Residues and metabolism of 19-nortestosterone laurate in steers†

The Analyst

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The illegal use of 19-nortestosterone (19NT; 4-estren-17β-ol-3-one; nandrolone) and its esters in livestock, for growth promotion purposes, has been widely reported in the European Union. The target residues for surveillance of abuse in bovine urine and bile samples are 17α- and 17β-19NT, although this choice of target residues is not based on *in vivo* radiotracer biotransformation data. In this study, four steers were administered [$^3H_2$]- and [$^2H_3$] 17β-19NT laurate (2 mg kg^{-1} body mass) by intramuscular injection and blood, urine, faeces and bile samples were taken for 30 d until slaughter, after which tissues were sampled for total residue analysis. Total plasma radiolabelled residues reached a maximum of 56.3 ± 15.9 pmol ml^{-1} at 36 h and were still appreciable (13.3 ± 1.6 pmol ml^{-1}) 30 d after treatment. Throughout the study period, total residue concentrations in bile (about 2–16 nmol ml^{-1}), urine and faeces (0.5–3 nmol ml^{-1} or g^{-1}) were higher than in other tissues sampled at slaughter. At slaughter there was evidence of residue accumulation in pigmented eye tissue (33.1 ± 6.1 pmol g^{-1}) and in white (13.4 ± 3.4 pmol g^{-1}) and black hair (28.9 ± 8.9 pmol g^{-1}). Evaluation of radio-HPLC profiles of urine and bile extracts generally indicated that 19NT and 19NT laurate residues were present in relatively small amounts among a complex mixture of metabolites. GC-MS analysis of glucuronidase-hydrolysed bile extracts indicated that the major metabolites were 5β-estrane-3α,17α-diol, 5α-estrane-3β,17α-diol, 5α-estran-3α-ol-17-one (norandrosterone) and estra-1,3,5(10)-triene-3,17α-diol (17α-estradiol).

Introduction

Anabolic hormones have been used to improve rates of protein deposition in livestock for many years. Although the use of steroids or steroid-like agents is licensed and widely used for this purpose in some countries, such applications are prohibited in the European Union. This ban may have contributed to the black market demand for hormones and hormone cocktails. Policing of illicit use is carried out under the auspices of the EU Directive 96/23/EC[1] and implemented through surveillance under the National Plan of individual member states. In the case of a licensed substance, appropriate residues and target tissues or fluids for surveillance are established as an outcome of pharmacokinetic studies, which form part of a product license submission; clearly, for unlicensed substances such information may be limited or non-existent. The synthetic anabolic steroid 19-nortestosterone (19NT) and its esters have been reported as widely used for growth promotion purposes in cattle.[2] The target residues generally investigated for surveillance of abuse are 17β-19NT and (primarily) the 17α-epimer in urine and bile; these analytes would be determined following incubation with hydrolytic enzymes to liberate free steroid from conjugates.[2–4] The evidence that the 17α-epimer is the primary metabolite in bovine is scant, since the only *in vivo* 19NT radiotracer study reported for cattle[5] did not involve the investigation of biotransformation products. Therefore, on the basis of extrapolation from metabolism studies in other species, a series of target metabolites were selected and investigated using gas chromatography-mass spectrometry (GC-MS) to determine their value in surveillance. Such metabolites of 19NT included 17α- and 17β-19NT, 5α-estran-3α-ol-17-one (19-norandrosterone), 5β-estran-3α-ol-17-one (19-noretiocholanone), 5α-estran-3β-ol-17-one (19-norepiandrosterone), 5α-estrane-3α, 17β-diol and 5α-estrane-3β,17α-diol.[6,7] There is added impetus to establish the nature of major metabolites in target samples given the recent EU Directive[1] requiring increased on-farm sampling, and reports that there may be endogenous sources of 19NT.[7–11]

In this study, radiotracer, chromatographic and mass spectrometric procedures were used to investigate the disposition and biotransformation of 19NT laurate (19NTL) in cattle, with the aim of establishing appropriate target residues and tissues for surveillance of 19NT use.

Experimental

Materials

$^3H_2$-labelled (1.7 TBq mmol^{-1}; possible label positions 2, 4, 6, 10 and 16) 17β-19NT were obtained from Amersham Life Sciences (Amersham, UK). All other chemicals were of analytical reagent grade or better unless specified otherwise. Steroid reference standards were obtained from Steraloids (New Barnet, UK) or Sigma (Poole, UK).

Synthesis of [$^2H_3$]17β-19-nortestosterone. [$^2H_3$]19NT was synthesised from 19NT (Sigma), essentially using the method described by Schänzer and Donike[12] for [$^2H_3$]testosterone. The product was a mixture of [$^2H_0$]-, [$^2H_1$]-, [$^2H_2$]- and [$^2H_3$]NT, in the ratio 24 : 8 : 4 : 64 m/m, with an overall yield of 37.6%.

Synthesis of unlabelled and [$^3H_2$]- and [$^2H_3$]17β-19-nortestosterone laurate. [$^3H_2$]19NT (925 MBq), [$^2H_3$]19NT (430 mg), lauric anhydride (1.67 g), triethylamine (0.6 ml) and a trace (catalytic) amount of dimethylaminopyridine were dis-

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solved in dichloromethane (80 ml). The mixture was stirred at ambient temperature for 16 h and 19NTL was isolated by repeated column chromatography on Kieselgel H (with hexane–ethyl acetate (6 + 1 v/v) as eluent. The chemical purity was checked using thin layer chromatography on silica gel 60 F_{254} aluminium TLC sheets (product 5554; Merck, Poole, UK) using ethyl acetate–hexane (1 + 1 v/v) as eluent, and C_{18} reversed-phase UV and radio-HPLC with a Model 1090 HPLC system with diode array detection (Hewlett-Packard, Bracknell, UK) and an in-line Radiomatic A525 radio-detector (Canberra Packard, Pangbourne, UK), using an acetonitrile–water gradient (1 ml min^{-1}, 80–100% acetonitrile over 2.5 min, 100% acetonitrile for 2.5 min, 100–80% over 1 min, then 80% for 5 min). The structure of the product was confirmed by atmospheric pressure chemical ionisation (APCI) LC-MS (conditions as in HPLC above) in the positive ion mode (Platform I, Micromass, Manchester, UK). Unlabelled 19NTL was synthesised in the same manner. A final concentration of labelled 19NTL was prepared in ethanol (295 MBq $mmol^{-1}$) by dilution of labelled with unlabelled 19NTL solution. This preparation was subsequently dissolved in arachis oil for administration to steers.

Animals and treatment

Friesian/Belgian Blue cross steers (130–140 kg, $n = 4$) were surgically prepared to provide indwelling gall bladder cannulae, as described by McEvoy,[13] and allowed a recovery period of at least 3 weeks prior to administration of 19NTL. A single intramuscular (rump muscles) injection of 19NTL in arachis oil (200 mg, including 140 MBq $^3H_2$ and 100 mg $^2H_3$ in 0.75 ml of ethanol, dissolved in 4.5 ml of arachis oil) was administered (2 April 1997 at about 0800 h) to each steer. The animals were then individually housed in metabolism crates for 7 d to allow collection of the total output of urine and faeces. Thereafter, animals were housed on straw bedding in stalls and re-introduced to the metabolism crates every 2–4 d for a period of about 7 h; this permitted representative sampling of excreta until euthanasia by captive bolt and exsanguination 30 d after 19NTL administration. Throughout the study, animals were fed a standard ration of hay and concentrates and allowed *ad libitum* access to water.

Animal sampling

All samples were stored at −20 °C as soon as practical after collection.

Blood, bile, urine and faeces. Blood (about 50 ml) was collected by jugular venepuncture and plasma was separated by centrifugation and stored in polypropylene tubes. Bile samples were collected periodically (it was not always possible to withdraw a sample) using a syringe, *via* the indwelling silicone rubber cannulae, and the volumes recorded. Urine was collected continuously while the steers were in the metabolism crates; urine was channelled into a polypropylene beaker *via* the funnelled flooring, and the total volume was determined at the time of sampling. To minimise contamination of urine, faeces were collected directly into polypropylene bags maintained in position using a harness; each sample was thoroughly mixed by manual manipulation of the collection bag prior to representative sampling. The total output of faeces was weighed at each collection.

Hair. Hair was clipped twice at each sampling time, before and 7 and 29 d following dosing. The first distal clippings removed hair to within about 7 mm of the skin and the residual proximal hair was removed with the second surgical clip. In addition, at 29 d, hair that had grown on the site clipped at 7 d was surgically clipped to provide regrowth samples.

Tissues at *post-mortem*. At *post-mortem*, liver, kidney, fat (kidney and mesenteric), rump muscle (contralateral to the injection site), brain, heart, lung, prostate, spleen, rumen, small intestine, spiral colon and eye (choroid and pigmented retinal epithelium), injection site, blood, bile, faeces and urine were sampled and packaged in polyethylene bags or polypropylene containers for storage.

Determination of total residues

Duplicate representative samples of plasma (1 ml), bile (0.1 ml), faeces (0.5 g), urine (1 ml) and tissues (500 mg) were placed in sample cones, combusted (Packard Oxidiser, Model 306M, Canberra Packard) and resulting tritiated water collected into glass vials containing scintillation cocktail (Monophase S, 10 ml, Canberra Packard), and the tritium content was determined by liquid scintillation counting. Plasma pharmacokinetic parameters were calculated for each animal using a compartmental modelling software program (TopFit 2.0[14]).

Extraction of urine and bile samples for HPLC analysis

Duplicate samples of urine and bile were extracted and analysed directly or following hydrolysis with *Helix pomatia* glucuronidase solution (10 mg ml^{-1}, in 0.025 M sodium acetate buffer, pH 4.5) and incubated for about 16 h at 37 °C. Urine samples (30 ml) were acidified with formic acid (6 μl), and bile (1 ml) samples were diluted with three volumes of 0.02% v/v formic acid, prior to concentration on to preconditioned (methanol, 10 ml, then 0.02% formic acid, 2 × 10 ml) C_{18} Mega Bond Elut cartridges (Varian, Walton on Thames, UK). Cartridges were washed three times with water (10 ml) and the extracts eluted with methanol (10 ml). The extracts were then evaporated to dryness under nitrogen. Sample extracts for hydrolysis were dissolved in methanol (0.25 ml) then 1.25 ml of β-glucuronidase solution was added and the mixture was incubated for 1 h at 50 °C, then extracted with diethyl ether (8 ml) and the organic phase was aspirated and evaporated to dryness. Hydrolysed and untreated extracts were dissolved in 0.02% v/v formic acid in methanol–water (1 + 1 v/v) (0.15 ml) for HPLC analysis.

Extraction of bile samples for GC-MS analysis

Bile samples were extracted essentially as described previously.[15] Following addition of internal standard ([^{2}H]5β-androstan-3α,17β-diol; 200 ng ml^{-1}), bile samples were adjusted to pH 6.8 and conjugated material was hydrolysed by incubation with *E. coli* β-glucuronidase (2000 units; 2 h at 50 °C). Hydrolysed samples (5 ml) were then extracted on preconditioned (methanol, 5 ml, then water, 5 ml) C_{18} Sep Pak Vac solid-phase cartridges (500 mg, 6 ml) (Waters, Watford, UK), and the cartridges were washed with water (5 ml) and then hexane (5 ml), drying the cartridges at each stage. Analytes were eluted with diethyl ether (5 ml). The ether extracts were washed with saturated sodium hydrogencarbonate (2 × 1 ml), dried over anhydrous sodium sulfate, then evaporated to dryness under oxygen free nitrogen (OFN) at 80 °C.

Oxime-*tert*-butyldimethylsilyl (oxime-TBDMS) derivatisation

Derivatisation was carried out essentially as described by Teal and Houghton.[15] Hydroxylamine hydrochloride (8% m/v in pyridine, 50 μl) was added to the ether extracts and heated at 80 °C for 30 min, then evaporated to dryness at 80 °C under OFN. Following addition of diethylamine hydrochloride (5% m/v in *N,N*-dimethylformamide, 50 μl) and *N*-(*tert*-butyldime-

thylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA, 50 μl), the samples were heated for 1 h at 80 °C. Water (5 ml) and diethyl ether (2 ml) were then added and the samples vortex mixed. After freezing at −30 °C, the ether layer was decanted off into clean tubes, evaporated to dryness at 80 °C under OFN, the residue was dissolved in undecane (25 μl) and the solution was analysed by GC-MS.

Methoxime-trimethylsilyl (MO-TMS) derivatisation

MO-TMS derivatives were formed essentially as described by Dumasia and Houghton.[16] Methoxyamine hydrochloride (8% m/v in pyridine, 25 μl) was added to extracts and the mixture was heated at 80 °C for 30 min and evaporated to dryness at 80 °C under OFN. Following addition of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA, 25 μl) and heating at 80 °C for 1 h, the sample was dissolved in chloroform–hexane (1 + 1 v/v, 1 ml) and passed through a mini-Sephadex LH-20 column. The eluate was evaporated to dryness at 80 °C under OFN, the residue was dissolved in undecane (25 μl) and the solution was analysed by GC-MS.

Radio-HPLC of urine and bile extracts

Acidified extracts of urine (50 μl) and bile (100 μl) were analysed by gradient HPLC using a C_{18} guard column (5 × 3 mm id, LiChroCART 50961; Merck) and an analytical column (Hypersil Elite, 5 μm particle size, 150 × 4.6 mm id) (Hichrom, Reading, UK). A dual pump HPLC system (two Model 2510 HPLC pumps and a Model 9500 autosampler; Varian) was used with an in-line flow scintillation detector (Radiomatic A500; Canberra Packard) at a flow rate of 1 ml min^{-1}.

The mobile phase solvents, 0.1% formic acid (A), and 0.1% formic acid in methanol (B), were used to run a linear gradient from 40 to 100% B in 12 min, remaining at 100% B until 22 min and re-equilibrating for 10 min with 40% B between runs.

LC-MS

Metabolite identification was evaluated by ion trap LC-MS (LCQ; Finnigan, Hemel Hempsted, UK) in the positive ion mode using APCI and electrospray ionisation (ESI) interfaces. The same chromatographic conditions were used as described for HPLC analysis.

GC-MS

Derivative preparations were analysed using a 25 m BPX5 column (25QC2/BPX5, 2 μm film thickness) (SGE, Milton Keynes, UK) by positive ion GC-MS using electron impact in the full scan mode (GCQ; Finnigan) to confirm the identity of metabolites (MO-TMS derivatives), or in the selected ion recording mode (MD800; Fisons, Wythenshawe, UK) for quantification of metabolites (oxime-TBDMS derivatives). For quantification of metabolites in bile, calibration standards and QCs were prepared in 0.1 M phosphate buffer (pH 6.8) and extracted alongside the samples and derivatised using the oxime-TBDMS method.

Results and discussion

Synthesis of [$^3H_2$]- and [$^2H_3$]17β-19-nortestosterone laurate

Kieselgel H column chromatographic purification of [$^3H_2$]- and [$^2H_3$]19NTL followed by concentration gave rise to a white solid (yield 87%). Characterisation by TLC gave a single spot (UV detection at 254 nm, R_f 0.6). A single peak was detected by HPLC using both UV detection (242 nm, retention time 4.85 min) and in-line radiochemical detection (retention time 5.1 min), indicative of 98.2 and 95.2% purity, respectively. LC-positive ion ESI-MS analysis gave rise to molecular ions at *m/z* 457 and 460, corresponding to $[M + H]^+$, for the unlabelled and deuterated products, respectively. Fig. 1 illustrates the structure of 19NTL, 19NT and potential metabolites.

Sampling of bile *via* gall bladder cannulae

Periodic sampling of bile *via* the indwelling cannulae provided a valuable means of assessing the hepatic output of biotransformation products throughout the study period. It should be noted, however, that it was not always possible to withdraw a bile sample at will, even following temporary withdrawal of feed; for some animals the ability to withdraw bile became more sporadic while animals were housed in metabolism cages. All cannulae were found to be patent at *post-mortem*. Following *post-mortem* investigation, the view was taken that the problem was associated with gradual thickening of the gall bladder wall, and, from time to time, occlusion of the cannulae by surrounding tissue. This could perhaps occur as a result of re-orientation of the gall bladder during the course of the day, such that the cannula inlet was not positioned in the bile pool.

Total plasma residues

Plasma pharmacokinetics of total radioactive residues were evaluated using two, three and four compartments models, without statistical weighting. On the basis of analysis of residuals, the four compartment model provided the best fit for the data, indicating the complex absorption/disposition pattern associated with steroid fatty esters administered in slow release oil formulation; parameters for individual animals and mean values using this model are given in Table 1 and the mean pharmacokinetic profile in Fig. 2. A mean ± *s* maximum concentration (C_{max}), of 56.3 ± 15.9 pmol ml^{-1} (about 15 pg

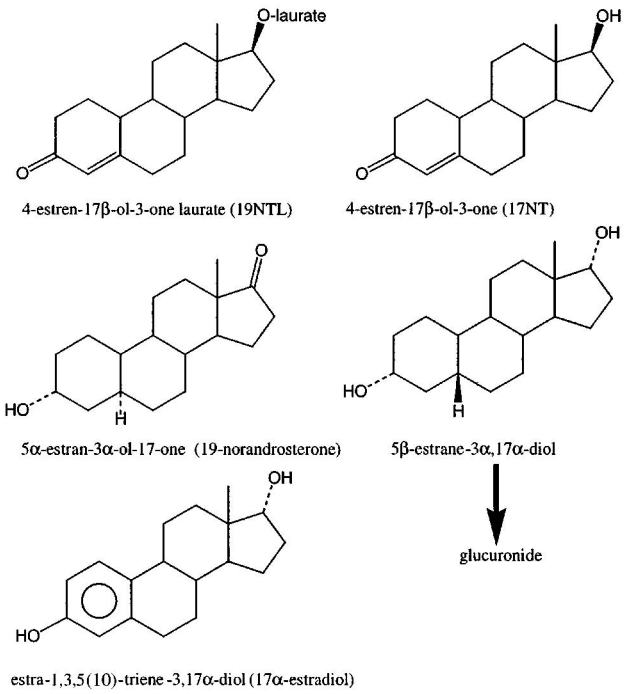

Fig. 1 Structures of 19-nortestosterone laurate and major metabolites.

19NT equivalents ml^{-1}) was found 36 h post-administration. Plasma half-lives (mean ± *s*) for α-, β-, γ- and δ-phases were 0.02 ± 0.03, 1.2 ± 1.2, 11.5 ± 3.1 and 437 ± 28 h, respectively. The total radiolabelled residue concentration (mean ± *s, n* = 4) at the termination of the study, 30 d after treatment (Fig. 2), was 13.3 ± 1.6 pmol ml^{-1} plasma. This indicates that specific target residues should be detectable in plasma at this time by methods such as immunoassay or GC-MS, even in the event that the predominant metabolite constituted as little as 10% of the total residues.

Total residues in tissues, fluids and excreta

At the point of first sampling following 19NTL administration, total radiolabelled residue concentrations were substantial, generally in the order bile (2–16 nmol ml^{-1}) ⩾ urine = faeces (0.5–3 nmol ml^{-1}), as shown in Fig. 3, and maintained throughout in these matrices at higher concentrations than those determined at slaughter in other tissues (Fig. 4 and 5). The exception to this was the injection site, where concentrations (mean ± *s, n* = 4) of 338 ± 373 nmol g^{-1} muscle were found; the large variation noted was associated with imprecision in sampling at the exact depth of injection in the target area. These data would indicate, in keeping with previous studies,[2,3] that for residue surveillance, where the focus is on slaughter-house sampling and when an injection site is not evident, bile, faeces or urine would provide the preferred target matrices. Of the edible tissues, concentrations were greatest in liver and kidney (Fig. 4). All gastro-intestinal tract samples posterior to the entry of the bile duct showed substantially lower concentrations following momentary washing of tissue (three times with about 10 volumes of Eagles buffered saline solution), indicating an element of surface contamination with intestine contents. Of note, pigmented eye tissue (choroid/pigmented retinal epithelium) contained appreciable and consistent concentrations of radiolabelled residues (33.1 ± 6.1 pmol g^{-1}; mean ± *s, n* = 4).

Evaluation of residues in hair, which was largely lacking in pigmentation, indicated appreciable accumulation of residues at 29 d after treatment. In all cases, concentrations were significantly greater in unwashed hair than in hair washed three times with 0.2% v/v aqueous dodecyl sulfate, as shown in Fig. 5. Although the washing process may have eluted residues from within the hair fibres, it is also possible that residues washed out may have originated from the hair surface, either from sebaceous secretions or surface contamination (*e.g.*, from excreta). In any event, these data indicate that hair could provide a useful non-invasive matrix for on-farm sampling, although it remains for the nature of target residues to be determined before the practical implications for surveillance can be established. The steers used in this study had predominantly white hair, although there were small dorsal regions of highly pigmented hair (remote from possible contamination by excreta) on two of the animals; comparison of hair from these regions, taken 29 d after administration, with white hair from the same animals indicated substantially higher concentrations (mean ± *s, n* = 2, determined in quadruplicate) of residues in black hair (28.9 ± 8.9 pmol g^{-1}) than in white hair (13.4 ± 3.4 pmol g^{-1}). This, with the data from the choroid/pigmented retinal epithelium of the eye (Fig. 6), provides an indication of preferential binding to pigments such as melanin.

Urine and bile metabolite profiles

The recovery (mean ± *s*) of total radio-labelled residues in urine and bile extracts prior to radio-HPLC was 84 ± 11% (*n* = 34) and 128 ± 30% (*n* = 24). Evaluation of extracts of urine and bile throughout the study period generally indicated the presence of only minor amounts of residual 19NTL, 19NT or 17α-19NT, among a complex mixture of metabolites, as shown in the representative radio-HPLC profiles in Fig. 6. HPLC of solutions of reference standards gave typical retention times for 17β- and

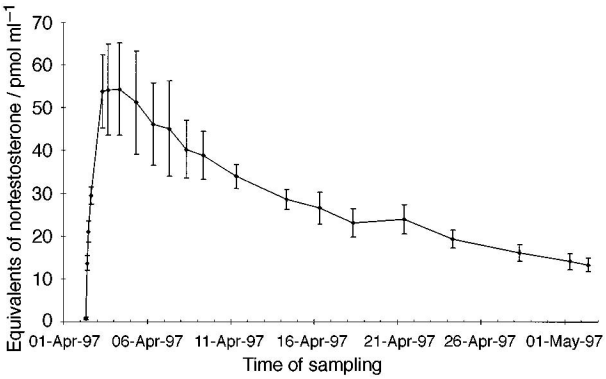

Fig. 2 Absorption and depletion of total radiolabelled residues of 19-nortestosterone laurate in plasma. Samples were collected over 30 d following a single intramuscular injection on 2 April 1997. Total radiolabelled residues (mean ± *s, n* = 4) were determined by liquid scintillation counting following sample combustion.

Table 1 Pharmacokinetics of total radiolabelled residues in plasma. Total radiolabelled residues were determined by liquid scintillation counting following sample combustion. Samples were collected over 30 d following a single intramuscular injection. Data were derived using a four compartment model, with no statistical weighting

	Steer					
Parameter[a]	770	772	783	785	Mean	*s*
$t_{1/2}\alpha$/h	0.0024	0.0007	0.0040	0.0691	0.019	0.033
$t_{1/2}\beta$/h	1.4	2.73	0.45	0.16	1.18	1.16
$t_{1/2}\gamma$/h	14.7	13.6	8.5	9.3	11.5	3.1
$t_{1/2}\delta$/h	412	459	413	463	437	28
MRT-abs/h	79.6	77.9	12.4	25.9	48.9	34.9
MRT-disp/h	457	554	586	639	559	77
MRT-tot/h	536	632	598	665	608	55
V_{ss}/l	7.5	12.3	12.4	10.3	10.6	2.3
Cl/ml min^{-1}	0.276	0.372	0.352	0.269	0.317	0.052
AUC/nmol ml^{-1} h	33.0	24.5	25.9	34.0	29.3	4.8
C_{max}/pmol ml^{-1}	78.3	45.0	44.5	57.6	56.3	15.9
T_{max}/h	47.4	44.2	22.1	30.3	36.0	11.9
r^2	0.999	0.998	0.997	0.998	0.998	0.001

[a] $t_{1/2}$ = half-life corresponding to the exponents for absorption or disposition; MRT = mean residence time for absorption (abs), disposition (disp) or for the complete system including lag-times (tot); V_{ss} = volume of distribution at steady state; Cl = total clearance; AUC = area under the curve from $t = 0$ to $t = \infty$; C_{max} = maximum plasma concentration; T_{max} = time taken to achieve maximum concentration; r^2 = coefficient of determination, describing the goodness of curve fit following least-squares non-linear regression analysis.

17α-19NT of 10.8 and 11.5 min, respectively; LC-MS retention times of other putative metabolites are given in Table 2. As expected, many of the more polar metabolites in samples were sensitive to hydrolysis by *Helix pomatia* β-glucuronidase, indicating extensive (glucuronide or sulfate) conjugation. Following hydrolysis, the majority of biotransformation products in urine and bile exhibited retention times longer than that

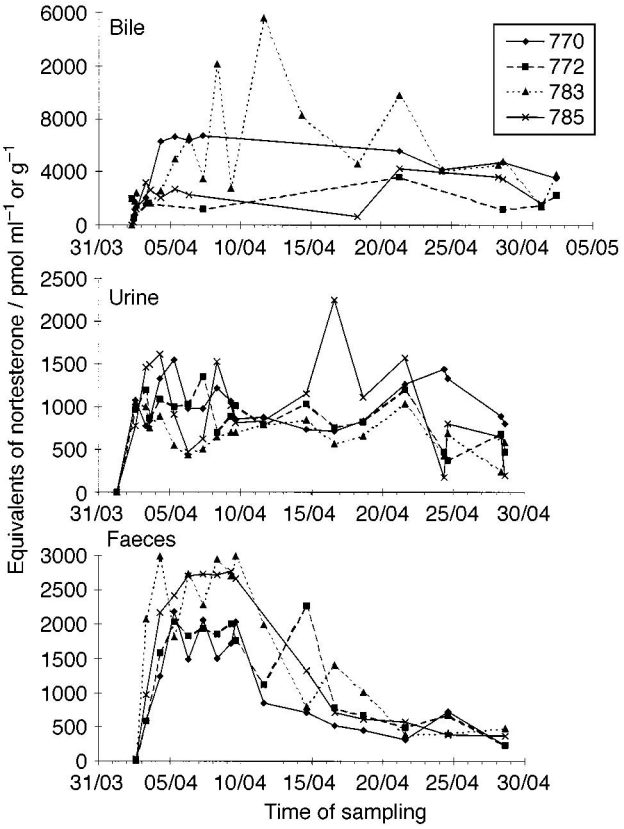

Fig. 3 Absorption and depletion of total radiolabelled residues of 19-nortestosterone laurate in bile, urine and faeces. Samples were collected over 30 d following administration. Total radiolabelled residues (values are mean of two determinations for each animal) were determined by liquid scintillation counting following sample combustion.

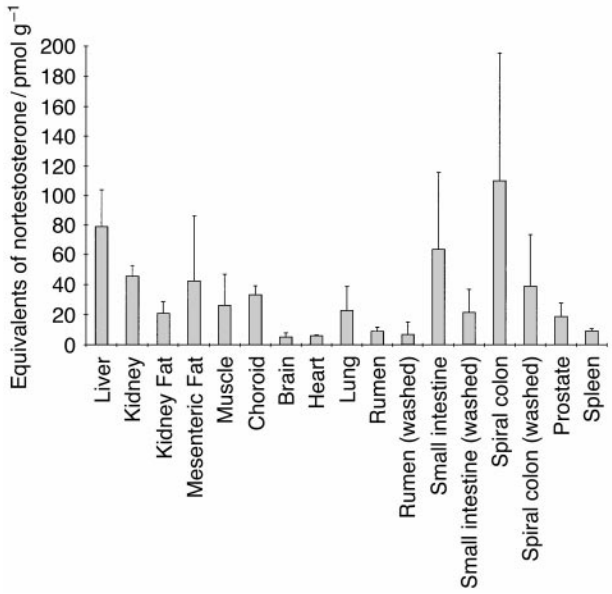

Fig. 4 Total radiolabelled residues of 19-nortestosterone laurate in tissues. Tissues were sampled from four steers *post-mortem* 30 d after 19-nortestosterone laurate administration and total radiolabelled residues (mean ± *s*, n = 4) determined by liquid scintillation counting following sample combustion.

those of 17β- and 17α-19NT; the retention times of products were more consistent with those of ring A reduced metabolites such as the epimers of estranolones and estranediols in urine, and of estranediols (*e.g.*, 5β-estrane-3α,17α-diol) in bile, as indicated by comparison of relative retention times (Fig. 6) with those in Table 2. In GC-MS studies, the major urinary metabolites in an adult cow 3 d after administration of 19NT undecanoate were found to be 5α-estrane-3β,17α-diol and 17α-

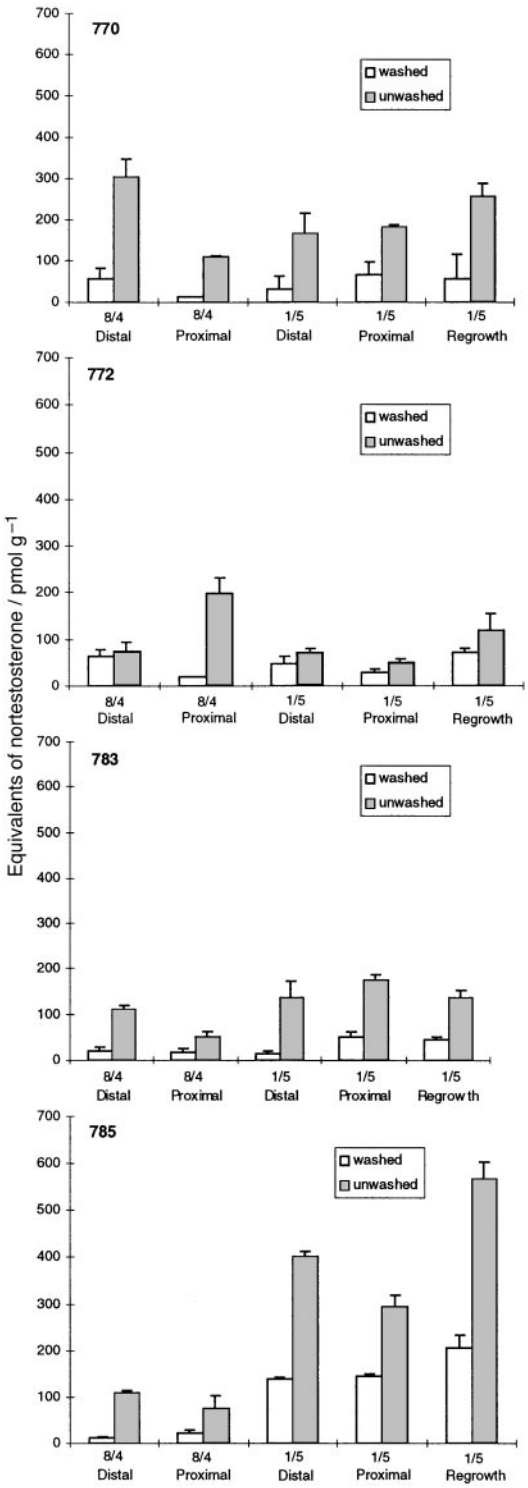

Fig. 5 Total radiolabelled residues of 19-nortestosterone laurate in hair. Hair samples were taken on days 7 and 29 following administration of 19-nortestosterone laurate. Samples were taken in two clippings. The first removed hair to within about 7 mm of the skin (distal) and the second the 7 mm proximal to the skin. A further sample (regrowth) was taken in a single surgical clipping at 29 d, from regions previously clipped on day 7. Samples were analysed untreated or, following washing, by liquid scintillation counting following sample combustion. Values are means ± *s* (n = 4).

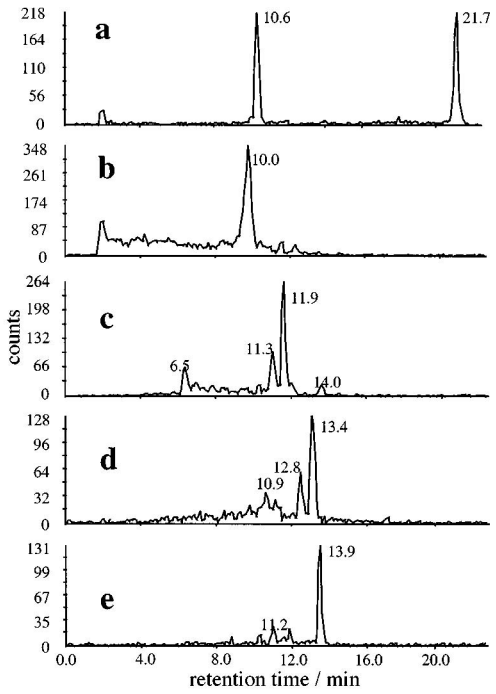

Fig. 6 Radio-HPLC metabolite profiles of urine and bile extracts. Representative urine and bile extracts were analysed untreated and following hydrolysis with *Helix pomatia* glucuronidase. (a) Standard mixture of nortestosterone (retention time 10.6 min) and nortestosterone laurate (retention time 21.7 min); (b) urine extract; (c) urine extract following treatment with β-glucuronidase; (d) bile extract; and (e) bile extract following treatment with β-glucuronidase.

19NT,[6] and in female calves treated with 19NT and 19NTL, 5α-estrane-3β,17α-diol, 17α-19NT and 5β-estran-17one-3α-ol were found to prevail.[7] Clearly, the present radio-HPLC procedures lack the resolution to confirm the presence of particular epimers in urine, although they provide evidence that 17α-19NT is not the predominant metabolite. Since the radio-HPLC profiles represent the total metabolites present (assuming minimal tritium exchange), it is possible that this disparity may

Table 2 HPLC retention times of steroid reference standards 20–1000 μg ml^{-1}. Steroid standards were prepared and analysed individually using chromatographic conditions described for HPLC, but with MS detection

Steroid	Retention time/min	Steroid	Retention time/min
4-Estren-17β-ol-3-one	10.8	5α-Estran-17β-ol-3-one,	12.0
4-Estren-17α-ol-3-one	11.5	5α-Estran-17α-ol-3-one,	12.5
4-Estren-17β-ol-3-one 17-laurate	21.7	5α-Estran-3β-ol-17-one	11.7
5α-Estrane-3β,17β-diol,	11.6	5α-Estran-3α-ol-17-one	10.8
5α-Estrane-3β,17α-diol,	12.0		
5β-Estrane-3α,17β-diol,	12.5		
5β-Estrane-3α,17α-diol	14.1	4-Estren-17α-ol-3-one 3-glucuronide	9.0
Estra-1,3,5(10)-triene-3,17α-diol	11.3	Estra-1,3,5-triene-3,17α-diol 3-sulfate	7.3
Estra-1,3,5(10)-triene-3,17β-diol (17β-estradiol)	11.0		

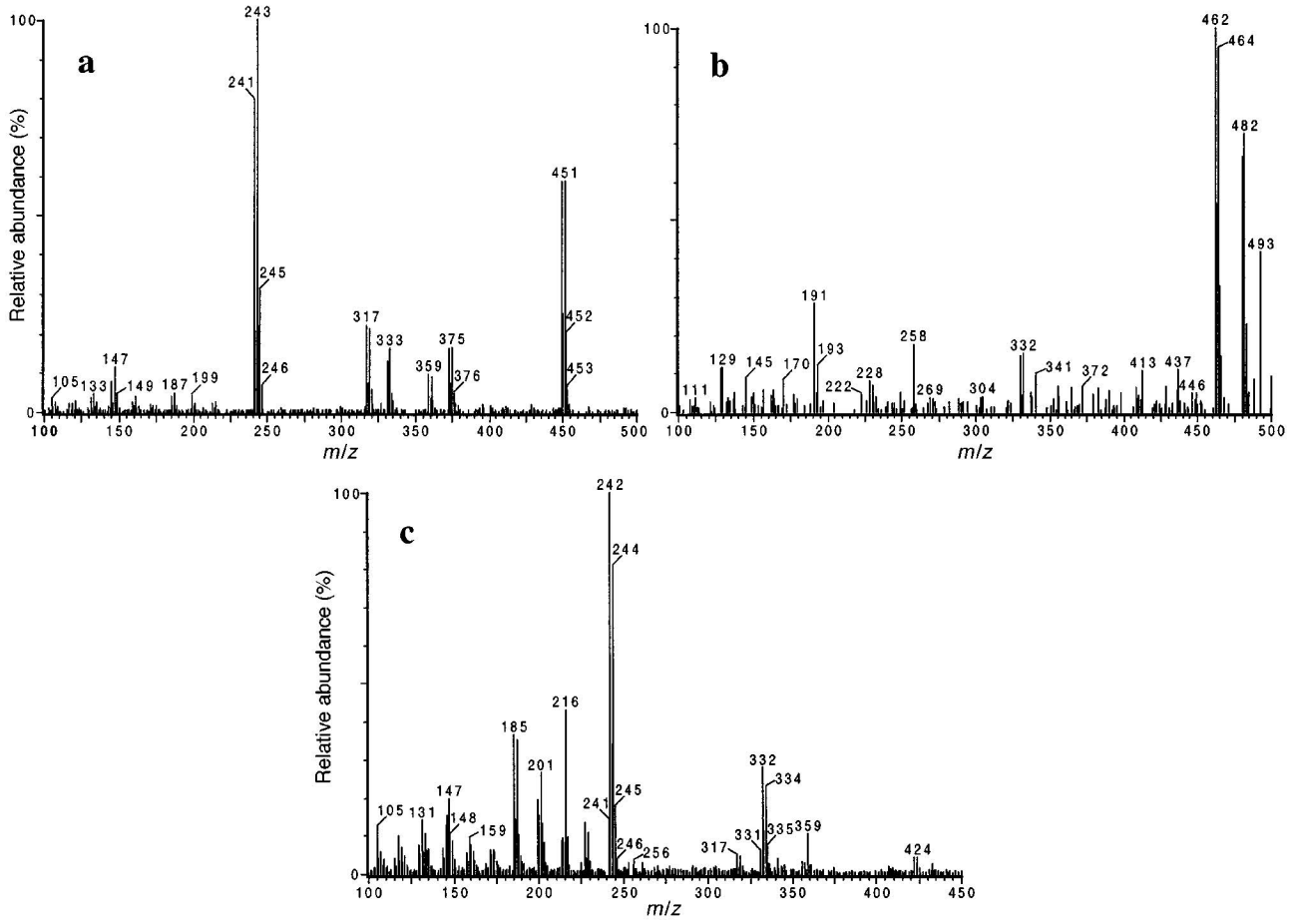

Fig. 7 GC-MS of major 19-nortestosterone laurate metabolites in bile. GC-MS spectra of oxime-TBDMS derivatives of (a) 5β-estran-3α,17α-diol (*m/z* 506 $[M]^{\bullet+}$) showing loss of *tert*-butyl (-57 Da) and two alkylsilanol groups (-265 Da), and (b) 5α-estran-3α-ol-17-one (*m/z* 519 $[M]^{\bullet+}$) showing loss of the *tert*-butyl group. The GC-MS spectrum (c) of the MO-TMS derivative of 5β-estran-3α,17α-diol indicates abundant fragment ions and provides for confirmation of metabolite identity.

represent differences in relative sensitivity of the GC-MS methods for the various metabolites. Differences may also exist as a result of variations in the nature of the 19NT treatment, the route of administration and between male and female and young compared with adult subjects. The latter view is partly supported by the data of Daeseleire *et al.*,[7] when compared with those of Benoit *et al.*;[6] the former found that 17α-NT residues were present at higher concentrations than 5α-estran-3β,17α-diol in urine of female calves administered intramuscular NTL but, interestingly, that the converse was true following daily 19NT treatment *via* the oral route. By comparison, studies in horses have revealed a predominance of estranediols (conjugates) in urine following administration of 19-NT.[17,18]

Identification of metabolites by LC-MS. In the present preliminary application of LC-MS, attempts to identify the major bile and urine metabolites were hindered by poor molecular ion formation produced by the estranes, which was possibly exacerbated by interference from matrix co-extractives. Some of the problems encountered have been addressed in parallel studies using hepatocytes to investigate biotransformation by the liver in steers;[19] these studies used positive ion LC-APCI-MS^2 and comparison with reference standards indicated biotransformation to 4-estren-17α-ol-3-one, 4-estrene-3,17-dione (major metabolite after 1 h), *n*-hydroxy-4-estrene-3,17-dione, *n*-hydroxy-4-estren-17-ol-3-one, 5β-estran-3α-ol-17-one and 5β-estrane-3α,17β-diol (major metabolite after 4 h of incubation) and (by ESI-MS^2) their respective glucuronide conjugates.

Identification of metabolites in bile by GC-MS. Sampling *via* gall bladder cannulae and analysis of bile samples by GC-MS permitted the identification and quantification of a number of 19NTL biotransformation products over the duration of the study. The recovery (mean ± *s*) of total radio-labelled residues from bile extracts prior to derivatisation was 36 ± 17% (n = 44). GC-MS of oxime-TBDMS derivatives of metabolites gave prominent fragment ions at *m/z* [M − 57]$^+$, consistent with facile loss of the *tert*-butyl moiety, as indicated in Fig. 7(a) for 5β-estrane-3α,17α-diol (for non-labelled product, *m/z* 506 [M]$^{\bullet+}$, giving rise to *m/z* 449 [M−57]$^{\bullet+}$ and, by loss of two alkylsilanol groups, *m/z* 241) and in Fig. 7(b) for 5α-estran-3α-ol-17-one (*m/z* 519 [M]$^{\bullet+}$ giving *m/z* 462 [M−57]$^{\bullet+}$); ions representing the loss of 189 and 265 Da (alkylsilanol groups) were typical for the majority of derivatives. MO-TMS derivatives, on the other hand, provided abundant fragment ions, as shown in Fig. 7(c) for 5β-estrane-3α,17α-diol (*m/z* 422 [M]$^{\bullet+}$, *m/z* 332 [M−90]$^{\bullet+}$, *m/z* 242 [M−90−90]$^{\bullet+}$, *m/z* 317 [M−15−90]$^{\bullet+}$, *m/z* 227 [M−15−90−90]$^{\bullet+}$ and so were used for confirmation of metabolite identity. Typical GC—MS retention times and relative retention times (compared with the internal marker [^{2}H]5β-androstane-3α,17β-diol) of derivatives of a range of putative metabolites are given in Table 3. Comparison of retention times from total ion chromatographs and selected ion recording experiments using MO-TMS derivatives of standards and bile sample extracts, as shown in Fig. 8, indicated that the major metabolite of 19NTL was 5β-estrane-3α,17α-diol (*m/z* 422 [M]$^{\bullet+}$), and that the other principle metabolites were 5α-estrane-3β,17α-diol, 5α-estran-3α-ol-17-one (norandrosterone, *m/z* 377 [M]$^{\bullet+}$), *m/z* 346 [M−31]$^{\bullet+}$, *m/z* 256 [M−31−90]$^{\bullet+}$ and 1,3,5(10)-estratriene-3,17α-diol (17α-estradiol, *m/z* 416 [M]$^{\bullet+}$). Co-administration of ^{2}H- and ^{3}H-labelled 19NTL allowed the ready discrimination of metabolites from endogenous steroid by the presence of twin ions in the mass spectra (Fig. 7) and permitted for instance, identification of 17α-estradiol (an endogenous steroid) as a

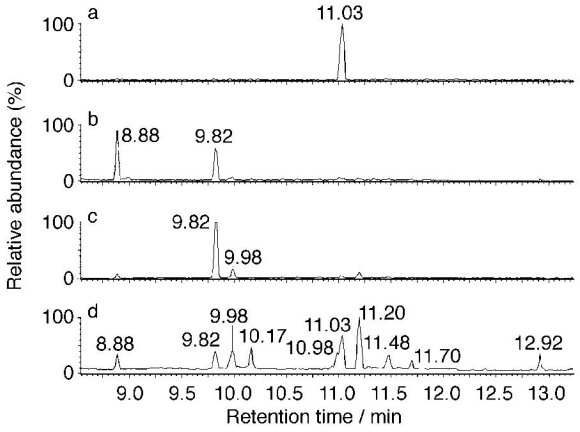

Fig. 8 Typical selected ion and total ion chromatographs indicative of the metabolites present in bile extracts following MO-TMS derivatisation. Selected ion chromatographs of (a) *m/z* 439, characteristic of [^{2}H]5β-androstane-3α,17β-diol [M]$^{\bullet+}$ ion, the internal marker, (b) *m/z* 332 characteristic of 5β-estrane-3α,17α-diol [M−90]$^{\bullet+}$ ion and (c) *m/z* 242 characteristic of 5β-estrane-3α,17α-diol [M−90−90]$^+$ ion. (d) The total ion chromatograph over *m/z* 100–700.

Table 3 GC-MS retention times (RT) of steroid standards and relative retention times (RRT), compared with the internal marker (RT/RT[^{2}H]5β-androstane-3α,17β-diol). Steroid oxime-TBDMS and MO-TMS derivatives were prepared and analysed as described for bile analysis

	MO-TMS derivatives		Oxime-TBDMS derivatives	
Steroid	RT	RRT	RT	RRT
[^{2}H]5β-Androstane-3α,17β-diol	11.13	1.0	17.03	1.0
5α-Estrane-3α,17β-diol	10.24	0.92	16.15	0.95
5α-Estrane-3β,17α-diol	10.36	0.93	16.22	0.95
5α-Estrane-3β,17β-diol	11.02	0.99	17.25	1.01
5β-Estrane-3α,17α-diol	9.56	0.86	14.31	0.84
5β-Estrane-3α,17β-diol	10.55	0.95	16.54	0.97
4-Estrene-3β,17β-diol	9.18	0.83	17.41	1.02
5(10)-Estrene-3β,17α-diol	10.28	0.92	15.5	0.91
4-Estren-17β-ol-3-one	12.34	1.11	19.57, 20.25	1.15[a], 1.19[a]
4-Estren-17α-ol-3-one	12.01	1.08	18.15, 18.43	1.07[a], 1.08[a]
Estra-1,3,5(10)-trien-3,17α-diol	12.08	1.09	18.17	1.07
Estra-1,3,5(10)-trien-3,17β-diol	12.35	1.11	19.24	1.13
Estra-1,3,5(10)-trien-3-ol-17-one	12.51	1.13	20.2	1.19
5β-Estran-3α-ol-17-one	11.19	1.01	18.22	1.07
5α-Estran-3α-ol-17-one	10.36	0.93	17.01	1.00
5α-Estran-3β-ol-17-one	11.21	1.01	18.2	1.07
5α-Estran-17β-ol-3-one	11.34, 11.4	1.02[a], 1.03[a]	18.39,19.03	1.08[a], 1.12[a]
5β-Estran-17β-ol-3-one	11.46, 11.49	1.03, 1.03[a]	18.26, 18.49	1.07[a], 1.09[a]
4-Estrene-3,17-dione	12.59	1.13	21.01, 21.32	1.23[a], 1.25[a]
5(10)-Estrene-3,17-dione			21.03, 21.32	1.24[a], 1.25[a]

[a] Denotes either *syn* or *anti* isomer for the derivative.

19NTL biotransformation product. 5β-Estrane-3α,17α-diol was subsequently quantified in all bile samples following oxime-TBDMS derivatisation, as indicated in Fig. 9. Concentrations in excess of 100 ng ml^{-1} were found throughout the study period, indicating the importance of this metabolite as a target residue in bile for at least 30 d after treatment.

Identification of metabolites in urine by GC-MS. Preliminary qualitative results from the analysis of urine samples by GC-MS support findings reported previously[6,7] and further quantitative studies are in progress to establish the relative merits of the metabolites present from the surveillance perspective.

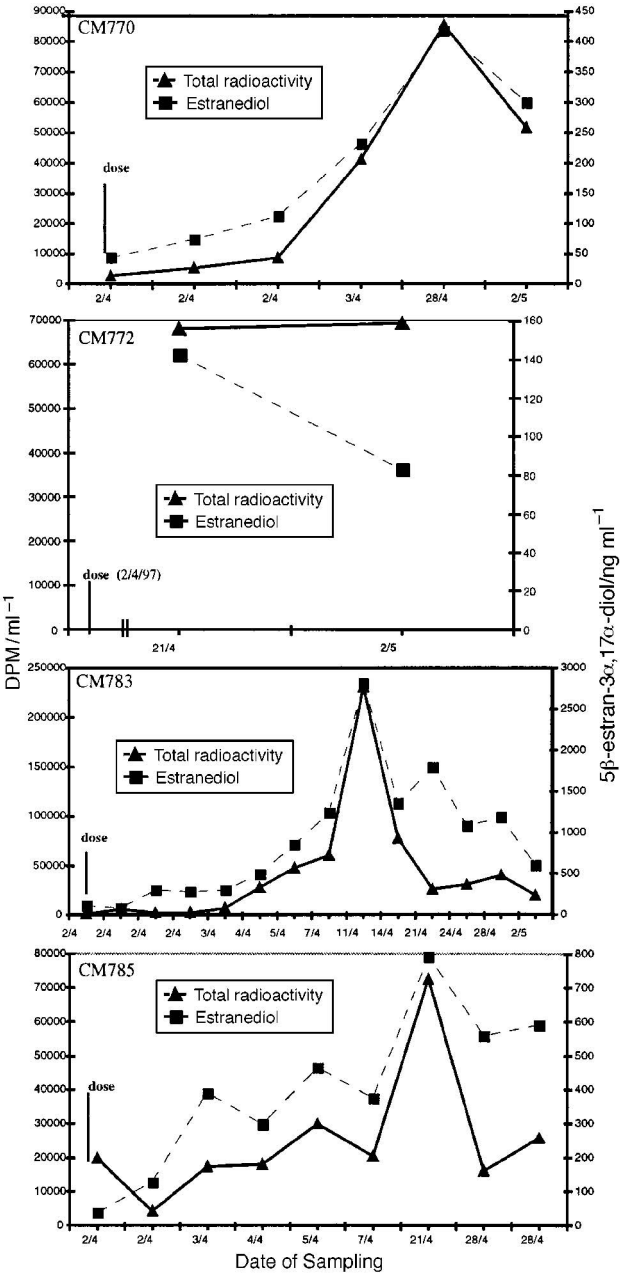

Fig. 9 Concentrations of 5β-estrane-3α,17α-diol in bile sampled from four steers throughout the study period. Total radioactivity was estimated by direct liquid scintillation counting of 50 μl of bile. Quantification was by GC-MS following oxime-TBDMS derivatisation.

Conclusions

The preliminary evaluation by radio-HPLC supports the view that estranediols (and conjugates) are the major biotransformation products in bile and urine, but not the contention that 17α-19NT or its glucuronide conjugate are the most abundant metabolites in steers. GC-MS analysis indicated that the major metabolite of 19NTL in bile was 5β-estrane-3α,17α-diol, with 5α-estrane-3β,17α-diol, 5α-estran-3α-ol,17-one and 17α-estradiol present as other principle products. The maintenance of appreciable total residues in plasma (about 10–50 pmol ml^{-1}) and hair throughout the study period indicated the potential importance of these analytical matrices for on-farm monitoring, given the ease of sampling compared with urine. Further work is under way to establish the nature of the target analytes in plasma, urine and hair.

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