylcimaterol (also called cimbuterol) have also been found to be illegally used as growth promoters, although their efficiency may be lower (Fig. 1). Moreover, some experiments have recently been reported on the potential effects on muscle accretion of compounds, such as ractopamine [7,8], which are structurally far from the β-agonists previously mentioned.

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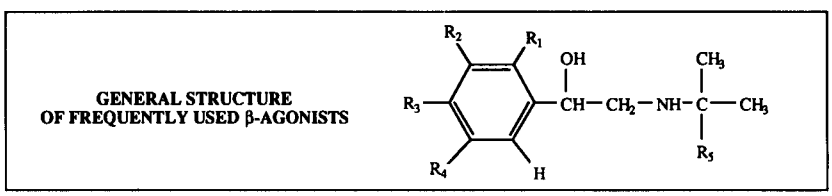
An accurate strategy of control is required, as the use of such compounds in meat-producing animals has great economic implications and also the toxic risk to consumers of meat from treated animals cannot be neglected, owing to the potent pharmacological activity of these molecules in humans. For this purpose, several methods have been developed, based mainly on enzyme immunoassay (EIA) [9–11] as a first screening step and gas chromatography–mass spectrometry (GC–MS) for confirmation: in the electron impact (EI) ionization mode [12–16] via different derivatization procedures, or with positive-ion [12,17,18] or negative-ion [19] chemical ionization (PCI or NCI). GC–MS–MS can also be used for confirmatory purposes [17].

Some laboratories have also used liquid chromatography (LC) with amperometric [20] or UV [21,22] detection for routine analysis. The additional use of postcolumn derivatization has been reported to increase the specificity and the sensitivity of the LC–UV methods [23]. However, the LC methods mentioned above were only suitable for clenbuterol, mabuterol or cimaterol determination. Further, as "new" β-agonists are frequently detected and identified in this field of growth promoters, it appears necessary to develop, as far as possible, multi-residue methods.

Unfortunately, owing to the varied chemical properties of these drugs, a multi-component extraction is difficult. First, a selective extraction technique based on diatomaceous earth-containing Chem Elut columns has been used [17,21–23]. This extraction technique has been used and adapted for the GC–MS of cyclic 2-(dimethyl) silamorpholine or dimethylsilylmethylene (DMS) derivatives in the EI mode [24]. It proved to be useful for detecting the presence of residues of clenbuterol, methylclenbuterol, mabuterol, methylmabuterol and tulobuterol.

Nevertheless, more polar β-agonists that contain additional hydroxy or alkylhydroxy groups on the aromatic ring could not be extracted in this way. The extraction of such β-agonists is complicated by the fact that these molecules are hardly extracted from water into organic solvents. Sev-



STUDIED β-AGONISTS

1 TULOBUTEROL	$R_1 = Cl / R_{2,3,4} = H / R_5 = CH_3$
2 MABUTEROL	$R_1 = H / R_2 = Cl / R_3 = NH_2 / R_4 = CF_3 / R_5 = CH_3$
3 METHYL-MABUTEROL	mabuterol analogue $R_5 = C_2H_5$
4 METAPROTERENOL	$R_{1,3,5} = H / R_{2,4} = OH$
5 TERBUTALINE	$R_{1,3} = H / R_{2,4} = OH / R_5 = CH_3$
6 CLENBUTEROL	$R_1 = H / R_{2,4} = Cl / R_3 = NH_2 / R_5 = CH_3$
7 SALBUTAMOL	$R_{1,4} = H / R_2 = CH_2OH / R_3 = OH / R_5 = CH_3$
8 CIMATEROL	$R_{1,4,5} = H / R_2 = CN / R_3 = NH_2$
9 METHYL-CLENBUTEROL	clenbuterol analogue $R_5 = C_2H_5$
10 METHYL-CIMATEROL	cimaterol analogue $R_5 = CH_3$
11 NA1141	clenbuterol analogue $R_5 = CH_2OH$
12 FENOTEROL	
13 RACTOPAMINE	

INTERNAL STANDARDS

14 METOPROLOL	β-blocking agent
15 ($^2H_6$) CLENBUTEROL	deuterated clenbuterol analogue (site of the deuterium label : tBu)
16 ($^2H_6$) SALBUTAMOL	deuterated salbutamol analogue (site of the deuterium label : tBu)

Fig. 1. General structure of frequently used β-agonists and list of the β-agonists and internal standards used in this work. Compound numbers correspond to those on the chromatograms.

eral extraction procedures, based mainly on ion-pair extraction using bis(2-ethylhexyl)phosphate (DEHP) [25], ion-exchange extraction [26] or solid-phase extraction (SPE) on octadecylsilica columns [27,28], and combinations of SPE and ion-pair extraction with DEHP [29], have been reported. However, these methods were carried out for toxicological experiments in humans, and they were proposed for the extraction of one or three β-agonists (i.e., terbutaline [25,26,27], bambuterol (a prodrug of terbutaline) [28], salbutamol [27,29] and fenoterol [27]).

To solve this problem of a multi-component extraction step, two main clean-up schemes can be adopted: immunoaffinity chromatography [5,12,30] and solid-phase extraction based on mixed-phase cartridges with both ion-exchange and lipophilic properties, depending on the pH conditions and the elution phase [14,17].

The object of this work, based on this latter extraction procedure, was to develop an improved method that provides multi-residue extraction for thirteen β-agonistic drugs of interest in the growth-promoting agent field. The extraction procedure described below provide in combination with GC–MS, a highly sensitive and selective multi-residue method. After a description of the extraction and derivatization procedures, the use of different derivatization and ionization techniques is discussed. When EI ionization alone is available, the use of cyclic DMS derivatives (Fig. 2), according to the method described by Dumasia and Houghton [14], can be useful for screening for or confirming the presence of clenbuterol-like compound residues. Moreover, these cyclic DMS derivatives can also be submitted for confirmation in the PCI mode. On the other hand, an improved and efficient method using TMS derivatives is described, both for screening for (EI mode) and for confirming (PCI mode) the presence of a broad range of β-agonistic drugs in urine samples at the low ng ml^{-1} level.

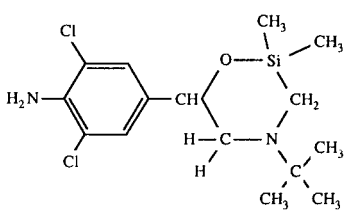


Fig. 2. Structure of cyclic 2-(dimethyl)silamorphine (DMS) derivative of clenbuterol.

EXPERIMENTAL

Standards

Clenbuterol hydrochloride was purchased from Interchim (Montluçon, France), salbutamol hemisulphate, terbutaline hemisulphate, metoprolol tartrate and metaproterenol hemisulphate from Sigma (St. Quentin, France). Cimaterol, methylclenbuterol, mabuterol, fenoterol hydrobromide and NA1141 were provided by Boehringer Ingelheim France (Reims, France), tulobuterol hydrochloride by Abbott (St. Remy sur Avre, France) and ractopamine hydrochloride by Lilly France–Elanco (St. Cloud, France). Methylcimaterol was synthesized (Chemistry Department, University of Rennes, France). Methylmabuterol was issued from a feed additive extract collected by LC (DGCCRF, Rennes, France); the concentration of the collected fraction was determined by comparison with a mabuterol standard solution. Hexadeuterated clenbuterol and salbutamol analogues were a generous gift from the State Institute for Quality Control of Agricultural Products (RIKILT–BFA, Wageningen, Netherlands). The stock solutions of these standard materials, prepared in methanol (1 mg ml^{-1}), were kept at −18°C.

Materials

All solvents and reagents were of analytical-reagent grade. Most of them and *Helix pomatia* juice (β-glucuronidase and arylsulphatase enzymes) were purchased from Merck (Nogent sur Marne, France). Chloromethyldimethylchlorosilane (CMDMCS) and toluene (GC grade) were supplied by Fluka (Mulhouse, France) and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) by Sigma. Solid-phase disposable extraction columns, Clean Screen DAU (CSDAU 506, 500 mg, 6 ml), made by Worldwide Monitoring (Horsham, USA), were supplied by Technicol (Stockport, UK).

Extraction and derivatization procedures

Enzymatic hydrolysis of conjugated metabolites. To a 10-ml sample of previously centrifuged urine (1700 *g*, 20 min) were added 4 ml of acetate buffer (0.2 M, pH 4.8) and the pH was adjusted to 4.8 if necessary. For the standard procedure, the internal standard(s) (4 ng ml^{-1}) were added at this stage. The urine sample was then treated with 50 μl of *Helix pomatia* juice and incubated overnight at 40°C.

Solid-phase extraction. The pH was adjusted to 6.0 by adding 4 ml of phosphate buffer (0.1 M, pH 6.0) and further adjusted if necessary. A Clean Screen DAU SPE column was preconditioned under a vacuum manifold by washing with successively 2 ml of methanol, 2 ml of purified water (> 14 MΩ cm) and 2 ml of phosphate buffer (0.1 M, pH 6.0). During this activation step, air must not reach the stationary phase. The sample was then applied to the SPE cartridge and drawn slowly through the column. Subsequently, 1 ml of 1.0 M acetic acid and 6 ml of methanol were passed through the column as a rinsing step. Finally, the analytes were eluted with 6 ml of ethyl acetate containing 3% (v/v) of concentrated (32%) ammonia solution.

The extract was evaporated to dryness under nitrogen (40°C) and the residue was, transferred into a screw-capped vial, using first 200 μl of methanol and then 100 μl of methanol. The methanol was then evaporated to dryness. For the study of recovery efficiency, metoprolol (6 ng ml^{-1}) was added at this stage as an external standard.

Derivatization. TMS derivatives were obtained by adding 50 μl of BSTFA to the dry residue. After vortex mixing, the sample was incubated at 75°C for 2 h.

For cyclic DMS derivatives, a CMDMCS mixture was prepared by adding 200 μl of diethylamine and 320 μl of CMDMCS to 4 ml of hexane. Owing to the hygroscopic character of the CMDMCS reagent, a white precipitate of ammonium chloride was formed. This mixture was vortex mixed and centrifuged at 2400 *g* for 15 min; 50 μl of the supernatant were added on the dry residue. After vortex mixing, the sample was incubated at 60°C for 40 min.

After cooling to room temperature, the reagents were removed under nitrogen (40°C). The dry TMS or cyclic DMS derivatized extract was dissolved in 25 μl of toluene.

Apparatus and GC–MS conditions

This experiment was carried out on two Hewlett-Packard GC–MS systems which consisted of two Model 5890 gas chromatographs and two Model 5971 low-resolution mass-selective detectors (MSDs). The two GC–MS systems were operated with an electron energy of 70 eV. One MSD was used in the EI mode and the other in the PCI mode. Each GC–MS system was interfaced to a Hewlett-Packard Model 7.0 Unix station. For GC, two identical OV-1 fused-silica capillary columns (30 m × 0.25 mm i.d.) (Ohio Valley; Interchrom, Montluçon, France), with a film thickness of 0.25 μm, were used with helium as the carrier gas at a flow-rate of 1 ml min^{-1}. The injector and transfer line temperatures were 260 and 275°C, respectively. The oven temperature was programmed as follows for the analysis of TMS derivatives: initial temperature, 70°C; initial hold, 2 min; first programming rate, 18°C min^{-1} up to 200°C; second programming rate, 5°C min^{-1} up to 245°C; then from 245 to 300°C at 25°C min^{-1}. The temperature programme used for the analysis of cyclic DMS derivatives was slightly different: the oven temperature was raised from 70 to 200°C at 18°C min^{-1}, then from 200 to 290°C at 10°C min^{-1} and finally from 290 to 300°C at 35°C min^{-1}. Under PCI conditions, methane, isobutane and ammonia were used as reagent gases. The gas was admitted via a gas-flow controller to an indicated analyser pressure of 1×10^{-4} Torr. A 2-μl aliquot was finally injected in the splitless mode (1-min delay) into the GC–MS system.

RESULTS AND DISCUSSION

Hydrolysis step and sample pretreatment

The hydrolysis step, which allows the deconjugation of glucuronide or sulphate ester forms of β-agonists, can be omitted for the specific determination of clenbuterol-like compounds (with no

hydroxylated substituents on the aromatic ring). On the other hand, this step was found to be essential for salbutamol. It must also be carried out for all β-agonistic drugs containing additional hydroxy or alkylhydroxy groups on the aromatic ring(s), although few data are available concerning the metabolites of β-agonistic drugs. Prolonged incubations, at a relatively high temperature, seem to be required.

The enzymatic hydrolysis step can be optionally followed by a methanol precipitation (with ca. 25 ml of methanol) under the acidic conditions required for enzyme activity. The sample is then treated in a rotary vaporizer (40°C) in order to evaporate the methanol phase and a small part of the aqueous phase. Subsequently, the remaining aqueous phase is transferred into a centrifugation tube and the glass-round-bottomed flask is rinsed with phosphate buffer (0.1 M, pH 6.0); the whole sample is then centrifuged (2400 *g*, 20 min). This procedure may be useful when there is need to concentrate urine samples containing very low levels of analytes, e.g., for confirmatory purposes. Moreover, it leads to a further preliminary clean-up, as a precipitate should appear after centrifugation. Consequently, this methanol precipitation step could be helpful for treating "dirty" urine samples from cattle. However, this procedure was not been used in the present experiment as calf urine samples were treated. Urine samples extracted directly on Clean Screen DAU columns have been found to be clean enough for GC–MS analysis. This observation fits well with previous results obtained with similar extraction procedures [14,17].

Extraction recovery study

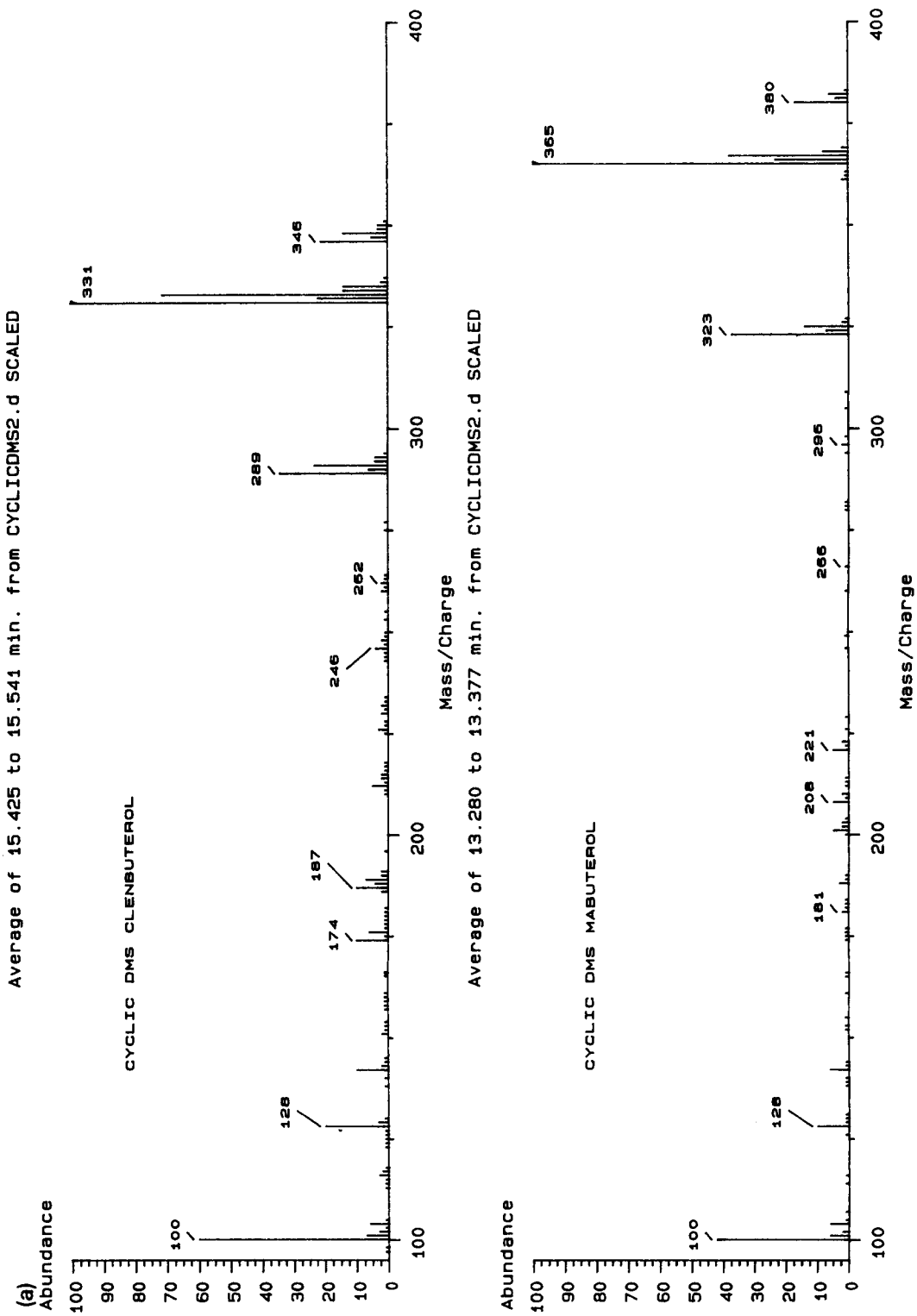
SPE columns such as Bond-Elut Certify or Clean Screen DAU cartridges contain a mixed phase with both ion-exchange and lipophilic properties, depending on the pH conditions and the elution phase. These copolymeric bonded-phase columns have recently been reported to provide an effective means for the co-extraction of β-agonistic drugs with various chemical properties. The extraction adapted in this study is a slightly mofidied form of the main procedure described previously [14,17,31]. The influence of the concentration of the concentrated ammonia solution in the elution phase was investigated. This experiment was carried out by GC–MS in the selected-ion monitoring mode. The recoveries were calculated by using metoprolol as external standard: the peak-area ratio of ions selected for

TABLE 1

Dependence of mean recoveries on the ammonia concentration in the elution phase using GC–MS results for 10-ml urine samples spiked at 5 ng ml^{-1}, with metoprolol as external standard (6 ng ml^{-1})

Compound	98 + 2 [a]		97 + 3 [a]		96 + 4 [a]	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
Tulobuterol	44	15.0	40	10.8	39	16.0
Mabuterol	83	9.1	73	6.8	59	9.6
Methylmabuterol	75	6.3	69	7.2	55	8.2
Metaproterenol	12	10.1	20	12.0	29	10.0
Terbutaline	25	5.6	36	9.2	40	12.5
Clenbuterol	69	8.0	68	5.5	62	6.4
Salbutamol	11	10.4	22	8.8	31	11.0
Cimaterol	37	3.5	46	3.4	41	15.4
Methylclenbuterol	64	8.7	60	3.5	56	9.5
Methylcimaterol	44	8.2	55	8.3	39	9.8
NA 1141	58	11.2	61	5.7	53	17.1
Fenoterol	52	14.9	59	9.9	48	11.0
Ractopamine	57	9.3	65	7.5	65	8.4

[a] Composition of ethyl acetate–concentrated (32%) ammonia elution phase (v/v); recovery (n = 10) and relative standard deviation (R.S.D. = 100 × S.D./mean).


(a)
Abundance
Average of 15.425 to 15.541 min. from CYCLICDMS2.d SCALED
CYCLIC DMS CLENBUTEROL
331
345
289
252
246
187
174
128
100
Mass/Charge
Average of 13.280 to 13.377 min. from CYCLICDMS2.d SCALED
CYCLIC DMS MABUTEROL
365
380
323
295
266
221
208
181
128
100
Mass/Charge

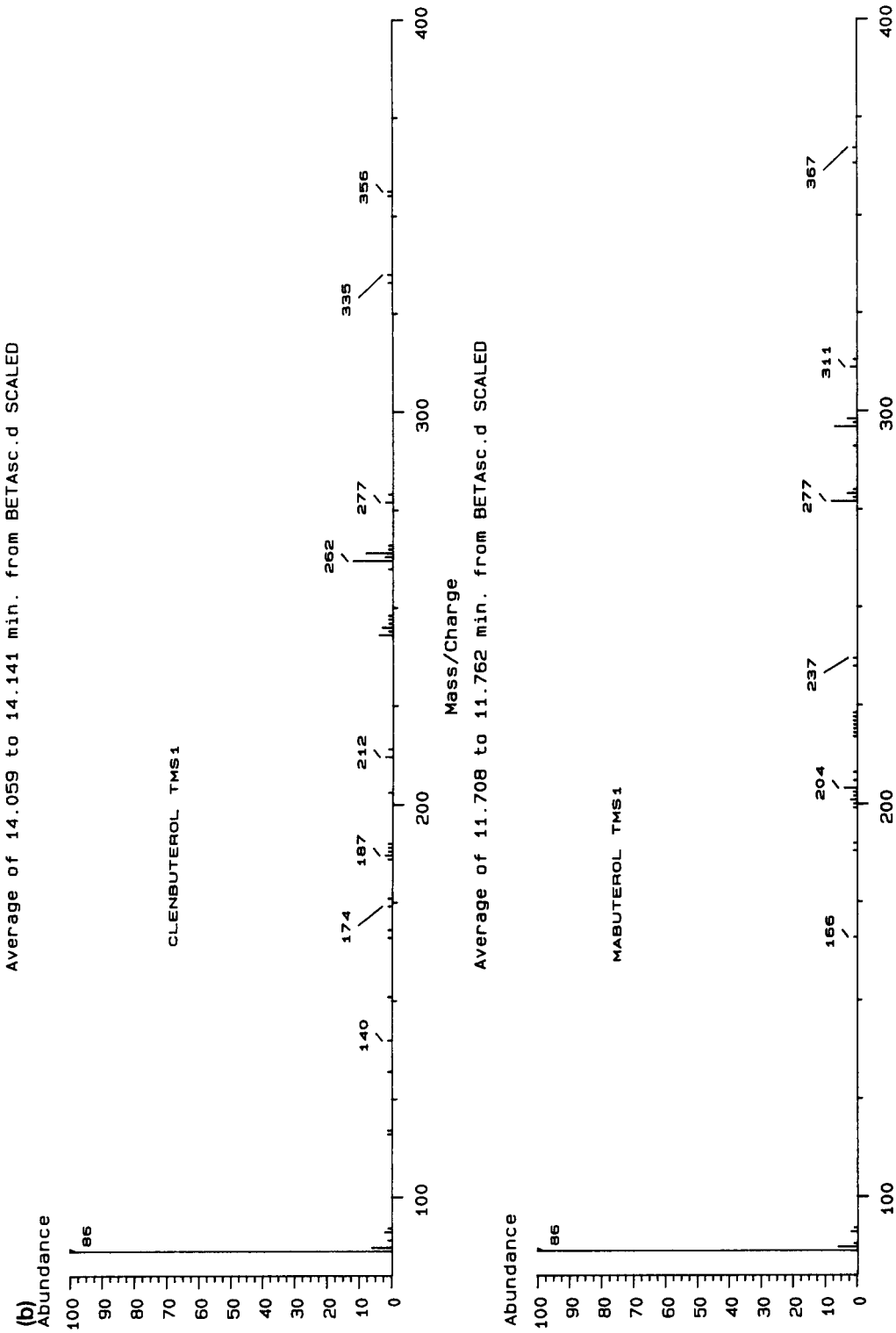


Fig. 3. Comparison between EI mass spectra of cyclic DMS derivatives of clenbuterol (a) and mabuterol and those of TMS derivatives of clenbuterol TMS1 and mabuterol TMS1 (b) (standard materials).

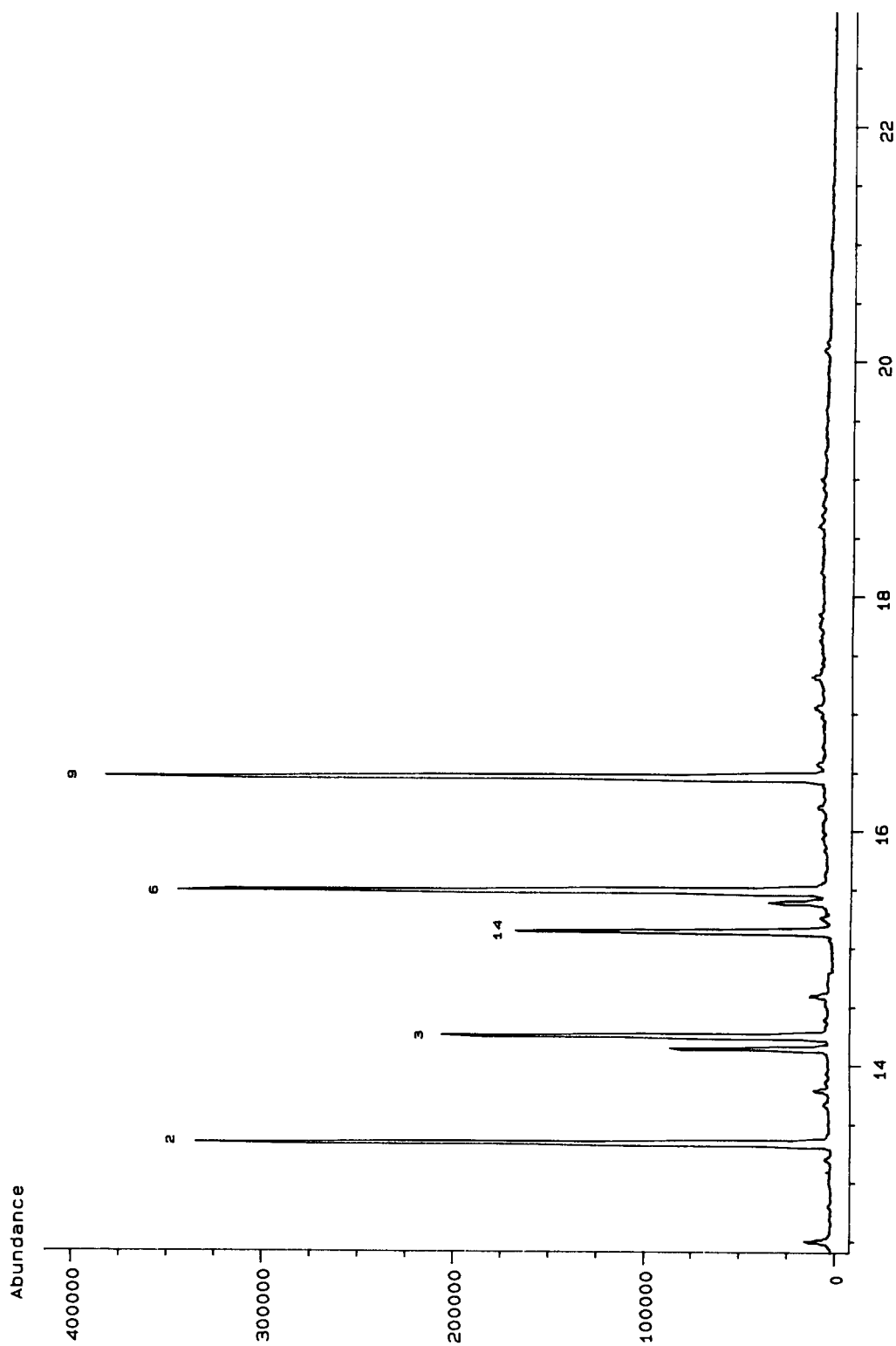


Fig. 4. Reconstructed EI chromatogram total ion current (TIC) obtained in the selected-ion monitoring mode of a cyclic DMS-derivatized urine extract: blank urine sample spiked at the 5 ng ml^{-1} level with mabuterol (2), methylmabuterol (3), clenbuterol (6) and methylclenbuterol (9). Metoprolol (14) was added as internal standard at 4 ng ml^{-1}. Abundance in arbitrary units.

each extracted analyte was compared with the corresponding ratio obtained for the standard material, on the assumption that the evaporated and derivatized standard material represented the 100% value. The results in Table 1 clearly reveal that the ammonia concentration has a great influence on the recoveries of the more polar β-agonists. A higher level of ammonia was shown to increase significantly the recoveries of compounds such as salbutamol, metaproterenol and terbutaline. It can also improve the recoveries of fenoterol, ractopamine, NA 1141 and cimaterol but the difference is not as great as with the salbutamol-like compounds. However, too high a concentration of ammonia (4%, v/v) seriously decreases the recoveries of most compounds (except salbutamol-like agonists), and especially of less polar β-agonists such as clenbuterol or clenbuterol-like agonists, e.g., methylclenbuterol, mabuterol and methylmabuterol. The relative standard deviations calculated for the recovery of each compound with this content of ammonia are also noticeably increased; this phenomenon could be partly explained by the fact that the high content of ammonia which must be driven off before the derivatization reagent is added could have adverse effects on the derivatization efficiency. Consequently, ethyl acetate–32% ammonia (97 + 3, v/v) was adopted as a good compromise for this study.

Cyclic DMS derivatization procedure

The cyclic DMS derivatives were prepared according to the method described by Dumasia and Houghton [14] for the GC–MS analysis of β-agonists, β-antagonists and their metabolites in horse urine. Hammar [32] first reported the use of such cyclized silyl derivatives for the GC–MS of β-hydroxyamines. In the above study, cyclic DMS derivatives were obtained by using 1,3-bis(chloromethyl)-1,1,3,3-tetramethyldisilazane (BCMTMDS)–CMDMCS (2 + 1, v/v). This latter procedure was also adapted in previous work [24]. However, the use of a catalyst such as BCMTMDS was found to make an evaporation step with the derivatization reagents impossible. Consequently, samples had to be analysed without further treatment. Unwanted reactions occurred and this derivatization procedure led to a higher background on the chromatograms compared with the results obtained with the method described here. Cyclic DMS derivatives proved to be useful for the analysis of clenbuterol-like compounds (mainly, clenbuterol, methylclenbuterol, mabuterol, methylmabuterol and tulobuterol). They were found to result in chromatographic peaks with acceptable resolution properties; in particular the peaks tailed less in comparison with the results obtained with other cyclized derivatives such as boronic acid derivatives [15,16]. These derivatives are stable for over 1 week in solution at 4°C. Unfortunately, this cyclic derivatization is not easily feasible for β-agonists containing additional hydroxy substituent(s), owing to the formation of side derivatives.

Screening and confirmatory analysis in the EI mode

The EI mass spectra obtained with cyclic DMS derivatives clearly show the interest of the latter in the CG–EI-MS analysis of clenbuterol-like compounds, especially when they are compared with the corresponding mass spectra obtained with TMS derivatives (Fig. 3). The use of cyclic DMS derivatives permits the detection of a large number of characteristic ions, of reasonable intensity. With EI ionization, they lead to a very specific fragmentation pattern, M^+, $[M-15]^+$, $[M-29]^+$, $[M-57]^+$, and provide far more informative data than the corresponding TMS derivatives. Consequently, these cyclic derivatives proved to be useful for both screening and confirming the presence of clenbuterol-like compound residues, particularly when no CI equipment (or other confirmation means) is available. Cyclic derivatized urine extracts recorded in the selected-ion monitoring mode lead to clean chromatograms (Fig. 4), as high-mass ions can be selected. As the sensitivity obtained is good and as the method is highly specific, unequivocal confirmation can be achieved at the 1 ng ml^{-1} level.

On the other hand, the EI mass spectra of TMS derivatives exhibit low abundances of the most specific ions. Indeed, these latter spectra show mainly a base peak formed by α-cleavage fragment ions. In most instances no or very poor

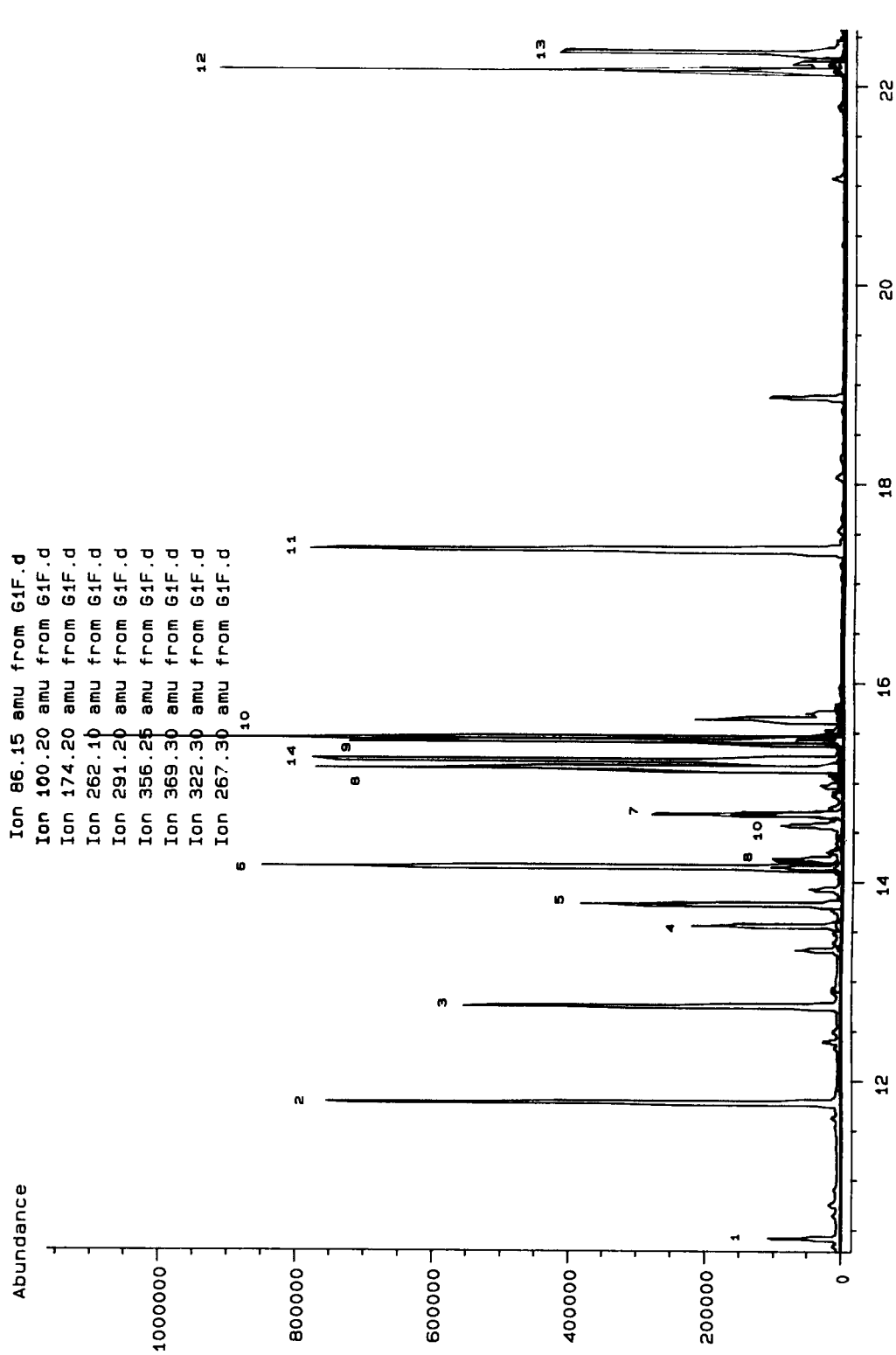


Fig. 5. EI–selected-ion current profiles of a TMS-derivatized urine extract obtained in the selected-ion monitoring mode: blank urine sample spiked at the 6 ng ml^{-1} level with all compounds except fenoterol and ractopamine (8 ng ml^{-1}); metoprolol was added as an internal standard at 4 ng ml^{-1}. From left to right on the chromatogram: tulobuterol TMS1 (m/z 86) (1), mabuterol TMS1 (86) (2), methylmabuterol TMS1 (100) (3), metaproterenol TMS3 (356) (4), terbutaline TMS3 (86, 356) (5), clenbuterol TMS1 (86, 262) (6), cimaterol TMS1 (72) (8), methylcimaterol TMS1 (86) (10), salbutamol TMS3 (86, 369) (7), cimaterol TMS2 (72, 291) (8), metoprolol TMS1 (72) (14), methylclenbuterol TMS1 (100, 262) (9), methylcimaterol TMS2 (86,291) (10), NA1141 TMS2 (174) (11), fenotereol TMS4 (322) (12), ractopamine TMS3 (267) (13). Abundance in arbitrary units.

molecular ions are detected. This phenomenon is further increased with clenbuterol or clenbuterol analogues. As it is well known that the selected-ion monitoring of low-mass ions leads to many interferences on chromatograms, the use of TMS derivatives for GC–EI-MS analysis is mainly useful for routine screening purposes. Owing to this lack of specificity, the decision limit (according to the EC quality criteria [33], four diagnostic ions, including whenever possible the molecular ion) is generally set very high, although the detection limit is very low (< 0.5 ng ml^{-1}) for most compounds. Consequently, this screening step can almost be considered as "improved chromatography". However, as the mass fragmentographic analysis of ions at m/z 86, 72, 100, 174, etc. (Fig. 5) in the EI mode offers high sensitivity, as few ions as possible should be recorded during this screening step to take advantage of the high sensitivity of the detection; monitoring of α,β-fragment ions appears to be sufficient.

Confirmatory analysis in the PCI mode

Cyclic DMS derivatives and TMS derivatives were analysed using the PCI mode, with methane, isobutane or ammonia as the reagent gas. Examples of full-scan data obtained with the different reagent gases for the two kinds of derivatives studied are shown in Fig. 6.

With regard to cyclic DMS derivatives, the mass spectra obtained do not offer a more specific fragmentation pattern than EI ionization, owing to the high stability of the cyclized derivative that is formed. The fragmentations observed when methane is used as the reagent gas are similar to those observed in the EI mode. However, PCI can nevertheless offer a good means of confirmation, especially when ammonia is used as the reagent gas. With this gas good protonated molecular ions can still be detected down to the 0.5 ng ml^{-1} level.

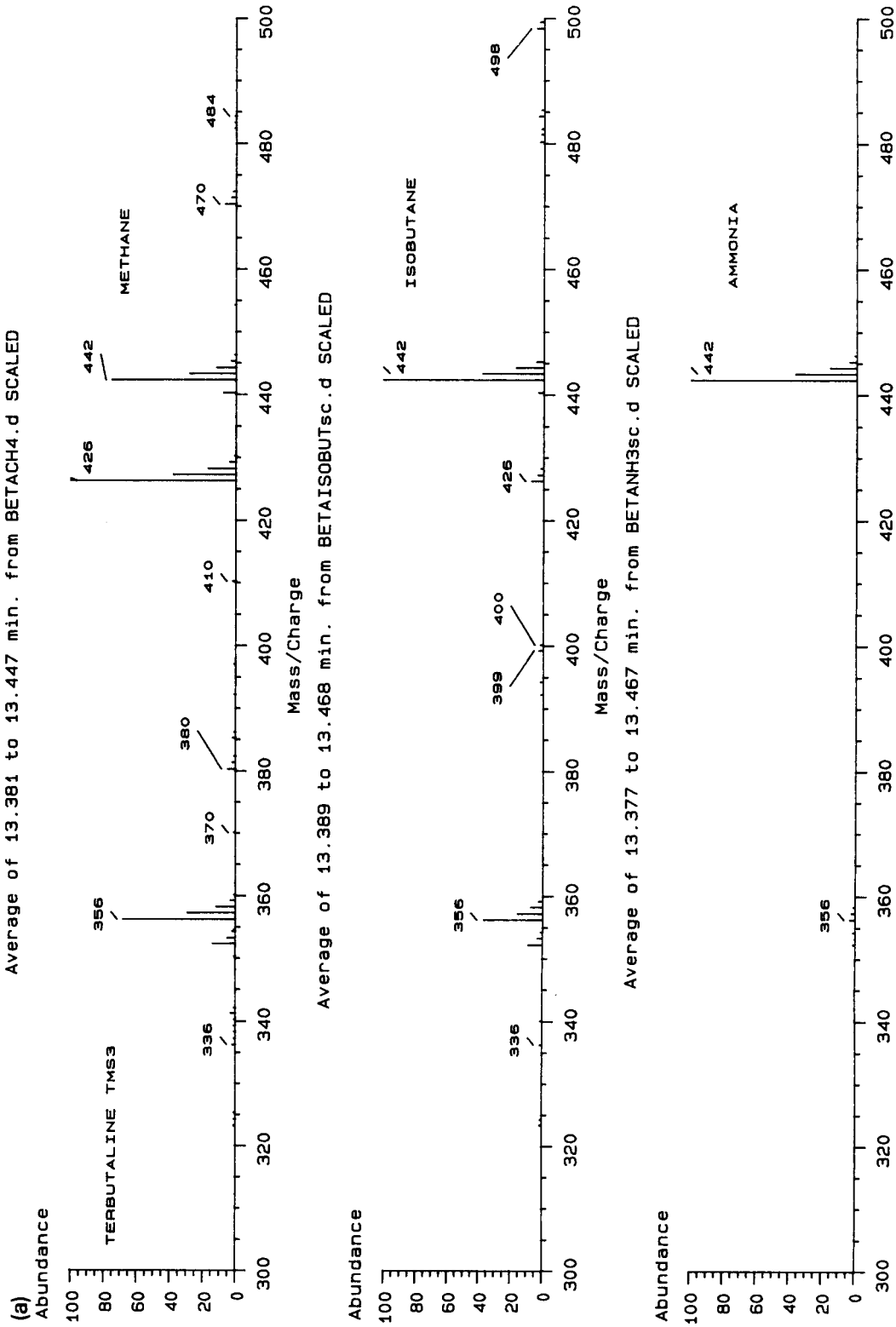
It is obvious that the use of PCI extensively increases the specificity of the low-resolution

TABLE 2

Reproducibility of the screening method (EI mode) using TMS-derivatized urine extracts and metoprolol as internal standard

Compound	Slope [a]		r^2	
	Mean value	R.S.D. (%)	Mean value	R.S.D. (%)
Tulobuterol	0.168	13.1	0.824	13.9
Mabuterol	0.462	12.7	0.960	2.0
Methylmabuterol	0.447	10.6	0.976	3.2
Metaproterenol	0.167	14.0	0.829	9.7
Terbutaline	0.262	10.1	0.874	2.1
Clenbuterol	0.646	7.6	0.997	0.2
Salbutamol	0.261	14.2	0.899	3.0
Cimaterol	0.514	9.4	0.990	0.9
Methylclenbuterol	0.626	6.8	0.997	0.1
Methylcimaterol	0.894	7.8	0.991	0.7
NA 1141	0.647	10.5	0.994	0.6
Fenoterol	0.264	11.7	0.968	3.9
Ractopamine	0.294	14.5	0.982	0.8

[a] Mean slopes ($n = 5$) and R.S.D.s of the calibration graphs obtained from urine samples spiked with 0.5, 1, 2, 4 and 6 ng ml^{-1} of each compound, except for tulobuterol, fenoterol and ractopamine (1, 2, 5, 8 and 10 ng · ml^{-1}); amount of internal standard (IS) added, 4 ng ml^{-1}. [b] Mean values of the squared correlation coefficients and corresponding R.S.D.s. For calibration graphs, whenever possible, the peak-area ratios of the most intense ions corresponding to the α, β-cleavage obtained under EI ionization were plotted against the concentration of the compounds, i.e. tulobuterol TMS1/internal standard (IS), (m/z 86)/(m/z 72); mabuterol TMS1/IS, 86/72; methylmabuterol TMS1/IS, 100/72; metaproterenol TMS3/IS, 356/72; terbutaline TMS3/IS, 356/72; clenbuterol TMS1/IS, 86/72; salbutamol TMS3/IS, 369/72; cimaterol TMS2/IS, 291/72; methylcimaterol TMS2/IS, 86/72; NA1141 TMS2/IS, 174/72; fenoterol TMS4/IS, 322/72; ractopamine TMS3/IS, 250/72.


(a)
Abundance
Average of 13.381 to 13.447 min. from BETACH4.d SCALED
TERBUTALINE TMS3
METHANE
Mass/Charge
Average of 13.389 to 13.468 min. from BETAISOBUTsc.d SCALED
ISOBUTANE
Average of 13.377 to 13.467 min. from BETANH3sc.d SCALED
AMMONIA

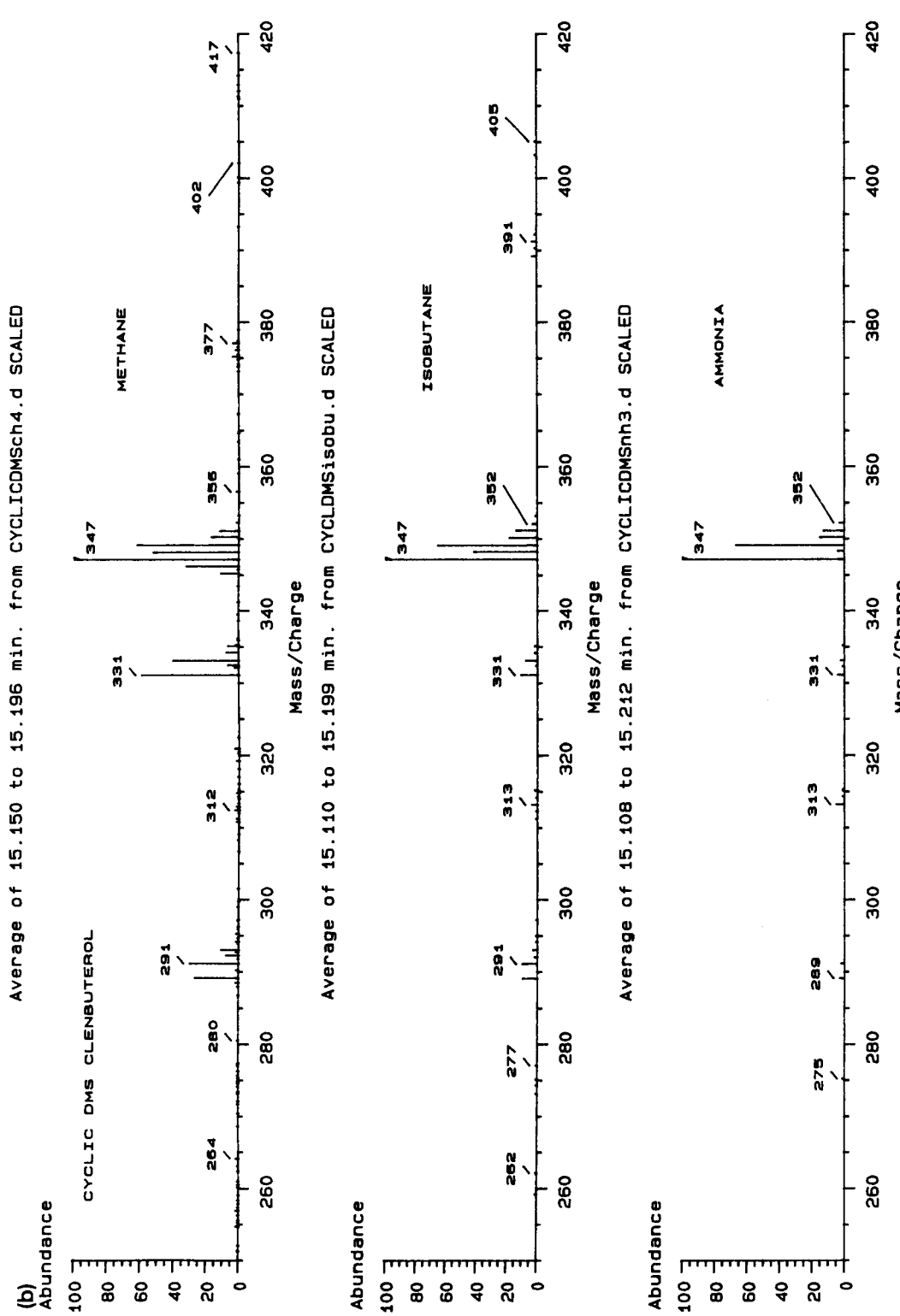


Fig. 6. PCI mass spectra of TMS derivative of terbutaline TMS3 (a) and those of cyclic DMS derivative of clenbuterol (b), using methane (top), isobutane (middle) and ammonia (bottom) as the reagent gas (standard materials).

GC–MS (GC–LRMS) of β-agonistic drug TMS derivatives. Whatever reagent gas is used, good protonated molecular ions are detected. Methane provides a very specific fragmentation pattern owing to fragment ions at $[M-15]^+$ and $[M-TMSO]^+$. With isobutane the specificity is lower; apart from protonated molecular ions, only $[M-TMSO]^+$ ions are detected, and with ammonia only protonated molecular ions are obtained. On the other hand, when focusing on protonated molecular ions and when compared with methane, ammonia gives a 6–12 times higher and isobutane a 1.5–4 times higher sensitivity, depending on the analyte.

■ *Quantification purposes*

Routine screening method via TMS derivatives under EI conditions. An experiment was carried out to test the within-laboratory reproducibility of the routine screening method described previously (Table 2). This reproducibility is reflected by the relative standard deviations of the mean slopes of the calibration graphs. The calibration graphs were established by linear regression analysis. As the intercepts were very close to zero, this was taken into account in calculating the calibration graph equations. This study revealed that the reproducibility of the method is sufficient as none of the relative standard deviations that were obtained was over 15%. For this study, metoprolol was used as an internal standard. The results in Table 2 show that metoprolol is well adapted for the analysis of clenbuterol-like compounds but is not so suitable for more polar β-agonists. Nevertheless, the aim of a routine screening method is not precise quantification.

Confirmatory method via TMS derivatives under PCI conditions. The repeatability and accuracy of the confirmatory method using methane as the reagent gas were evaluated for clenbuterol and salbutamol (Table 3). The applicability of hexadeuterated salbutamol and clenbuterol analogues instead of metoprolol for reliable quantification can be clearly seen. Consequently, deuterated analogues improve the accuracy and repeatability of analysis, especially in the CI mode. In contrast to the EI mode (many interferences at m/z 92), the use of $[M+H]^+$ or $[M-CH_3]^+$ ions in the selected-ion monitoring mode leads to no restriction on the site of the deuterium label in the internal standard.

In conclusion, the use of a rapid SPE step with mixed-phase disposable columns followed by TMS derivatization leads to an easy, rapid, inexpensive and reliable method for screening for and confirming the presence of several β-agonists by GC–MS. The first screening step of the TMS derivatives with EI ionization should nevertheless be followed by a confirmatory step under "soft" PCI conditions, which provide good protonated molecular ions. The choice of the reagent gas is

TABLE 3

Repeatability and accuracy of the PCI (methane) confirmatory method using metoprolol and deuterated analogues as internal standards

Compounds	Calibration graph [a]	r^2 [b]	Amount found [c] (mean ± S.D.) (ng ml^{-1})	R.S.D. [d] (%)	Δ [e]
Clenbuterol/metoprolol (349/340)	$0.616x + 0.0137$	0.990	1.93 ± 0.030	1.6	−0.07
Clenbuterol/[$^2H_6$]clenbuterol (349/355)	$1.280x - 0.0082$	0.999	1.98 ± 0.015	0.8	−0.02
Salbutamol/metoprolol (440/340)	$0.192x + 0.0018$	0.898	2.16 ± 0.070	3.3	+0.16
Salbutamol/[$^2H_6$]salbutamol (440/446)	$1.250x + 0.0155$	0.998	2.03 ± 0.022	1.1	+0.03

[a] Equations of the calibration graphs obtained from urine samples spiked with 0.5, 1, 2, 4 and 6 ng ml^{-1} of clenbuterol and salbutamol; amount of internal standard added, 4 ng ml^{-1}. For calibration graphs, peak-area ratios of the ions with the indicated m/z values were plotted against clenbuterol and salbutamol concentrations. [b] Squared correlation coefficients obtained for the four calibration graphs. [c] Five urine samples spiked with 2 ng ml^{-1} (= true value) of clenbuterol and salbutamol (+internal standard, 4 ng ml^{-1}) were then tested on the four calibration graphs, the mean amounts ($n = 5$) of which were found are indicated (±S.D.) [d] R.S.D.s of the mean amounts found. [e] Accuracy: Δ = mean amount found − true value.

highly dependent on the nature of the analyte and the sensitivity level that is needed. Ammonia can be used when low detection levels (< 0.5 ng ml^{-1}) are required; unfortunately, only protonated molecular ions can be detected in that case. Otherwise, in spite of the lower sensitivity that it provides, methane is to be preferred, whenever possible, to isobutane or ammonia, owing to the specific fragmentation pattern obtained, which allows an unequivocal confirmation at 1 ng ml^{-1} for most compounds.

With regard to cyclic DMS derivatives and in addition to the fact that they proved to be useful for the EI-MS of clenbuterol-like compounds, they could also be confirmed in the PCI mode using ammonia as the reagent gas. Further, PCI adds information to the data obtained with EI ionization when one is attempting to elucidate the structure of a "new" β-agonist. Cyclic DMS derivatives appear to be helpful for this kind of experiment. Moreover, cyclic boronic acid derivatives may also be used for this purpose [15,16,34].

Over the past 3 years, a broad range of analytical means, including GC–EI-LRMS and GC–PCI-LRMS, have been developed for detecting β-agonistic drugs in biological samples and feedstuffs. Therefore, the reliable analysis of β-agonists may be achieved, in most instances, when several methods are carried out in combination. Other techniques such as NCI-LRMS, LRMS–MS or high-resolution MS should further increase the sensitivity and/or specificity of β-agonistic drug detection and identification.

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Determination of clenbuterol in bovine urine by enzyme immunoassay following concentration and clean-up by immunoaffinity chromatography

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Abstract

A method for the determination of clenbuterol in bovine urine is described, which involves clean-up and concentration of the sample by immunoaffinity chromatography, followed by microtitre plate enzyme immunoassay of the extract. Both the chromatographic procedure and the assay use ovine antiserum raised against clenbuterol. Enzyme-labelled clenbuterol was prepared by conjugation of a clenbuterol metabolite [4-amino-3,5-dichloro-α-(2-hydroxy-1,1-dimethyl)ethylamino methylbenzyl alcohol] with alkaline phosphatase using a diazotization procedure. The procedure provided good recovery and linearity of response, and an acceptable limit of determination of 10 pg ml^{-1}. The coefficient of variation of the assay ranged from 28% at 20 pg ml^{-1}, to 4.5% at 0.5 ng ml^{-1}. The procedure proved sufficiently robust to enable heavily pigmented samples to be analysed without undue non-specific effects.

Keywords: Enzymatic methods; Immunoassay; β-Agonists Bovine urine; Clenbuterol; Immunoaffinity; Urine

The β_2-adrenoreceptor agonist, clenbuterol, is licensed in the UK for the use in the relief of respiratory disorders and as a tocolytic. Potential health hazards to consumers associated with illegal use of clenbuterol as a repartitioning agent have given rise to much international concern, and incidents of food poisoning, in which clenbuterol was implicated, have been reported recently in both Spain [1] and France [2]. The ability to detect low concentrations of residues in samples taken from live animals would do much to help combat this problem. Of the samples which may be readily taken from the live animal, urine is perhaps the most appropriate: urine is the main route of excretion and it has been found that concentrations in urine are far higher than in blood following drug withdrawal [3].

To enable effective monitoring of clenbuterol use by urine analysis, it is appropriate to establish the urinary concentration likely to be achieved when the drug is used legitimately at the therapeutic dose. It may be useful to take account of the maximum residue limit (MRL) set for the liver in the UK (0.5 ng g^{-1}), and how this relates to urinary concentration, to suggest an appropriate target detection limit for a urine assay. Published data indicate that during withdrawal, concentrations of clenbuterol in urine are appreciably lower than in liver. It is not possible to establish a generalised relationship between urinary and liver clenbuterol concentrations from these data [3], however, since urinary clenbuterol concentrations were not normalised to take account

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of the extent to which the urine was concentrated in vivo. For the present study, it was considered appropriate to develop an assay with a limit of determination of at least 0.05 ng ml^{-1}, one tenth of the UK MRL for liver.

A direct enzyme immunoassay (EIA) method for urinary clenbuterol has been reported previously [4]; however the limit of detection of the procedure (0.2–0.5 ng ml^{-1}) is insufficient for the present application. Concentration of sample prior to EIA, achieved by employing solid-phase extraction with C_{18}-silica, has enabled appreciable improvement in assay sensitivity to be attained [5,6], but the accuracy of analyses of highly pigmented samples may be affected by interference from co-extractives. This may limit the applicability of such procedures. The objective of the present study was to investigate the use of immunoaffinity adsorption chromatography to lower the limit of detection of the assay and to reduce the effects of co-extractives on the EIA, thereby enabling reliable quantitation of clenbuterol in urine down to 50 pg ml^{-1}. An alkaline phosphatase derivative of the clenbuterol metabolite 4-amino-3,5-dichloro-α-(2-hydroxy-1,1-dimethyl)ethylamino methylbenzyl alcohol was used in the EIA.

EXPERIMENTAL

Materials

Reagents. Clenbuterol hydrochloride, tritium-labelled clenbuterol, and metabolite NA1141 were obtained from Boehringer (Ingelheim); sodium azide, and ethanolamine from Sigma (Poole); gelatin from Difco (Detroit, MI); sodium hydrogencarbonate from Fisons (Loughborough); CNBr-activated Sepharose 4B from Pharmacia LKB (Uppsala); Ultima Gold XR liquid scintillation cocktail from Packard (Merident, CT). Sodium chloride, sodium dihydrogenorthophosphate dihydrate, disodium hydrogenorthophosphate dihydrate, sodium acetate trihydrate, tris(hydroxymethyl)methylamine (Tris), hydrochloric acid, methanol, glycine, and ammonium sulphate were from BDH (Poole), and were all of AnalaR grade. Sheep anti-clenbuterol-ovalbumin serum (SC248/5) was raised at the Central Veterinary Laboratory, as described previously [5].

Buffers. Distilled, deionised water was used to prepare all aqueous solutions. Phosphate buffers (0.1 M; pH 7.0) were prepared with sodium dihydrogenorthophsophate dihydrate and disodium hydrogenorthophosphate dihydrate, with sodium chloride (0.9%, w/v), sodium azide (0.2%, w/v for PAS buffer), and gelatin (0.1%, w/v for PAS-gelatin buffer) added. Ethanolamine buffer (1 M) was prepared at pH 9.0; carbonate–saline buffer (pH 8.3) contained sodium hydrogencarbonate (0.1 M) and sodium chloride (0.5 M). Acetate–saline buffer (pH 4.0) contained sodium acetate (0.1 M) and sodium chloride (0.5 M). Tris–saline buffer (pH 8.0) contained Tris (0.1 M) and sodium chloride (0.5 M). Methanolic glycine buffer was prepared on the day of use by diluting glycine buffer (pH 2.0; 0.1 M) 20-fold in methanol, and readjusting the pH to 2.0 with 10 M HCl.

Preparation of γ-globulin fraction of anti-clenbuterol serum

The γ-globulin fraction of antiserum bleed number SC248/5 was prepared by precipitation using 33% saturated ammonium sulphate solution (final concentration), according to the method of Garvey et al. [7].

Preparation of immunoaffinity gel

The immunoaffinity gel was prepared by coupling excess anti-clenbuterol γ-globulin with CNBr-activated Sepharose 4B, essentially following the manufacturer's instructions. The activated Sepharose (15 g) was swollen in 1 mM HCl (55 ml), then washed with 1 mM HCl (3 l), gently dried under reduced pressure and resuspended in carbonate–saline buffer (75 ml). Anti-clenbuterol γ-globulin (≡ 1 ml serum) was added to the suspended gel and mixed by rotation overnight at 4°C. The suspension was washed with buffer and dried under reduced pressure. Excess binding sites were blocked by addition of ethanolamine buffer (100 ml). The gel was then washed three times alternately with sodium acetate–saline buffer and Tris–saline buffer, and stored in PAS buffer at 4°C until required.

Immunoaffinity columns were prepared by transferring gel suspension containing 0.5 g dry gel equivalent into 25 ml 'Bond Elut' reservoirs (Analytichem, Harbor City, CA).

Extraction procedure

A trace quantity of ^{3}H-clenbuterol (6.7 Bq; 0.002 ng ml^{-1} urine) was added to each urine sample to enable recovery of clenbuterol and column performance to be assessed. PAS storage buffer was removed from the column by application of gentle pressure, 10 ml of sample was added, and mixed slowly for 10 min by mechanical rotary inversion. The extracted urine was then eluted through the gel, which was then washed with 3 × 10 ml water. Excess water was removed under gentle pressure after each wash. Methanol–glycine buffer (10 ml) was then added, and mixed by inversion for 20 min at ambient temperature (15–20°C). The column eluate was subsequently collected, dried under nitrogen at 40°C, and the extract redissolved in PAS-gelatin buffer (500 μl). Aliquots of the original urine sample (250 μl), the column washings (250 μl) and the final extract (50 μl) were taken for analysis by liquid scintillation spectroscopy (LSS): this enabled procedural losses to be monitored and quantitated.

Between each use, the columns were washed with water (10 ml), Tris–NaCl buffer (2 × 20 ml), acetate–NaCl buffer (2 × 20 ml), leaving to soak each time for 10 min, then finally washed with PAS buffer (10 ml) before storing in PAS buffer (20 ml).

Urine samples

Control urine was prepared by pooling urine samples from four untreated lactating cows. This was filtered through a Whatman No. 1 filter paper, and stored frozen (−20°C) in aliquots of 50 ml until required.

Urine samples ($n = 10$) were obtained from untreated animals following natural evacuation of the bladder; these were routinely collected as part of the UK National Surveillance Scheme for residues in meat. The samples were selected on the basis of visual appearance, varying from pale yellow to opaque dark brown. A high proportion were highly pigmented in order to represent samples which could, with inadequate clean-up, give rise to high blank values in EIA.

Enzyme immunoassay

The EIA employed alkaline phosphatase-labelled clenbuterol metabolite and was performed as described by Sauer et al. [5]. Standards were prepared in PAS–gelatin buffer in the range 0–10.0 ng clenbuterol per ml and added to wells in quadruplicate (20 μl per well). Sample extracts and quality control samples (20 μl per well) were added in duplicate. End-point absorbance values for standards and samples were expressed as a percentage of the mean absorbance value in the absence of competing clenbuterol (B/B_0). Results were calculated from the standard calibration curve either by manual interpolation or using a microcomputer following application of a four-parameter logistic curve fit [9].

RESULTS

Binding capacity of the immunoaffinity gel

The binding capacity of the gel was determined, by titration of clenbuterol (10 ng ml^{-1} in water) against the immunoaffinity gel, to be 260 ng clenbuterol per g dry gel equivalent (130 ng per column). Immunoaffinity columns were reused at least ten times during the period of this study. Assessment of the column washings by LSS throughout this period provided no evidence of diminished binding capacity.

Analytical recovery

Analytical recovery of ^{3}H-clenbuterol from fortified samples, as assessed by LSS was found to be 79.7 ± 3.53% (mean ± S.D.; $n = 90$); the recovery was independent of the clenbuterol concentration in the range 0.002–0.5 ng ml^{-1} (Student's T-test; $t = 0.23$; $P = 0.82$). For all analyses, concentrations determined by EIA were corrected for mean recovery as determined by LSS.

Assay sensitivity

The limit of determination was assessed by extracting control urine spiked at 0, 0.01 and 0.02

ng clenbuterol per ml; mean concentrations (n = 4) determined by EIA were < 0.005, 0.01 (C.V. = 6%) and 0.018 (C.V. = 8%) ng ml^{-1}, respectively.

The limit of detection of the EIA was determined from the standard deviation of the B/B_0 values given by negative urine extracts throughout the study. The mean B/B_0 was 101.6 ± 5.66% (mean ± S.D.; n = 30), giving a limit of detection at the 99% confidence limit (mean + 3S.D.) of 0.2 ng clenbuterol per ml extract (0.01 ng ml^{-1} urine). Of the control samples analysed (n = 30), three gave positive results, in the range 0.014–0.021 ng ml^{-1}; the samples were all highly pigmented (opaque, dark brown).

Precision and accuracy

The precision and accuracy of the assay were determined by extracting control urine spiked with clenbuterol at 0, 0.02, 0.1 and 0.5 ng ml^{-1}. Concentrations determined by EIA, precision and accuracy are shown in Table 1.

Linearity of assay response

Control urine: The linearity of response was determined for duplicate extracts of control urine spiked with clenbuterol in the range 0.01–0.2 ng ml^{-1}, and for duplicate extracts of a degraded, pigmented urine sample spiked in the same range. Analysis of covariance showed that the responses were not different, and so the concentrations determined by EIA for each matrix were pooled. The linear plot gave the equation $y = 1.103x + 0.001$, $r = 0.9981$, $n = 20$, where y is the concentration determined and x is the concentration added, both in ng ml^{-1}.

Clenbuterol-incurred sample

Further linearity assessment was carried out on a sample from an animal in which clenbuterol (intravenous administration; 1 μg kg^{-1} body weight) had been incurred. Serial two-fold dilutions of this sample were prepared (control urine was used as the diluent), extracted, then analysed by EIA. Linear regression analysis of concentration found (y axis, ng ml^{-1}) against sample dilution (x axis) gave $y = 0.844x + 0.048$; $r = 0.9840$, $n = 13$. From this line, the concentration in the original sample was determined to be 0.892 ng ml^{-1}.

Selected urine samples

To assess the possible influence of interfering co-extractives in urine, ten negative urine samples of varying degree of pigmentation were blind-spiked with clenbuterol in the range 0 to 0.2 ng ml^{-1}, extracted and analysed by EIA. Linear regression analysis of concentration found (y axis) against concentration expected (x axis), both in ng ml^{-1}, gave $y = 0.827x + 0.013$; $r = 0.9915$. One clenbuterol negative sample gave rise to a response in the assay (0.021 ng ml^{-1}).

Conclusions

The current study shows that immunoaffinity clean-up and concentration enable urinary clenbuterol to be determined by EIA down to 0.01 ng ml^{-1}, providing an assay with a linear response up to 0.5 ng ml^{-1}. The procedure proved well suited to use in routine screening procedures where a high degree of sensitivity may be required.

The method compares favourable with procedures described elsewhere which used liquid–liquid extraction [8,10] or C_{18}-silica extraction [5,6]: limits of detection of about 0.26 ng ml^{-1} [8], 0.15 ng ml^{-1} [10] and 0.01 ng ml^{-1} [5,6] were reported. The latter procedures (5,6), although highly sensitive, were reported to give rise to high

TABLE 1

Precision and accuracy of urinary clenbuterol concentrations determined by EIA following immunoaffinity extraction

Concentration added (ng ml^{-1})	n	Concentration determined (ng ml^{-1})	Mean recovery (%) (by LSS)	Corrected concentration (ng ml^{-1})	Accuracy (% of true value)	C.V. (%) (precision)
0.02	11	0.018	80.7	0.022	110	28
0.1	9	0.082	81.4	0.101	101	32
0.5	12	0.379	79.5	0.477	95	4.5

blank values when urine samples had become degraded.

The extraction procedure described here proved reliable in removing the majority of interfering co-extractives and thus allowed accurate determination across the working range of the assay. Three control samples, selected because they contained high concentrations of brown pigmentation, gave rise to positive response in the assay. The values determined, however, were near the limit of determination of the assay and an order of magnitude lower than blank values determined in similar samples, which were analysed by EIA following C_{18}-silica extraction [6]. Sample deterioration of this type which results in such high concentrations of pigmentation, should only occur rarely, however, when collection or storage procedures are highly inappropriate. Confirmation, or otherwise of low concentrations of clenbuterol found in "false-positive" control samples in the present study should be possible using gas chromatography–mass spectrometry (GC–MS) procedures such as described by Girault and Fourtillan [11]: however GC–MS facilities available in this laboratory were insufficiently sensitive for this purpose.

The low limit of determination achieved by the current immunoaffinity extraction procedure should, by virtue of low residual co-extractive concentrations, enable clenbuterol to be determined for longer periods after drug withdrawal than is possible using other extraction methods. Where fresh samples which have a relatively low degree of pigmentation are analysed, the possibility exists of concentrating extracts further, with a concomitant further improvement in assay sensitivity.

The immunoaffinity procedure helps to resolve many of the problems associated with the determination of low concentrations of clenbuterol in urine and, by providing effective sample clean-up, circumvents complications which may arise when urine samples are subjected to either prolonged, or inappropriate storage conditions. It is envisaged that sample extracts prepared by this method would be well suited to confirmatory analysis by GC–MS. Application of this procedure to the analysis of tissue homogenates is currently being investigated.

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Extraction and clean-up of the β-agonist salbutamol from liver and its determination by enzyme immunoassay

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Abstract

A method has been developed for the extraction and immunoassay of salbutamol in liver. The procedure involves digestion of liver with Subtilisin protease at pH 10.5, clarification by acid precipitation and centrifugation. The salbutamol in the supernatant was concentrated onto a 2-g C_{18} "Bond Elut" cartridge at pH 9.1 ± 0.3, eluted with 99.5% methanol and the eluate concentrated and quantitated by enzyme immunoassay. The method was validated using tissues in which salbutamol had been incurred and tissues spiked with salbutamol. The mean recovery from liver spiked at 1 μg kg^{-1} was 57 ± 8% (S.D.). Statistical analysis of the results for 18 batches of samples enabled the calculation of the probability of false negatives (sensitivity) and of false positives (specificity) of the method. The probability of false negatives among samples containing 1 μg kg^{-1} was 1% and that of false positives was 2.5%. The method was used successfully on incurred tissues, and is used routinely in this laboratory for screening samples taken in compliance with the requirements of the EC Residues Directive (86/469).

Keywords: Enzymatic methods; Immunoassay; β-Agonists; Liver tissue; Salbutamol

The administration of high doses of β_2 adrenoceptor agonists (β-agonists) such as clenbuterol, terbutaline and salbutamol to food animals has been shown to increase carcase lean/fat ratios [1]. Although such use is illegal throughout the European Community, incidences of human food poisoning where β-agonists were implicated have been reported [2]. Consequently, interest has focused in recent years on the abuse of β-agonists both in the United Kingdom and in Europe. Under the UK "Residues in Meat" programme, liver samples from randomly selected animals are monitored for the presence of these compounds.

Several workers have devised methods for the analysis of salbutamol in plasma by liquid chromatography (LC) [3–8], and by gas chromatography–mass spectrometry (GC–MS) [9–12] but plasma is inappropriate for the surveillance of β-agonists because of the low concentrations present [13].

Investigation of LC with amperometric and UV absorbance detectors, combined with a variety of sample clean-up and concentration approaches, failed to show the combination of freedom from co-extractives and high recovery which is necessary if residues are to be detected at 1 μg kg^{-1} in liver (unpublished data). The particular problems found may have been related to particular chemical attributes of salbutamol. For example, because it is a basic compound it may be subject to protein binding and consequent poor recoveries, especially if protein precipitation techniques are used to prepare extracts [14]. Therefore we have investigated the use of a Subtilisin Carlsberg protein digestion step [14] in the

present study. Additionally, salbutamol is charged at all pH values and does not readily lend itself to simple, specific back-extracting procedures. This severely restricts the options for sample clean-up.

Immunoassay offers a sensitive alternative to LC for analysis of sample extracts. This approach is investigated here, using the cross-reaction of a clenbuterol enzyme immunoassay (EIA) to enable analysis of liver extracts, following their concentration on octadecyl silane extraction cartridges.

The procedure developed for salbutamol analysis was applied to the analysis of 180 randomly chosen bovine and porcine liver samples.

EXPERIMENTAL

Materials

Salbutamol (free base), Subtilisin Carlsberg and *p*-nitrophenyl phosphate, were obtained from Sigma. Tritium labelled salbutamol was a gift from Glaxo (Ware, UK), and tritium-labelled clenbuterol was a gift from Boehringer-Ingelheim (Bracknell, UK).

Alkaline phosphatase (ALP1-12G) was from Biozyme Las. (Blaenavon, UK). Ultima gold XR liquid scintillation fluid was obtained from Canberra-Packard (Reading). The alkaline phosphatase–clenbuterol conjugate and the clenbuterol antiserum were prepared as described elsewhere [15].

Other chemicals were of analytical reagent grade and were purchased from BDH Merck. Salbutamol standard solutions were prepared in water and stored at 4°C. The diethanolamine (DEM) buffer (1 M, pH 9.8) also contained magnesium chloride (0.5 mM) and sodium azide (0.2 g l^{-1}). The phosphate saline gelatin buffer (PAS–gelatin) consisted of 0.1 M phosphate buffer containing gelatin (1 g l^{-1}), sodium chloride (9 g l^{-1}), sodium azide (0.2 g l^{-1}) adjusted to pH 7.0. The *p*-nitrophenyl phosphate (0.02 M in DEM buffer) was prepared 20 min before use and was kept protected from light until needed.

The LC system consisted of two Pye Unicam Model 4015 pumps with an interface, a Model 4850 controller and data handling system (Unicam, Cambridge) and a Severn Analytical 6500 UV detector set at 233 nm. The heated magnetic stirrer with water bath and thermostatic probe were made by IKA and supplied by Sartorius Instruments.

Extraction procedure

Liver samples (10 g) were prepared in batches of twelve: two samples from untreated animals (controls) were included in each batch, one of which was spiked at 1 μg kg^{-1} with radiolabelled salbutamol (3500 dpm). Samples were homogenised in 10 ml Tris buffer (1 M, pH 10.5). Protease (10 mg), and a further 20 ml of Tris buffer and a magnetic stirring bar were then added to each tube, the tubes were capped and incubated in a water bath on a heated magnetic stirrer for one hour at 55°C. During this incubation the sample was completely solubilized apart from a small amount of connective tissue. The protease was subsequently deactivated by immersing the tubes in boiling water for 15 min. When cool, the digest was clarified by acidifying with concentrated hydrochloric acid (3.5 ml) and centrifugation (30 min, 1500 *g*). The supernatant was decanted into a fresh tube and its pH was adjusted to 9.1 with NaOH (10 M) prior to solid phase extraction. This pH has been found to be optimal for the retention of salbutamol on the bond Elut cartridge.

Solid phase extraction

C_{18} Bond Elut cartridges (2 g, Analytichem; Code 1125-6015) were fitted with 75-ml reservoirs containing a 20-μm plastic frit and set up on a "Vac-Elut" box. The columns were prewashed with 5 ml of methanol and 10 ml of water. The samples were poured into the reservoir and allowed to percolate slowly (not more than 5 ml min^{-1}) through the column either under gravity or reduced pressure (maximum 30 mmHg). The columns were washed with water (10 ml) followed by acetonitrile (4 ml). Finally, the salbutamol was eluted from the column with 99.5% methanol (6 ml).

The eluate was dried under nitrogen at 37°C and then redissolved in 0.5 ml PAS–gelatin buffer prior to determination by enzyme immunoassay.

Enzyme immunoassay

Salbutamol standards and three batches of 12 samples were analysed on each microtitre plate using the enzyme immunoassay described by Sauer et al. [15]. A calibration graph covering the range from 10 to 5000 pg salbutamol per well was used to interpolate salbutamol concentration.

LC clean-up

Aliquots of the extract (200 μl) were injected onto a 3 μm Hypersil ODS column (100 × 4.6 mm i.d.) with a 5 μm Lichrosorb RP18 guard column (5 × 3 mm i.d.). The mobile phase was 15% methanol in 0.2% orthophosphoric acid pumped at a flow-rate of 1 ml min^{-1} and a fraction collected from 1 min before the retention time of Salbutamol until 2 min after. The fraction was buffered to pH 7 ± 1 with 11 μl of sodium hydroxide solution (10 M) and taken to dryness on a Gyrovap centrifugal evaporator (45°C, 3 h). It was redissolved in 100 μl of water and assayed by the enzyme immunoassay.

RESULTS AND DISCUSSION

Survey of field samples

The method was used to analyse 18 batches of samples from the UK "Residues in Meat" programme. The recovery of the radiolabel in these batches enabled estimation of the reproducibility of the extraction procedure: the mean recovery was 57% with a coefficient variation of 8% (n = 14).

It was also possible to obtain an estimate of the variability of B/B_0 (%) [this is a measure of the enzyme labelled clenbuterol bound in the presence of competing salbutamol (B) expressed as a percentage of that bound in the absence of competing salbutamol (B_0)] values for the negative liver extracts in this population of field samples (i.e., those not containing salbutamol or any other cross-reactants): this was done by analysis of variance within the batches following the removal of outliers (results outside three standard deviations from the batch mean) and was found to be 5.64%. The B/B_0 (%) values given by liver samples, fortified to contain 1 μg kg^{-1} salbutamol, were far enough outside this range for numbers of false positives and false negatives to be acceptable. The mean difference between B/B_0

TABLE 1

Specificity and sensitivity of the method

B/B_0 (%) units below mean of tissue blanks [a]	Specificity				Sensitivity			
	No. of tissue blanks per batch				No. of tissue blanks per batch			
	1	2	3	4	1	2	3	4
1	0.747	0.778	0.792	0.800	0.994	0.998	0.999	0.999
2	0.785	0.819	0.833	0.841	0.992	0.997	0.999	0.999
3	0.820	0.855	0.869	0.876	0.989	0.996	0.998	0.998
4	0.851	0.885	0.899	0.906	0.985	0.994	0.996	0.997
5	0.878	0.911	0.923	0.930	0.979	0.991	0.994	0.995
6	0.902	0.932	0.943	0.949	0.972	0.987	0.991	0.993
7	0.922	0.949	0.959	0.963	0.963	0.981	0.986	0.989
8	0.938	0.962	0.971	0.974	0.952	0.974	0.980	0.983
9	0.952	0.973	0.979	0.982	0.939	0.964	0.972	0.976
10	0.963	0.981	0.986	0.988	0.922	0.951	0.960	0.965
11	0.972	0.987	0.991	0.992	0.903	0.914	0.926	0.933

[a] B/B_0 (%) is a measure of the enzyme labelled clenbuterol bound in the presence (B) of competing salbutamol expressed as a percentage of that bound in the absence of competing salbutamol.

TABLE 2

Residues of salbutamol in livers of treated animals [Animals were injected (intravenous) with salbutamol sulphate (Ventolin) 1 h prior to slaughter]

Dose ($\mu g\ kg^{-1}$)	0.2	1.0	5.0
Salbutamol measured ($\mu g\ kg^{-1}$)	1.5	1.6	4.5

(%) for the negative samples and fortified samples was shown to be 21.5%.

Table 1 shows the specificity and sensitivity [16] obtained when different values below the blank control tissue were taken to define the positives threshold. Using these thresholds is likely to underestimate the sensitivity of the method because in practise many positive samples will contain more than 1 μg salbutamol kg^{-1} and would have lower B/B_0 (%) values.

Since we wish to detect at least 99% of all positive samples (sensitivity = 0.99) the threshold value was set at 5 B/B_0 (%) "units" below the mean tissue blank [i.e., samples with B/B_0 (%) values less than this are taken as positive]. According to Table 1 this will give rise to a false positives rate of 12% (specificity of 0.88).

Liver samples from 3 animals that had been dosed with salbutamol were analysed and the results are shown in Table 2.

Confirmation

Samples with B/B_0 (%) values below the threshold were repeated in a batch containing four blank control tissues. This enables the threshold to be set at 8 B/B_0 units below the mean of these four tissue blanks and would theoretically give rise to a false positives rate of only 2.5%.

The very small number of samples which still appeared positive after this treatment were subjected to the LC clean-up described above. This reduces the possibility that clenbuterol or other interfering or cross-reacting compounds will be present during the EIA. We demonstrated the separation of clenbuterol from the salbutamol by collecting the salbutamol fraction of an extract containing radiolabelled clenbuterol. Only 0.1% of the radiolabel appeared in the salbutamol fraction.

Following LC clean-up all suspect positives were found to be negative except the spiked samples and those containing incurred salbutamol.

The method described has therefore proved to be reliable in routine use and has enabled the screening of several hundred samples of bovine and porcine liver for the presence of salbutamol. Its effectiveness at differentiating negative samples from those spiked at 1 $\mu g\ kg^{-1}$ and from samples containing incurred residues has been demonstrated.

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Determination of salbutamol in rats at low concentrations using liquid chromatography with electrochemical detection

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Abstract

A simple and sensitive method for the determination of salbutamol in rat tissues using liquid chromatography with electrochemical detection is described. The development of the method involves optimization of sample clean-up (immunoaffinity chromatography or solid-phase extraction) and of the chromatographic and detection conditions (column type, mobile phase composition, oxidation potentials). Optimum results were obtained using immunoaffinity extraction for sample clean-up, a CN-type column with 3.5 mM phosphate buffer (pH 4)–acetonitrile (25 + 75) as the mobile phase for chromatographic separation and an electrode potential of +1.15 V for electrochemical detection. The recovery of salbutamol from samples was 64.3% and the limit of detection was 0.1 ng g^{-1} in lyophilized carcass samples. The method was applied to the analysis of lyophilized carcass samples from rats treated subcutaneously with salbutamol and concentrations ranging from 8.3 to 26.8 ng g^{-1} were found.

Keywords: Liquid chromatography; Sample preparation; Biological samples; Salbutamol; Rats

Drugs that act on β_2-adrenergic receptors are widely used for the treatment of bronchial asthma and for the prevention of premature labour in medicine and in veterenary science [1]. Salbutamol [2-*tert*-butylamino-1-(4-hydroxy-3-hydroxymethylphenyl)ethanol] [2] is a β_2-agonist that, with clenbuterol and cimaterol, has been also studied as a growth promotor in meat-producing animals. The metabolic effects of these substances (also called repartitioning agents) are an increase in proteic deposition together with a decrease in body fat accumulation, by mechanisms that are not yet well known [3–6]. Taking into account that these agents do not lose their activity in the digestive tube of ruminants and monogastrics and that they are active in extremely low doses (0.25–4 mg kg^{-1} body weight), it can be assumed that their utilization in cattle breeding is easy and convenient [7,8]. However, the utilization of these drugs as growth promoters in animals is prohibited in all European Economic Community (EEC) countries.

Methods reported for the determination of salbutamol have generally been applied to pharmacokinetic studies and are based on the use of liquid chromatography (LC) with electrochemical

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[9–13] or fluorescence [14–17] detection. For the determination of salbutamol residues, the methods described are based on immunoenzymatic detection for urine samples [18] and on gas chromatography–mass spectrometry for urine and liver sample [19,20].

In this paper a method based on LC with electrochemical detection for the determination of low levels of salbutamol in lyophilized carcass samples is described. The development of the method includes the optimization of sample clean-up by immunoaffinity chromatography (IAC) or solid-phase extraction (SPE) and of the chromatographic and detection conditions.

EXPERIMENTAL

Reagents and apparatus

Salbutamol was obtained from Sigma (St. Louis, MO). All other chemicals were purchased from Merck (Darmstadt, Germany). LC-grade water was obtained with a Milli-Q purification system (Millipore, Bedford, MA) and nitrogen was obtained from Arliquido (Oporto, Portugal).

Immunoaffinity columns (Multi Prep III HC) were purchased from Genego (Gorizia, Italy) and Sep-Pak C_{18} cartridges from Waters (Milford, MA).

The LC instrument was obtained from Gilson (Villiers-le-Bel, France) and consisted of a Model 307 pump and a Model 141 electrochemical detector with a thin-layer-type cell. The injector was a Model 7125 injection valve (Rheodyne, Cotati, CA) with a 50-μl loop. The chromatographic columns were 250 mm × 4.6 mm i.d., packed with 5-μm Spherisorb ODS-2 or Spherisorb-CN particles (Phase Sep, Clwyd, UK). Data were recorded with a Spectra-Physics (San Jose, CA) integrator Data Jet.

A Mettler (Zurich, Switzerland) balance Model AE 200, a Selecta (Barcelona, Spain) Macrotronic centrifuge, a Selecta Agimatic S magnetic mixer, a Type KTH water-bath at 37 ± 1°C (Edmund Bühler, Tübingen, Germany) and a Crison (Barcelona, Spain) pH meter were also used.

Standard solutions

A 25.0-mg amount of salbutamol was weighed accurately into a 50-ml volumetric flask and dissolved in water (solutions of salbutamol are light sensitive and must be prepared under diffused ligth and stored in dark). This stock solution is stable for several months if stored at 4°C. Working solutions were prepared by diluting the stock solution with water to salbutamol concentrations of 5 μg ml^{-1} and 500, 250, 100, 50, 20 and 10 ng ml^{-1}. The 5 μg ml^{-1} solution was used for the study of the chromatographic conditions and the other solutions for calibration and for the study of clean-up procedures.

Animals

Eight adult female rats were divided in two groups, a control group and a salbutamol group. The rats in the salbutamol group were treated subcutaneously for 15 days with 1 mg kg^{-1} body weight of salbutamol in 0.9% NaCl solution. The rats in the control group were treated in the same way but with 0.9% NaCl solution only. After this time the animals were killed and their carcasses lyophilized [3].

Sample preparation

A 500-mg amount of a lyophilized sample of rat tissue was reconstituted with 10 ml of water and mixed for 30 min with a magnetic stirrer at 500 rpm. The sample was then centrifuged at 3000 *g* for 10 min and the supernatant aqueous phase was removed.

For SPE, the aqueous extracts were adjusted to pH ≈ 10–11 with 0.1 M NaOH and then, after pretreatment of Sep-Pak C_{18} cartridges (according to the manufacturer's instructions), were loaded and allowed to drain into the cartridges. Subsequently, the Sep-Pak cartridges were washed with 5 ml of water and 5 ml of water–methanol (50 + 50, v/v). Salbutamol was finally eluted with 3 ml of methanol and the eluates were evaporated to dryness at 37°C under a stream of nitrogen.

For immunoaffinity extraction the aqueous extracts of tissues were diluted with 10 ml of Multi Prep III HC extraction buffer (1 + 9) and then processed following the manufacturer's instruc-

tions. Briefly, the diluted sample solutions were transferred to the immunoaffinity columns, which were washed with 8 ml of Multi Prep III HC extraction buffer (1 + 9) and then with 2 ml of Multi Prep III HC extraction buffer (1 + 99). The analyte was eluted twice with 1 ml of 96% ethanol and the eluate was evaporated to dryness at 37°C under a stream of nitrogen [20].

Chromatography

The residues of the two clean-up procedures were dissolved in 200 μl of different mobile phases and injected into the chromatographic system. The mobile phases consisted of 1.0, 3.5 or 5.0 mM diammonium hydrogenphosphate in LC-grade water buffered with orthophosphoric acid to pH 3.6, 4.5 or 5.2, mixed with acetonitrile (25 : 75, 20 : 80 and 15 : 85, v/v). The two different Spherisorb columns were used at a flow-rate of 1.5 ml min^{-1}.

Detection was effected at cell electrode potentials of +0.85, +1.00 and +1.15 V. Daily, the working electrode was polished and the reference electrode was refilled with a freshly prepared solution of 3 M NaCl.

Each time the mobile phase was changed the column was conditioned overnight using the same conditions that would be used on the next day.

RESULTS AND DISCUSSION

The effects of the chromatographic conditions are shown in Table 1. Increasing the buffer ionic strength, with a constant pH and buffer : acetonitrile ratio, decreases the retention time and increases the peak height of salbutamol [21]. Increasing the relative amount of buffer in the mobile phase, at constant pH and ionic strength, decreases the retention time and increases the peak height. Increasing the pH of the buffer in the mobile phase, at constant ionic strength and buffer : acetonitrile ratio, increases the retention time and peak height. The CN column, for the same conditions, gives a better result than the ODS column, based on peak heights. For detection, a better absolute response is obtained with a higher cell electrode voltage.

TABLE 1

Effects of the chromatographic conditions [a]

	Parameter	Value	Retention time (min)	Peak height (mm)
A	Ionic strength (mM)	1.0	11.87	36.0
		3.5	4.30	89.5
		5.0	3.24	87.0
B	Buffer (%)	15.0	5.96	53.3
		20.0	4.86	82.0
		25.0	4.30	89.5
C	Buffer pH	3.6	4.30	89.5
		4.5	5.34	112.3
		5.2	5.36	117.0
D	Potential (V)	+0.85	4.30	28.0
		+100	4.30	59.5
		+1.15	4.30	89.5
E	Column	ODS	4.30	89.5
		CN	6.57	141.0

[a] Column: ODS (A,B,C,D). Mobile phase: 3.5 mM phosphate buffer (B,C,D,E), pH = 3.6 (A,B,D,E)–acetonitrile (25 + 75) (A,C,D,E). Detection: +1.15 V (A,B,C,E). Range: 100 nA V^{-1} (A,B,C,D,E).

The recoveries of salbutamol from fortified samples and the relative standard deviations (n = 5), based on peak heights, are given in Table 2. It can be seen, for instance, that a sample without salbutamol, spiked with a 200.0 ng g^{-1} amount, had recoveries of 69.3 and of 64.3% with the SPE and IAC sample clean-up procedures, respectively. The salbutamol recoveries indicate that the SPE and IAC procedures give almost identical performances. However, from the chromatograms for a salbutamol lyophilized rat group sample (Figs. 1 and 2), it is concluded that SPE does not

TABLE 2

Mean recoveries of salbutamol in spiked lyophilized carcass samples [a]

Concentration present (ng g^{-1})	Concentration added (ng g^{-1})	Mean recovery (%) [b]		R.S.D. (%) [b]	
		SPE	IAC	SPE	IAC
0.0	200.0	69.3	64.3	8.8	13.0
20.8	80.0	65.7	65.4	9.5	15.1
26.8	40.0	–	68.1	–	20.9

[a] Column: CN. Mobile phase: 3.5 mM phosphate buffer (pH 3.6)–acetonitrile (25 + 75). Detection: +1.15 V. Range: 20 nA V^{-1}. [b] n = 5.

achieve an efficient extract purification. Therefore, the method adopted for the determination of salbutamol residues involved an aqueous extraction, IAC purification and the following LC conditions: CN column, 3.5 mM phosphate buffer (pH 3.6)–acetonitrile (25 + 75) as mobile phase, a flow-rate of 1.5 ml min^{-1} and a potential of +1.15 V for electrochemical detection. Under these conditions, the limit of detection was 0.1 ng g^{-1} [22].

Salbutamol concentrations determined in four different rats using the described method were 8.3, 16.5, 20.8 and 26.8 ng g^{-1}.

The method used to determine residues of salbutamol in lyophilized rat carcass samples is acceptable. However, it should be tested on other tissues, mainly crude tissues.

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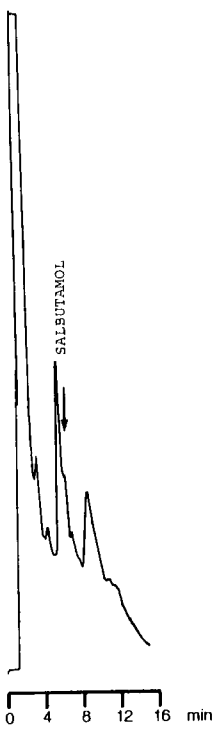

Fig. 1. Chromatogram of a lyophilized rat carcass sample with salbutamol (20.8 ng g^{-1}) subjected to SPE. Conditions as in Table 2.

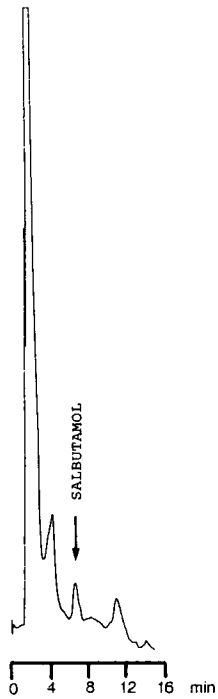

Fig. 2. Chromatogram of a lyophilized rat carcass sample with salbutamol (20.8 ng g^{-1}) subjected to IAC. Conditions as in Table 2.

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Development and optimization of a liquid chromatographic method for the determination of gentamicin in calf tissues

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Abstract

A simple and sensitive method for the determination of gentamicin in calf tissues (muscle, liver, kidney and fat) based on liquid chromatography (LC) is described. A 5% (w/v) trichloroacetic acid solution was used to precipitate proteins. After centrifugation of the sample, the pH and ionic strength were adjusted for further purification by liquid–solid ion-exchange extraction using CM-Sephadex gel. A 100-μl aliquot of the eluate from the ion-exchange cartridge (in 0.05 M NaOH) was injected on to the LC column after adjustment of the pH and counter ion concentration. The chromatography consisted of an ion-pairing system with a mobile phase containing camphorsulphonate as the counter ion, a reversed-phase column (LiChrospher 100 RP-18 end-capped) and postcolumn derivatization with *o*-phthalaldehyde and 2-mercaptoethanol followed by fluorimetric detection. The three major components of gentamicin were eluted as a single peak. Recoveries ranged from 68 to 98% in tissue samples fortified with gentamicin at 0.05–3.2 μg g^{-1} levels. The limit of quantification was 50 ng g^{-1} in muscle and fat and 100 ng g^{-1} in liver and kidney.

Keywords: Liquid chromatography; Antibiotics; Calf tissues; Gentamicin

Antibiotics are widely used to treat serious infections in livestock and to prevent disease. These practices imply that drug residues may persist in edible tissues derived from treated animals. Such residues, if animals have been heavily dosed, may be a risk to the consumer. Consequently, withdrawal periods must be determined.

Aminoglycosides are a therapeutically important class of antibiotics but possess significant potential for toxicity. However, gentamicin, together with streptomycin, dihydrostreptomycin and neomycin have been approved for veterinary use in food-producing animals [1]. Gentamicin, a multi-component aminoglycoside antibiotic, was first isolated by Weinstein et al. [2] from *Micromonospora purpurea* and was introduced as a therapeutic agent in 1969. It has a broad spectrum of activities against both Gram-positive and Gram-negative bacteria. It is composed of three major components, C_1, C_2 and C_{1a}, which have similar antibacterial properties [3].

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The US Food and Drug Administration (FDA) has established limits of tolerance (or maximum residue limits, MRLs) for antibiotics in foods that move in international trade. MRLs must be toxicologically acceptable in terms of estimated intake by consumers.

Microbiological methods have traditionally been used to detect residues of antibiotics but lack specificity and fail to distinguish amongst different aminoglycosides. Barends et al. [4] compared liquid chromatography (LC) and microbiological methods to determine gentamicin. The concentrations tested were above 1 μg ml^{-1} and concluded with a preference for LC methods, especially where reproducibility was concerned. LC has become the method of choice because of its specificity, sensitivity and precision. It has been applied to determine gentamicin in biological fluids [5–7] and in milk and tissues [8,9], with a limit of detection (LOD) of 200 ng g^{-1} for the latter application.

In this work, a method was developed to determine gentamicin in calf tissues (muscle, liver, kidney and fat) under conditions allowing detection of residual amounts which met with the MRLs established by the FDA (between 100 and 400 ng g^{-1}) and a limit of quantification (LOQ) of half the MRL.

EXPERIMENTAL

Reagents, chemicals and standards

All chemicals and solvents were of analytical-reagent grade. Gentamicin sulphate (potency equivalent to 620 μg gentamicin mg^{-1}) was obtained from Virbac SA Laboratories (Carros, France). A 1.621 mg ml^{-1} gentamicin sulphate solution was prepared in 1×10^{-3} M disodium EDTA (Merck) and stored at 5°C for up to 1 month in a polypropylene container. Further dilutions were prepared in 1×10^{-3} M EDTA to spike muscle, liver and kidney to give concentrations as free base of 0.5, 1.0, 1.5, 2.0, 4.0, 8.0, 16.0 and 32.0 μg ml^{-1}. Spiking solutions used to fortify fat samples were prepared in acetonitrile–1×10^{-3} M EDTA (50 + 50, v/v) at the same concentration as for the other tissues.

Sodium *dl*-camphor-10-sulphonate was purchased from Aldrich. *o*-Phthalaldehyde (OPA) (Fluka) solution was prepared as follows: 5.3 g of boric acid (Merck) and 7.5 ml of 30% NaOH ($d^{20} = 1.33$) were placed in a 250-ml volumetric flask and dissolved in ca. 200 ml of doubly distilled water. A 0.2-g amount of OPA was dissolved in 10 ml of 95% ethanol and added to the above solution, together with 0.5 ml of 2-mercaptoethanol (Aldrich) and 1 ml of 30% Brij-35 solution (Sigma). The volume was adjusted to 250 ml with doubly distilled water, degassed by ultrasonication for 5 min prior to use and discarded if not used within 24 h.

Preparation of samples

Tissue samples were left to thaw at room temperature, minced and 5.0 ± 0.1-g samples were weighed in 50-ml Virtis glass vials, then fortified using 500 μl of the appropriate spiking solution. A 20-ml volume of a 5% (w/v) trichloroacetic acid (TCA) solution containing 1×10^{-3} M EDTA was added to the fortified samples 30 min later and the mixture was blended for 10 min using a Virtis Model 45 mixer fitted with U-shaped blades. The blended tissues were transferred into a 40-ml Teflon centrifuge tube and centrifuged for 15 min at 8000 rpm (7000 *g*) at 5°C. The supernatant was transferred into a 50-ml volumetric flask, the pH was adjusted to 7.0 ± 0.2 with 30% NaOH solution and the contents were diluted to volume with 0.1 M Na_2SO_4–0.1 M K_2HPO_4 (pH 7). A 10-ml volume of the mixture, corresponding to 1 g of tissue, was used for the separation of gentamicin from interfering compounds by solid-phase extraction: 5 g of CM-Sephadex C-25 (a weak cation exchanger from Pharmacia) were left to swell overnight in 50 ml of 0.2 M Na_2SO_4–1×10^{-3} M EDTA (pH 7) containing 0.02% (w/v) sodium azide, then 1-ml aliquots of the slurry were used to fill Pasteur pipettes plugged at the bottom with hydrophilic cotton (Pharmacopoeia grade). These cartridges were washed with 2×1 ml of 0.2 M Na_2SO_4–1×10^{-3} M EDTA using a rubber bulb. A 10-ml volume of the above tissue sample was passed through the cartridge in fractions of 1 ml, followed by 2×1 ml of 0.2 M Na_2SO_4–1×10^{-3} M

EDTA, 1 ml of doubly distilled water and 250 μl of 0.05 M NaOH. Gentamicin was eluted directly into an autosampler injection vial while applying pressure until the bed ran dry, using 1 ml of 0.05 M NaOH. Volumes of 100 μl of 1 M HCl and 100 μl of 0.5 M camphorsulphonate (pH 2.2) were added to the eluate before mixing and direct injection into the chromatograph (injection volume 100 μl).

Standards containing no biological material were processed in the same way, but omitting the blending and centrifugation steps.

Chromatographic equipment

The LC system consisted of a solvent-delivery pump fitted with a membrane pulse damper (Spectroflow 400; Applied Biosystems, Foster City, CA), an autosampler (Model 507; Beckman, San Ramon, CA) equipped with a 100-μl sample loop and a column oven, a guard column (4 × 4 mm i.d.) and an analytical column (125 × 4 mm i.d.) prepacked with LiChrospher 100 RP-18 end-capped (particle size 5 μm).

A second pump of the same model and a low-dead-volume tee (Model 5-8283; Supelco, St. Germain-en-Laye, France) were used for addition of the OPA solution to the mobile phase. Derivatization took place in a Teflon knitted open-tubular (KNOT) reactor (3 m × 0.5 mm i.d.) (Model 5-9206; Supelco).

The fluorescence detector (Shimadzu Model RF-551; Touzart et Matignon, France) was equipped with a 150-W xenon short arc lamp. All data capture and calculations were performed using a Winner data station (Spectra-Physics, Les Ulis, France).

Conditions for chromatography and detection

The mobile phase was 0.05 M sodium *dl*-camphor-10-sulphonate in 1×10^{-4} M EDTA (pH 2.2, adjusted with hydrochloric acid)–methanol (45 + 55, v/v), and was filtered through a 0.2-μm nylon filter prior to use. The column and the KNOT reactor were kept at 45°C during analysis. The flow-rates of the mobile phase and postcolumn derivatization reagent were 1.2 and 0.5 ml min^{-1}, respectively.

Routine replacement of the guard column was done after every 100 injections. The analytical column was regenerated on a weekly basis by initial flushing with methanol–doubly distilled water (60 + 40, v/v), then running a gradient up to 100% methanol in 15 min and flushing with 100% methanol for 15 min (flow-rate 1.2 ml min^{-1}). The analytical column was replaced after every 300 injections to prevent any alterations to the peak shape or area.

Fluorescence detection was performed at an excitation wavelength of 340 nm and an emission wavelength of 440 nm. The sensitivity selected was "high" and the gain was set to 1.

RESULTS AND DISCUSSION

Development of the method

Preparation of biological samples. Tissue sample pretreatment in drug residue analysis by LC commonly involves two stages [10]: first, blending in buffer or protein precipitation using organic solvents or precipitating agents; another approach is alkaline [11] or enzymatic digestion of the tissues; and second, extraction of the analytes by liquid–liquid or liquid–solid extraction, or more recently by immunoaffinity methods [12,13]. These two steps must reflect the prime necessity in trace analysis, i.e., to minimize sample dilution.

For gentamicin, each of its three major C components contains five basic amino groups. Because of their similar basic strength, an apparent pK_a value of 8.2 is obtained [14].

In the first stage of the analytical protocol, a number of approaches were used, comparing gentamicin recoveries after grinding of the samples in phosphate buffer (pH 6.4) and in acidic solutions ($HClO_4$, TCA). The recoveries from muscle were 39.2, 78.5 and 85.9%, respectively, for a concentration of gentamicin of 0.5 μg g^{-1}. Alkaline digestion is said to enhance the recovery of gentamicin from kidney [11], but the digestate would need be highly diluted before being loadable on a weak cation exchanger for further clean-up. The most efficient treatment with respect to the cleanness of the samples, recovery of gentamicin and adaptability to further treatment on ion exchangers was obtained after precipita-

tion of the sample using TCA, although Peng et al. [5] and Shaikh and Allen [1] advised against the use of precipitating reagents, reporting that gentamicin was precipitated together with the proteins when TCA or tungstic acid was used for deproteination. EDTA was added at a concentration of 1×10^{-3} M for all solutions used in sample treatment, as gentamicin is reported to chelate doubly charged metal ions [15].

Of several possible extraction procedures, liquid–solid extraction was chosen. The hydrophilicity of gentamicin prevents efficient partitioning into organic solvents and liquid–liquid extraction did not appear to allow any effective recovery from the samples. The wide use of CM-Sephadex C-25 [6,8,16,17] can be explained by the fact that weak cation exchangers on osidic supports (crossed-linked dextran for CM-Sephadex) are well adapted to good elution of polar aminoglycosides. Other supports have been tested (e.g., silica or polymeric resins). Experiments with weak cation exchangers on silica (Bond Elut) or on polymeric resins (Fractogel) failed to give a satisfactory recovery of gentamicin (less than 55%). Silica modified by apolar groups (C_8, phenyl and C_{18}) allowed good recoveries (55–81% for a concentration of gentamicin of 0.5 μg g^{-1} in muscle), but the lack of specificity towards endogenous compounds precluded their use.

The method originally described by Anhalt and Brown [6] was selected and modified in the following ways: the volume loaded is 10 ml instead of 1 ml to make up for the dilution entailed by the grinding of the sample; and final elution is achieved using 0.5 M NaOH as had been developed for amikacin in plasma [18] rather than a solution of high ionic strength, as such an alternative was shown to be unsuitable for injection into the LC system (alteration of peak shape, peak area and retention time of gentamicin). Adding 1 M HCl (100 μl) and 0.5 M camphorsulphonate solution (100 μl) to the 1-ml eluate made possible injection into the chromatograph of a 100-μl aliquot of this mixture, the composition of which is very close to that of the mobile phase. Such modifications have been used before [18].

The recoveries of gentamicin were above 65% for any concentration in any tissue tested when compared with standards that had been processed according to the same protocol.

Optimization of chromatographic conditions. Chromatographic conditions usually described for the elution of gentamicin are based on the separation of its major components (C_1, C_2 and C_{1a}) in an attempt to control the relative composition of pharmaceutical formulations [19]. Originally described by Anhalt in 1977 [16] and utilized as such or slightly modified to determine gentamicin in biological fluids [20] or in pharmaceutical formulations [19] for concentrations greater than 1 μg ml^{-1}, a three-peak profile LC method for gentamicin using sodium pentanesulphonate as counter ion, when applied to tissue samples, failed to allow the determination of the C_{1a} component because of interfering endogenous compounds.

Further, a multi-peak system could not possibly allow the determination of gentamicin at levels below 200 ng g^{-1}. In addition, a chromatographic system eluting gentamicin as a single peak was more appealing for achieving good selectivity, especially towards other aminoglycosides, and a better LOQ.

A single-peak approach for the determination of gentamicin using camphorsulphonate as the counter ion has been briefly described [21] with detection by refractometry and a limit of detection of about 10 μg per injection. Its application was limited to standards. When tested with coupling with OPA postcolumn derivatization and fluorimetric detection, it appeared to be in good agreement in the following respects: for the same concentration of gentamicin, the peak area was equal to the sum of the three major component peak areas obtained using pentanesulphonate as counterion; the claimed LOQs (50–100 ng g^{-1}) were easily achieved; good selectivity towards endogenous compounds and other aminoglycosides was achieved; and a short run time (7 min) was possible as the chromatograms showed no late-eluting peaks and a high frequency of assays could be achieved (8 h^{-1}).

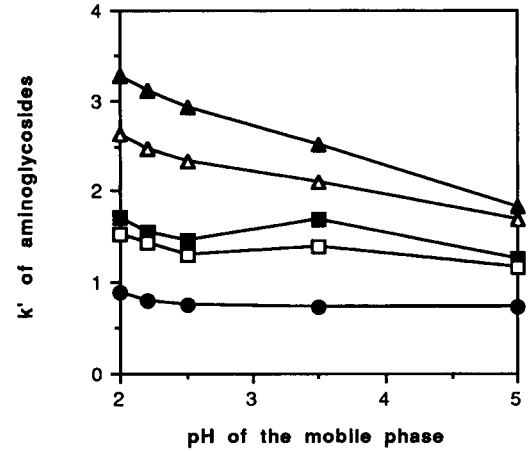
The chromatographic conditions were optimized for the retention time of gentamicin with respect to methanol content (45–60%, v/v), counter ion concentration (0.01–0.10 M for camphorsulphonate), pH of the mobile phase (2.0–

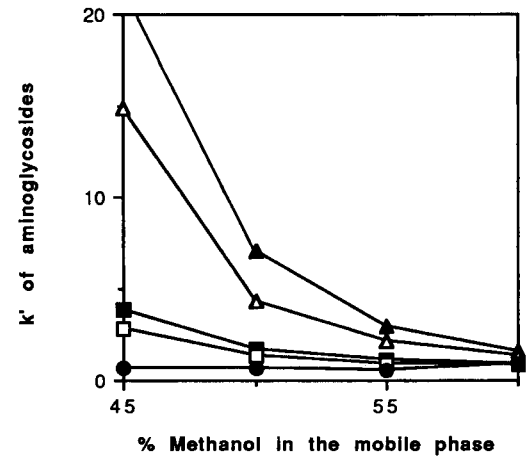
5.0) and temperature of the analytical column (25–55°C).

Figure 1 shows the variations of the capacity factors of gentamicin and other aminoglycosides (neomycin, kanamycin, amikacin and dihydrostreptomycin) with percentage of methanol in the mobile phase, temperature of the analytical column and pH of the mobile phase.

The number of primary and secondary amino groups present in the molecule tested can explain their elution pattern: six, five, four and four amino groups for neomycin, gentamicin, kanamycin and amikacin, respectively. Under all conditions tested, dihydrostreptomycin was eluted in the void volume, the selectivity for gentamicin was very good and gentamicin always eluted as a single peak. The pH values tested were always below the pK_a values of all the molecules tested. In choosing a value of 2.2 for pH and not below, the stability of the stationary phase was preserved. Increasing the counter-ion concentration entailed a greater retention of gentamicin; this levelled off above about 0.05 M camphorsulphonic acid.

Concerning the choice of the organic modifier, the use of acetonitrile separated gentamicin into its different components. The retention time of gentamicin appeared to be very sensitive to slight variations of the methanol content; 55% methanol was an acceptable proportion as gentamicin did not co-elute with any of the other aminoglycosides or with the tissue endogenous compounds. Its retention time allowed high-frequency analy-





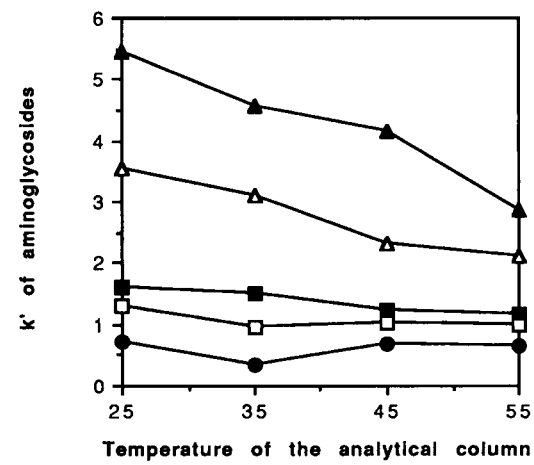


Fig. 1. Variations of the capacity factors (k') of (△) gentamicin, (▲) neomycin, (■) kanamycin, (□) amikacin and (●) dihydrostreptomycin as a function of pH of the mobile phase (temperature 45°C, methanol content 55%), methanol content (pH of the mobile phase 2.2, temperature 45°C) and temperature of the analytical colum (°C) (pH of the mobile phase 2.2, methanol content 55%). Column, LiChrospher RP-18 end-capped; mobile phase, 0.05 M sodium *dl*-camphor-10-sulphonate, in 1×10^{-4} M EDTA–methanol flow-rate 1.2 ml min^{-1}; postcolumn derivatization reagent, OPA–2-mercaptoethanol–Brij-35, flow-rate 0.5 ml min^{-1}; temperature of KNOT reactor, 45°C; detection, $\lambda_{ex} = 340$ nm; $\lambda_{em} = 440$ nm; amount of each aminoglycoside injected, 50 ng.

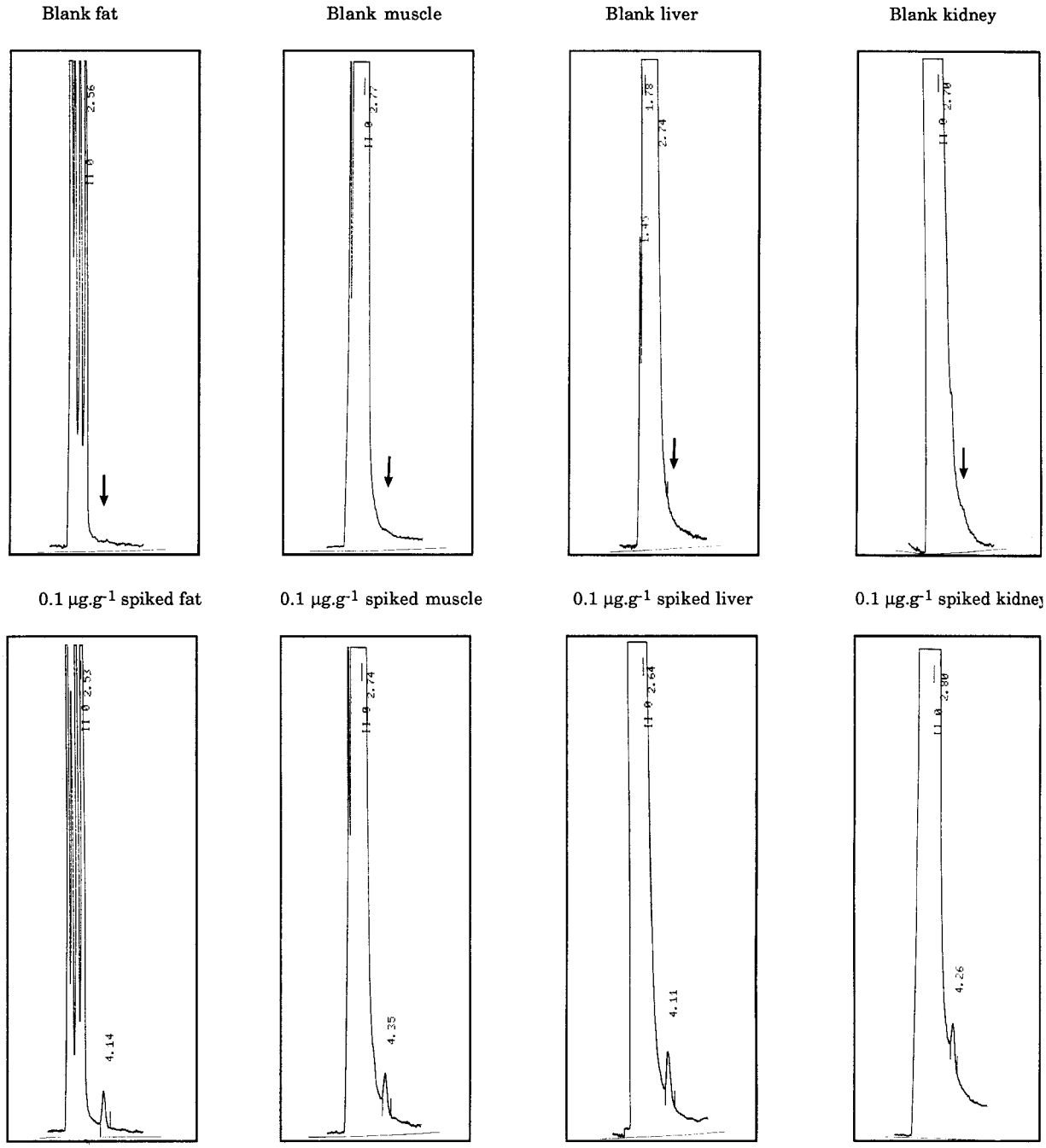


Fig. 2. Typical chromatograms obtained for gentamicin in calf tissues. Column, Li Chrospher RP-18 end-capped; mobile phase, 0.05 M sodium *dl*-camphor-10-sulphonate and 1×10^{-4} M EDTA–methanol (45–55, v/v), pH 2.2, flow-rate 1.2 ml min^{-1}; postcolumn derivatization reagent, OPA–2-mercaptoethanol-Brij-35, flow-rate 0.5 ml min^{-1}; temperature of analytical column/KNOT reactor, 45°C; detection, $\lambda_{ex} = 340$ nm; $\lambda_{em} = 440$ nm.

TABLE 1

Chromatographic parameters for gentamicin peak

Parameter	Concentration in standards ($\mu g\ ml^{-1}$)	
	0.05	0.5
Retention time (min)	4.42	4.40
Capacity factor (k')	2.54	2.54
No. of theoretical plates	5280	16225
Tailing factor	1.36	1.17

ses; for the same reason, a temperature of 45°C was selected.

EDTA was added to the mobile phase at a concentration of 1×10^{-4} M. Reversed-phase ion pairing developed for LC was effective in eliminating the interferences from endogenous primary amines with low molecular weight that remain in the eluate from the ion exchanger. Gentamicin could be well separated by using a hydrophobic counter ion (camphorsulphonate), then being more retained on the reversed-phase material than are other compounds with fewer amino groups. This approach has already been used successfully for the assay of another aminoglycoside, amikacin, in plasma [22].

Optimization of derivatization conditions. A major obstacle to the LC determination of gentamicin has been the problem of detection. Gentamicin exhibits no significant UV bands above 190 nm and has no native fluorescence. Hence the several methods that have been developed involve derivatization of the drug to form dansyl [5], precolumn OPA [23], postcolumn OPA [16,19] or 1-fluoro-2,4-dinitrobenzene derivatives [17].

Another type of derivatization for amines has been described that enhances their LOD; this uses naphthalenedicarboxaldehyde (NDA) and cyanide as derivatization agents [24–26]. It has been described for precolumn derivatization only and needs separate dissolution of NDA in organic solvents because of its instability in aqueous media, necessitating an additional solvent-delivery system if used in a postcolumn mode.

Detection was optimized with respect to reactor configuration, nature of the additives in the OPA reagent (thiol, detergent), its flow-rate and temperature for the derivatization reaction, with regard to the peak area for gentamicin.

A KNOT reactor was used as it further develops radial mixing with subsequent reduction in band broadening.

Various reagents (i.e., Brij-35, Triton X-100, sodium dodecyl sulphate, cetyltrimethylammonium bromide and β-cyclodextrin) have been tested in other studies to enhance the fluorescence response for the derivatives. Only Triton X-100 is said to have a positive effect [27]. When Triton was used as the detergent in the OPA reagent, although the derivatization yield was slightly higher than with Brij-35, it was more sensitive to minor variations of temperature. Triton appeared to enhance the signal for low concentrations (0.05 $\mu g\ ml^{-1}$) more than for higher concentrations (0.5 $\mu g\ ml^{-1}$), thus restricting the linear calibration range. Ethanethiol and 2-dimethylaminoethanethiol hydrochloride were test-

TABLE 2

Validation parameters for gentamicin assay in spiked calf tissues

Tissue	Correlation coefficient of regression curve [a]	Recovery from tissue samples (%)		Relative standard deviation (%) [a]	
		0.1 $\mu g\ g^{-1}$	0.8 $\mu g\ g^{-1}$	Low concentration (0.1 $\mu g\ g^{-1}$)	High concentration (0.80 $\mu g\ g^{-1}$)
Standard	0.9999	100	100	3.2	2.2
Liver	0.9986	98	70	11.8	5.6
Kidney	0.9974	68	83	12.7	8.2
Fat	0.9991	79	77	6.1	4.3
Muscle	0.9989	88	74	9.9	8.2

[a] $n = 7$.

ed in place of 2-mercaptoethanol as nucleophiles, but did not improve the signal-to-noise ratio.

The ideal flow-rate for OPA was 0.50 ml min^{-1}; both 0.25 and 0.75 ml min^{-1} led to a 10% decrease in the signal.

Heating and thermostating of the KNOT reactor improved the fluorescence signal and reproducibility; 45°C gave the higher response between 20 and 75°C. Similar results on the influence of temperature have been reported [18].

Chromatographic parameters for the gentamicin peak in the selected system for concentrations of 0.05 and 0.5 $\mu g\ ml^{-1}$ in standards are summarized in Table 1.

Assay validation

The method was tested for concentrations of gentamicin ranging from 0.05 $\mu g\ g^{-1}$ (for muscle samples) or 0.10 $\mu g\ g^{-1}$ (for liver, kidney and fat samples) to 3.20 $\mu g\ ml^{-1}$. Figure 2 shows typical chromatograms obtained for a blank and a concentration of 0.10 $\mu g\ g^{-1}$ in fat, muscle, liver and kidney samples.

Validation parameters. Correlation coefficients for calibration graphs for standards, liver, kidney, fat and muscle samples are summarized in Table 2. The LOD measured was 25 ng g^{-1} in muscle and fat samples (signal-to-noise ratio = 3) and 50 ng g^{-1} in kidney and liver samples. LOQs were 50 ng g^{-1} and 100 ng g^{-1}, respectively. Recoveries calculated by interpolating concentrations on peak areas for biological standards spiked at identical concentrations ranged from 68 to 98%, depending on the type of tissue examined, when compared with standards processed as biological samples. These recoveries do not take into account the loss of gentamicin on CM-Sephadex, this being $8 \pm 2\%$ on comparing the peak areas obtained with those for solutions of gentamicin in mobile phase. The validation on CM-Sephadex-treated standards seemed useful to confirm that this step did not entail any disturbance of the linear response of the method used.

Ruggedness of the method. After protein precipitation and further treatment on ion-exchange cartridges, the samples were stable for up to at least 24 h. No variation in the recovery of gentamicin was noted when new batches of the CM-Sephadex slurry were prepared. To ascertain the reliability of the system, every experiment was started with a daily suitability test, consisting of three replicate injections of a 0.8 $\mu g\ ml^{-1}$ solution of gentamicin in the mobile phase. The relative standard deviation calculated on corresponding peak areas should not exceed 2%. No variations in retention time or peak area were noted on preparing fresh reagents or changing the guard or analytical column. About 300 injections could be performed without altering the column performance. Blank tissues from different animals were tested and gave identical chromatographic profiles. After spiking, the resolution of gentamicin from endogenous compounds was always identical. The method proved applicable to real samples from calves administered orally with gentamicin (data not shown).

Conclusion

The method proposed is selective and rapid and only requires a one-step liquid–solid extraction, identical for any tissue tested. Gentamicin can be determined with good selectivity, sensitivity and reliability. Gentamicin is stable for up to at least 24 h in the modified eluate from CM-Sephadex and over 50 samples can be analysed per day with a fully automated LC system. The method can be adapted to other aminoglycosides, provided that minor changes in sample treatment and the mobile phase are introduced.

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Routine detection of buprenorphine in horse urine: possibilities and limitations of the combined use of radioimmunoassay, liquid chromatography and gas chromatography–mass spectrometry

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Abstract

A radioimmunoassay developed for the determination of buprenorphine in human urine samples was examined for its applicability to screening post-race equine urine specimens. The detection limit of the immunoassay in equine urine is 0.1 ng ml^{-1}. Confirmation methods using liquid chromatography with electrochemical detection and gas chromatography–mass spectrometry have detection limits of 0.2 ng ml^{-1} (unchanged buprenorphine and *N*-desalkylbuprenorphine) and 0.1 ng ml^{-1} (after hydrolysis), respectively. The procedures were validated after in-field administration of 300 μg of buprenorphine to a test horse. The procedures were also applied to screening a large number of random post-race equine urine samples.

Keywords: Gas chromatography; Immunoassay; Liquid chromatography; Mass spectrometry; Buprenorphine; Doping; Equine urine; Urine

Buprenorphine {[5a,7a(*S*)]-17-(cyclopropylmethyl)-α-(1,1-dimethylethyl)-4,5-epoxy-18,19-dihydro-3-hydroxy-6-methoxy-α-methyl-6,14-ethenomorphinan-7-methanol} is a synthetic derivative of thebaine. This product is structurally related to etorphine, which, at very low doses (about 100 μg per horse) acts as a stimulating agent. Etorphine has been reputed as a doping agent [1–4] and is commercially known as Immobilon [5] or "elephant-juice" [6]. Buprenorphine was first mentioned as a possible doping agent for greyhounds by McLinden [7]. It was expected that low doses (300 μg per horse) would also induce the specific locomotor activity similar to the response seen with other analgesics, including etorphine, morphine and fentanyl [8]. A radioimmunoassay (RIA) method developed for the detection of buprenorphine in human urine samples was examined for its applicability to equine urine specimens. The use of liquid chromatography (LC) with electrochemical detection (ED) for the confirmation of positive samples was examined. This method has previously shown its ability to detect therapeutic levels of unchanged buprenorphine and its *N*-desalkyl derivative in human urine samples [9]. Alternatively, a method based on gas chromatography–mass spectrometry (GC–MS) with selected ion monitoring (SIM) was developed for the detection of low concentrations of buprenorphine, following enzymatic hydrolysis, liquid–liquid extraction and silylation of equine urine samples.

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EXPERIMENTAL

Materials

Radioimmunoassay. Buprenorphine, *N*-desalkylbuprenorphine and *N*-ethylbuprenorphine (internal standard) were synthesized from thebaine [10]. Sodium [^{125}I]iodide (IMS 30) was obtained from Amersham International (Amersham, UK). Goat antiserum to rabbit γ-globulin, normal rabbit serum and Freund's complete adjuvant were obtained from Calbiochem (San Diego, CA). Radioactivity was counted on a γ counter (BF 5300, Berthold, Wildbad, Germany). Bovine serum albumin (BSA), fraction 5, and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide were obtained from Janssen Chimica (Beerse, Belgium). Samples were analysed before and after hydrolysis with β-glucuronidase (40 U ml^{-1})–aryl sulphatase (20 U ml^{-1}) (*Helix pomatia*) (Merck, Darmstadt).

LC–ED. All glasware was silanized with trimethylchlorosilane (TMCS) (5% in toluene). Toluene and acetonitrile were of LC grade. To prepare buffer solutions, water was previously purified using a Seral Seralpur PRO90CN system (Merck-Belgolabo, Overijse, Belgium). Sodium 1-heptanesulphonate (10 mmol) and 0.01% tetrabutylammonium sulphate were added to phosphate buffer (0.02 mol l^{-1}; pH 4). The apparatus and chromatographic conditions were similar to those described previously [9], except that LC was carried out on a LiChrospher CN (5 μm) column (25 cm × 0.4 cm i.d.) instead of a LiChrosorb CN column (5 μm).

GC–MS. Derivatization was carried out with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) (Pierce, Rockford, IL). Selected ion monitoring was performed on a Hewlett-Packard Model 5971A detector linked to a Vectra Model 486-33T computer. Gas chromatography was car-

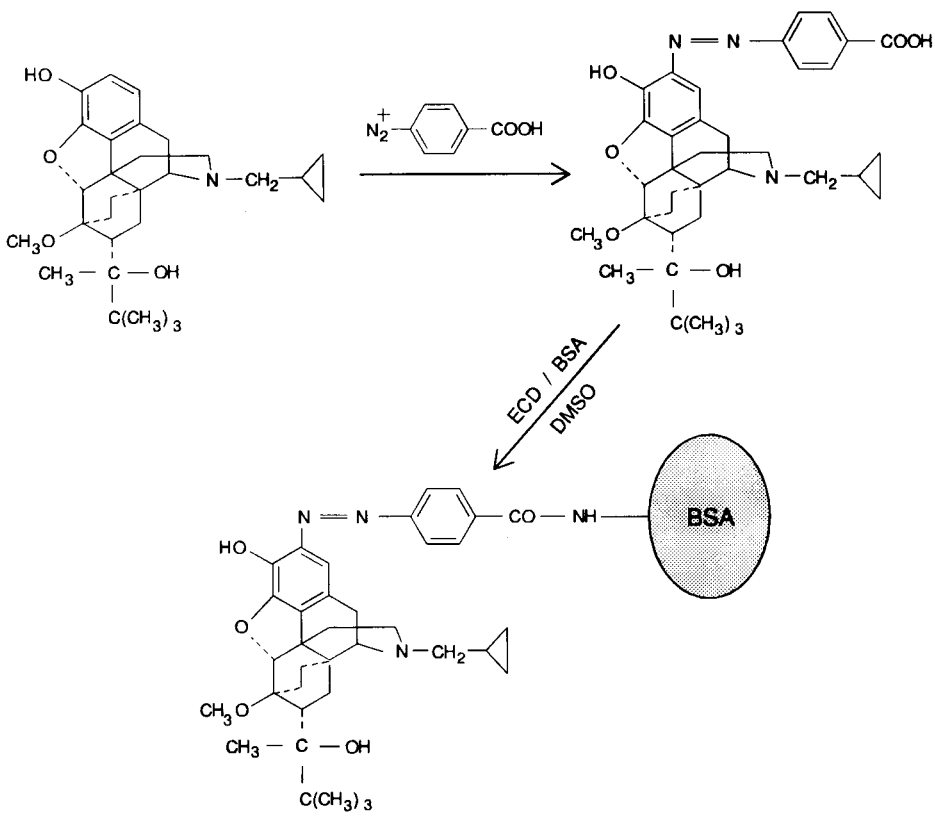

Fig. 1. Preparation of a specific buprenorphine immunogen.

ried out on an HP 5890 gas chromatograph equipped with a 12 m × 0.2 mm i.d. capillary column (HP-1).

Methods

Radioimmunoassay. The RIA developed for buprenorphine was described recently [11]. High specificity of the antibodies was obtained through haptenization of buprenorphine *ortho* to the phenolic group. The synthesis of the immunogen is illustrated in Fig. 1. Intramuscular injection of the immunogen into rabbits resulted in the production of antibodies which were able to detect small amounts of buprenorphine in urine samples.

The iodination of buprenorphine and subsequent purification [12] resulted in a tracer with a high specific activity (920 Ci $mmol^{-1}$) (Fig. 2).

For testing, antisera were diluted (1 + 399) with assay buffer. The separation of bound and free tracer was carried out by a double antibody method that reduced interferences from materials associated with equine urine. The calibration graph was constructed by adding 100 μl of standard solutions (0.1, 0.5, 1, 5, 10 and 25 ng ml^{-1} of buprenorphine in drug-free equine urine) to the incubation mixture and analysing the standards with the immunoassay. In a first step, equine urine samples (100 μl) were assayed directly. To 300 μl of assay buffer (2% BSA in 0.1 mol l^{-1} phosphate buffer; pH 7.4), 100 μl of tracer solution and 100 μl of antiserum dilution were added. The mixture was incubated at room temperature for 1.5 h, followed by addition of 50 μl of normal rabbit serum (1 + 99 in distilled water) and 50 μl of goat anti-rabbit γ-globulin (1 + 11.5 in phosphate buffer). After vortex mixing, incubation was continued for another 6 h. The precipitate was centrifuged for 15 min at 3000 *g* and counted with the γ detector for 1 min.

Alternatively, equine urine samples (0.5 ml) were hydrolysed with 50 μl of β-glucuronidase at pH 5.5 (citrate buffer; 0.5 ml) at 37°C for 12 h. A 100 μl aliquot of the mixture was analysed by the RIA.

LC–ED. For the confirmation of positive RIA results, a previously described LC method with electrochemical detection was applied [9]. The chromatographic conditions were slightly modified. The mobile phase consisted of acetonitrile–buffer (32 + 68) and was pumped at 1 ml min^{-1} through a LiChrospher CN column. A similar extraction procedure was used, except that 4 ml of equine urine were analysed instead of 2 ml of human urine.

GC–MS. Urine samples were first hydrolysed with β-glucuronidase and then purified by the liquid–liquid extraction procedure as described in the LC method. The purified extracts were analysed either directly with GC–MS or following an additional purification step, by injecting them previously into an LC apparatus and collecting the "buprenorphine-peak", e.g., the fraction that eluted between 11.0 and 12.0 min. The collected mobile phase was dried under a stream of nitrogen, concentrated in silanized microvials and derivatized with 100 μl of MSTFA prior to the analysis. Derivatization was performed at 70°C for 15 min followed by evaporation of the excess of derivatizing reagent under a stream of nitro-

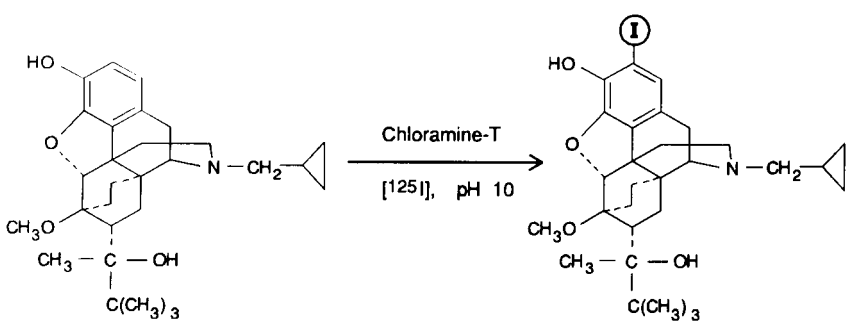

Fig. 2. Synthesis of the tracer [2-^{125}I]iodobuprenorphine.

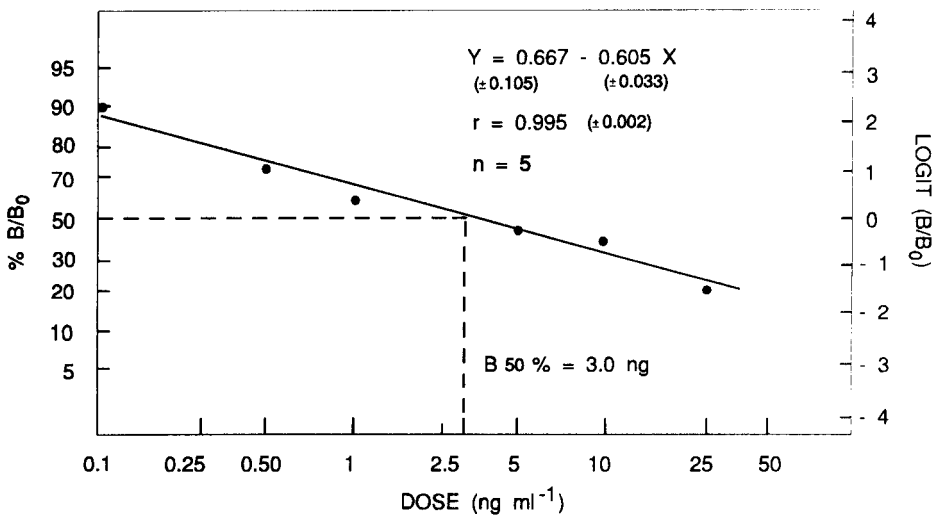

Fig. 3. Calibration graph for buprenorphine ($n = 5$).

gen. The residue was dissolved in 10 μl of toluene and 1 μl aliquots were injected into the chromatograph. The following operating conditions were established: column temperature, programmed from 70 to 270°C at 35°C min^{-1}; and temperatures of the injection port and detector interface, 260 and 280°C, respectively. The SIM mode was tuned on 450 and 539 m/z. The multiplier was set at 2350 V and the ionization potential at 70 eV.

Animal administration

One healthy horse (mare, portbred, ca. 1.70 m, 10 years old, chestnut) was dosed by rapid intravenous injection of 300 μg of buprenorphine (vena jugularis) (Temgesic 0.3 mg injection). Urine samples were collected by catheterization of the urinary bladder, immediately before dosing and at time intervals of 0.5, 1, 2, 5, 8 and 24 h. These urine specimens were all analysed by RIA, LC and GC–MS. Alternatively, a field study was undertaken with 100 post-race urine samples collected at random from track horses.

RESULTS

Figure 3 shows the calibration graph for buprenorphine. The curve was linearized with the logit–log method [13] and fitted by least-squares regression. The effects of the hydrolysis step on the values of the calibration graph were examined as follows: a calibration graph was first constructed with standard solutions of buprenorphine in drug-free equine urine. These samples were then hydrolysed with 50 μl of β-glucuronidase and again measured by the RIA. No shift was observed for the values of the calibration graph.

The specificity of the test towards several structural analogues of buprenorphine was examined (Fig. 4). The major metabolite *N*-desalkylbuprenorphine shows a cross-reactivity of about 90% over a large concentration range. The 3-*O*-

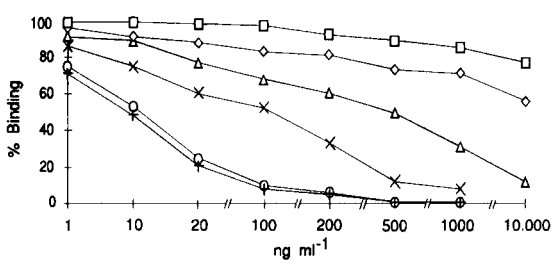

Fig. 4. Specificity of the immunoassay. Several compounds were incubated, at different concentrations, with a fixed amount of tracer and antiserum. + = Buprenorphine; ○ = *N*-desalkylbuprenorphine; × = *O*-methylbuprenorphine; △ = etorphine; ◇ = diprenorphine; □ = thebaine.

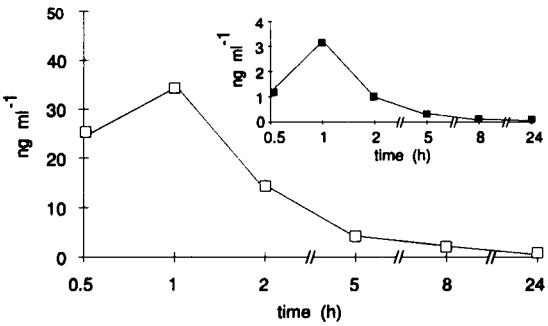

Fig. 5. Urinary concentrations of buprenorphine equivalents after intravenous administration of 300 μg of buprenorphine. ■ = Data for non-hydrolysed urine samples and □ = data for the hydrolysed urine samples (mean; $n = 5$).

methyl derivative of buprenorphine, only cross-reacts about 12% with the antibodies. When urine samples were hydrolysed with β-glucuronidase, the apparent buprenorphine concentration increased on average by a factor 20. These observations clearly demonstrate that, to a certain extent, the antibodies are able to recognize the substituents on the phenolic group of buprenorphine. The structurally related compounds etorphine and diprenorphine show small cross-reactions of 4.0% and 1.2%, respectively. It was previously shown that other important drugs such as fentanyl, morphine, naloxone, cocaine, codeine, methadone, pentazocine and phencyclidine are not detectable by the immunoassay at a concentration of 10 μg ml^{-1} [11].

The intra- and inter-assay precisions for three urine samples collected from the test horse with concentrations of 3.1, 1.1 and 0.6 ng ml^{-1} were counted ($n = 3$). The intra-assay precision for the three selected urine samples was 3.4, 4.8 and 8.9%, respectively, and the inter-assay precision was 6.2, 8.2 and 10.6%, respectively.

The accuracy of the test was examined by comparing the results obtained by LC (x) with those obtained by the RIA (y). Three urine specimens from the test horse, with measurable concentrations for the LC method, were analysed ($n = 3$). The results of the two methods were related by the regression line $y = 0.68 + 1.72\ x$. The minimum detectable dose of the assay was assessed with a one-sided Student's t-distribution test [14]. During the analysis of several equine urine samples, large fluctuations for the results

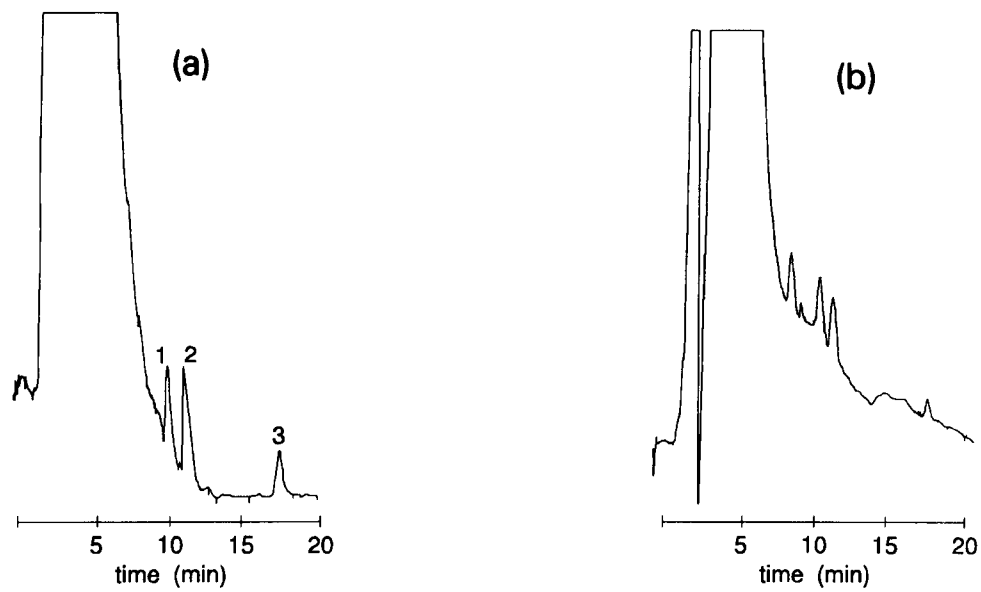

Fig. 6. (a) Liquid chromatogram of spiked urine sample, containing internal standard (1), buprenorphine (2) and N-desalkyl-buprenorphine (3), 1 ng ml^{-1} of each. (b) Liquid chromatogram of an equine urine sample after the administration of 300 μg of buprenorphine to a test horse.

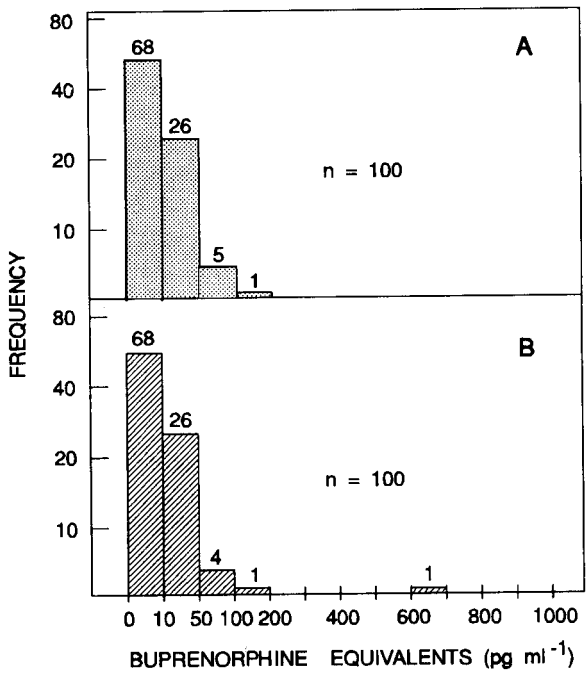

Fig. 7. Buprenorphine equivalents of 100 post-race equine urine samples determined (A) before and (B) after hydrolysis.

for the blank urines were observed. In some blank urines, apparent buprenorphine concentrations ranged from 50 to 100 pg ml^{-1}, probably as a result of endogenous materials. For this reason, the minimum detectable dose of the assay is higher than for human urine samples. Two populations (0 and 100 pg ml^{-1} tubes) were subjected to a "one-sided" Student's t-distribution test ($P = 0.01$, 18 degrees of freedom). It was found that a 100 pg ml^{-1} sample was significantly different from a blank sample in 99% of the cases.

The urine samples from the test horse were assayed by the RIA and the results are illustrated in Fig. 5. The large increase in apparent buprenorphine concentration measured after hydrolysis of the samples makes it possible to detect buprenorphine for at least 24 h after administration of 300 μg. This is fully sufficient for routine post-race drug screening.

The results for the urine samples from the test horse were confirmed with the LC–ED method. Figure 6a shows a chromatogram of a blank urine sample, spiked with internal standard (N-ethyl-

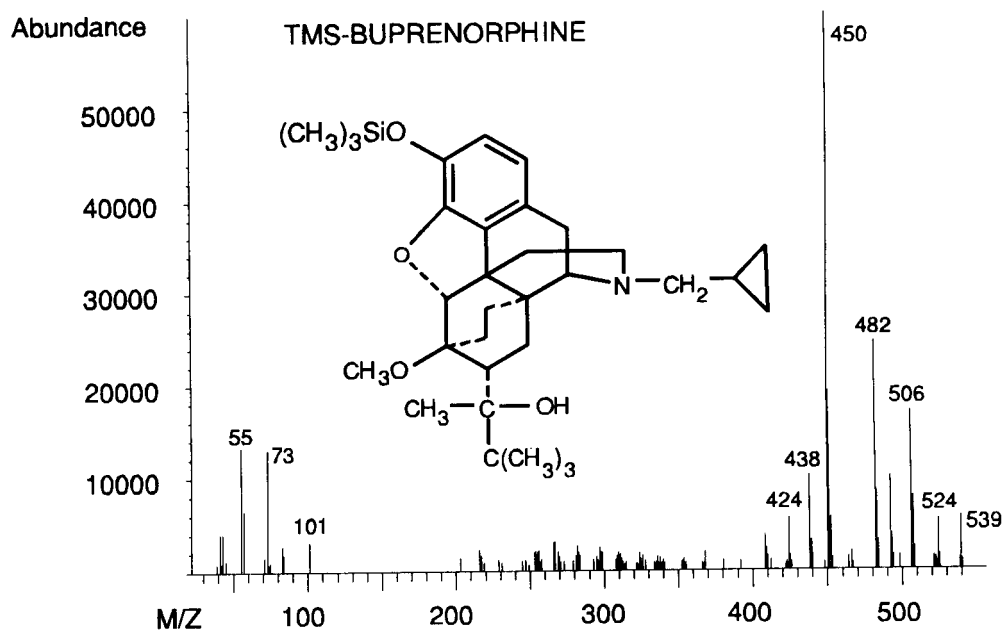

Fig. 8. Full-scan mass spectrum of the TMS derivative of buprenorphine.

buprenorphine), buprenorphine and *N*-desalkylbuprenorphine (1 ng ml^{-1} of each). The internal standard, buprenorphine and the metabolite *N*-desalkylbuprenorphine elute at 10.3, 11.6 and 17.9 min, respectively. In Fig. 6b, a chromatogram is shown of an extract obtained from an unhydrolysed urine sample collected 1 h after the administration of 300 μg of buprenorphine. The concentrations of unchanged buprenorphine and *N*-desalkylbuprenorphine are 1.0 and 0.4 ng ml^{-1} respectively. Because of the huge amounts of co-extracted impurities, it was impossible to apply the LC–ED method to urine samples previously hydrolysed with β-glucuronidase.

When the RIA was applied to a large population ($n = 100$) of post-race equine urine samples, a higher background interference was observed than for human urine samples. The results of the

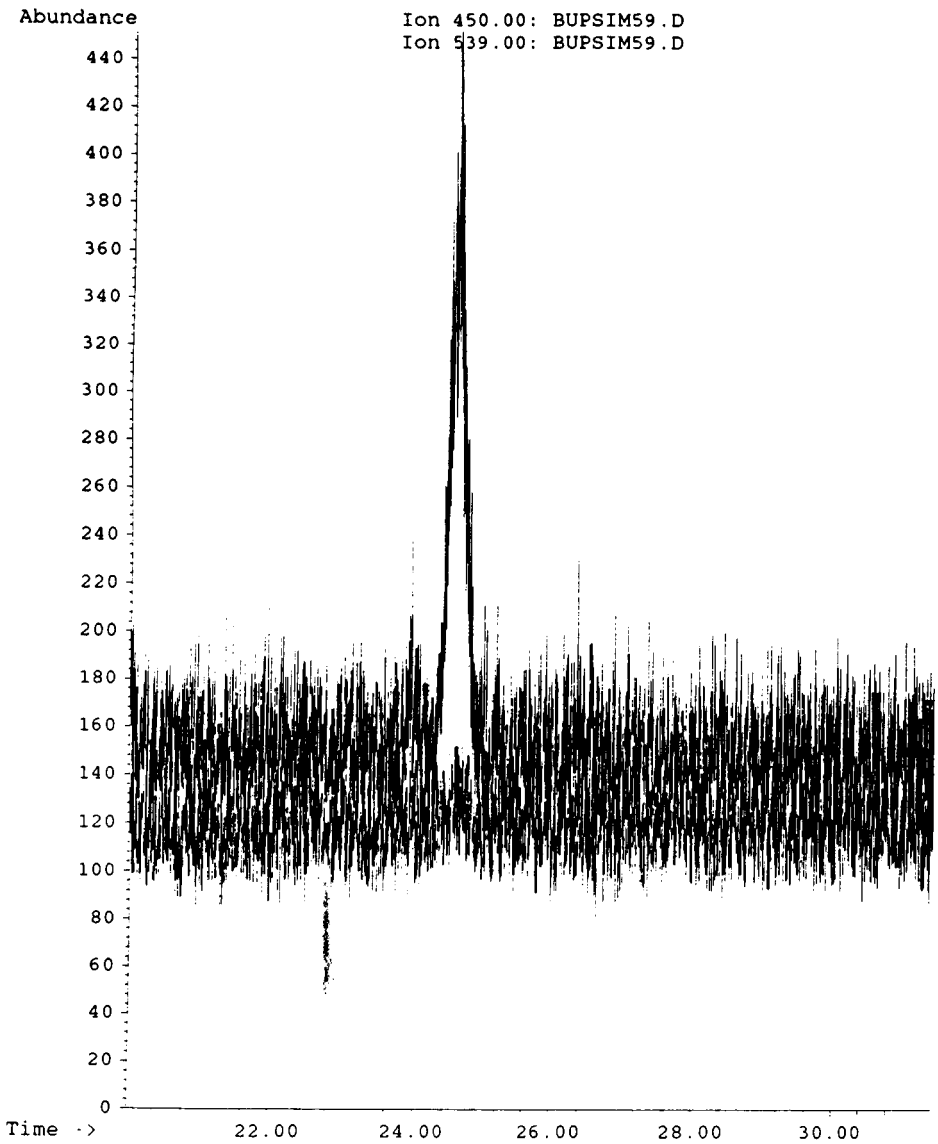

Fig. 9. SIM chromatogram of an unknown equine urine sample.

RIA screening of 100 post-race urine samples are illustrated in Fig. 7. The samples were analysed (A) before and (B) after hydrolysis with β-glucuronidase. One sample was found to be positive with the RIA (near the cut-off value) and was examined further. The confirmation with LC was not conclusive. On the other hand, the GC–MS (SIM) method allowed the presence of buprenorphine in the sample to be confirmed. The sample was first submitted to enzymatic hydrolysis and extracted with toluene as described previously [9]. It was further shown that an additional purification step by LC did not provide advantages over liquid–liquid extraction.

The full-scan mass spectrum of trimethylsilyl-buprenorphine is illustrated in Fig. 8. The molecular ion peak (M) is at m/z 539 ($C_{32}H_{49}NO_4Si$). The mass spectrum is closely related to that of buprenorphine (MW 467, $C_{29}H_{41}NO_4$) and shows significant peaks at m/z 55, 57, 101, 438 (M − 101; loss of $C_6H_{13}O$), 450 (M − 89; loss of CH_3OH and C_4H_9) and 482 (M − 57; loss of C_4H_9). Figure 9 shows the signal obtained with SIM of the unknown equine urine sample; this signal corresponds to ca. 0.4 ng ml^{-1}. The recovery with the GC–MS procedure was assessed by spiking drug-free equine urine samples with 1 ng ml^{-1} buprenorphine and comparing the area under the curve with that for an injected standard solution of 1 ng ml^{-1} buprenorphine. A yield of 52% (S.D. 8%; $n = 5$) was obtained.

DISCUSSION

The methods described permit the detection of buprenorphine in equine urine at levels down to 100 pg ml^{-1}. The sensitivity of the procedure allows the detection of buprenorphine in equine urine up to 24 h after the intravenous administration of 300 μg of the drug. The detectability of the LC method is limited to 0.2 ng ml^{-1} of unchanged buprenorphine. This method can be used to detect buprenorphine up to 5 h after the administration of 300 μg (Fig. 5), while the GC–MS procedure allows the confirmation of buprenorphine up to 24 h after administration. Although it was expected that an intravenous injection of 300 μg of buprenorphine would induce the specific locomotor response, the observed locomotor activity was very low for this dose. Higher concentrations of buprenorphine are probably necessary to obtain the same effects as those described for an intravenous injection of etorphine (100 μg per horse). However, two comments can be made concerning the very low concentration detected in the positive field sample. First, the precise moment of collection following the administration of buprenorphine and also the dose that was given were not known. On the other hand, the actual experiments were conducted on an aged horse, which may react differently to the same dose in comparison with thoroughbred horse. The results of the field study on post-race equine urine samples confirmed the assumption that buprenorphine is being used as a doping agent for horses.

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Methods for the assay of chloramphenicol and two of its metabolites in broiler tissues using two liquid chromatographic methods

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Abstract

The extraction of chloramphenicol (CAP) and of its two metabolites, deacetylchloramphenicol (NAPD) and chloramphenicol glucuronide (CAPG), from fortified broiler tissues (liver, kidney, fat, muscle, skin) and two liquid chromatographic (LC) methods for their determination were developed. The first method allows the simultaneous assay of CAP and CAPG after purification using an automatic advanced sample preparation system. The quantification limits range between 0.250 and 0.050 $\mu g\ g^{-1}$ and the detection limits from 0.015 to 0.080 $\mu g\ g^{-1}$. In the second method, for CAP and NAPD only, the processes are based on liquid–liquid extraction using the physico-chemical properties of the active compounds and their behaviour versus solvents. Reversed-phase LC separation with an ion-pair mobile phase is applied. The values obtained for the quantification limit are 0.050 or 0.100 $\mu g\ g^{-1}$ and the detection limits range from 0.015 to 0.025 $\mu g\ g^{-1}$. Recoveries were established by comparison of results obtained for a 0.500 $\mu g\ ml^{-1}$ standard and those obtained with tissues fortified at 0.05, 0.100, 0.500, 1.00 and 10 $\mu g\ g^{-1}$ (ten assays per concentration), and ranged between 64% and 95%, depending on the tissue.

Keywords: Liquid chromatography; Broilers; Chloramphenicol; Extraction; Tissues

From a public health viewpoint, complete information about the pharmacokinetics of chloramphenicol in broilers is desirable, because for this drug the maximum residue level recommended by the council regulation EEC No. 675/92 is 10 $\mu g\ kg^{-1}$ for meat. As there was no information about the fate of chloramphenicol (CAP) in broilers, it was necessary, before undertaking residue studies in broilers, to have a sensitive method for the determination of this compound and two of its metabolites, deacetylchloramphenicol (NAPD) and chloramphenicol glucuronide (CAPG), in broiler tissues. The aim of this study was to develop a suitable method for the qualitative and quantitative measurement of these three compounds.

Although physico-chemical methods for pharmacokinetic and residue analysis of choramphenicol and its degradation products have already been reported for dairy cow milk and tissue residues [1], rat urine [2] and blood [3], the two methods developed here used either the automatic advanced sample preparation system (AASP) or liquid–liquid extraction. The first method (method 1) allows the simultaneous assay of CAP and CAPG after purification using an AASP system. The second (method 2) allows the assay of CAP and NAPD only. The processes are

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based on liquid–liquid extraction and reversed-phase liquid chromatographic (LC) separation with an ion-pair mobile phase.

EXPERIMENTAL

Apparatus and reagents

A Jouan K81 centrifuge, a rotatory Heildolph-type magnetic stirrer, a vortex-type magnetic stirrer and an electric chopper (Black and Decker) or a manual rasp (Mouli-grater, Moulinex) were used.

The liquid chromatograph consisted of a Varian Model 5020 gradient system with a 200-μl sample loop, a Varian Model 2050 variable-wavelength UV detector set at 278 nm, a 4 mm × 4 mm i.d. precolumn packed with RP-18 (LiChrospher 100, end-capped, 5 μm; Merck), a 12.5 cm × 4 mm i.d. column packed with RP-18 (LiChrospher, end-capped, 5 μm) and a Model D2500 computer.

Two mobile phases were used: for method 1, diammonium hydrogenphosphate (0.66 g l^{-1})–methanol (73 + 27) of pH 7.98 ± 0.01; and for method 2, sodium octylsulphate (0.002 M in 0.2% sulphuric acid)–acetonitrile–methanol (71 + 14.5 + 14.5).

The pneumatic AASP preparation set (AASP PrepStation) and cassettes for storage of samples,

TABLE 1

Extraction diagram of CAP and CAPG from tissues by method 1

Parameter/step	Tissue		
	Muscle	Liver, kidney	Fat, skin
Sample weight	1 g	1 g	1 g
Fortification volume	1 ml	1 ml	1 ml
Incubation time	30 min	30 min	30 min
Incubation temperature	Ambient	0°C	37°C
Extraction			
Temperature	Ambient	0°C	Ambient
Add	None	1 ml $CoCl_2$ (10 g l^{-1})	None
Mix for 10 s	No	Yes	No
Add 1.5 ml of 8 M urea. Mix for 10 s with vortex mixer.			
Allow to settle for 15 min.			
Add 2 ml of water. Mix for 10 s with vortex mixer.			
Centrifuge for 10 min at 4000 *g*.			
For fat and skin: discard the layer of fat on the surface of the aqueous layer.			
Transfer 2 ml of the supernatant into a haemolysis tube.			
Add 400 μl of 1 M oxalic acid. Shake.			
Place the tube in a refrigerator (4°C) or in ice (Kidney, Liver).			
Allow to settle for 5–10 min. Centrifuge for 5 min at 4000 *g*.			
Drop the supernatant on to the cassette containing cartridges packed with C_{18}.			
Purification on the cassette			
Preconditioning of the cassette:			
Put the cassette on the pneumatic station.			
Drop 0.5 ml of THF on to each cartridge. Elute under pressure (100 kPa).			
Drop 0.5 ml of acetonitrile. Elute under pressure (100 kPa).			
Drop 1 ml of ultra-pure water. Elute under pressure (100 kPa).			
Add 0.250 ml of Tris buffer (0.1 M, pH 7). Elute under pressure (100 kPa).			
Pour simultaneously 0.250 ml of Tris buffer + 1 ml of tissue extract.			
Gently mix the sample and the buffer with a Pipetman (before being drawn through the sorbent).			
Elute under pressure (100 kPa).			
Transfer the cassettes into the AASP system.			

containing ten separate cartridges (packed with C_{18}, Al 12229002), were obtained from VARIAN.

All chemicals were of analytical-reagent grade (Merck).

Standard solutions. Weigh 25 mg of each of the three compounds (NAPD, Le Petit batch 4540015/8; CAP, Le Petit batch 426025/8; CAPG, Sigma C9899) into a 50-ml volumetric flask. Add 2.5 ml of methanol, shake to effect complete dissolution and adjust the volume to 50 ml with ultra-pure water. Dilute aliquots of this stock standard solution with ultra-pure water to give working standard solutions with concentrations ranging from 10 to 0.05 $\mu g\ ml^{-1}$.

Tissue grinding. Take about 20 g of thawed control tissue (muscle, liver, kidney, fat), cut it into small parts and grind for 30 s. For skin, rasp about 20 g of frozen tissue (−80°C) with a manual rasp. Distribute 1-g amounts, exactly weighed, into centrifugation tubes fitted with glass caps. Deep freeze at −20°C pending fortification.

Tissue fortification. The tissue fortifications were done every day on aliquot fractions of tissues according to the procedure described in Table 1. For the control tissue, 1 ml of ultra-pure water was added to the ground tissue.

Extraction and purification

In method 1, proteins in the ground tissues were denatured with 8 M urea. After elimination of the urea using oxalic acid, the two compounds were separated after being deposited on a cartridge packed with C_{18} cassette. Table 1 summarizes the different steps of the extraction for all the tissues.

For method 2, the ground tissues were placed in a basic medium, then extracted with ethyl acetate. After evaporation to dryness and purification with carbon tetrachloride–hexane mixtures, the compounds were dissolved in methanol–water (1 + 4, v/v) (Table 2).

TABLE 2

Extraction of CAP and NAPD from tissues by method 2

Parameter/step	Tissue		
	Muscle	Liver, kidney	Fat, skin
Sample weight	1 g	1 g	1 g
Fortification volume	1 ml	1 ml	1 ml
Incubation time	30 min	30 min	30 min
Incubation temperature	0°C	0°C	37°C
Extraction			
Temperature	Ambient	0°C	Ambient
Add	1 ml water	None	5 ml hexane
Shake	10 s, vortex mixer	No	10 min, Heidolph stirrer
Centrifuge for 10 min at 4000 *g*	No	No	Yes
Discard the hexane phase	No	No	Yes
Recover the aqueous phase	No	No	Yes
Add 1 ml of THF. Mix for 10 s with vortex mixer.			
Add 500 μl of 1 M NaOH. Mix for 10 s.			
Add 5 ml of distilled ethyl acetate.			
Mix with a Heildolph rotatory stirrer at 100 rpm for 15 min.			
Centrifuge for 10 min at 4000 *g*.			
Place 4.8 ml of the upper layer in a round-bottomed flask.			
Evaporate to dryness with a Buchi evaporator.			
Dilute with 800 μl of CCl_4–hexane (1 + 1).			
Add 800 μl of methanol–water (1 + 4).			
Mix for 10 s with a vortex mixer.			
Centrifuge for 10 min at 3500 *g*.			
Inject the supernatant into the loop.			

Validation

The concentration range studied was between 10 and 0.05 $\mu g\ g^{-1}$. The validation of the two methods was performed by confirming linearity coupled with a study of the reproducibility and repeatability after analysis of extracts obtained from fortified tissues. As the response of the detector to these compounds is known, the amounts in fortified samples were calculated by comparing the chromatographic peak areas with

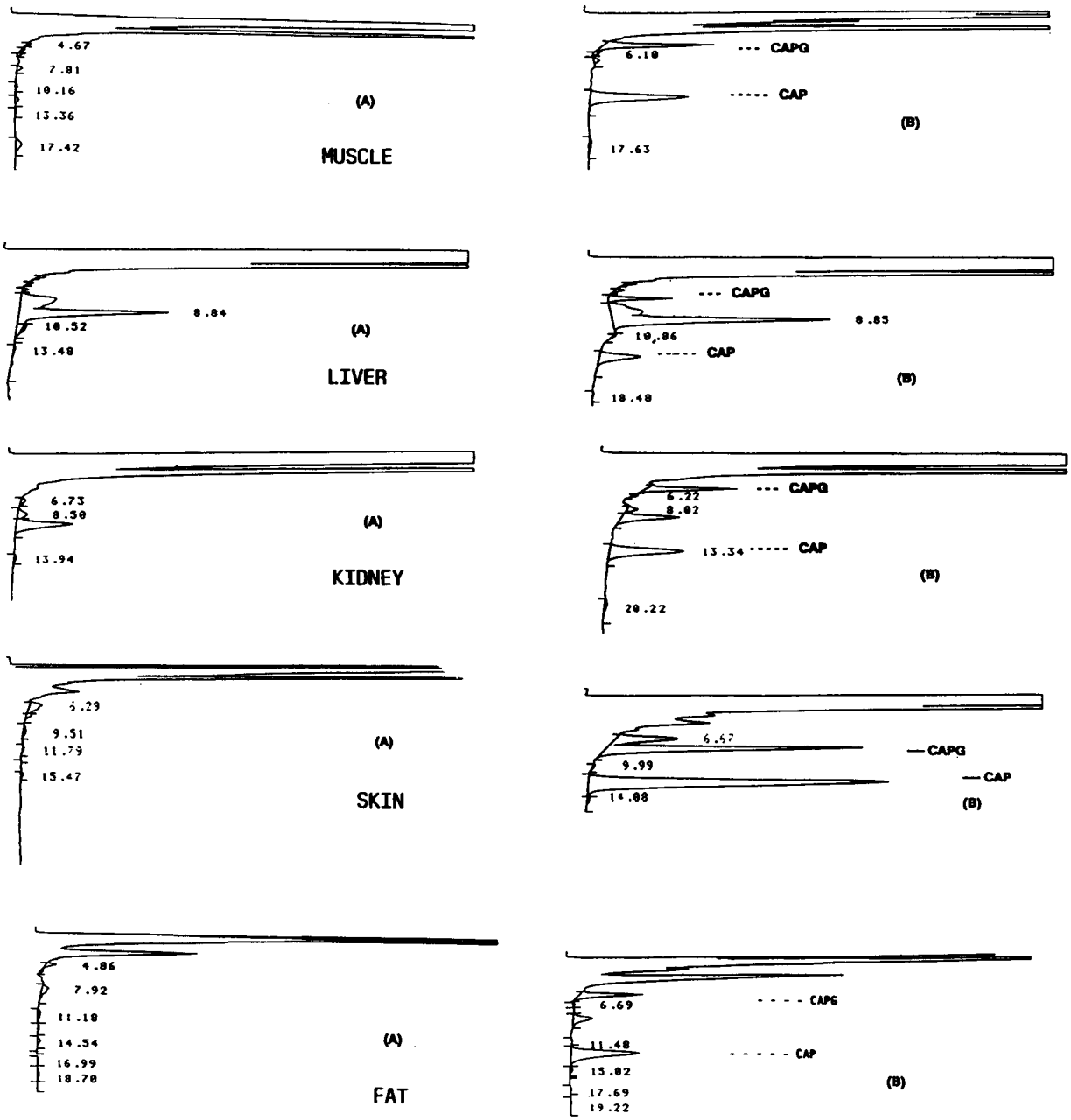

Fig. 1. Chromatograms of (A) control tissues and (B) tissues fortified with CAP and CAPG at 1 $\mu g\ g^{-1}$ (method 1).

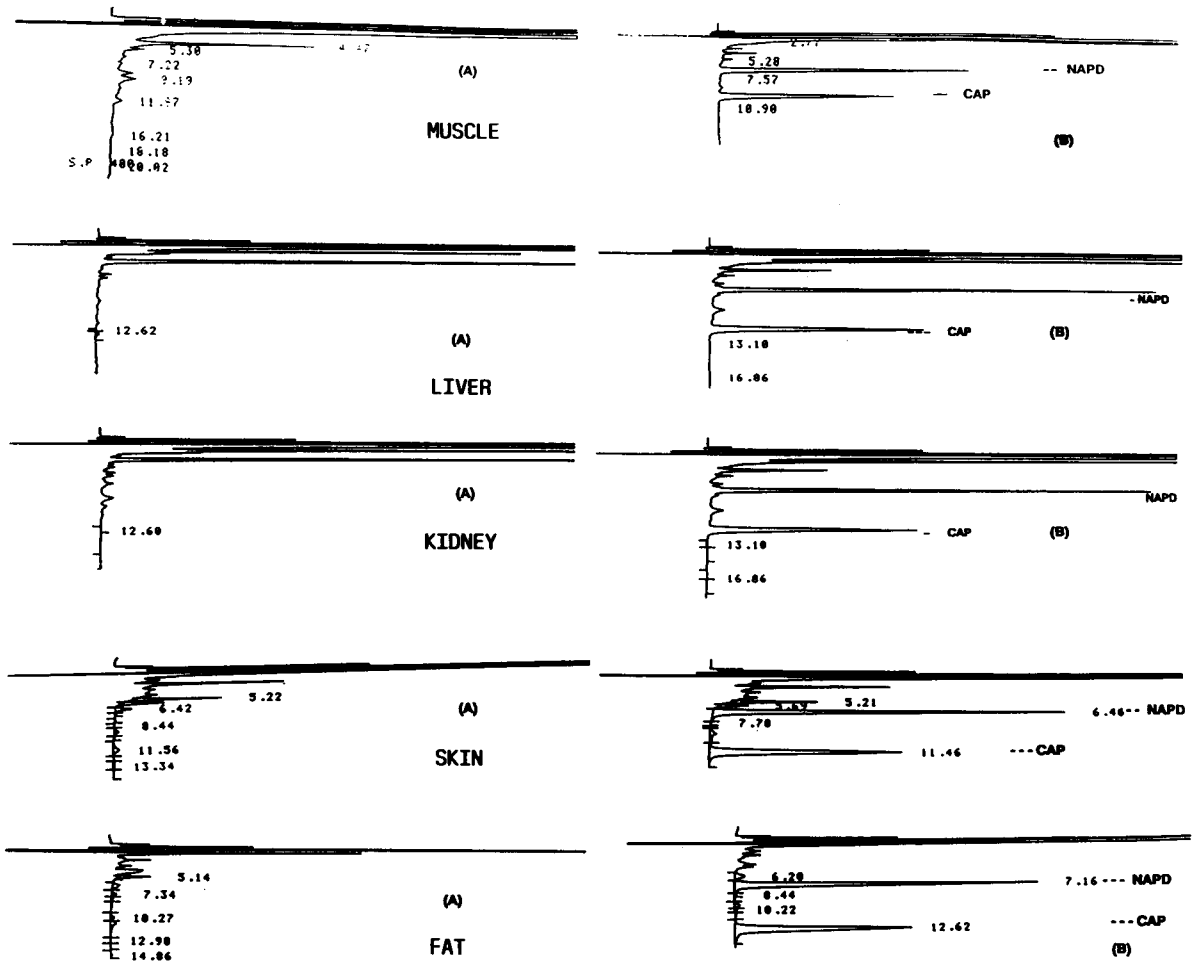

Fig. 2. Chromatograms of (A) control tissues and (B) tissues fortified with CAP and NAPD at 1 $\mu g\ g^{-1}$ (method 2).

TABLE 3

Confidence intervals (expressed in %) obtained for the concentration range studied (0.05–10.0 $\mu g\ g^{-1}$) [a]

Tissue	Method 1		Method 2	
	CAP1	CAPG1	CAP2	NAPD2
Muscle	20% ≥ 0.10 30% < 0.10	20% ≥ 0.250 50% < 0.250	20%	20%
Kidney	20% ≥ 0.10	20% ≥ 0.20	20%	20% ≥ 0.1 30% < 0.1
Liver	20% ≥ 0.265	20% ≥ 0.200	25%	20% ≥ 0.10
Fat	20%	25% ≥ 0.250	20% ≥ 0.100 30% < 0.100	20%
Skin	15% ≥ 0.10 30% < 0.10	20%	20%	20%

[a] ≥ means confidence intervals valid for concentrations higher than or equal to the value indicated; < means confidence intervals valid for concentrations lower than the value indicated. When no sign is given, the confidence intervals are valid for the whole concentration range studied.

TABLE 4

Quantification (QL) and detection limits (DL) ($\mu g\ g^{-1}$)

Tissue	Method 1				Method 2			
	CAP1		CAPG1		CAP2		NAPD2	
	Q	D	Q	D	Q	D	Q	D
Muscle	0.050	0.025	0.100	0.035	0.050	0.025	0.050	0.025
Kidney	0.185	0.055	0.200	0.050	0.050	0.015	0.050	0.015
Liver	0.265	0.080	0.200	0.050	0.050	0.015	0.100	0.015
Fat	0.050	0.015	0.250	0.030	0.050	0.020	0.050	0.010
Skin	0.050	0.015	0.100	0.030	0.050	0.020	0.050	0.010

that of a standard. These tests were carried out by the same technician using the same equipment on different days or with new cassettes.

Statistical analysis

Statistical analysis of the measured peak areas was performed to verify the linearity and the sensitivity [4]. Repeatability and reproducibility were tested according to the recommendations of the norm NF X 06–041 [5]. The detection limit and the quantification limit were calculated as recommended by Aarons et al. [6].

For method 1, the calculation of different parameters was done by comparing the peak area obtained for the fortified tissue with that measured for the fortified tissue at 1 $\mu g\ g^{-1}$ which is the mean concentration in the range of concentrations tested. For method 2, these parameters were calculated by comparing the measured peak area with the peak area of the corresponding standard injected into the loop.

The recoveries were evaluated by comparison of the peak areas of fortified tissues and standards. For these calculations, weighing factors were used [6].

RESULTS

Specificity

Some examples of the chromatograms obtained for control and fortified tissues are shown in Figs. 1 and 2. The chromatograms of the control tissues do not show any interferents with similar retention times to those found for the investigated compounds. The retention times of the tested compounds are only approximate times; as the LC system was not thermostated, they vary according to the atmospheric conditions.

Fidelity

The results of the fidelity study are expressed as confidence intervals. Generally, 20% could be in nearly all instances. A summary of the results is given in Table 3.

TABLE 5

Recoveries (%) [a]

Tissue	Method 1		Method 2	
	CAP1	CAPG1	CAP2	NAPD2
Muscle	61.27 ± 4.82	72.67 ± 11.7	99.12 ± 7.24	69.70 ± 7.06
Kidney	71.36 ± 3.48	78.60 ± 2.36	94.84 ± 6.82	71.16 ± 5.81
Liver	64.67 ± 3.99	70.44 ± 6.84	88.21 ± 7.15	68.19 ± 6.38
Fat	81.50 ± 7.10	94.11 ± 16.92	98.40 ± 7.10	81.96 ± 4.55
Skin	78.19 ± 5.40	71.18 ± 4.79	96.27 ± 6.33	80.24 ± 4.28

[a] Means ± standard deviations ($n = 50$).

Limits of quantification

The limits of quantification were evaluated from the linear calibration graphs obtained for the fortified tissues for each of the active compounds. They are defined as $10 \times$ S.D.(min)/slope, where S.D.(min) is the standard deviation obtained for the measurement of the peak area of the lowest concentration tested. The results are given in Table 4 and can be summarized as follows: CAP1, 0.050 $\mu g\ g^{-1}$ for muscle, fat and skin, 0.185 $\mu g\ g^{-1}$ for kidney and 0.265 $\mu g\ g^{-1}$ for liver; CAPG1, 0.100 $\mu g\ g^{-1}$ for muscle and skin, 0.200 $\mu g\ g^{-1}$ for liver and kidney, and 0.250 $\mu g\ g^{-1}$ for fat; CAP2, 0.050 $\mu g\ g^{-1}$ for all tissues; and NAPD2, 0.050 $\mu g\ g^{-1}$ except for liver (0.100 $\mu g\ g^{-1}$).

Limits of detection

The limits of detection depend on the tissue. They were evaluated from the linear calibration graphs obtained for the fortified tissues for each of the active compounds. They are defined as equal to $3 \times$ S.D.(min)/slope, where S.D.(min) is defined as above. The results are given in Table 4, and can be summarized as follows: CAP1, 0.015–0.080 $\mu g\ g^{-1}$ (the highest value corresponding to liver); CAPG1, 0.030–0.050 $\mu g\ g^{-1}$; CAP2, 0.015–0.025 $\mu g\ g^{-1}$; and NAPD2, 0.010–0.025 $\mu g\ g^{-1}$.

Recovery

For each concentration, ten assays of fortified tissue and five assays of standard were carried out. The results are given in Table 5.

The average extraction recoveries, evaluated for concentrations above the limit of quantification, were as follows: between 61.27 and 81.50% for CAP1 (the lowest value was obtained for muscle); ca. 75% for CAPG (except for the fat, where it reaches 94.11%); $\geqslant 90\%$ for CAP2; and ca. 70% (muscle, liver, kidney) and ca. 80% (skin, fat) for NAPD2.

Conclusion

The proposed methods are suitable for the detection of CAP, CAPG and NAPD in tissues of broilers treated with chloramphenicol. They cannot determine the analytes at the maximum residue level (10 $\mu g\ kg^{-1}$ for meat) but are suitable for evaluating the kinetics of these compounds in broiler tissues after administrations of chloramphenicol.

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Detection of streptomycins in raw milk by an antibody-capture immunoassay

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Abstract

An indirect competitive enzyme-linked immunosorbent assay carried out as an antibody-capture test was developed for the determination of streptomycins in milk. The antiserum was raised in rabbits using streptomycin linked to a bacterial protein as immunogen. The antibodies were concentrated by threefold ammonium sulphate precipitation and reacted equally with streptomycin and dihydrostreptomycin. No other cross-reactions were observed. To perform the test microtitre plates were coated overnight with streptomycin (1 mg ml^{-1}). The milk to be examined was skimmed and treated with oxalic acid. Antiserum and milk samples were mixed and incubated for 1 h. Depending on the amount of streptomycin or dihydrostreptomycin in the sample, more or less antibody molecules remain free to be bound to the streptomycin coat. The sensitivity of the test is better than 1.6 $\mu g\ kg^{-1}$. Quantification is possible up to 100 $\mu g\ kg^{-1}$ without sample dilution.

Keywords: Enzymatic methods; Immunoassay; Antibiotics; Antibody-capture test; Enzyme-linked immunosorbent assay; Milk; Streptomycins

Streptomycins are routinely applied in the intramammary treatment of bovine mastitis. The withholding period after treatment is based on a concentration range of residues in milk between 100 and 200 $\mu g\ kg^{-1}$ (ADI concept). Widely used microbiological inhibitor tests with *Bacillus stearothermophilus* var. *calidolactis* or *Streptococcus thermophilus* have detection limits between 500 and 10000 $\mu g\ kg^{-1}$ and can only meet the requirements for the technological safety of milk [1].

The Codex Committee on Residues of Veterinary Drugs in Food (CCRVDF) has suggested maximum residue limits (MRLs) for a number of antibiotics, which are based on toxicological evaluations. MRLs for streptomycin and dihydrostreptomycin will be discussed in the future [Joint FAO/WHO Expert Committee on Food Additives and Contaminants (JECFAC)] [2]. Results are expected in 1993 at the level of 100 $\mu g\ kg^{-1}$ (FDA safe level = 125 $\mu g\ kg^{-1}$) [3].

The aim of this study was to develop a rapid and sensitive screening method for the detection of streptomycins in milk to be used in routine laboratories for the examination of large numbers of samples.

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EXPERIMENTAL

Antibodies

Antisera were raised in Zika-hybrid rabbits (3-month-old females) using a streptomycin–bacterial protein conjugate (undisclosed composition; Charm Sciences, Malden, MA) as vaccine, applicated in ascending concentrations from 100

to 400 μg per animal. Additionally the vaccine contained Alu Gel S (Serva, Heidelberg, Germany), complete Freund's adjuvant (Difco, Detroit, MI) for the first injection and incomplete Freund's adjuvant (Difco) for further injections. Intracutaneous injections (multiple point at the back) were made at weekly intervals for 4 weeks. Booster injections were performed monthly with a vaccine containing 300 μg of immunogen. Sera were collected 10 days after the last immunization. Antibodies were concentrated by threefold ammonium sulphate precipitation [4].

Sample preparation

Raw milk samples of 10 ml each were skimmed by centrifugation (10 min at 2200 *g*). To 5 ml of skimmed milk 556 μl of 4% (w/v) oxalic acid (Merck) were added with subsequent gentle shaking at room temperature for 10 min. After centrifugation (20 min at 2200 *g*), to 2 ml of the clear supernatant 60 μl of 1 mol l^{-1} NaOH (Merck) and 50 μl of Tween 20 (2.1 mmol l^{-1}) in distilled water (Merck–Schuchardt, Hohenbrunn, Germany) were added. Standards were prepared by adding dihydrostreptomycin sesquisulphate · 3/2 H_2SO_4 (Sigma, St. Louis, MO) to antibiotic-free bulk milk (research farm) at concentrations of 1.6, 3.1, 6.3, 12.5, 25, 50 and 100 μg kg^{-1}. Standards were prepared as indicated above. The prepared samples could be stored at 4°C for several (4–5) days and at −18°C for up to 6 months.

Enzyme-linked immunosorbent assay

Microtitre plates (Nunc Maxi Sorp) (Nunc, Roskilde, Denmark) were coated overnight at 37°C with 100 μl per well of dihydrostreptomycin (1 mg ml^{-1} in 0.1 M carbonate buffer, pH 9.6). Blocking was performed with 0.5% (w/v) chicken albumin (Sigma, grade III) in 0.01 M phosphate-buffered saline (PBS)–0.5 M NaCl–0.05 mM Tween 20 (pH 7.4) (300 μl per well) at 37°C for 45 min. Prepared samples (300 μl) and antibodies diluted in PBS (as above, but without chicken albumin) (50 μl) were mixed to give a final concentration of antibodies of 1:3000 and were incubated at room temperature for 1 h on a rotary shaker (250 rpm). Neutralized samples (100 μl per well) were transferred to the microtitre wells and incubated at 37°C for 90 min to allow free antibodies to be immobilized on the dihydrostreptomycin coat. A pig anti-rabbit–antibody enzyme conjugate (alkaline phosphatase; Dakopatts, Glostrup, Denmark) diluted 1:1000 (v/v) in PBS (100 μl per well) was added and incubated at 37°C for 90 min. As a chromogenic substrate, 150 μl per well of 1 mg ml^{-1} nitrophenyl phosphate (Merck) in 0.1 M hydrogencarbonate buffer–0.001 M $MgCl_2$ (pH 9.8) were added and incubated for 1 h at 37°C. The absorbance was measured at 405 nm (SLT 400 AT ELISA reader).

Four thorough washings of the microtitre plate were performed between each step of the procedure using 0.5 M NaCl and 0.05 mM Tween 20 in distilled water.

The titres of the antisera were determined using a direct ELISA with dihydrostreptomycin as coating and the anti-rabbit–antibody enzyme conjugate for antibody detection as indicated above. Specificity was tested with the same direct ELISA using different inhibitors as coating substance. All serum fractions during the immunization schedule were tested in the same way.

Evaluation

To compensate for fluctuations in different tests, the relative absorbance (% absorbance) was calculated (absorbance of zero sample = 100%). Positive and negative samples were discriminated by using three times the standard deviation (3*s*) of analytical results referring to zero samples.

RESULTS

Antibodies

Antibodies used in the test reached titres up to 1:64 000 after seven booster injections. The specificity was enhanced by boosting. Inhibitors tested for cross-reactivity were as follows (cross-reactivity in %): dihydrostreptomycin 100.0, streptomycin 90.0, tobramycin 1.7, paromamycin 1.8, oxytetracycline 1.7, polymyxin B 1.9, gentamicin 1.8, neomycin 1.7, kanamycin 1.8, penicillin G 1.7, ampicillin 1.8, tetracycline 1.9, erythromycin 1.8, chloramphenicol 2.0 and sul-

TABLE 1

Repeatability determined using 20 independently prepared milk samples containing different concentrations of dihydrostreptomycin ($\mu g\ kg^{-1}$) (all results % absorbance)

Parameter [a]	Concentration ($\mu g\ kg^{-1}$)							
	0	1.6	3.1	6.3	12.5	25	50	100
$\bar{x}_A$	98.9	50.6	29.5	20.5	15.5	11.7	8.6	6.2
s_A	5.7	3.1	2.2	1.6	1.5	1.1	1.0	0.8
C.V. (%)	5.8	6.1	7.5	7.8	9.7	9.4	11.6	12.9

[a] $\bar{x}_A$ = arithmetic mean; s_A = standard deviation; C.V. = coefficient of variation.

TABLE 2

Discrimination ranges for positive and negative results applying three times the standard deviation (all results % absorbance)

Parameter [a]	Dihydrostreptomycin ($n = 20$)		Streptomycin ($n = 12$)	
	0 $\mu g\ kg^{-1}$	1.6 $\mu g\ kg^{-1}$	0 $\mu g\ kg^{-1}$	1.6 $\mu g\ kg^{-1}$
$\bar{x}_A$	98.9	50.6	100.0	66.3
$\bar{x}_A - 3s$	81.8	41.3	82.9	58.2
$\bar{x}_A + 3s$	116.0	59.9	117.1	74.4

[a] $\bar{x}_A$ = arithmetic mean; s = standard deviation.

phadimidine 1.8. The cross-reactivity of dihydrostreptomycin and streptomycin was 90%. The other substances did not react at a significant level.

ELISA

The repeatability of the ELISA was tested with identically prepared and repeatedly examined raw milk samples containing different concentrations of dihydrostreptomycin ($n = 20$ of each) (Table 1).

The discrimination range for positive and negative results was defined using samples containing 1.6 $\mu g\ kg^{-1}$ of dihydrostreptomycin ($n = 20$) or streptomycin ($n = 12$), compared with corresponding numbers of zero samples (Table 2) by applying the $3s$ criterion. It was found that there is a range of relative absorbance values between positive and negative results of 81.8–59.9% absorbance for dihydrostreptomycin and 82.9–74.4% absorbance for streptomycin. According to the low concentrations detectable and the expected MRL of 100 $\mu g\ kg^{-1}$, it was decided to

TABLE 3

Quantitative evaluation of 120 blind samples spiked with different concentrations of dihydrostreptomycin

Spiked concentration ($\mu g\ kg^{-1}$)	n	Classification ranges ($\mu g\ kg^{-1}$)							
		0	1.6	3.1	6.3	12.5	25	50	100
0	30	30	–	–	–	–	–	–	–
1.6	21	–	21	–	–	–	–	–	–
3.1	15	–	–	14	1	–	–	–	–
6.3	12	–	–	2	9	1	–	–	–
12.5	12	–	–	–	–	9	3	–	–
25.0	9	–	–	–	–	–	6	3	–
50.0	12	–	–	–	–	–	–	11	1
100.0	9	–	–	–	–	–	–	–	9

TABLE 4

Evaluation of field samples, performed either for streptomycin or for dihydrostreptomycin as analyte

Proposed analyte	Negatives	Positives ($\mu g\ kg^{-1}$)						
		< 1.6	~ 1.6	~ 3.1	~ 6.3	~ 12.5	~ 25	~ 50
Streptomycin	135	14	18	12	2	7	8	1
Dihydrostreptomycin	135	31	13	3	11	4	–	–

assign samples fitting these ranges as probably being negative.

To confirm the accuracy of the test, spiked samples were used because of the lack of a physico-chemical method of the required sensitivity. One milk sample was divided into 120 aliquots and spiked with different concentrations of dihydrostreptomycin (30 negatives and 90 positives). All aliquots were prepared as blind samples. Qualitative evaluation showed no false-positive or false-negative results. Quantitative evaluation of the samples is given in Table 3.

Field samples

In October 1991, 197 bulk milk samples were taken and examined by the described ELISA. Evaluation was performed either for streptomycin or for dihydrostreptomycin as analyte (Table 4). Because of the cross-reactivity of these two substances, the content of one or the other can only be proposed by comparison with a homologous standard dilution.

DISCUSSION

The method described allows a clear positive–negative classification of raw milk samples containing streptomycins at the level of 1.6 μg kg^{-1}. Quantification is possible up to 100 μg kg^{-1} without sample dilution.

Because of the cross-reactivity of streptomycin and dihydrostreptomycin it is impossible to decide whether the component in a sample is one or the other. The positive–negative classification is not influenced, however, but for quantification the result for a sample has to be compared with a homologous standard dilution. Hence the quantification results will differ if either streptomycin or dihydrostreptomycin is the proposed analyte. Concerning the field samples, the results for dihydrostreptomycin are more probable considering the application of the two antibiotics in veterinary medicine in Germany.

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Determination of dimetridazole residues in bovine tissue by liquid chromatography

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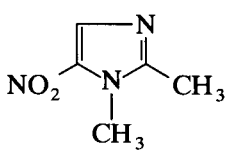
Abstract

An analytical procedure for the detection and identification of dimetridazole (DMZ) residues in muscle tissue is described. The tissue was homogenised with dichloromethane and the extract reduced to a small volume by rotary evaporation. The sample was further cleaned-up by solid phase extraction on silica cartridge and DMZ was eluted with ammonium acetate buffer (pH 4.3). The eluate was extracted with ethyl acetate and the organic phase evaporated in the presence of the mobile phase. DMZ was quantified and confirmed by reversed-phase liquid chromatography with UV–visible diode array spectrophotometric detection at 320 nm. The average recovery from spiked muscle was 84% in the concentration range 10–50 $\mu g\ kg^{-1}$. The confirmation limit was calculated to be 5 $\mu g\ kg^{-1}$.

Keywords: Liquid chromatography; Bovine tissue; Dimetridazole; Muscle tissue

Dimetridazole (DMZ, 1,2-dimethyl-5-nitroimidazole) is a nitroimidazole active against both anaerobic bacteria and protozoal parasites. It is used for the treatment of swine treponemal disentery, histomoniasis in turkeys and coccidiosis in rabbits [1]. In addition to their antimicrobial effects, nitroimidazoles are also useful as growth promotants.



dimetridazole

In the European Community (EC) the nitroimidazole drugs have been approved for use as nutritional feed additives in poultry to control blackhead infection. Concentrations in feed are allowed in the range of 50–200 mg kg^{-1}. Additionally, DMZ is marketed as medicinal feed additive for the control of swine disentery at concentrations of 200–450 mg kg^{-1}.

Dimetridazole is suspected to be carcinogenic and mutagenic and therefore the Commission of European Communities published in March 1992 a Directive with a maximum residue level of 10 $\mu g\ kg^{-1}$ for DMZ in meat, fat, kidney and liver.

Metabolism studies [2] have shown that DMZ is almost totally degraded (particularly in the liver) rather than conjugated with some other groups. As a consequence, very little deposition occurs in the tissues. However, the residue levels could be significant in cases of gross or accidental overfeeding or impaired metabolism and, therefore, a suitable method should be available to the analyst.

Several methods have been developed to quantitate DMZ in feed or meat. In 1970, a spectrophotometric method was published, which was

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able to detect 150 mg kg^{-1} of DMZ in feed [3]. About 10 years later, gas chromatography was used to measure DMZ in feed at the 50 mg kg^{-1} level [4], while liquid chromatographic (LC) methods with UV or electrochemical detection were able to detect DMZ in tissue at concentrations of 10 $\mu g\ kg^{-1}$ and 0.3 $\mu g\ kg^{-1}$, respectively [5,6]. In most cases a combination of multiple liquid–liquid extraction is necessary and requires extensive and time consuming sample clean-up.

The present work proposes a simple and rapid extraction method for quantitation and confirmation of DMZ by LC with UV–visible diode array spectrophotometric detection at the 5 $\mu g\ kg^{-1}$ level.

EXPERIMENTAL

Apparatus

The instruments used were an Ultra Turrax T25 (Janke–Kunkel IKA, Germany), an ultrasonic bath (Selecta, Madrid), a Macrotonic centrifuge (Selecta), a rotary evaporator (Büchi, Flawil, Switzerland), a sample concentrator with nitrogen stream (Techne LD, Duxford) and a Moulinex homogenizer (Moulinex, Bilbao). The LC equipment consisted of a Waters Model 6000A pump, a Waters injector (Model U6K) and a diode array detector (Waters 990). The LC column used throughout this work was a Novapak C_{18} columnn (4 μm) in a radial compression module of Waters. The flow-rate was 1 ml min^{-1}.

Reagents

Water was purified by demineralization (MilliQ, Millipore Bedford, MA). DMZ was obtained from Sigma, acetonitrile (for liquid chromatography) and anhydrous sodium sulphate from Pan-Reac (Montplet and Esteban, Barcelona), ammonium acetate, dichloromethane (for residue analysis) and ethyl acetate (for residue analysis) from Merck) (Darmstadt). A Sep-pak silica cartridge of Waters (Milford, USA) was used. DMZ standard solutions were prepared by dissolving 210 mg of DMZ in 100 ml of methanol. Working standards for LC were prepared in the range of 0.105–0.42 $\mu g\ \mu l^{-1}$ by diluting the standard solution with mobile phase.

The mobile phase solvent for LC was acetonitrile–0.05 M ammonium acetate buffer pH 4.3 (30:70, v/v). The mobile phase was filtered through 0.45-μm Millipore filter membranes.

Note: in order to avoid photolysis of the analyte all DMZ containing vessels were shielded from light.

Procedure

Weigh a tissue sample (10 g) and 50 g anhydrous sodium sulphate into a screw-topped glass centrifuge tube (250 ml) and add 50 ml of dichloromethane. Homogenise the mixture using an Ultra Turrax homogeniser for 10 min and centrifuge at 2800 *g* for 10 min. Transfer the dichloromethane layer to a round-bottomed flask and repeat the extraction of the residue with two further 50 ml portions of dichloromethane. Combine the dichloromethane layers and decrease the volume to approximately 5 ml on a rotary evaporator under vacuum at 30°C. Wash the silica cartridge with 5 ml of a mixture of acetonitrile and water (20:80, v/v), 5 ml of acetonitrile, 5 ml of dichloromethane and dry the pretreated cartridge by means of a gentle stream of nitrogen, during 30 min.

Press the sample extract through the dry pretreated cartridge with a glass syringe. Rinse the round-bottomed flask with 2×5 ml of dichloromethane and apply it through the cartridge. After drying the cartridge with a stream of nitrogen for 30 min, elute DMZ with 7 ml of 0.05 M ammonium acetate (pH 4.3) into a screw-topped amber glass tube. Then add 2 ml of ethyl acetate to the eluate and shake the mixture. Transfer the upper layer to another screw-topped amber glass tube and repeat the extraction with ethyl acetate three times. Evaporate the combined organic portions in presence of 200 μl of mobile phase and fill the aqueous residue up to 1 ml with the LC eluent.

RESULTS AND DISCUSSION

Most of the nitroimidazole extraction procedures described in the literature use an organic

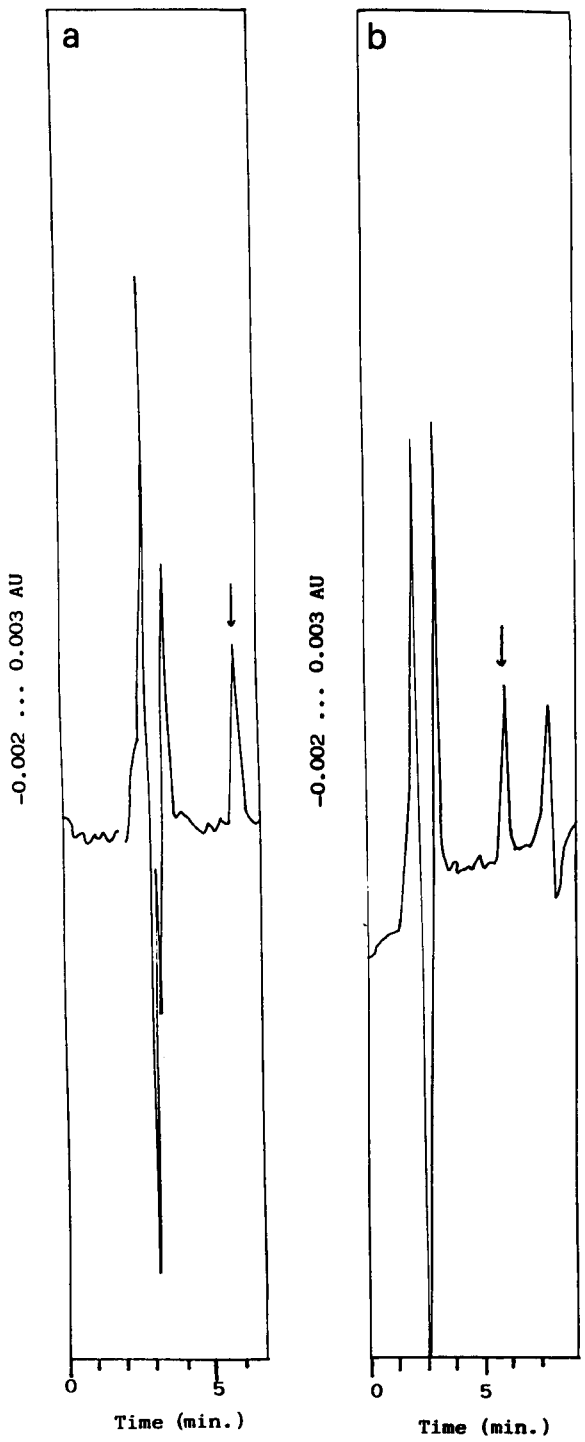

Fig. 1. Chromatograms at 320 nm of 2.6 ng standard DMZ (a) and muscle sample spiked with a corresponding amount (b).

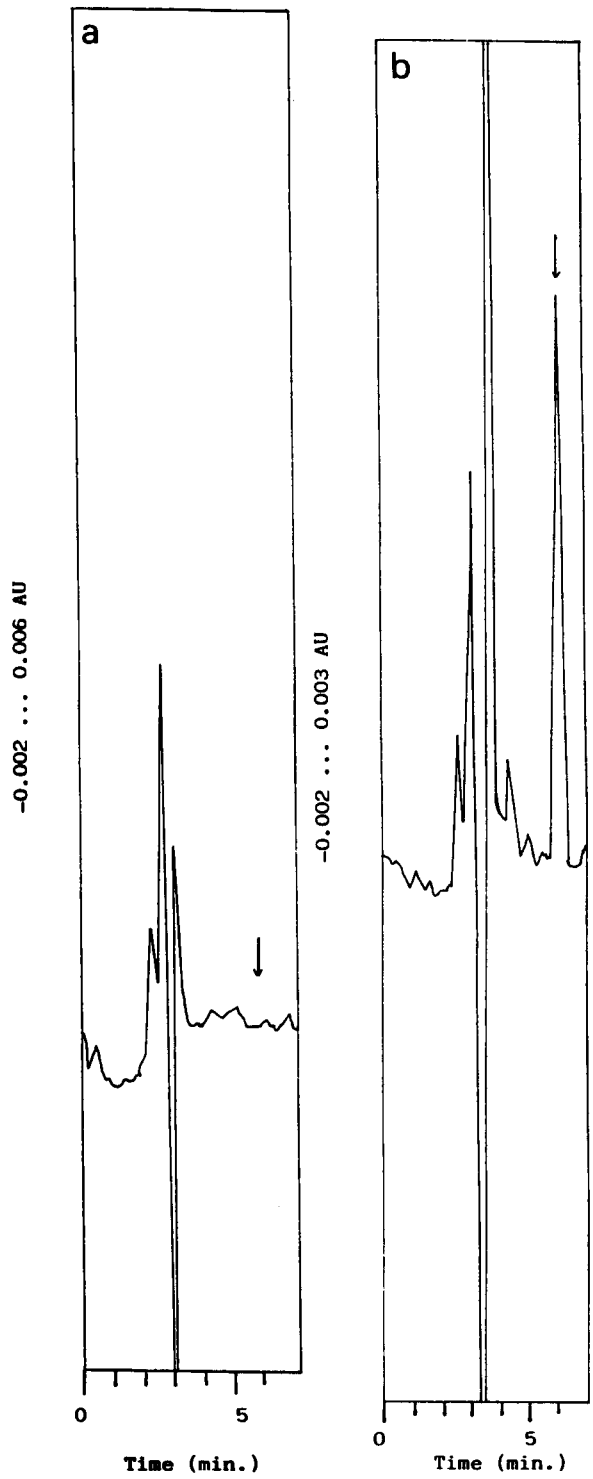

Fig. 2. Chromatograms obtained in the described LC conditions of blank tissue (a) and muscle sample spiked with 21 $\mu g\ kg^{-1}$ (b).

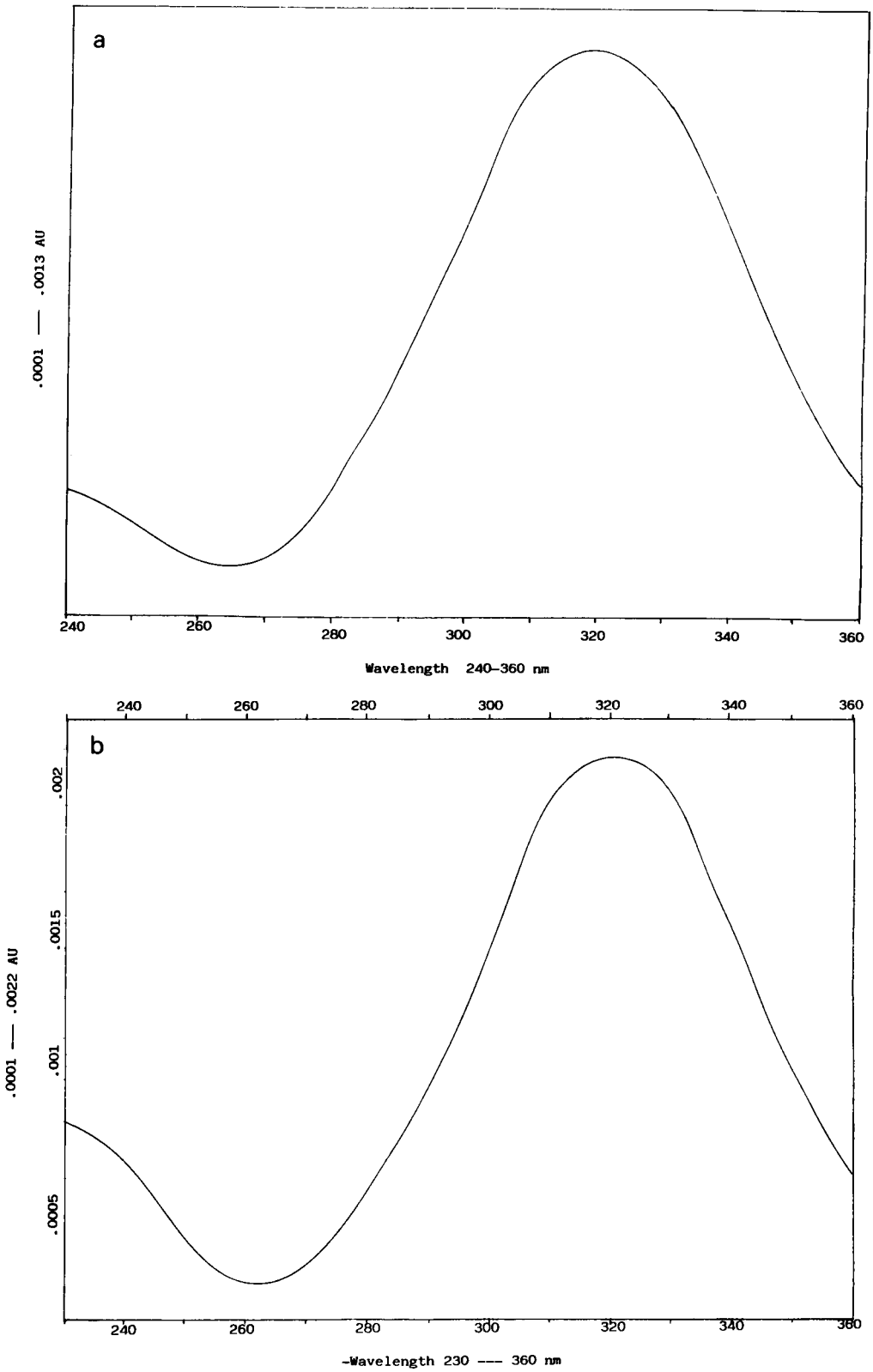

Fig. 3. Spectra between 240 and 360 nm of standard DMZ (5.25 ng) and the spiked sample peak (21 μg kg^{-1}).

solvent, e.g., benzene or dichloromethane from which the drug is then re-extracted into an acidic aqueous solution. Further clean-up is carried out making the aqueous phase alkaline and then again extracted into the organic phase. These extraction procedures may frequently produce emulsions and losses of drug residues. As an alternative, we used an organic extraction with dichloromethane, followed by solid phase extraction. The silica cartridge was used for the purification step, avoiding multiple liquid–liquid extraction and therefore improving the DMZ recovery.

Dichloromethane was selected as extraction solvent because of its relatively low boiling point, which should prevent losses in the evaporation step [7].

The recovery was found to be greatly influenced by an excessive evaporation. For this reason we avoided dryness during the evaporation of the sample extracts. Linearity of response using peak heights at 320 nm vs. injected quantity, was studied by successive injections ($n = 2$) of 50-μl aliquots of working solutions: 0.105, 0.21, 0.315 and 0.42 ng μl^{-1} corresponding to, respectively, 5.25, 10.5, 15.75 and 21 ng injected.

The calibration graph was calculated using the least squares method and can be expressed as: $y = 0.7x - 0.024$, where y = peak height in mm at 0.008 absorbance units full scale; x = ng of DMZ injected.

The linearity was excellent with a correlation coefficient $r = 0.9999$ ($n = 8$). With the proposed method, 2.6 ng DMZ injected can be detected at 320 nm corresponding 5.25 μg kg^{-1} in tissue samples (Fig. 1). As is shown in this figure, at this concentration well-defined peaks were obtained, which indicates a detection limit of ca. 1 μg kg^{-1}. Figure 2 illustrates chromatograms of blank (a) and spiked (b) tissue with 21 μg kg^{-1} DMZ.

For recovery studies blank muscle tissue samples were spiked at levels of 10.5, 21 and 42 μg kg^{-1}. The results are shown in Table 1, and as can be seen, mean recovery at all levels is higher than 75%.

The use of a diode array detector permits confirmation of suspicious peaks, compairing its spectra with a standard spectrum. Figure 3 shows the LC-diode array UV spectrum of 5.25 ng of standard DMZ and the spectrum obtained from the DMZ peak of the spiked sample of Fig. 2b (21 μg kg^{-1}). As can be seen, both spectra are practically identical, confirming the identity of the peak.

TABLE 1

Recovery of DMZ added in methanol solution to muscle tissue

DMZ added (μg kg^{-1})	% Recovery ($n = 6$)		C.V. (%)
	Mean	σ	
10.5	87.2	8.5	9.8
21.0	87.2	8.0	9.2
42.0	78.0	8.1	10.4

With the proposed clean-up procedure, only a few matrix compounds absorb at these high wavelengths. For this reason, the detection and confirmation limits for DMZ in tissue samples, using the proposed method, are the same as for the DMZ standard, and are in the range of 5 μg kg^{-1}.

Conclusion

The proposed method of DMZ determination in bovine muscle by LC after a rapid clean-up procedure based on liquid–liquid and solid phase extraction, permits detection, quantitation and confirmation of DMZ at the maximum residue levels established by the European Community (EC).

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Determination of sulphamethazine in animal tissues by enzyme immunoassay

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Abstract

An enzyme immunoassay for sulphamethazine was developed using an antiserum raised in rabbits by immunization against a sulphamethazine diazo derivative coupled to bovine serum albumin. Horseradish peroxidase coupled to sulphamethazine by a carbodiimide method was selected as a tracer. The dose of sulphamethazine that caused 50% binding inhibition was 3.8 ng per well with a 2-h incubation time at 37°C and 1.2 ng per well with a 16-h incubation time at 4°C. The assay was evaluated by using control and sulphamethazine-fortified muscle and liver samples. These samples were treated by the matrix solid-phase dispersion method for the subsequent isolation of the drug. The method allowed a detection limit well below the tolerance limit (0.1 mg kg^{-1}) generally applied for sulphamethazine.

Keywords: Enzymatic methods; Immunoassay; Animal tissues; Sulphamethazine; Tissues

Sulphonamides have a broad spectrum of antibacterial activity and are frequently incorporated in feeds as a practical method for the prevention or treatment of a variety of so-called "confinement diseases" in animals, mainly pigs. However, there are concerns for human health because of the possible presence of residues of the drug in meat [1]. The US Food and Drug Administration (FDA) requires a 15-day withdrawal period of feed containing sulphamethazine before slaughter and has established its tolerance limit in tissues for human consumption at 0.1 mg kg^{-1} [2–5]. Although different sulphonamide–antibiotic combinations have been approved for use in swine feed, sulphamethazine has been identified as the major problem in ca. 95% of all sulphonamide tissue violations [6]. This paper reports a competitive enzyme immunoassay (EIA) for routine screening of sulphamethazine in tissues.

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EXPERIMENTAL

Reagents and equipment

Sulphamethazine was obtained from Janssen Chimica (Geel/Belgium), sulphaguanidine, sulphachloropyridazine, sulphaquinoxaline, sulphamerazine and sulphadimethoxine from Sigma (St. Louis, MO), sulphanilamide, sulphadiazine and sulphathiazole from Aldrich (Milwaukee, WI),

bovine serum albumin (BSA) from Serva (Heidelberg, Germany), horseradish peroxidase (HRP) from Boehringer (Mannheim, Germany), 3,3′, 5,5′-tetramethylbenzidine (TMB) microwell peroxidase system from Kirkegaard & Perry (Gaithersburg, MD), Tween 20, cytochrome *c* and thimerosal (merthiolate) from Merck (Darmstadt, Germany), aprotinine (trasylol) from Bayer (Brussels), Freund's complete adjuvant from DIFCO (Brussels) and bulk C_{18} (40 μm, 18% load, end-capped) octadecylsilyl-derivatized silica from Analytichem International (Harbor City, CA). All other chemicals were of analytical-reagent grade or better and were used as received. Deionized water was purified with a Milli-Q system (Waters).

Buffers were prepared as follows: (A) carbonate–hydrogencarbonate buffer solution ("coating" buffer): 50 mM pH 9.6 Na_2CO_3 (1.59 g), $NaHCO_3$ (2.99 g) and distilled water (to 1 l); (B) phosphate-buffered saline (PBS)–gelatine solution (0.01 M PBS–0.2% gelatine) (EIA buffer): NaCl (8.6 g), $Na_2HPO_4 \cdot 2H_2O$ (1.6 g), $NaH_2PO_4 \cdot H_2O$ (0.14 g), thimerosal (0.1 g), gelatine (2 g) and distilled water (to 1 l); (C) washing solution: NaCl (8.76 g), Tween 20 (0.5 ml), distilled water (to 1 l); (D) stopping solution: 6 M H_2SO_4.

All stock and working standard solutions were stored at 4°C.

ELISA plates (Nunc-Immuno Plate Maxisorp F96) were obtained from Nunc (Roskilde, Denmark). Absorbance of plate wells was measured with a Multiscan MCC/340 microplate reader from ICN Flow Laboratories.

Immunogen preparation

Sulphamethazine was diazotized and coupled to bovine serum albumin [7,8].

Preparation of the diazo derivative. Sulphamethazine (57 mg, 0.27 mmol) was dissolved in 4 ml of 0.5 M H_2SO_4 and the solution was left overnight with stirring at 4°C. A solution of sodium nitrite (19 mg, 0.27 mmol) in distilled water (1 ml) was added dropwise to this mixture with stirring in the dark at 4°C. The formation of diazotized sulphamethazine was detected by the appearance of a deep yellow colour after reaction with *N*,*N*-dimethylaniline [9]. The formation of the diazo derivative was also monitored by thin-layer chromatography on silica gel 60 Alu Rol sheets (Merck) after migration in the solvent mixture ethyl acetate–ammonia solution–methanol (30 + 1 + 15, v/v/v). After spraying of the sheet with a 0.1% solution of 2,7-dichlorofluoresceine in ethanol (Merck), it appeared that the R_F value of sulphamethazine in this system was 0.79, whereas its diazo derivative did not migrate. The reaction was continued until the disappearance of the sulphamethazine spot. The reaction was stopped by addition of a solution of ammonium sulphamate (7 mg) in water (140 μl).

Coupling to bovine serum albumin. The solution of the diazo derivative was added in 50-μl portions to 4 ml of carbonate buffer (pH 10) containing 100 mg of BSA. The reaction mixture was left for 18 h at 4°C. Unreacted material was separated from the conjugate by chromatography on Sephadex G-50 with 0.05 M ammonium carbonate solution (pH 8). The conjugate was then lyophilized.

Antibody preparation

The immunogen was used to prepare an antiserum. Five rabbits (New Zealand breed) were immunized by the protocol of Vaitukaitis et al. [10] with 1 mg of diazotized sulphamethazine–BSA conjugate in complete Freund's adjuvant. The animals received five injections with an interval between injections of 1 month. After the third injection, blood samples were taken from the ear vein at 10-day intervals to follow the appearance of the antibodies.

The crude antiserum obtained was divided into aliquots and stored at −20°C after dilution in 0.1 M PBS–glycerol mixture (serum–PBS–glycerol, 1 + 9 + 10) corresponding to a final dilution of 1 + 19.

Enzyme conjugate preparation

The sulphamethazine horseradish peroxidase conjugate was prepared by a modified carbodiimide method [11,12]. Sulphamethazine (10 mg) was dissolved in a mixture of pyridine (400 μl) and distilled water (400 μl) and the solution was added to horseradish peroxidase (20 mg) dissolved in distilled water (2 ml). A 10-mg amount

of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was added and the solution was stirred at 4°C for 18 h. Excess of sulphamethazine was eliminated by chromatography on Sephadex G-25 column in PBS buffer; the column was first washed with PBS buffer, the solution was then added to the column and the enzyme conjugate preparation was eluted with 3.5 ml of PBS buffer.

This conjugate was then diluted (1 + 20, v/v) in a buffer containing 80 vol.% of a solution of 75 ml of PBS + 2 g of BSA + 125 ml of glycerol + 20 mg of thimerosal, 20 vol.% of aprotinine and 0.1 wt.% of cytochrome *c*.

Pretreatment of the samples

The muscle and liver samples were treated according to the matrix solid-phase dispersion method [13]. The material (C_{18}, octadecylsilyl-derivatized silica) was washed using a vacuum box successively with 30 ml each of hexane, dichloromethane and methanol to remove contaminants, then 0.5 g of tissue was added to 2 g of the prewashed C_{18} material in a glass mortar. The sample was gently blended with a glass pestle to produce a semi-dry, homogeneous material. This was poured into a syringe barrel column (10 ml) containing two filter-paper discs (Whatman No. 1). Two other filter-paper discs were placed on the top of the material and a syringe plunger was used to compress the sample to a volume of 4 ml. A 100-μl disposable pipette tip was attached to the end of the column to increase the residence time of the eluting solvent in the column. The column was washed with 8 ml of hexane by gravity flow (excess of hexane was removed by applying a positive pressure) and sulphamethazine was eluted with 8 ml of dichloromethane. The eluted fraction was evaporated to dryness under nitrogen and the residue was dissolved in 250 μl of PBS–gelatine (buffer B).

Assay procedure

The sulphamethazine antiserum diluted 1 + 15 000 in coating buffer A (100 μl) was added to the inner 60 wells of a polystyrene microtitration plate (the well to well variations are greatly reduced by omitting results from the perimeter wells). The plate was covered with a plastic film (plate sealer from Tech Gen, Zellik, Belgium) and stored at 4°C for 16 h. The coating solution was aspirated, then 50 μl of standard solutions of sulphamethazine (range 0.2–100 ng) or tissue extracts from control or spiked samples were dispensed, in triplicate, into individual wells on the microtitration plate already coated with the specific antibodies. Subsequently, 100 μl of the sulphamethazine–enzyme conjugate solution diluted 1 + 10 000 in PBS–gelatine (buffer B) was added. The plate was covered with a plate sealer and, after shaking, incubated overnight at 4°C or for 2 h at 37°C. The wells were washed three times with washing solution C to remove unbound material. TMB solution (150 μl) was then added to each well and the plate was incubated at 37°C in the dark. After 30 min the reaction was stopped by adding 50 μl of 6 M H_2SO_4 to each well, in the same order and at the same rate as the substrate solution was added so as to keep the reaction time constant for all of the samples. The plate was gently shaken before absorbances were measured at 450 nm with the microtitre plate reader.

The cross-reactivities (*CR*) of the antiserum were determined by preparing dose–response curves for a range of sulphonamides and were calculated with the equation

$$CR = \frac{\text{nmol sulphamethazine required to reduce colour production by 50\%}}{\text{nmol other drug required to reduce colour production by 50\%}}$$

Calibration. The percentage binding was calculated from the absorbance obtained in the absence (B_0) and the presence (B) of sulphamethazine in standards and samples as follows: binding (%) = $(B - NS/B_0 - NS) \times 100$, where *NS* represents the non-specific binding, i.e., the absorbance measured in wells uncoated with the antiserum. Calibration graphs were prepared by plotting log(sulphamethazine concentration) against percentage binding or against logit(percentage binding) (logit–log plot).

Calculation. Results were calculated according to the method of Rodbard and Frazier [14] by interpolation from a calibration graph where the bound activity, expressed as the logit of the ratio (in percent) between absorbance increase per 30

min, at each concentration of sulphamethazine (B) divided by the bound activity in the absence of unlabelled sulphamethazine (B_0), was plotted against log(sulphamethazine concentration). The Rodbard and Frazier procedure of calculation was adapted (Logivet, Wodecq, Belgium) to enzyme immunoassay using EXCEL (Microsoft) on a Macintosh computer.

RESULTS

Typical calibration graphs obtained (a) after a short incubation time (2 h at 37°C) and (b) after a long incubation time (overnight at 4°C) are presented in Fig. 1. The day-to-day variability of the logit–log plot was determined from ten calibration graphs for both types of incubation. For 2-h competitions at 37°C, the mean slope ± standard deviation (S.D.) and relative standard deviation (R.S.D.) was $-(0.62 \pm 0.02)$ (3%) and the mid-point of the curve at 50% binding inhibition (ED50) was (3.8 ± 0.25) ng per well (6.6%). For competitions overnight at 4°C, these values were respectively $-(0.69 \pm 0.01)$ (2%) for the slope and (1.2 ± 0.05) ng per well (4%) for the mid-point ($n = 10$).

Nine sulphonamides were assessed for cross-reactivity with the anti-sulphamethazine antiserum. The cross-reactivities (CR) of the antibody are given in Table 1. It is clear that other commonly used sulphonamides, except sulphamerazine, had no significant effects. Compared with sulphamethazine, sulphamerazine, with the unique difference of one methyl group at the pyrimidinyl C-4 position, showed 13% cross-reactivity.

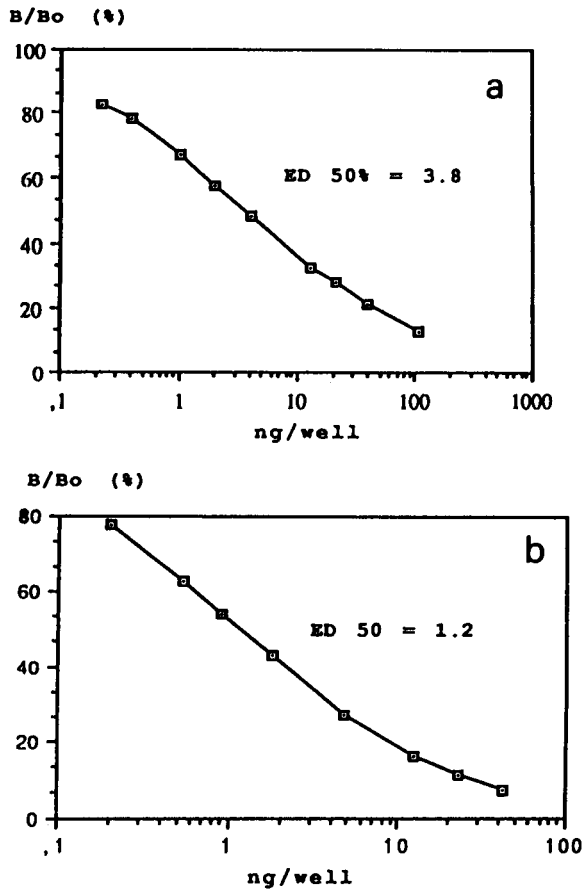

Fig. 1. Calibration graphs for the enzyme immunoassay of sulphamethazine: (a) 2-h incubation at 37°C; (b) 16-h incubation at 4°C.

Evaluations of the accuracy and reproducibility of the assay in muscle and liver samples are given in Table 2. The accuracy of the method was checked by recovery experiments performed on

TABLE 1

Percentage cross-reaction of the antiserum anti-diazotized sulphamethazine coupled to bovine serum albumin

Substance	Cross-reaction (%) [a]	Substance	Cross-reaction (%) [a]
Sulphamethazine	100	Sulphachloropyridazine	< 0.01
Sulphamerazine	13.2	Sulphathiazole	< 0.01
Sulphadimethoxine	0.1	Sulphanilamide	< 0.01
Sulphaguanidine	< 0.01	Sulphadiazine	< 0.01
Sulphaquinoxaline	< 0.01		

[a] Cross-reaction (%) = $\frac{\text{nmol sulphamethazine required to reduce colour production by 50\%}}{\text{nmol other drug required to reduce colour production by 50\%}}$.

TABLE 2

Accuracy and reproducibility of the assay in muscle and liver samples [a]

Muscle samples		
Accuracy		
Added ($\mu g\ kg^{-1}$)	Measured ($\mu g\ kg^{-1}$)	Recovery (%)
100	81.3 ± 3.4	81
250	222.5 ± 9.3	89
Reproducibility		
Added ($\mu g\ kg^{-1}$)	R.S.D. (%)	
	Intra-assay ($n = 8$)	Inter-assay ($n = 8$)
100	7.4	11.3
250	8.2	12.2
Liver samples		
Accuracy		
Added ($\mu g\ kg^{-1}$)	Measured ($\mu g\ kg^{-1}$)	Recovery (%)
100	91.8 ± 4.2	92
250	235.3 ± 11.2	94
Reproducibility		
Added ($\mu g\ kg^{-1}$)	R.S.D. (%)	
	Intra-assay ($n = 8$)	Inter-assay ($n = 8$)
100	8.1	10.2
250	7.8	12.4

[a] Sulphamethazine concentrations were determined by enzyme immunoassay after addition of known amounts of sulphamethazine to a muscle or liver sample from an untreated pig. Values are means of eight determinations (blank values subtracted) ± S.D.

spiked tissue samples. Known amounts of sulphamethazine were added to tissue samples at two concentrations and assayed in triplicate.

The recoveries were near to 100% and the difference between the true value and the mean of the determinations was well below the limits (−20% to +10% for real concentrations > 10 $\mu g\ kg^{-1}$) recommended by the EEC Commission Decision (87/410) [15]. The reproducibilities also fitted the recommendations of the EEC (for real concentrations > 10 $\mu g\ kg^{-1}$, R.S.D. < 0.15%).

When used to analyse muscle samples, the limit of detection (mean determined concentration for 20 blank samples + 3 times the S.D.) [16] of this assay was established to be 4.8 $\mu g\ kg^{-1}$ and the limit of determination (mean of 20 blank samples + 6 times the S.D.) was 5.4 $\mu g\ kg^{-1}$. In liver tissue, the limit of detection and limit of determination were calculated to be 5.1 and 5.7 $\mu g\ kg^{-1}$, respectively.

DISCUSSION

The enzyme immunoassay developed for the determination of sulphamethazine residues in pig tissues extracts is based on a competition between sulphamethazine present in the samples and sulphamethazine labelled by horseradish peroxidase. Its principle is similar to that of assays previously set up for the analysis of artificial anabolics [17] and clenbuterol [18]. Associated with the matrix solid-phase dispersion procedure of extraction, this assay could be used as a routine method for the screening of sulphamethazine in exported and imported liver or muscle of pigs. Indeed, the present screening control in Belgium is based on thin-layer chromatography [19], which can occasionally lead to false-positive results. The enzyme immunoassay has a limit of detection of about 5 $\mu g\ kg^{-1}$, which is well below the tolerance limit of 0.1 mg kg^{-1} that has been established for the concentration of sulphamethazine residues in edible tissues. Numerous immunochemical methods have already been published for the determination of sulphonamides in blood, animal tissues and urine. Further, several test kits are commercially available. However, these kits are expensive for use in screening of large series of samples and for this reason the present system was developed. Its performance was found to be similar to that described by McCaughey et al. [11] for the determination of sulphadimidine in pig urine.

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Detection of eight sulphonamides and dapsone in milk by a liquid chromatographic method

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Abstract

A liquid chromatographic method for the detection of eight sulphonamide drugs (sulphadiazine, -thiazole, -merazine, -methizole, -dimidine, -methoxypyridazine, -doxine and -methoxazole) and the sulphone dapsone in milk was developed. The method is based on a solid-phase extraction on cation-exchange columns using phosphoric acid, acetonitrile and methanol for elution. Analysis was performed after evaporation (concentration 5:1) with a Superspher RP-18 column. The mobile phase was an isocratic mixture of methanol, acetonitrile and sodium acetate and detection was performed at 275 nm. The recovery at the 25 $\mu g\ kg^{-1}$ level was 62–85%, the repeatability at the 25 $\mu g\ kg^{-1}$ was 1.69–10.35% and the sensitivity was 5 $\mu g\ kg^{-1}$, with the exception of sulphadiazine, -dimidine and -methoxazole (10 $\mu g\ kg^{-1}$). This method fulfils the requirements of EEC regulation 675/92 and the US FDA "safe level". Out of 74 sulpha-suspicious raw milk samples (microbial receptor assay), dapsone was detected in four samples and sulphadimidine in nine samples. In no case was the EEC limit of 100 $\mu g\ kg^{-1}$ exceeded. One sample contained more than 25 $\mu g\ kg^{-1}$ of dapsone.

Keywords: Liquid chromatography; Dapsone; Milk; Solid-phase extraction; Sulphonamides

Sulphonamides and dapsone are widely used in veterinary medicine for both the treatment and prevention of infectious diseases. Residues of these substances in milk can be detected by microbial and/or immunological screening methods, which determine either inhibitors (e.g., agar diffusion tests), sulphonamides (e.g., microbial receptor Charm II test) or a special sulphonamide (e.g., sulphadimidine enzyme-linked immunosorbent assay) [1]. Positive and/or suspicious findings ought to be confirmed, e.g., by liquid cromatography (LC). The demands for the development of such a confimation method are based on the requirements to fulfil regulatory limits of detection (Table 1) and to detect and determine several sulphonamides of practical importance.

In this paper an LC method with solid-phase extraction (SPE) for the detection of sulphadiazine, -thiazole, -merazine, -methizole, -dimidine, -methoxypyridazine, -doxine and -methoxazole and the sulphone dapsone at the 10 $\mu g\ kg^{-1}$ level is presented.

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EXPERIMENTAL

Materials and equipment

The sulphonamides were obtained from Serva (Heidelberg), with the exception of sulphamethizole and -methoxazole (Sigma, Deisenhofen). Dapsone (4,4'-diaminodiphenylsulphone) was purchased from Merck–Schuchardt (Darmstadt). Reagents and chemicals were supplied by Merck (Darmstadt).

The vacuum manifold was an adsorbex solid-phase extraction unit (Merck). The SPE columns contained aromatic sulphonic acid (Baker,

TABLE 1

Limits of detection: requirements for sulpha and dapsone residues in milk ($\mu g\ kg^{-1}$)

Requirement due to	Substances	Concentration ($\mu g\ kg^{-1}$)
German drug regulation [2]	Sulphonamides including N^4-acetyl metabolites	100
	Dapsone	1
JECFAC (MRL) [a] [3]	Sulphadimidine and metabolites	50
	Sulphadimidine	25
FDA ("safe level") [4]	Sulphadimidine	10
	Sulphadimethoxine	10
	Sulphamerazine	10
	Sulphathiazole	10
	Sulphadiazine	10
EEC Regulation No. 657/92 [5]	Sulphonamides	100
	Dapsone	25

[a] JECFAC = Joint Expert Committee on Food Additives and Contaminants (WHO/FAO); MRL = maximum residue limit.

Gross-Gerau). A Model L-6200 gradient pump, L-4200 UV–visible detector, AS-200 autosampler, D-6000 interface, LC organizer and I-5025 column thermostat were supplied by Merck. The analytical cartridges were LiChroCART, Superspher 100 RP-18, 250 mm (Merck).

Sample preparation

Mixture of 10 ml of milk and 2 ml of oxalic acid (7.5%, w/v) (pH 1.8–2.0) was centrifuged, 10 ml of distilled water were added to the supernatant and the mixture was centrifuged and filtered.

Solid-phase extraction (SPE) and concentration

Column conditioning was carried out with 3 ml of methanol and 3 ml of LC-grade water and column washing with 3 ml of (10%) phosphoric acid, 3 ml of phosphoric acid–acetonitrile (75 : 25), 3 ml of phosphoric acid–methanol (90 : 10) and 1.5 ml of LC-grade water.

Sample elution was effected with 4.5 ml of sodium acetate–calcium chloride buffer–methanol (50 : 50). The eluate was concentrated 5 : 1 in a water-bath (80°C) to a volume of 1.8 ml, then 0.04 ml of acetic acid glacial was added to adjust the pH to 4.5–4.7. The volume was made up to 2.0 ml with LC-grade water, followed by centrifugation.

LC analysis

The mobile phase was 0.01 M sodium acetate (pH 4.6)–acetonitrile–methanol (78 : 17 : 15) at a flow-rate of 0.7 ml min^{-1}. The column was thermostated at 25°C, the injection volume was 50 μl, the detection wavelength was 275 nm and the analytical time was 25–30 min. Samples were analysed successively without and with addition of a standard solution (25 $\mu g\ kg^{-1}$ per substance) in order to compensate for changes in retention time.

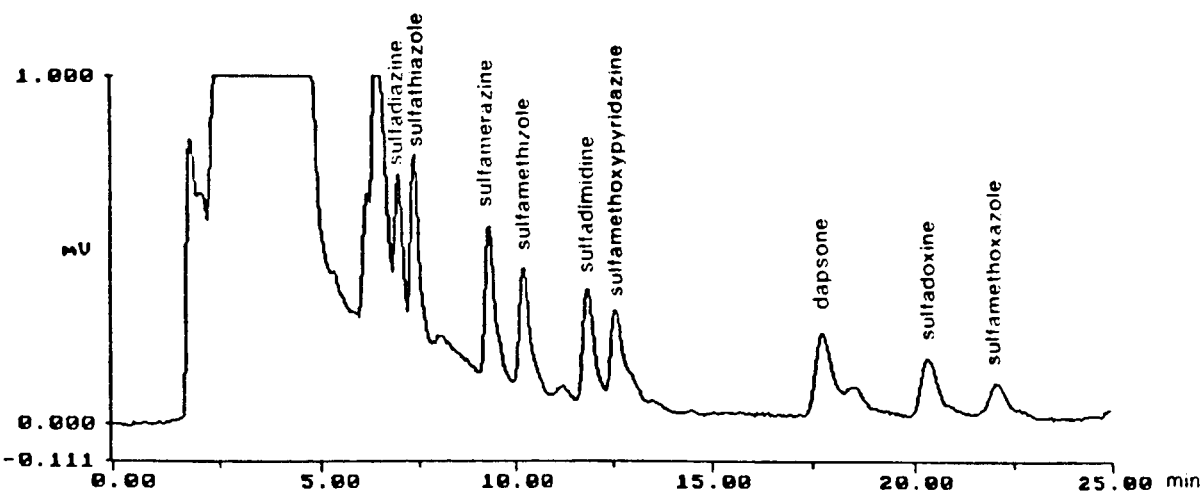

Fig. 1. Chromatogram of a standard milk sample (10 $\mu g\ kg^{-1}$ per substance).

Evaluation

Peak areas were multiplied by correction factors derived from the analysis of aqueous standard solutions and mean recovery rates.

RESULTS AND DISCUSSION

Chromatograms

Figure 1 shows a chromatogram of a standard milk sample (10 μg kg^{-1}) and Fig. 2 a chromatogram of the raw milk containing dapsone residues lined with the chromatogram of a milk sample plus standard solution.

Retention times

The retention times of the substances under study, summarized in Table 2, show that the substances are distinctly separated. As can be seen from Figs. 1 and 2, the sulphadiazine peak is very close to the matrix peaks, which might complicate the evaluation. By application of a different analytical column (RP Select B Superspher) the separation in the front area can be improved.

Recovery

To UHT milk samples different concentrations (2.5, 5, 7.5, 10, 20, 25, and 50 μg kg^{-1}) of the drugs were added. The drugs were dissolved in 1 M NaOH and diluted with distilled water. Recoveries at the 25 μg kg^{-1} level are summarized in Table 2.

TABLE 2

Retention times, recoveries and repeatability

Substance	Retention time (min)	Recovery (%) [a]	Repeatability (R.S.D.) (%) [a]
Sulpha-			
-diazine	6.6	76	1.75
-thiazole	7.0	74	1.99
-merazine	8.6	83	5.08
-methizole	9.6	77	4.53
-dimidine	10.7	85	1.69
-methoxypyridazine	11.6	83	5.54
-doxine	18.3	76	2.19
-methoxazole	20.8	62	2.62
Dapsone	17.2	82	10.35

[a] 25 μg kg^{-1} level.

Repeatability

Three spiked raw milk samples were successively analysed. The relative standard deviations at the 25 μg kg^{-1} level are summarized in Table 2.

Sensitivity

To three raw milk samples sulphonamides and dapsone were added in 5 μg kg^{-1} steps and

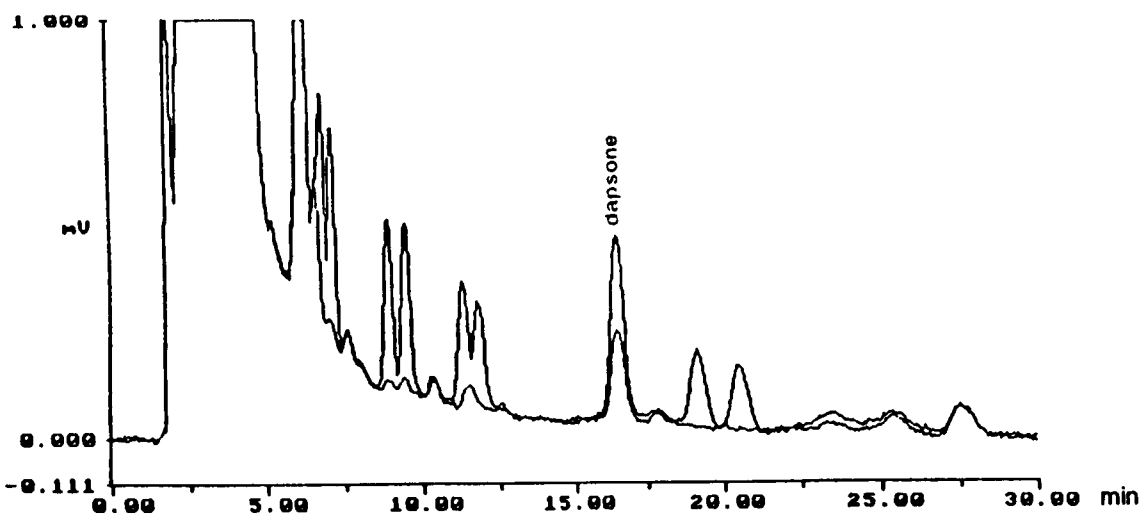

Fig. 2. Chromatogram of a raw milk sample containing dapsone, analysed without and with addition of standard solution.

analysed once. With respect to 2σ limits the sensitivity is 5 μg kg^{-1} with the exception of sulphadiazine, -methizole, -dimidine and -methoxazole, which can be differentiated at the 10 μg kg^{-1} level (see Figs. 3 and 4).

Detection of other substances (specificity)

Experiments with other sulphonamides gave retention times for < 6.5 min for sulphaguanidine, -anilamide, -isomidine and -acetamide, which means that owing to the matrix peaks these substances cannot be identified. The retention times of sulphapyridine (8.1 min), -meter (11.9 min), -chlorpyridazine (16.3 min) and -isoxazole (19.1 min) differ from those of the substances under study and could be detected by this method. The retention times of two sulphonamides were > 30 min (sulphadimethoxine 42 min, -quinoxaline 46 min).

Three N^4-acetyl metabolites (sulphamerazine, -dimidine and -dimethoxine) were added to milk and analysed. The N^4-acetyl metabolite of sulphamerazine could not be detected and those of sulphadimidine and -dimethoxine had retention times of 10.1 and > 30 min, respectively.

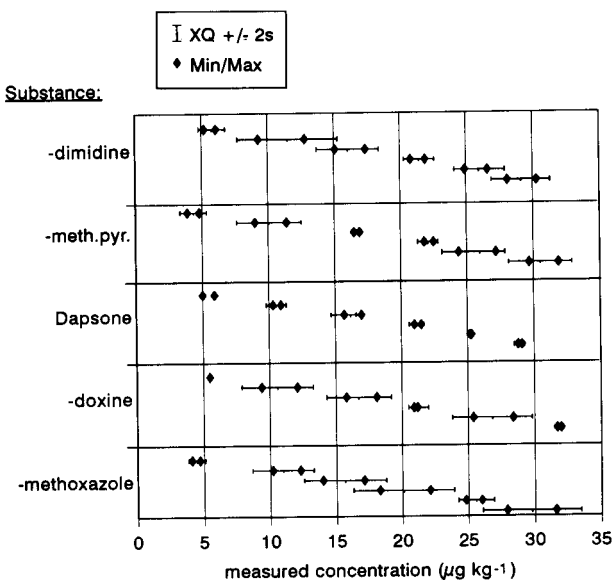

Fig. 3. Sensitivity of sulphonamide detection by LC. Addition in 5 μg kg^{-1} steps; $n = 3$ per concentration and substance.

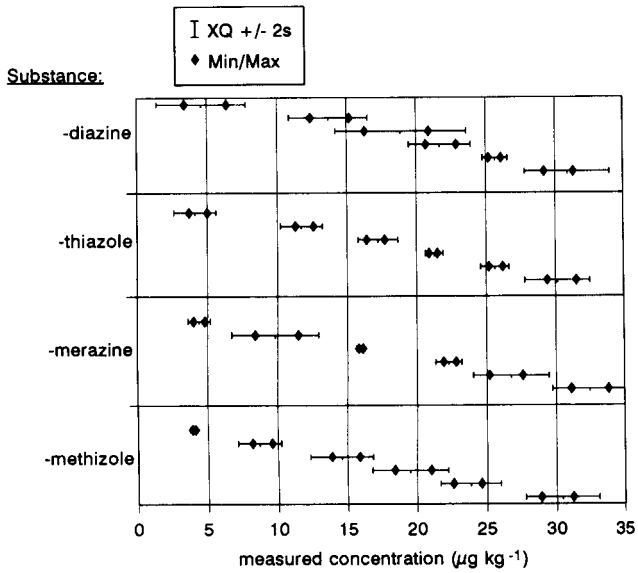

Fig. 4. Sensitivity of sulphonamide and dapsone detection by LC. Addition in 5 μg kg^{-1} steps; $n = 3$ per concentration and substance.

Other antibiotics (penicillin, tetracycline, erythromycin, streptomycin, novobiocin, gentamicin) and trimethoprim, which were added to milk in concentrations between 100 and 500 μg kg^{-1}, gave no signal. The retention time of chloramphenicol was > 30 min.

Examination of bulk raw milk

About 400 bulk raw milk samples from the northern part of Germany were analysed by different tests. The sensitivity of inhibitor tests with *Bacillus stearothermophilus* as test microorganism (modification of the Brillantblack reduction test, Delvo test SP) was sulphadimidine ≤ 250 μg kg^{-1}, sulphamerazine ≤ 75 μg kg^{-1} sulphadimethoxine ≤ 75 μg kg^{-1} and dapsone ≤ 10 μg kg^{-1}, and that of the microbial receptor assay (Charm II test) according to the manufacturer claim was ≤ 10 μg kg^{-1} sulphadimidine. As the microbial receptor assay for sulphonamides might be influenced by unspecific factors, samples with a count of $\leq 65\%$ of the control milk were evaluated as sulpha suspicious [6]. Samples that were positive and suspicious in one test were examined by LC analysis. The results were as follows:

Agar diffusion test with *Bacillus stearothermophilus*:
inhibitor positive: $n = 3$ (= 0.8%)
microbial receptor assay (Charm II test):
sulpha suspicious: $n = 74$ (= 18.7%)
LC analysis of 74 samples:
dapsone: $n = 4$ (= 1.0%)
$< 10\ \mu g\ kg^{-1}$ $n = 1$
$10–25\ \mu g\ kg^{-1}$ $n = 2$
$25–50\ \mu g\ kg^{-1}$ $n = 1$
sulphadimidine: $n = 9$ (= 2.3%)
$< 10\ \mu g\ kg^{-1}$ $n = 7$
$10–25\ \mu g\ kg^{-1}$ $n = 2$

In no case was the EEC limit of $100\ \mu g\ kg^{-1}$ of sulphonamides exceeded. One sample contained more than $25\ \mu g\ kg^{-1}$ of dapsone.

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Ion chromatographic determination of perchlorate in cattle urine

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Abstract

Much research has been done over the years on the subject of thyreostatic drugs of the thiouracil type in cattle fattening. These substances increase body weight by enlarged filling of the gastro-intestinal tract and by augmented water retention. On the other hand, little work has been done on the elimination and the presence of residues of inorganic thyreostatics such as perchlorate. The aim of this study was to evaluate the elimination of ammonium perchlorate in cattle urine according to the concentration given and the duration of administration. Perchlorate concentrations were measured by mobile phase ion chromatography. The detection limit was 0.1 mg kg^{-1} for tenfold diluted urine.

Keywords: Ion chromatography; Cattle; Perchlorate; Thyreostatic drugs; Urine

Thyreostatic drugs (TS) are used to cause fraudulently increased weight gain in cattle by inhibition of the thyroid gland. The weight gain consists mainly of an increased filling of the gastro-intestinal tract and augmented extracellular water retention in slaughtered animals [1]. Meat from TS-treated animals may be exudative and thus of inferior quality. The presence of residues of these highly potent antithyroid drugs in meat may constitute a human health hazard. Therefore, the use of these drugs for cattle fattening is prohibited in the European Economic Community (EEC).

Thyreostatic drugs were commonly abused in cattle fattening in the 1970s. The most frequently used substances in those days were of the thiouracil type, such as methyl-, propyl- and phenylthiouracil and tapazole [2,3]. These drugs can be analysed for by high-performance thin-layer chromatography and gas chromatography–mass spectrometry [4]. A large number of inorganic anions also show thyreostatic activity, and perchlorate salts belong to this group of inorganic thyreostatic drugs [5]. For regulatory control of the abuse of these thyreostatics, a method of analysis had to be developed and more information was needed on the excretion and the residue levels.

Data on inorganic TS originate mainly from East European countries. Previous investigations have concerned mostly the zootechnical effects of these compounds, but little work has been done on the possible residues in meat and the thyroid gland and the excretion of these inorganic growth promoters in urine [6–13].

The aim of this study was to investigate whether perchlorate could be found in cattle urine after administration of a single dose of ammonium

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perchlorate to a cow and, if so, how the elimination occurred for different doses of a single oral ingestion. After these preliminary trials, the withdrawal period was determined after prolonged administration of a daily dose of 4 g of ClO_4^- for 10 days.

EXPERIMENTAL

Reagents and reference compounds

Ammonium perchlorate (NH_4ClO_4) and potassium perchlorate ($KClO_4$) were obtained from Janssen Chimica (Geel, Belgium) and Aldrich-Chemie (Steinheim, Germany), respectively. The eluent consisted of a mixture of sodium carbonate (Na_2CO_3) (Riedel-de Haën, Seelze, Germany), 0.1 M tetrabutylammonium hydroxide (TBAOH) (Dionex, Sunnyvale, CA) and acetonitrile (ACN) (Merck, Darmstadt, Germany). All other reagents were of analytical-reagent grade and used as received.

Solutions

Stock standard solutions of ammonium and potassium perchlorate were prepared by dissolving 1 g of these products in 1 l of LC-grade water (Merck). These stock standard solutions were diluted 100-fold to give working standard solutions of 10 mg kg^{-1}. From the latter solutions, dilutions equivalent to 1, 0.8, 0.6, 0.4, 0.2, 0.1 and 0.05 mg kg^{-1} were prepared.

Chromatographic conditions

The analyses were carried out with a Dionex DX-100 ion chromatograph. The method is based on mobile phase ion chromatography (MPIC).

MPIC is a patented technique that utilizes a hydrophobic column packing, a hydrophilic mobile phase (1 mM Na_2CO_3–2 mM TBAOH–27% ACN) containing an ion-pairing reagent (TBAOH) and a suppressor (12.5 mM H_2SO_4) to lower the mobile phase background conductivity before entering a conductivity detector. The eluent flow-rate was 1 ml min^{-1} and the regenerant flow-rate was ca. 4 ml min^{-1}. Separation by MPIC depends on the partitioning of neutral ion pairs between the hydrophilic mobile phase and the hydrophobic stationary phase.

The column was a neutral polystyrene resin that contained no fixed ion-exchanging sites. For this reason the column can be used for either anion or cation determinations by appropriate choice of the pairing reagent.

The retention time of perchlorate, whether the ammonium or the potassium salt was used, was ca. 10.00 min.

The data were recorded with a Shimadzu C-R5A Chromatopac integrator coupled to the ion chromatograph.

Procedure

One 3-year-old black-pied cow was used for these experiments. Several doses were given in ascending order of concentration (2, 4, 6 and ten times 4 g). Between each dosing a rest period of about 2 weeks, starting at the time when the analytical results for the previous experiment were below the detection limit, was inserted. This period allowed the cow to recover slightly and to ensure that it was free from residues from the previous dose before starting a new experiment. Urine samples were taken at several times after ingestion (1, 7, 12, 24, 36, 48, 72 h, etc.). Each sample consisted of ca. 80 ml of urine collected by catherization of the bladder.

For analysis, 1 ml of urine was diluted tenfold with water. For macroscopically visible contaminants, the urine was first filtered and subsequently diluted. This diluted filtrate was loaded directly into the ion chromatograph using a 1-ml tuberculin syringe. Only 25 μl of this volume was retained in the 25-μl loop of the injection pump. By switching the valve system the loop was connected with the eluent flow and the sample was taken up in the eluent flow.

RESULTS

Preliminary experiments

Before starting the experiments on the elimination of perchlorate after oral administration to a cow, a method of analysis had to be developed. Dionex proposed certain analytical conditions

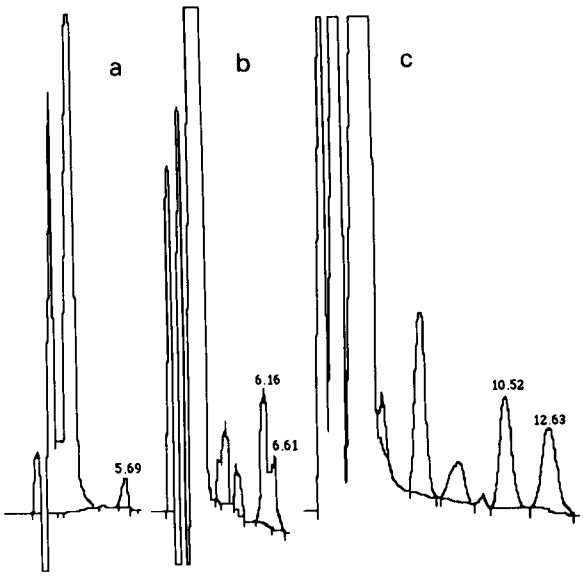

Fig. 1. (a) Chromatogram of porcine urine spiked with ClO_4^- ($t_R = 5.69$ min) (eluent containing 35% ACN); (b) chromatogram of bovine urine spiked with ClO_4^- ($t_R = 6.61$ min), interfering peak with $t_R = 6.16$ min (eluent containing 35% ACN); (c) chromatogram of bovine urine spiked with ClO_4^- ($t_R = 10.52$ min), interfering peak with $t_R = 12.63$ min (modified eluent containing 27% ACN).

based on studies with human urine spiked with perchlorate (eluent, 1 mM Na_2CO_3–2 mM TBAOH–35% ACN at a flow-rate of 1 ml min^{-1}; regenerant, 12.5 mM H_2SO_4 at a flow-rate of 3–4 ml min^{-1}). Under these conditions perchlorate could be determined within 6 min (Fig. 1a). However, using these conditions on cattle urine, perchlorate could not be separated from an endogeneous substance inherent in the urine (Fig. 1b). It has not been possible to identify this compound.

The next step was to search for an appropriate composition of the eluent that would enable the interference from this unknown peak to be eliminated. The best resolution and separation were obtained with an eluent of almost the same composition but with a slightly different acetonitrile content, i.e., 27% instead of 35%. Under these conditions perchlorate was virtually baseline separated from the interfering peak (Fig. 1c).

The results are expressed as mg ClO_4^- per kg urine. The detection limit (3σ) was ca. 0.1 mg kg^{-1} for a tenfold dilution of urine.

Elimination of ammonium perchlorate after oral administration of three different single doses

This first study used one 3-year-old black-pied cow to explore the possibility of analysing cattle urine for perchlorate residues after oral administration of a single dose of ClO_4^-. From a practical point of view the ammonium salt was used because of its greater solubility in water than magnesium or potassium perchlorate, so that the whole dose could be given at one time in a maximum volume of 50 ml.

The elimination of ClO_4^- residues was followed after oral administration of three different single doses of ClO_4^-, viz., 2, 4 and 6 g administered at intervals of ca. 40 days to the same cow. The results are shown in Fig. 2.

Excretion starts rapidly after oral ingestion, independent to the dose given. Maximum ClO_4^- levels in the urine were found 5–12 h after ingestion depending on the concentration administered. The interval in which the maximum ClO_4^- level was reached varied with the dose (Table 1). The maximum residue level after oral ingestion of 2 g of ClO_4^- was observed 5 h after administration. For a 4-g dose it was found after 9 h, and for 6 g after 12 h.

The rate of elimination was equivalent to the concentration, so that the concentration of ClO_4^- in the urine decreased to ca. one third of the maximum amount for all three doses 24 h after oral ingestion (33/104 for 2 g; 80/220 for 4 g; 130/414 for 6 g). The higher the dose given, the

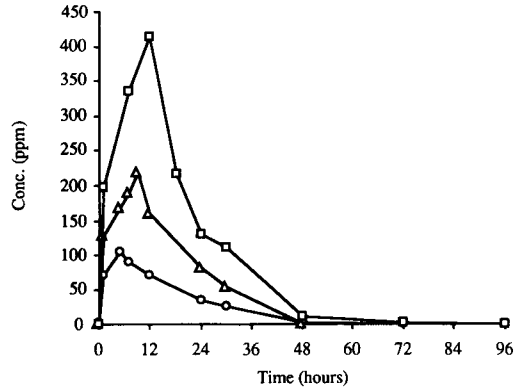

Fig. 2. Elimination curves for three different doses of ClO_4^- (○ = 2 g; △ = 4 g; □ = 6 g).

TABLE 1

Comparison of some elimination characteristics of the three different single doses and the multiple dose treatment

Parameter	Dose of ClO_4^-			
	2 g	4 g	6 g	10×4 g
Maximum concentration of ClO_4^- residues (mg kg^{-1})	104.5	219	414	151.4
Time of maximum excretion (h)	5	9	12	12
Concentration of ClO_4^- at 24 h (mg kg^{-1})	33.4	81	129	43.6
Rate of elimination until 24 h (mg kg^{-1} h^{-1})	3.7	9.2	23.8	9.0
Time of elimination (h)	48	48	72	72

faster was the rate of elimination. The rates of elimination for the three different doses are summarized in Table 1.

The lowest dose of 2 g of ClO_4^- reached its maximum value in 5 h and decreased to about one third of this maximum in 19 h (= 3.7 mg kg^{-1} h^{-1}). When 4 g of ClO_4^- were ingested, maximum excretion was obtained after 9 h, and 24 h later this amount was reduced to ca. one third at a rate of 9.2 mg kg^{-1} h^{-1}. For the highest concentration of 6 g of ClO_4^- the rate of elimination, calculated after reaching its maximum excretion level, was about 23.8 mg kg^{-1} h^{-1}. This concentration-linked elimination rate occurred until 48 h after administration, at which time the residue level of the 4-g dose was undetectable. For the 2-g dose small traces of ClO_4^- residues could be detected. After administration of 6 g of ClO_4^-, traces of ClO_4^- were still present 72 h after ingestion; a further 24 h later no ClO_4^- residues were found.

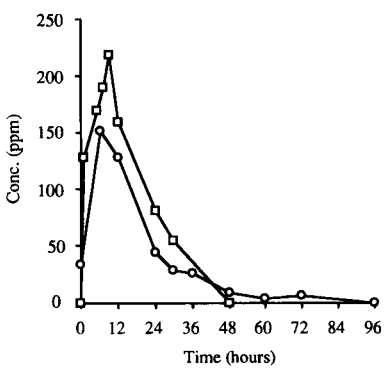

Fig. 3. Elimination curve for 4 g of ClO_4^- in (○) a single dose and (□) ten doses.

Hence there is evidence for a concentration-linked elimination rate in the first phase of the elimination (until 48 h) and in a second phase the rate of elimination decreased, so that the higher the concentration given, the longer small traces of residues could be found in the urine.

Elimination of ammonium perchlorate after prolonged administration

In a second experiment, a single cow was treated with a daily dose of 4 g of ClO_4^- for ten successive days. The results obtained are shown in Fig. 3.

After this treatment period, the ClO_4^- level observed in urine was in the same range as after the ingestion of a single dose. After withdrawal of the drug, an almost identical elimination rate as for the single dose was observed. In the first 2 days after stopping the treatment, the ClO_4^- levels in urine decreased very rapidly. The amounts and the rate of excretion were comparable to those after ingestion of a single dose. The difference between application of a single dose and repeated doses during 10 days was that residues were detected until 72 h after stopping the treatment in the case of the long-term administration whereas for the single dose the residue levels were below the detection limit of 0.1 mg kg^{-1} after 48 h.

This evolution of excretion probably results from the fact that small amounts of ClO_4^- were stored for some time in the thyroid gland and were released after stopping the treatment. On the other hand, results for urine samples taken during the period of administration indicated that a concentration of 4 g of ClO_4^- per day was not sufficient to obtain a steady-state. This lack of accumulation was probably also the consequence of the very rapid decline of ClO_4^- in the urine.

DISCUSSION

Data on the excretion and distribution of inorganic thyreostatics are scarce. Most of them origi-

nate from East European countries and were concerned with the zootechnical effects of these drugs in cattle fattening. The results differ from West European opinion on the quality of the meat after treatment with TS. According to some East European researchers, the use of inorganic TS, i.e., ammonium or magnesium perchlorate or a combination, increased the average daily weight gain of cattle in a short time and reduced the feed costs without decreasing the meat quality [10,11]. In contrast, in pigs they even decreased the moisture content and increased the intramuscular fat and protein concentration. They improved the biological value of pig meat [6,9]. Also in cattle, meat quality regarding tenderness and moisture content was ameliorated using ClO_4^- [7,8]. On the other hand, in some investigations no difference among treatments was found; it was concluded that the use of ClO_4^- in cattle fattening did not give a significant gain in daily liveweight [13,14].

When using ammonium or magnesium perchlorate as a growth promoter, an optimum dose of 1.5–5.0 mg kg^{-1} liveweight is recommended, the feeding period should not exceed 3 months and the animals should be slaughtered at least 5 days after the withdrawal of the salts from the diet [11]. Apart from these biological and zootechnical data, little has been published concerning the distribution and the elimination of perchloric acid salts in farm animals. In calves given ClO_4^- at a concentration of 200, 20 or 2 mg kg^{-1}, peak concentrations of ClO_4^- in blood occurred 5 h after application. Excretion in urine was up to 8.5% of that given. The amount of the anion retained in the body increased as the amount given was reduced [12]. This was not confirmed in this work, where a higher concentration gave residues for a longer time.

During the period of treatment, no significant changes in the behaviour or reactions of the animal were observed. Repulsive reactions against oral administration occurred after a few days. At this high concentration of 10 mg kg^{-1} in the present experiments (4 g), the excretion was characterized by a rapid start with a maximum concentration after 12 h. The results confirmed the fact that only slight accumulation occurred during prolonged administration of even this high concentration of 10 mg kg^{-1} liveweight [11,12]. During the whole period of administration a steady state was never reached because of the rapid excretion of ClO_4^-. At the time of the following daily dose, the residue level had returned to a concentration of approximately one third of the maximum concentration. At this elimination rate, storage of ClO_4^- residues seemed almost impossible.

Conclusions

Perchlorate was excreted in the urine after oral administration of a single dose of ClO_4^- and excretion started very rapidly. The three different doses (2, 4 and 6 g) showed an elimination curve equivalent to the dose given. The rate of elimination was dose dependent, so that the highest amount gave small residue amounts for a longer time. The higher the dose, the longer was the time during which residues could be found in the urine. ClO_4^- levels in urine showed a biphasic elimination: after an initial rapid decline, observed in the first 2 days after treatment, the ClO_4^- excretion slowed by a factor 16 depending on the treatment. Prolonged ClO_4^- administration at a dose of 4 g per day for 10 days resulted in a longer excretion period in the urine. Long-term treatment influenced the second phase of elimination. Small traces of ClO_4^- could be found for a longer time. This suggests that ClO_4^- administered over a longer period, as practised in cattle fattening, accumulates in the body and give residues in urine for longer periods than found with single doses. ClO_4^- as a growth promoter has the unfortunate characteristic (for analysts) of a rapid decline of ClO_4^- residues in cattle urine, which makes it difficult to detect and control its abuse in cattle fattening. Lowering the detection limit may result in a longer period during which residues can be found. Therefore, adjusted clean-up procedures are needed in order to lower the detection limit in this preliminary technique.

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Development of an immunoaffinity column and an indirect immunoassay with a biotin–streptavidin detection system for aflatoxin M_1 in milk

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Abstract

A sensitive and rapid screening method for the estimation of aflatoxin M_1 (AFM_1) in milk has been developed. Milk samples were first purified by immunoaffinity chromatography (IAC) using polyclonal antibodies raised in rabbits against aflatoxin M_1–bovine serum albumin (AFM_1–BSA) and coupled to an activated sepharose matrix. The eluate was analyzed in an indirect competitive streptavidin–biotin modified enzyme linked immunoassay (EIA). Microtitre plates were coated with AFM_1–BSA that competes with the analyte in the sample for binding with the biotinylated antibody. Bound biotinylated antibody was detected using a streptavidin biotinylated horseradish peroxidase complex. The limit of detection of the EIA is 2 ng kg^{-1}. By combination of IAC and EIA, spiked milk samples were analyzed. In conclusion, IAC–EIA is a sensitive and rapid method for the estimation of AFM_1 in milk.

Keywords: Immunoassay; Aflatoxins; Biotin; Milk; Streptavidin

Aflatoxin B_1, B_2, G_1 and G_2 are frequently occurring contaminants in human food and animal feed [1]. AFM_1 is a hydroxylated metabolite of aflatoxin B_1 (AFB_1) which is found in the milk of animals fed AFB_1 contaminated feed. The carryover of AFB_1, ingested by feed, into AFM_1 in the milk was experimentally determined and is 1–2%. It varies from animal to animal, from day to day and from one milking to the next [2]. Because of the toxicity of aflatoxins and the importance of milk in the human diet, especially for infants and young children, milk has the greatest demonstrated potential for introducing aflatoxin residues from edible animal products into that diet [3]. Many countries have regulated the amount of AFM_1 in dairy products. A tolerance level of 0.05 μg kg^{-1} is generally accepted.

It was our aim to develop a rapid prepurification system and a sensitive assay with minimum interferences from the matrix.

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EXPERIMENTAL

Apparatus

The microtitre plate photometer was from Eurogenetics (Tessenderlo). The microtitre plate washer was from Flow (Helsinki). Radioactivity was counted in a Rackbeta Model 1711 liquid scintillation counter LKB Wallac (Turku).

Materials

All reagents were analytical grade. AFM_1–oxime–BSA, aflatoxin M_2 (AFM_2), aflatoxin B_2

(AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin Q_1 (AFQ_1), aflatoxicol (AFOL), bovine serum albumin (BSA), tetramethylbenzidine (TMB), biotine-ε-aminocaproyl-*N*-hydroxysuccinimide were purchased from Sigma (Mont Louis, Brea, CA). AFB_1 and AFM_1 standards in chloroform (1 μg ml^{-1}) were kindly supplied by Dr. H.P. van Egmond, RIVM (Bilthoven). PD-10 columns, CNBr-activated sepharose, Dextran T-70 were purchased from Pharmacia (Uppsala). Rabbits were from Laboratoire d'Hormonologie (Marloie). ^{3}H-Aflatoxin B_1 (^{3}H-AFB_1) with a specific activity of 18 Ci $mmol^{-1}$ was from Moravek (Brea, CA). Streptavidin biotinylated horseradish peroxidase complex was from Amersham (Ghent). Filter columns (1 ml) were purchased from Baker (Deventer). Microtitre plates Maxisorp were from Nunc (Roskilde). Scintillation fluid RIALUMA was from Lumac (Olen). Proclin 300 was a gift from Rhomn and Haas (Antwerp). Dimethylsulfoxide, dimethylformamide, NaCl, Na_2HPO_4, H_2O_2, $(NH_4)_2SO_4$ and citric acid were from Merck (Darmstadt).

Production and characterization of antibodies

Specific polyclonal antibodies were raised in rabbits. Four rabbits (New Zealand) were immunized by multiple site intradermal injections of 250 μg AFM_1–oxime–BSA dissolved in 0.5 ml of saline (0.154 M NaCl), emulsified with 0.5 ml Complete Freund's adjuvant. Two booster injections were administered with an interval of 2 weeks. Afterwards, 4 injections were administered at 4-week intervals. For the booster injections the same emulsion was administered as for the first immunization. Ten days after injection blood was taken from the marginal ear vein. Antibodies were obtained as described by Harder and Chu [4]. Antibodies were precipitated thrice with $(NH_4)_2SO_4$ at a final saturation of 33.3%. The precipitate was redissolved in 0.01 M PBS (0.01 M Na_2HPO_4, 0.15 M NaCl, 0.02% Proclin 300, pH 7.4) to the volume of the original serum sample and applied to a PD-10 column as described by the manufaturer. The antibodies were stored at −20°C.

Antibody titres of the different antisera fractions of the rabbits were determined by radioimmunoassay (RIA). The titre was defined as the reciprocal of the antibody dilution required for 50% binding of the tracer. A protocol described by Evrard et al. [5] was followed. Appropriate dilutions of the different purified antisera fractions were made in RIA buffer (0.2 M Na_2HPO_4, pH 7.4, containing gelatin, 5 g l^{-1}). 50 μl of the antibody dilution and 50 μl of ^{3}H-AFB_1 (10000 cpm or 0.92 nmol ^{3}H-AFB_1) were added to 150 μl of RIA buffer. The tubes were incubated at 37°C during 30 min and subsequently for 2 h at 4°C. 250 μl of dextran coated charcoal (Norit Charcoal, 5 g l^{-1}; dextran, 0.5 g l^{-1} in RIA buffer) was added and the tubes were incubated for 15 min at 4°C. After centrifugation at 4000 rpm for 10 min the supernatant was added to 2.5 ml of scintillation fluid.

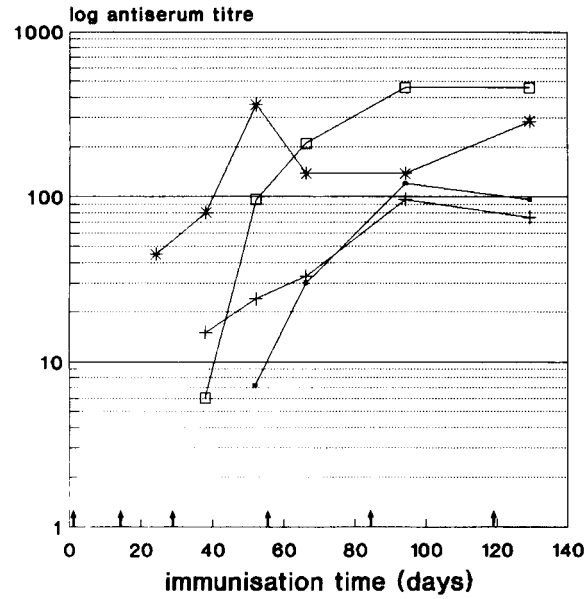

Fig. 1. Antibody titres of 4 rabbits immunized with AFM_1. Key to symbols: ■ = V6; + = V7; * = V8; □ = V9.

In Fig. 1 the titres of the different antibody fractions for each rabbit are plotted against the immunization schedule. The booster injections are indicated by arrows.

Preparation of an immunoaffinity column

Antibodies were coupled to a CNBr-activated Sepharose matrix according to the instructions of

the manufacturer. Finally the gel was equilibrated with 30 ml PBS. Immunoaffinity columns were made containing 0.5 ml (capacity of the gel was 2.5 μg AFM_1 ml^{-1} gel) in filter columns and stored at −4°C.

Biotinylation of antibodies

Antibodies were biotinylated as described by Strasburger and Kohen [6]. The purified immunoglobulines were dialyzed against PBS overnight at 4°C. The IgG were diluted to a concentration of 1 mg ml^{-1} with PBS. A 2-ml portion was made slightly basic by adding 90 μl of 1 M sodium phosphate, pH 9, and 20 μl of biotin-ϵ-caproyl-*N*-hydroxysuccinimide dissolved in dimethylformamide at a concentration of 1 mg per 40 μl was added with stirring. The mixture was stirred for 6 h at room temperature and dialyzed against PBS. The biotinylated antibodies (AB-B) were stored in fractions of 1 ml at −20°C.

Streptavidin-biotin based ELISA

An indirect competitive EIA was developed. Microtitreplates were coated with AFM_1–BSA. 50 μl of AFM_1–BSA in PBS (1 μg ml^{-1}) was added to the wells and the plates were dried overnight at 37°C and stored at −20°C until use. The plates were washed 4 times with washing solution (Tween 20, 0.5 g l^{-1} in PBS). To reduce the non-specific binding 200-μl aliquots of 3% BSA dissolved in PBS was added to the wells and the plates were left for 30 min on a shaker. The solution was removed and the plates were washed 4 times. Standard solutions were made in PBS–methanol (9 + 1). 50 μl of the standards and 50 μl of a 2.4×10^{-6} dilution of AB-B in assay buffer (0.1% BSA in PBS) were added to the wells in duplo. The plates were incubated overnight at 4°C. The plates were washed 4 times and 100 μl streptavidin biotinylated horseradish peroxidase complex diluted 10^{-3} in assay buffer was added and reacted for 30 min. After washing the plates 6 times with washing solution, the plates were rinsed with water before adding 100 μl of substrate solution (substrate A: 0.1 M Na_2HPO_4, 1% H_2O_2 adjusted to pH 5.5 with citric acid; and substrate B: 0.5 g tetramethylbenzidine dissolved in 40 ml of dimethylsulfoxide, diluted with bidistilled water to 1 l and adjusted with citric acid to pH 2.4; before use equal volumes of A and B were mixed). The plates were left for 15 min on a shaker at room temperature. 50 μl of 1 M H_2SO_4 was added and the colour was read with a microtitreplate photometer at 450 nm.

Sample preparation

A 10-ml volume of skimmed milk was diluted to 20 ml with PBS and applied onto the IAC. The column was washed with 18 ml of PBS and the toxins were eluted with 5 ml of PBS–methanol (1 + 9, v/v). The eluate was evaporated to dryness under reduced pressure and the residue was dissolved in 5 ml of PBS–methanol (9 + 1, v/v). The IAC was equilibrated with 20 ml of PBS.

RESULTS

In Fig. 2 a standard curve for AFM_1 obtained by the competitive EIA with a working range between 0.25 pg per well (90% B/B_0) and 125 pg per well (20% B/B_0) is shown. The detection limit, defined as the minimum quantity of the analyte which yields a signal two standard deviations below the signal for zero pg on the standard curve was 2 ng kg^{-1}.

The cross-reactivity of different aflatoxin analogues was determined by the EIA. Standard solutions of the different cross-reacting toxins (AFM_2, AFB_1, AFB_2, AFG_1, AFG_2, AFQ_1 and AFOL) were made and were added to the wells.

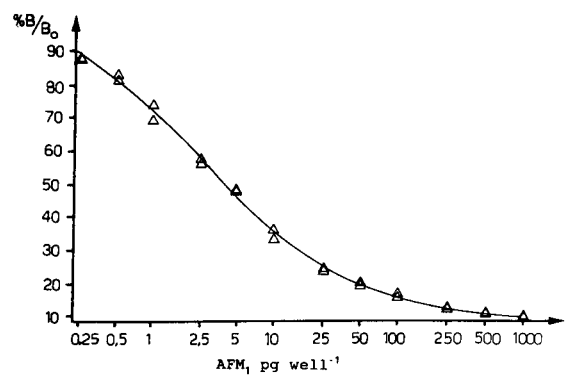

Fig. 2. Aflatoxin M_1 standard curve.

The protocol described above was followed. The results were then plotted in a series of standard curves. The cross-reactivity was arbitrarily defined as the ratio of the weight of the specific antigen (AFM_1) required to reduce binding by 50% to the weight of the cross-reactant required to reduce binding by 50%, multiplied by 100 [7]. In Fig. 3 the cross-reacting curves are shown. The antibody was specific for AFM_1, the cross-reactivities were 10.26%, 29.62%, 0.34%, 5.70%, 0.21%, 0.60% and 0.10% for aflatoxin M_2, B_1, B_2, G_1, G_2, Q_1 and aflatoxicol, respectively. Recovery experiments were done by spiking skimmed milk (fat content ≤ 3 g kg^{-1}) at different levels of contamination: 0.05 μg kg^{-1}, 0.08 μg kg^{-1}, 0.1 μg kg^{-1}, 0.12 μg kg^{-1} and 0.15 μg kg^{-1}. The results are presented in Table 1.

In conclusion, a high sensitive and rapid screening method for AFM_1 was developed. By the use of the IAC up to 30 samples could be purified in one day and analyzed by EIA. Only a limited number of spiked milk samples have been tested, the method will be further validated by analyzing naturally contaminated milk samples.

TABLE 1

Recovery experiments of aflatoxin M_1 contaminated milk

AFM_1 spiked into milk (μg kg^{-1})	AFM_1 by ELISA (μg kg^{-1})	Coefficient of variation (%)	Number of samples
0.05	0.049	17.8	9
0.08	0.082	6.6	4
0.10	0.1049	12.6	8
0.12	0.117	29	3
0.15	0.136	13.4	5

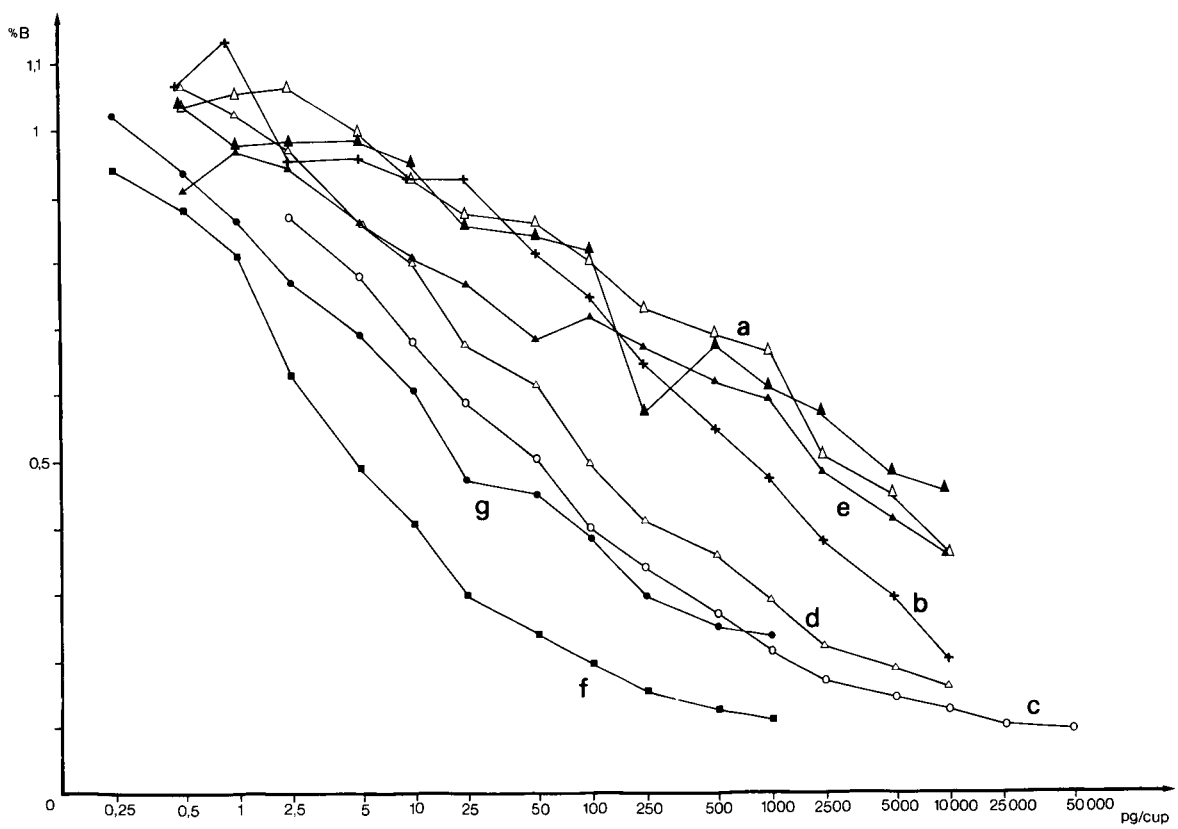

Fig. 3. Cross-reactivity curves of the antiserum raised against AFM_1–BSA with other aflatoxin analogues. Key to symbols: a, AFG_2; b, AFQ_1; c, AFM_2; d, AFG_1; e, AFB_2; f, AFM_1; g, AFB_1; h, AFOL.

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Strategy for the development and operation of rapid screening methods for residues analysis

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Abstract

A programme of method development is underway at the Central Veterinary Laboratory to screen meat, kidney, liver, bile and urine for residues. The classes of analytes include antibiotics, β-agonists, hormones and anthelmintics with the majority being detected by immunoassay. A unified approach has been employed both for the operational use of the screening methods and in the development of the assays themselves. This allows a high degree a standardisation of the methodology and the facile incorporation of appropriate quality assurance protocols necessary to provide defined, accurate data. The advantages and application of bulk production of coated plates, enzyme conjugates and quality assurance materials as well as the use of standardised assay formats are demonstrated by reference to analytical data obtained in the routine operation of analyses for chloramphenicol, cloxacillin, streptomycin and sulphadimidine and penicillin G.

Keywords: Immunoassay; Residue analysis; Screening methods

The advantages in terms of cost, throughput and simplicity of enzyme immunoassays (EIAs) for screening assays are well documented. There is, however, a considerable number of different formats, reagents and protocols applied to methods being both developed in-house and available commercially in kit form. A standardised approach to method development as well as to operational use of those methods was undertaken in order to reduce the variability between assays, to tailor detection limits to the requirements of maximum residue limits (MRLs), to enable a greater degree of automation to be employed and to reduce the numbers of different reagents required. In addition the specifications of the assays included the ability to detect the residue at the MRL in tissue homogenate or raw milk without recourse to any further sample preparation or analyte concentration, thus considerably reducing cost.

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METHOD DEVELOPMENT

Table 1 lists the analytes for which EIAs have already been developed and validated and those in preparation. The assigned limits of determination are calculated from validation data as the lowest concentration of analyte giving a reduction in signal of > 2.0 standard deviations from zero dose. The unified approach taken for method development includes: ovalbumin as the hapten carrier protein; antisera raised in sheep; common immunisation schedules; direct competitive EIA format using antisera coated plates; horseradish peroxidase–hapten conjugates; tetramethyl benzidine substrate; rapid analysis (15–30 min); assay carried out directly on tissue homogenates; and

TABLE 1

Analytes for which EIAs have been developed [a]

Analyte	Limit of determination (ng per g tissue)
Penicillin G	20
Cloxacillin	1.0
Neomycin	10
Clenbuterol	0.5
Gentamycin	10
Chloramphenicol	0.2
Salbutamol	1.0
Sulphamethazine	2.0
Trenbolone	2.5
Cephaloglycine	25
Zeranol	1.5
Cephataxime	50
Streptomycin	2.5
β-Lactams [b]	100

[a] Under development: tetracyclines (generic), nitrofurans (generic), virginiamycin M1 and S1, 11 sulphonamides, benzimidazoles (generic, except thiabendazole), thiabendazole and β-agonists in addition to those above. [b] Receptor assay.

characterisation of immunogens by UV absorption or radiotracer analysis.

Ovine polyclonal antisera is preferred and coated directly onto microtitration plates, following glycine treatment [1], in 0.05 M Tris buffer (pH 8.5). Plates are washed in 0.05 M sodium phosphate buffer containing 0.5% sucrose, 0.1%

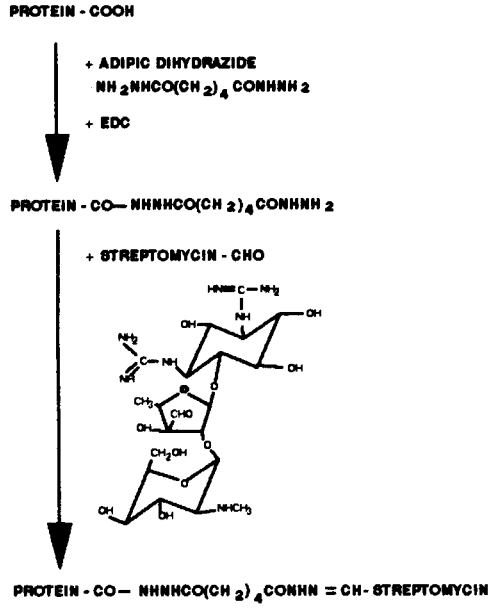

Fig. 2. Preparation of streptomycin immunogen.

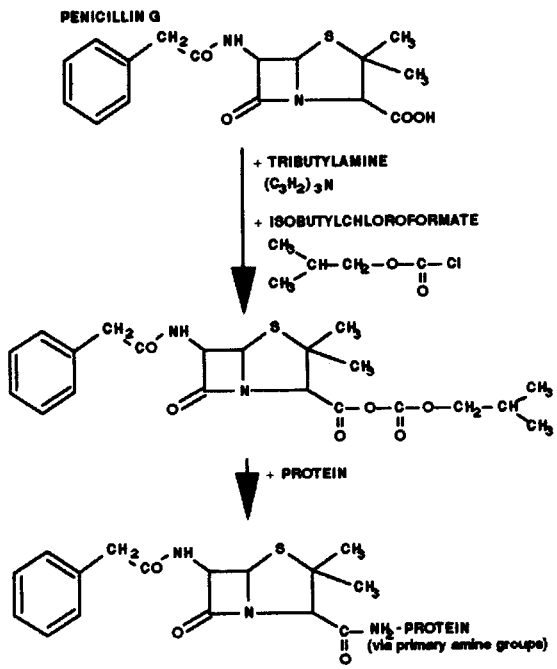

Fig. 1. Preparation of penicillin G immunogen.

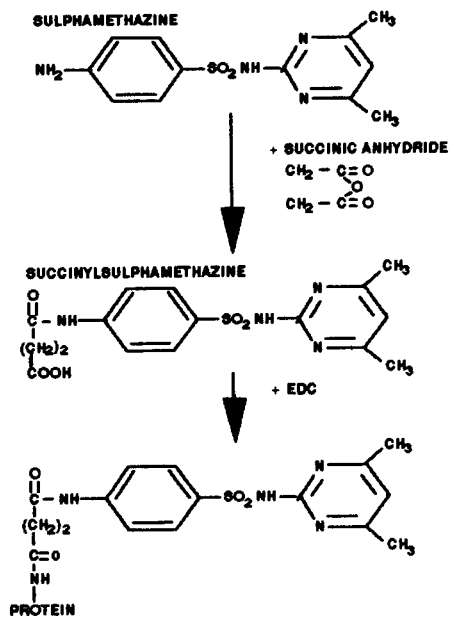

Fig. 3. Preparation of sulphamethazine immunogen.

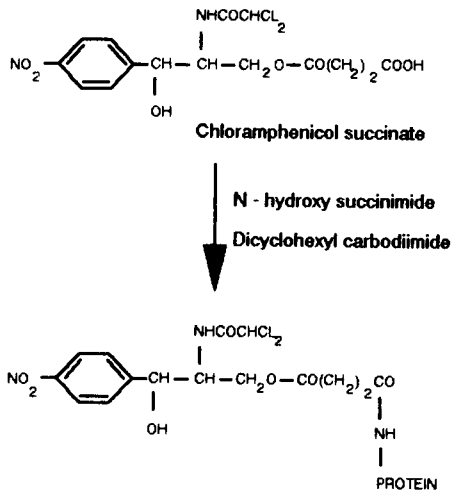

Fig. 4. Preparation of chloramphenicol immunogen.

bovine serum albumin (BSA) and 8.5% sodium chloride, dried and stored at 4°C. The signal generation for the direct competitive EIA utilises

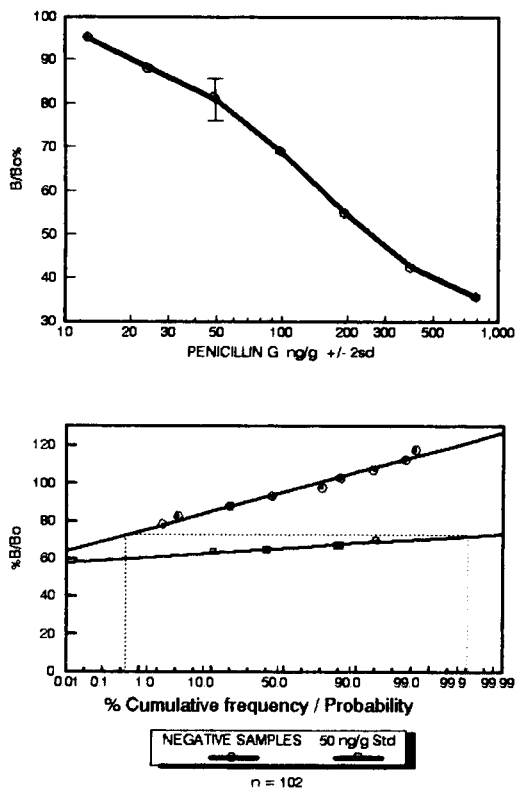

Fig. 5. Dose response curve for penicillin G, and probability curves.

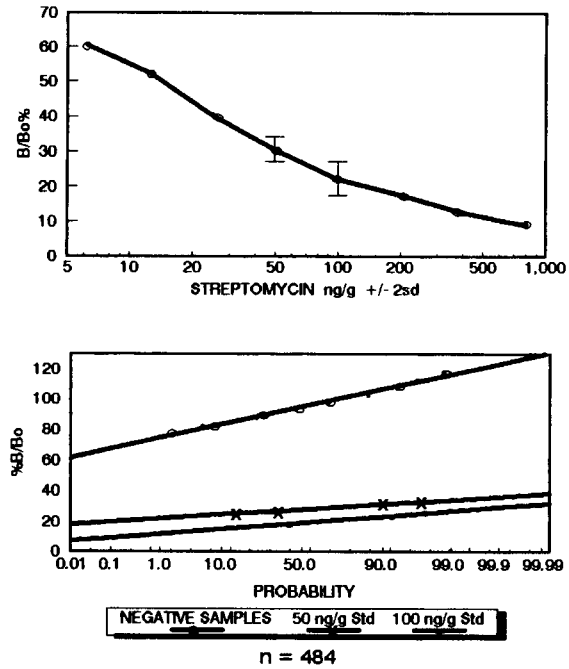

Fig. 6. Dose response curve for streptomycin, and probability curves.

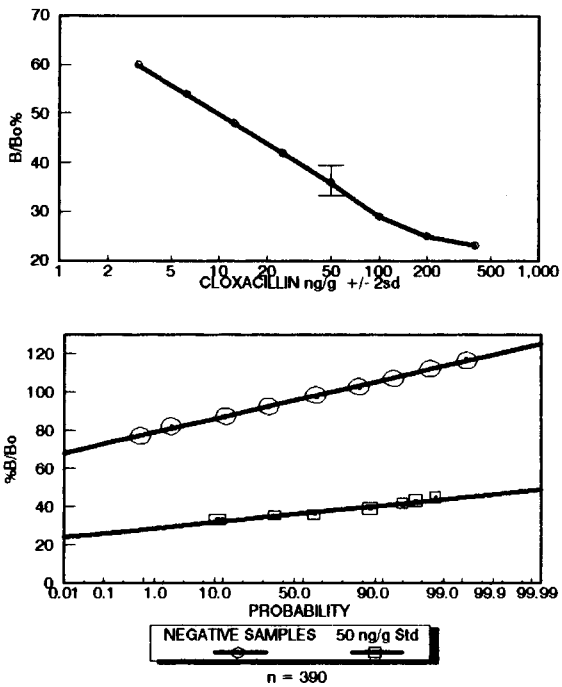

Fig. 7. Dose response curve for cloxacillin, and probability curves.

horseradish peroxidase (HRP) as the enzyme of choice which may be aminated [2] for hapten conjugation. Again a primary requirement for these HRP conjugates is long term stability so that large batches of quality assured material can be manufactured and stored (often as freeze dried aliquots).

Only reactions involved in the preparation of hapten immunogens and conjugates is necessarily different depending on available or introduced reactive groups on the hapten or enzyme. The reactions used in the procedures utilised for penicillin G, streptomycin, sulphamethazine, cloxacillin and chloramphenicol are shown in Figs. 1–4.

A primary aim of the programme was to decrease considerably, or even eliminate, the need for sample preparation and analyte concentration. A considerable proportion of overall assay cost is attributable to this area as well as having significant affect on assay variability. All the data given here is for EIAs carried out on simple 10% homogenates of kidney samples with no further

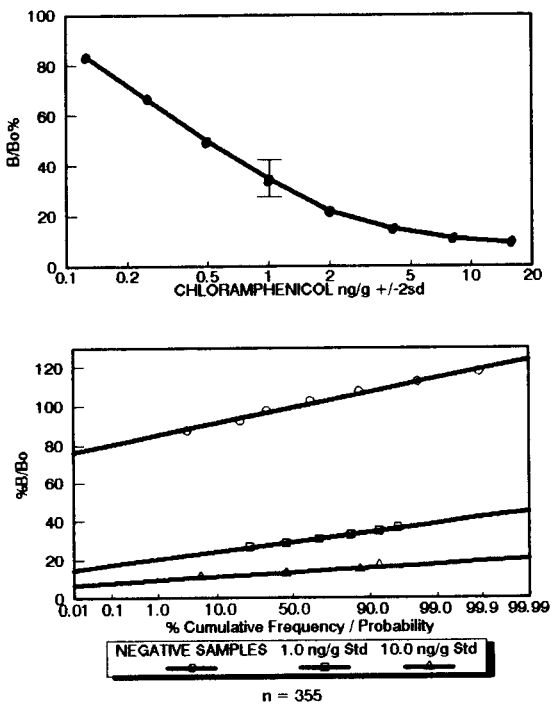

Fig. 8. Dose response curve for chloramphenicol, and probability curves.

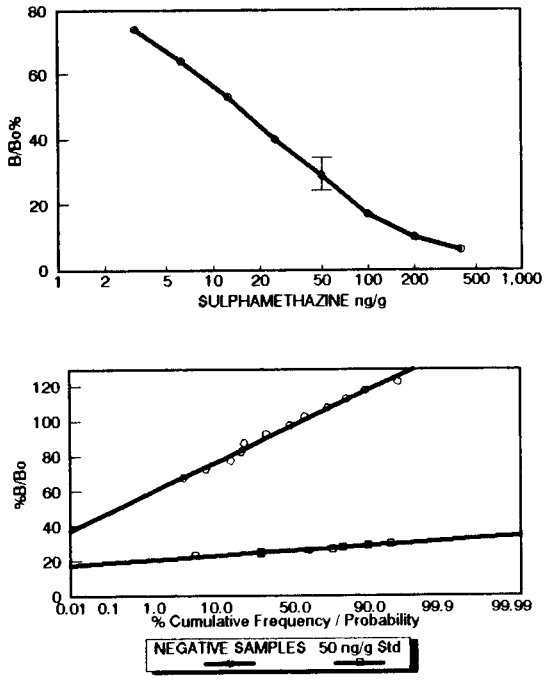

Fig. 9. Dose response curve for sulphamethazine, and probability curves.

TABLE 2

Assay variance

Analyte	Dose	Intra-assay		Inter-assay	
		n	C.V. (%)	*n*	C.V. (%)
Streptomycin	0	484	5.4		
	50	60	2.3	15	7.7
	100	60	2.1	15	6.4
Penicillin G	0	102	4.6		
	50	56	3.7	17	3.2
	100	56	5.0	16	3.6
Chloramphenicol	0	355	4.9		
	1.0	148	4.4	36	10.8
	10	148	4.1	36	12.6
Cloxacillin	0	390	3.9		
	50	144	4.8	35	3.1
Sulphamethazine	0	1364	6.1		
	50	292	5.0	73	5.7
	100	20	4.6	5	9.1

preparation other than a brief centrifugation step to remove cell debris. It is therefore an essential part of the assay validation process, in addition to specificity, sensitivity and reproducibility, to characterise the performance of the method with data obtained from large numbers of different samples over an extended period of time. By plotting the percentage cumulative distribution for the range of B/B_0 values obtained for both negative and spiked samples against each B/B_0 value, it is possible to define the probabilities for the occurrence of false negative and false positive results (e.g., Fig. 5). By introducing a horizontal line on the probability graph, parallel to the y axis at any given $\%B/B_0$ value (dotted line), a line perpendicular to the y axis at the point of intersection with the line of distribution of the negative samples gives the probability of a false positive. Similarly a perpendicular at the intersection point of the spiked sample distribution gives the expected probability of a false negative.

The requirement for screening assays is for minimal false negatives: the information obtained from the probability plots may thus be used to specify and quantify each assay performance at either the limit of determination or the MRL as appropriate.

RESULTS AND DISCUSSION

Examples of standard curves for spiked tissue homogenates are given in Figs. 5–9. As no extraction of analyte is necessary there is no need for correction for recovery. Error bars for $\pm 2 \times$ S.D. are included at the MRL or the chosen cut-off point for samples to be transferred to confirmatory analysis.

Performance characteristics from the probability curves (Figs. 5–9) show confidence limits for the penicillin G assay to be 0.35% false positives at a $< 0.05\%$ false negative level and cloxacillin, sulphamethazine, chloramphenicol and streptomycin, $< 0.05\%$ false positives at $< 0.01\%$ false negative level. Assay variance is given in Table 2 for all five analytes and were well within acceptable limits.

All the immunoassays developed are also applicable for the analysis of untreated, raw milk samples and several have been validated for muscle and liver homogenates thus providing further evidence for the application of a consistant and standardised approach to the development and operation of immunoassay based residue screening programmes.

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Rapid screening method for ivermectin residue detection in cattle muscle and liver by liquid chromatography with UV detection

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Abstract

A rapid screening method for the determination of residue of ivermectin in edible bovine tissue and liver is described. The analyte is extracted from the tissue with a methanol–water mixture. The clean-up procedure is based on solid phase extraction on a Sep-Pak C_{18} cartridge. Analysis is performed by isocratic elution with methanol–acetonitrile–water (9:9:2, v/v/v) on a Novapak C_{18} 5-μm column and UV–diode array spectrophotometric detection at 254 nm. The average recovery from spiked bovine muscle was 78.6% (S.D. = 15.0%) and from spiked liver sample 78.5% (S.D. = 10.9%) in the concentration range 14–275 $\mu g\ kg^{-1}$. The method has a detection limit of 5–10 $\mu g\ kg^{-1}$.

Keywords: Liquid chromatography; Bovine tissue; Cattle; Ivermectin; Liver tissue; Muscle tissue

Ivermectin is a very potent broad spectrum antiparasitic drug. It is a mixture of two homologous macrocyclic lactone disaccharides, containing not less than 80%, 22,23-dihydroavermectin B_{1a} (H_2B_{1a}) and not more than 20% 22,23-dihydroavermectin B_{1b} (H_2B_{1b}) (Fig. 1). The drug is effective in very low dosage against nematodes and arthropod parasites in cattle [1] and has been widely used in the treatment of endo- and ectoparasites in sheep, horses and cattle. Since 1991 Spain includes the analytical control of ivermectin residues in the National Residue Program. Therefore, a rapid screening method is needed for the analysis of meat tissues and liver.

Tolerance levels (25–100 $\mu g\ kg^{-1}$) are known in the U.S.A. Residue Program, where the detection levels of the analytical method have to be able to detect 15–20 $\mu g\ kg^{-1}$ of ivermectin in liver depending on the species. Recently the E.C. published their residues levels and put the tolerance levels for ivermectin at 15 $\mu g\ kg^{-1}$ in meat and 20 $\mu g\ kg^{-1}$ in liver [2].

Liver is the target tissue for residue control as was shown by Prabhu et al. [1] who studied the ivermectin distribution in fat, liver, muscle and kidney after oral and in-feed administration of ivermectin in swine. Although the metabolism of ivermectin is different in swine from that in cattle, the unaltered parent drug was shown to be the major residue component in both animals [3].

Several analytical procedures have been used to measure ivermectin in animal tissues. The first reported methods for determining avermectins in animal plasma [4,5] were based on UV detection, the first after Florisil clean-up of the plasma, and the second method using a tedious liquid–liquid-

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extraction clean-up followed by normal phase liquid chromatography (LC). A very rapid reversed-phase method for determining concentrations in serum, also with UV detection uses two cartridges (C_{18} and silica) for clean-up of the sample which makes the procedure more rapid, but also more expensive [6]. Methods for the determination of ivermectin in tissues have been reported using LC with fluorescence detection after clean-up procedures based on two cartridges [7] or several liquid–liquid solvent partition steps [1] followed by derivatization of ivermectin to a fluorescent product and separation and quantitation by LC with fluorescence detection.

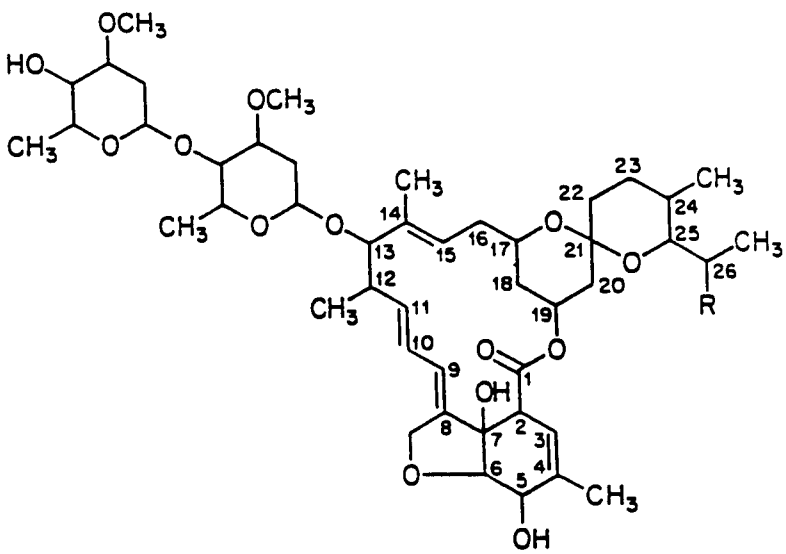
The method reported here is based on extraction of ivermectin with acetonitrile and clean-up of the extract with a C_{18} cartridge. The residue, evaporated till dryness, is injected in an LC system with detection of absorbance at 254 nm. In the described method, ivermectin will be used as the drug name. However, dihydroavermectin H_2B_{1a} has been used in residue studies because it is the major compound and metabolized at a lower rate than H_2B_{1b}.

EXPERIMENTAL

Apparatus and reagents

Ivermectin in glycerol formal with 1.38% H_2B_{1a} and 0.21% H_2B_{1b} was obtained from Merck, Sharp and Dohme (Rahway, NJ). Acetonitrile (Panreac) and methanol (Romil, Loughborough) was HPLC grade. Sep-Pak C_{18} cartridges (Millipore-Waters, Milford, MA) were used. Water was Milli-Q (Millipore-Waters) deionized. Ultraturrax (Janke-Henkel, Heidelberg) was used for homogenization.

The LC system (Millipore-Waters) was composed of a Model 510 dual liquid chromatographic pump, a universal injector (model U6K), a Model 441 UV detector of fixed wavelength (254 nm) (Waters) with a Data Module integrator (Waters). For confirmation purposes, the photodiode array spectrophotometric detector with the corresponding Model 990 printer–plotter (Millipore-Waters) was used. The chromatographic column used throughout this work, at room temperature, was packed with Novapak 5-μm C_{18} (150 × 3.9 mm i.d.) (Millipore-Waters) and acetoni-



I: R = CH_3CH_2
II: R = CH_3

Fig. 1. Structure of (I) 22,23-dihydroavermectin B_{1a} and (II) 22,23-dihydroavermectin B_{1b}.

trile–methanol–water (9:9:2, v/v/v) was the mobile phase. The flow-rate was 1 ml min^{-1}.

Extraction and clean-up of samples

Samples of bovine muscle and liver tissue were minced and mixed in a Braun homogenizer. A 5-g mass of the homogenate was placed into a screw-topped glass centrifuge bottle (250 ml), 3.5 ml of water and 45 ml of acetonitrile were added and the content was thoroughly mixed using an ultra-turrax. The bottles were centrifuged at 2800 *g* for 10 min and supernatants were transferred to round-bottomed amber glass flasks and extractions were repeated with a fresh water–acetonitrile mixture. The combined supernatants were evaporated to ca. 6 ml in a rotary evaporator with vacuum and a waterbath temperature of 60°C. The aqueous suspensions were applied to Sep-Pak C_{18} cartridges (Millipore-Waters), which were pretreated with 4 ml of acetonitrile and 4 ml of acetonitrile–water (1:1) using plastic disposable syringes. The flow-rate of solvents through Sep-Pak should be about 10 ml min^{-1}. The cartridges were eluted with 3 + 2 ml of acetonitrile and the eluate, combined in screw-topped amber glass tubes, was evaporated to dryness at 50°C

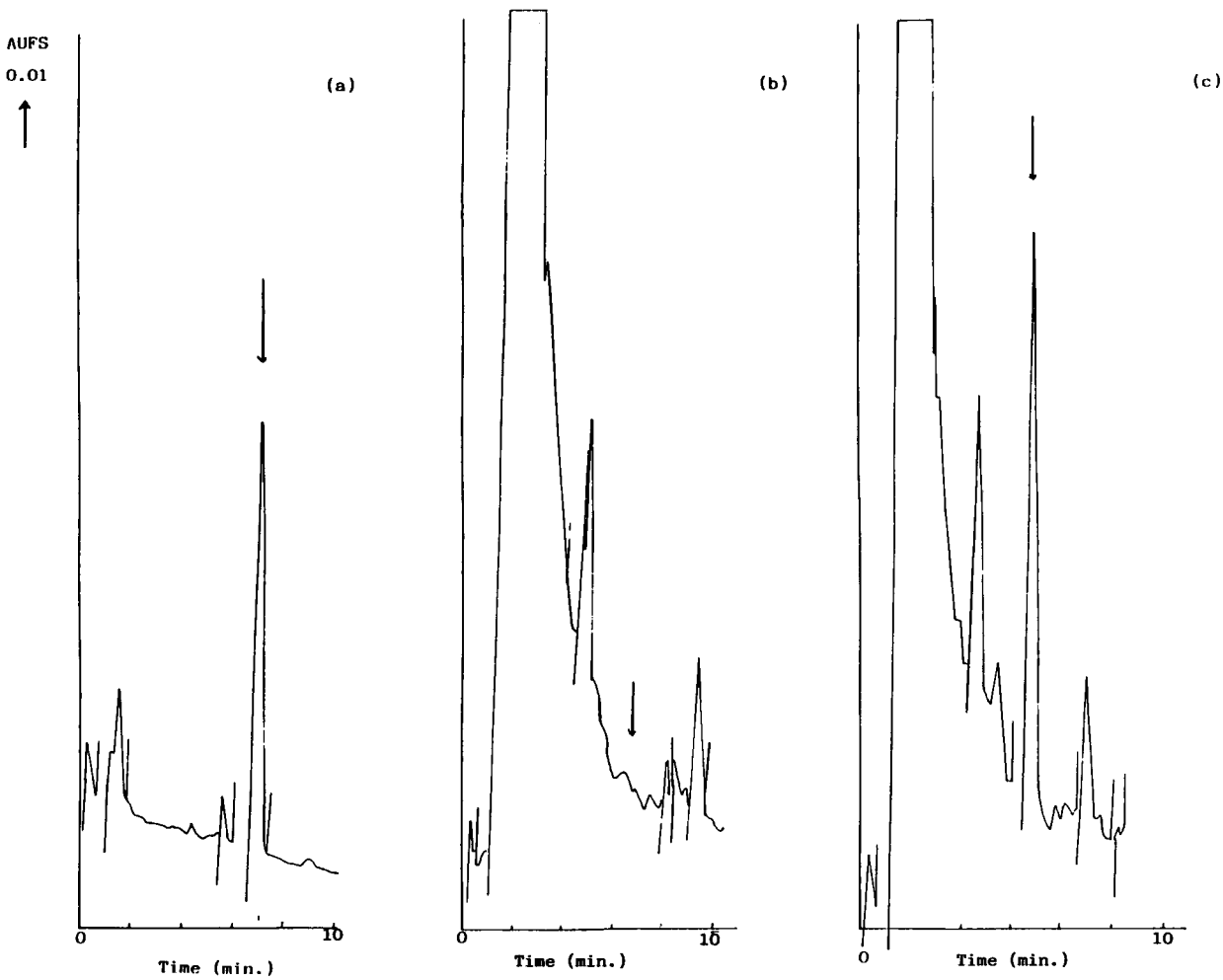


Fig. 2. Typical chromatograms of (a) standard ivermectin (138 ng), (b) blank and (c) spiked (207 $\mu g\ kg^{-1}$) muscle sample. LC conditions as described in Experimental.

under a gentle stream of nitrogen. The dry residue was dissolved in 200 μl of methanol.

Detection and identification by LC

A standard stock solution of 13.8 ng ml^{-1} dihydroavermectin B_{1a} was prepared in methanol (1 : 1000 dilution of the commercial standard) and stored in a freezer (−20°C). A working standard solution was prepared diluting the standard solution ten-fold (1.38 ng μl^{-1}) in mobile phase. With both solutions, a standard curve was made in the concentration range of 6–250 ng per injection by plotting peak height at 0.01 absorbance full scale versus injected amount in the chromatographic system described.

The extraction method was validated with control and fortified samples of bovine meat and liver in the 10–200 $\mu g\ kg^{-1}$ range.

The identity of peaks at the ivermectin retention time may be confirmed with a diode array spectrophotometric detector, using a wavelength range of 220–300 nm.

RESULTS AND DISCUSSION

Previously reported methods for the determination of ivermectin in tissue are sensitive and specifically based on LC separation of fluorescent ivermectin derivatives and fluorescence detection, or unspecific, based on LC with UV detection, generally with complicated extraction and time consuming clean-up procedures.

As ivermectin is quantitatively extractable with organic solvents from liver and muscle tissue, the proposed method here uses extraction of the sample with water–acetonitrile, which does precipitate the proteins.

First of all recovery of ivermectin in the solid phase clean-up step was tested. As was described in Ref. 7, the residual silanol groups in reversed-phase material could cause irreversible absorption of ivermectin on the cartridges, however, we obtained recoveries of ivermectin H_2B_{1a} of 95–100% after its purification on Sep-Pak C_{18} in the described conditions. The linearity of the detector response at 254 nm was controlled, injecting various amounts of ivermectin, in the range of 6–120 ng.

The regression line passed close to the origen, with a slight negative intercept: $y = 0.96x - 0.46$, where x = ng of injected ivermectin, and y = peak height in mm at 0.01 absorbance full scale (= 235 mm). The correlation coefficient was 0.9955 (n = 16). Peak-height measurement was preferred rather than peak-area because not always a good peak integration was obtained, as in this screening method various other peaks may interfere in a

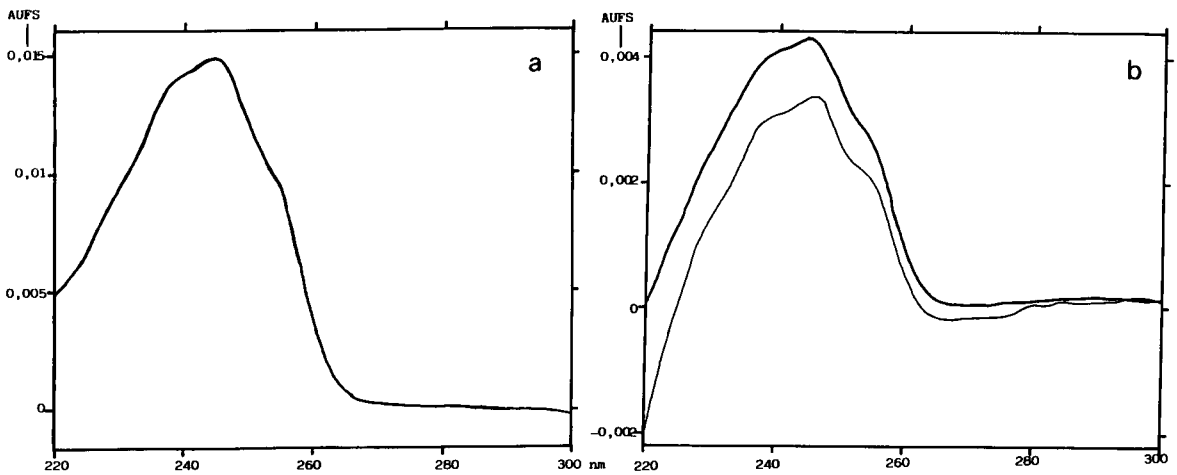


Fig. 3. Spectra of (a) standard ivermectin (138 ng) and (b) of the ivermectin peak of muscle samples spiked with 69 and 138 $\mu g\ kg^{-1}$.

good integration. In Fig. 2a, ivermectin H_2B_{1b} can also be indicated in the chromatogram of the standard solution.

Recovery assays in bovine muscle samples were carried out with spiked samples at the 14–275 μg kg^{-1} level. Results are presented in Table 1, and as can be seen the mean recovery value ($\pm$S.D.) of 26 determinations is 78.6 $\pm$ 15.0%. A typical chromatogram of standard ivermectin (138 ng), blank muscle sample and spiked (207 μg kg^{-1}) muscle sample is shown in Fig. 2, and as may be observed, the detection limit of the method for ivermectin in meat samples is about 5 μg kg^{-1}. However, confirmation of the presence of ivermectin using diode array spectrophotometric detection is not possible at this level. A satisfactory spectrum can only be obtained at levels higher than 20–30 μg kg^{-1}. An example is shown in Fig. 3.

TABLE 1

Recovery of ivermectin added to muscle tissue at the 276 μg kg^{-1} level [a]

Tissue	(μg Kg^{-1}) Spiked	n	Recovery (%)	S.D. (%)
Blank muscle tissue	–	5	–	–
Muscle tissue	14	4	65	12
	28	4	74	10
	69	5	79	11
	138	5	85	15
	276	8	89	12
Average			78.6	15.0

[a] Limit of detection = 5 μg kg^{-1}.

Recovery assays were also carried out with

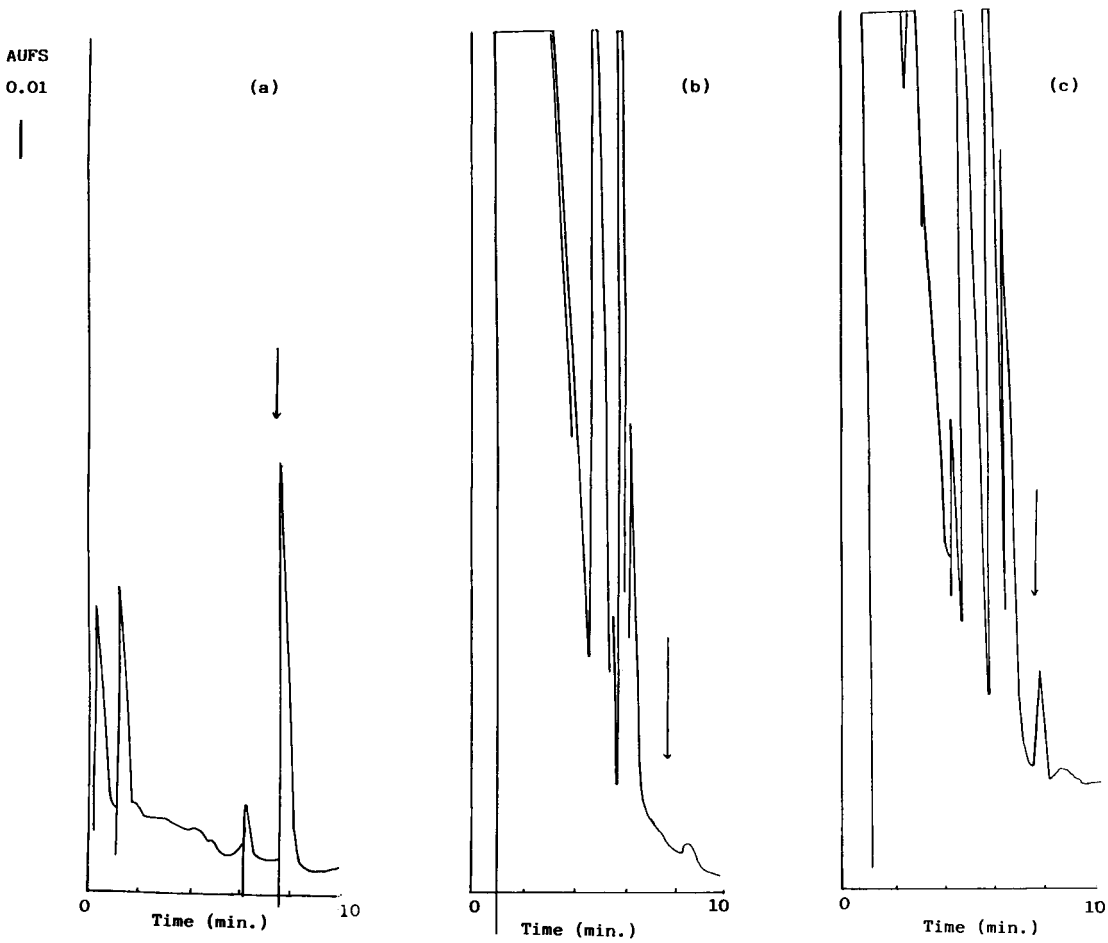


Fig. 4. Typical chromatograms of (a) standard ivermectin (69 ng), (b) blank liver sample and (c) spiked (41 μg kg^{-1}) liver sample.

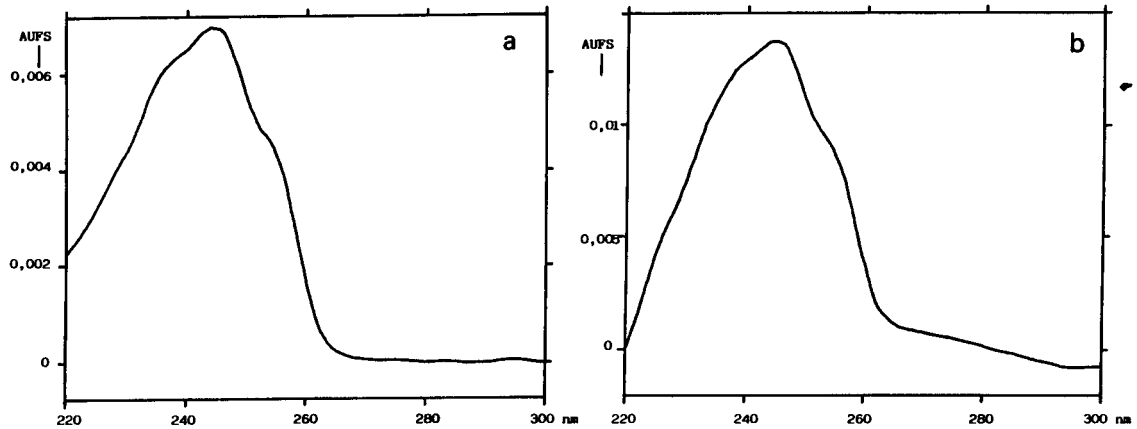


Fig. 5. Spectra of (a) standard ivermectin (69 ng) and (b) liver sample spiked with 276 $\mu g\ kg^{-1}$ ivermectin.

liver sample, the target tissue of ivermectin residue control. Table 2 shows the obtained values of 25 determinations yielding a mean recovery of 70.5 ± 10.9%, at the range of 25–275 $\mu g\ kg^{-1}$. A typical chromatogram of standard ivermectin (69 ng), blank and spiked (41 $\mu g\ kg^{-1}$) liver sample is shown in Fig. 4, from which may be concluded that the detection limit of this method for liver samples is 5–10 $\mu g\ kg^{-1}$. Confirmation by diode array spectrophotometry using the same LC system may be achieved at levels higher than 50 $\mu g\ kg^{-1}$.

An example of peak confirmation is shown in Fig. 5, where a spectrum of directly injected ivermectin (69 ng) is compaired with the ivermectin peak spectrum of a spiked liver sample (276 $\mu g\ kg^{-1}$).

TABLE 2

Recovery of ivermectin added to liver tissue at the 28–276 $\mu g\ kg^{-1}$ level [a]

Tissue	Spiked ($\mu g\ kg^{-1}$)	n	Recovery (%)	S.D. (%)
Blank liver	–	5	–	–
Liver tissue	28	4	80	11
	69	10	72	6
	138	6	80	12
	276	5	89	5
Average			78.5	10.9

[a] Limit of detection = 5–10 $\mu g\ kg^{-1}$.

The analytical method was successfully applied to samples provided by the Inspection Service for the National Residue Program. Till now, no positive samples were found. The proposed method may be used as screening method for ivermectin residue detection in meat and liver samples at the 5–10 $\mu g\ kg^{-1}$ level and at levels higher than 20–50 $\mu g\ kg^{-1}$ (meat and liver) peaks may be confirmed by diode array spectrophotometric detection, compairing the standard spectrum with those of the suspicious peaks.

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