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4th Symp. on Handling of Environmental and Biological Samples in Chromatography Basle, April 27–29, 1988

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CONTENTS:

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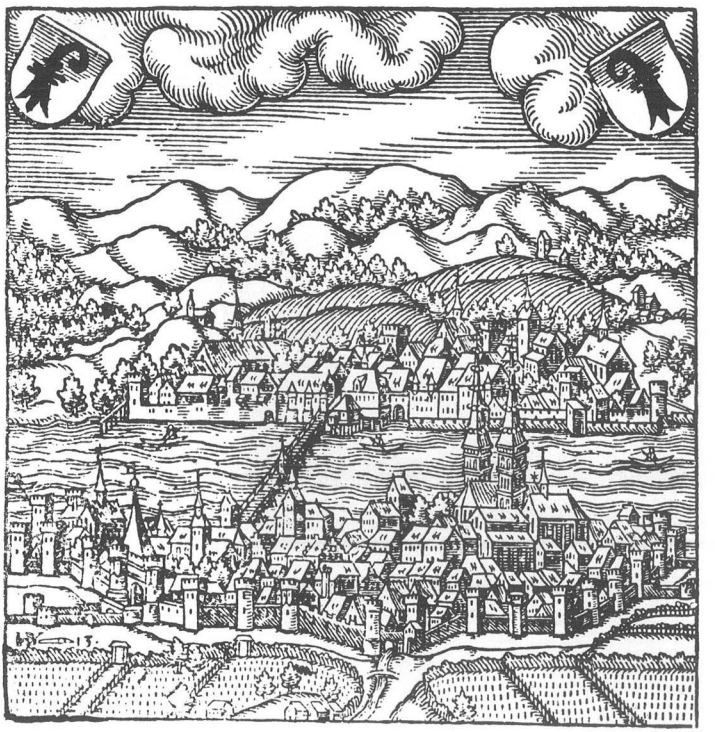

FOURTH SYMPOSIUM ON HANDLING OF ENVIRONMENTAL AND BIOLOGICAL SAMPLES IN CHROMATOGRAPHY

Basle, April 27–29, 1988

Guest Editor

R. W. FREI

(Amsterdam)

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CONTENTS

4TH SYMPOSIUM ON HANDLING OF ENVIRONMENTAL AND BIOLOGICAL SAMPLES IN CHROMATOGRAPHY, BASLE, APRIL 27–29, 1988

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PREFACE

This Proceedings volume contains a collection of papers on the theme of sample handling presented at the *4th Symposium on Handling of Environmental and Biological Samples in Chromatography*, held in Basle (Switzerland), April 27–29, 1988.

The relevance of the theme was clearly shown by the attendance at the event of over 200 participants and the presentation of 38 high-quality posters and 30 oral papers. Robotics was launched as a new theme in this series of symposia by an extra half day devoted to this topic. However, opinions on the usefulness of robotics in an analytical laboratory as opposed to other automatable techniques were rather divergent and partly negative. Nevertheless, the consensus was that robotics offered a complementary rather than a competitive approach to routine sample handling procedures.

The use of solid–liquid extraction techniques based on on-line or off-line precolumn technology has gained wide acceptance even in many routine laboratories owing to the inherent simplicity and automatability of such an approach. Column switching and disposable cartridge systems were proposed for on-line procedures and several commercial systems are now available. Although much of the work (>90%) in solid–liquid extraction is based on hydrophobicity interaction, the use of more selective adsorbents ranging from ion exchangers to complexing or bioselective surfaces has also been discussed. The adaptation of several pre-columns in series such as size exclusion–reversed phase or hydrophobic interaction–ion exchange has proved to be a powerful approach. Automation possibilities in all these instances received high priority throughout the symposium and so did validation, the use of expert systems, computer optimization of phase combinations, etc.

Another message that came through clearly was the importance of considering the interplay between sample handling, separation and detection (total or integrated systems approach). The impact and importance of a proper choice of the detection mode and the possible introduction of chemistry as either a pre- or post-column derivatization step were clearly realized. The inherent selectivity of the reaction plus the enhanced detection properties of the derivative (analyte) can contribute significantly to the overall performance of the analytical system. The automation possibilities of such derivatization techniques were underlined by several speakers and commercial equipment for this line of methodology is being more widespread. The use of "clever chemistries" was propagated by some participants to the extent that elimination of the chromatographic column was suggested, *viz.*, the use of flow injection in place of robotics and/or a chromatographic separation.

In spite of the impression that certain sample handling methodologies have reached a high degree of maturity, several new technologies were also discussed. These included more selective adsorption and desorption techniques, on-line coupling of precolumns with capillary gas chromatography, sample purification by zone electrophoretic techniques, on-line coupling of dialysers with a precolumn for deproteination and even supercritical fluid extraction. Many of these "techniques on the horizon" will certainly appear in force in the symposium on this topic planned for 1990, ensuring that the frontier spirit in this field will be maintained.

R. W. FREI

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REVIEW

SYSTEMATIC APPROACH TO COLUMN SWITCHING

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CONTENTS

1. INTRODUCTION

Modern liquid chromatography becomes more attractive and more powerful if the number of separation columns is increased. Usually a liquid chromatographic system contains a simple flow-through sequence. If this sequence is branched and switching devices are used to interface individual columns to a chromatographic network[1–3], liquid chromatography becomes a versatile and powerful separation system.

Column switching leads to an on-line approach to sequential column chromatography. A fraction of the effluent from a primary column is selectively transferred to a secondary column for further separation. Highly selective separations are achieved by changing the operating basis by using different transfer techniques and/

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or switching functions or by changing the chromatographic modes of separation during the overall process.

The chromatographic modes usually refer to the separation mechanism which the chromatographer intends to provide. The chromatographic mode or separation mechanism depends on the overall interactive relationships between the analyte, the mobile phase and the stationary phase. Neither the chromatographic modes, *e.g.*, multi-dimensional, solvent switching or post-column acceleration, nor the objectives of the chromatographic modes applied, *e.g.*, trace enrichment, front cut or heart cut, are well suited to describe column switching networks. The operating bases of the transfer techniques and the switching functions used throughout the network are unrelated to the chromatographic modes and therefore best suited to define clearly the column switching network.

It is the aim of this paper to help disentangle related or equivalent terms used to describe column switching techniques by well defined transfer techniques and switching functions. By this systematic approach, appropriate column networks may be set up in a easily surveyed manner.

2. DEFINITION OF THE TERM COLUMN SWITCHING

The term column switching is used in modern liquid chromatography for different operating modes without a strictly defined sense. Synonymous expressions such as "multiple column chromatography", "sequential chromatography", "multi-channel chromatography", "split chromatography", "coupled column chromatography", "isomodal, bi- or heteromodal chromatography", etc., are used for column switching, but with different objectives for chromatographic separations.

The term column switching[4] includes in the widest sense all techniques by which the direction of flow of the mobile phase is changed by valves, so the effluent from a primary column is passed to a secondary column for a defined period of time. The use of valves means that the chromatographic system involves not one, but a number of columns forming a network. Switching within this network may be effected manually or by automated controllers.

The objectives of column switching are (1) to increase the chromatographic resolution and selectivity; (2) to enrich trace amounts of sample; (3) to protect sensitive detectors (*e.g.*, electrochemical detectors) from contamination by coextractives; (4) to prevent destabilization of the chromatographic equilibrium of the column by coextractives; and (5) to achieve further objectives or a combination of several objectives within one chromatographic network.

Throughout this paper "primary column" indicates the column from which the analyte fractions are transferred to the following "secondary column". Primary and secondary eluents are the respective mobile phases used for elution of the corresponding columns.

3. TRANSFER TECHNIQUES AND SWITCHING FUNCTIONS

Four basic techniques are used to transfer a sample fraction from primary to secondary columns. Depending on the direction of flow of the mobile phase during the transfer period, a direct or a reversed flow direction can be distinguished. The

direction of flow and the origin of the transfer mobile phase, whether it is used as a primary or a secondary column eluent, are thereafter the parameters used to characterize the transfer techniques.

In addition to the different transfer techniques there are several switching functions, which are used to optimize chromatographic parameters. Commonly used column switching functions are column backflusing, column selection and recycling chromatography.

3.1. Direct transfer technique

Fig. 1a shows the switching valve arranged in the direct transfer technique. The primary mobile phase enters the valve (IN) and flushes the column. The moment the analyte starts to elute the column, the switching valve is rotated into the transfer position (ON) (Fig. 1b). The analyte fraction separated is directed through the OUT port with the primary mobile phase on to the secondary column. After the analyte fraction of interest has eluted completely from the primary column, the valve is rotated back and the secondary mobile phase (PUMP port) starts to elute the analyte from the secondary column. No reconditioning of the primary column is needed as the column is always flushed with the same mobile phase.

3.2. Indirect transfer technique

Fig. 2a shows the switching valve arranged for executing the indirect transfer technique. The primary mobile phase enters the valve through the IN port and flushes the column. The primary mobile phase separates the analytes, but the analytes of interest do not elute from the column. After rotating the switching valve (Fig. 2b) into the transfer position (ON), the secondary mobile phase with a higher elution power than the primary mobile phase is directed on to the primary column. The analytes now elute from the column and are transferred on to the secondary column through the OUT port. After elution of the analytes is completed, the valve is rotated back

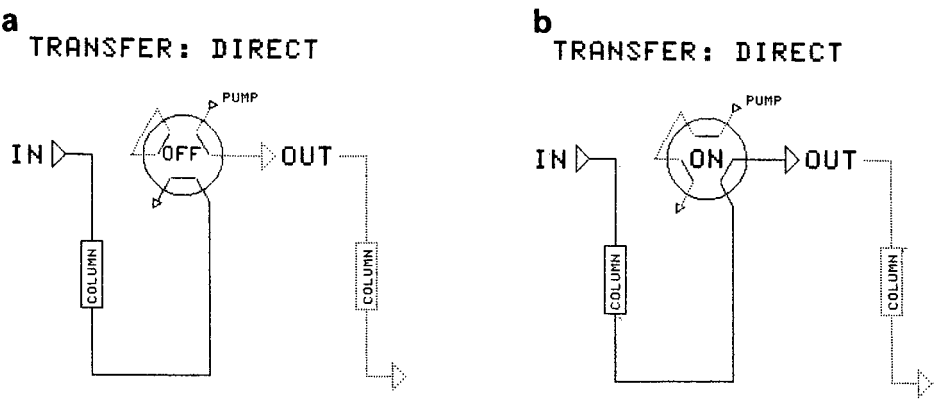

Fig. 1. (a) Elution of primary column. (b) Transfer on to the secondary column with primary eluent. The following notations are used in all figures: IN = primary eluent entrance of the primary column, flow from previous system part (*e.g.*, injection valve); OUT = eluent exit, flow on to the secondary column; PUMP = eluent pump of the secondary column; OFF = valve in the standby position; ON = valve in the switching position; straight line shows the actual flow, dotted lines are flow lines of the secondary column or bypass flow lines.

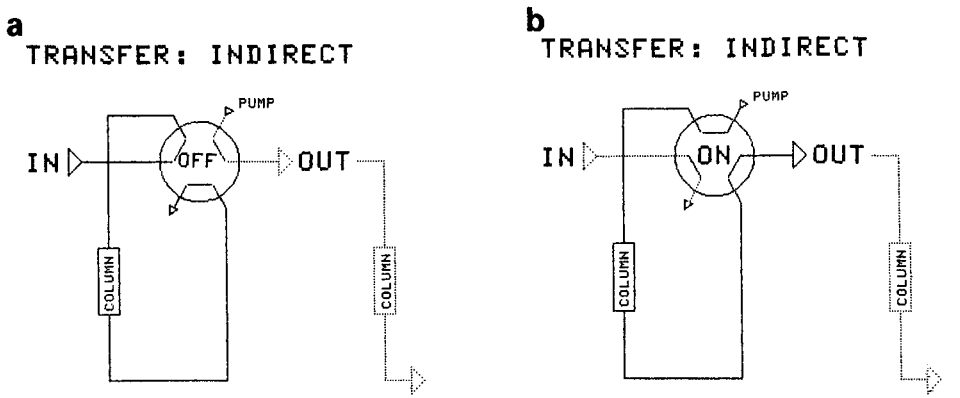

Fig. 2. (a) Elution of the primary column. (b) Transfer on to the secondary column with the secondary eluent.

(position OFF, Fig. 2a). The secondary mobile phase now separates the analyte fraction on the secondary column, while the primary column is reconditioned with the primary mobile phase.

3.3. Reversed transfer technique

Fig. 3a shows the basic setup of a chromatographic network for reversed transfer of the analytes on to the secondary column. The analytes are not necessarily separated with the primary mobile phase on the primary column. The fraction of interest does not elute from the column. After rotating the switching valve into the transfer position ON (Fig. 3b) the secondary mobile phase, which is an eluent equal to or stronger than the primary mobile phase, is delivered through the pump entrance and transfers the fraction of the analyte of interest by reversing the flow direction of the primary column through the OUT port on to the secondary column. When the transfer of the analyte is completed, the switching valve is rotated back (Fig. 3a). The

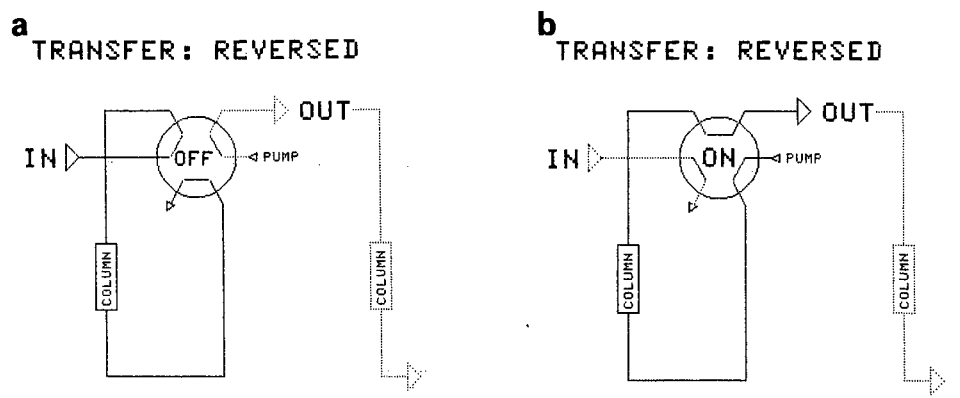

Fig. 3. (a) Elution of the primary column. (b) Transfer in reversed flow direction on to the secondary column with the secondary eluent.

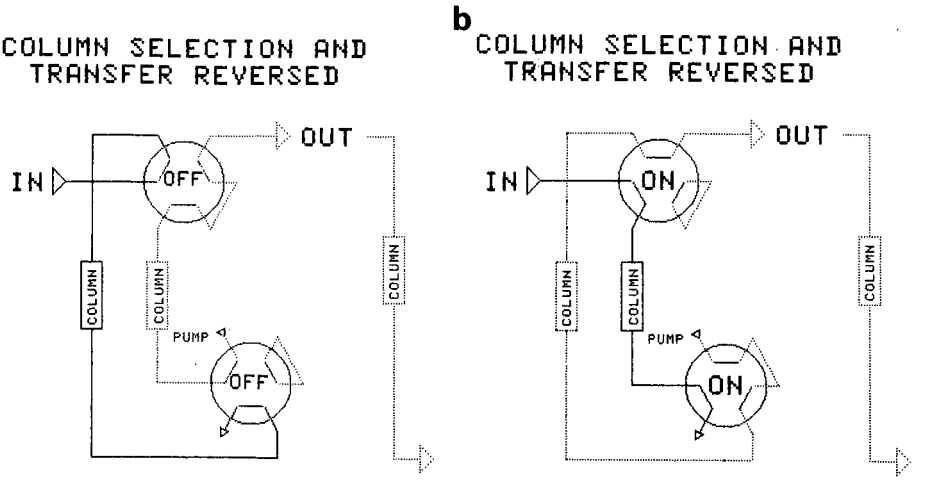

Fig. 4. (a) Elution of the left-hand primary column and transfer of the right-hand primary column in reversed flow direction on to the secondary column with the secondary eluent. (b) Elution of the right-hand primary column and transfer of the left-hand primary column in reversed flow direction on to the secondary column with the secondary eluent.

secondary mobile phase now separates the analyte fraction on the secondary column, while the primary column is reconditioned.

Fig. 4. shows a special setup for reversed transfer which uses two primary columns alternately. The left column is connected in-line to the sampling eluent stream (IN) (Fig. 4a). The right primary column is flushed with the eluent of the secondary column in the reversed transfer technique and the analytes are separated on the secondary column. After rotating both valves into the ON position (Fig. 4b) the secondary eluent flows in a reverse direction through the left column and the right column is loaded with the analyte of the next sample[5].

3.4. Loop transfer

Fig. 5a shows a modification of the direct transfer technique. The primary and secondary columns are not connected on-line during the transfer period. This transfer technique avoids excessive pressure on the primary column during the transfer period. The effluent from the primary column is collected in a loop and re-injected into the secondary column (Fig. 5b). A disadvantage of the loop transfer technique is that the volume of the transfer fraction is given by the loop volume so the components of interest to be transferred must elute from the primary column in a sufficiently high concentration to allow the transfer of all or at least the major part of the sample fraction.

The loop transfer technique is principally possible as a modification of each basic transfer technique. The indirect or reversed transfer techniques necessitate an additional loop valve.

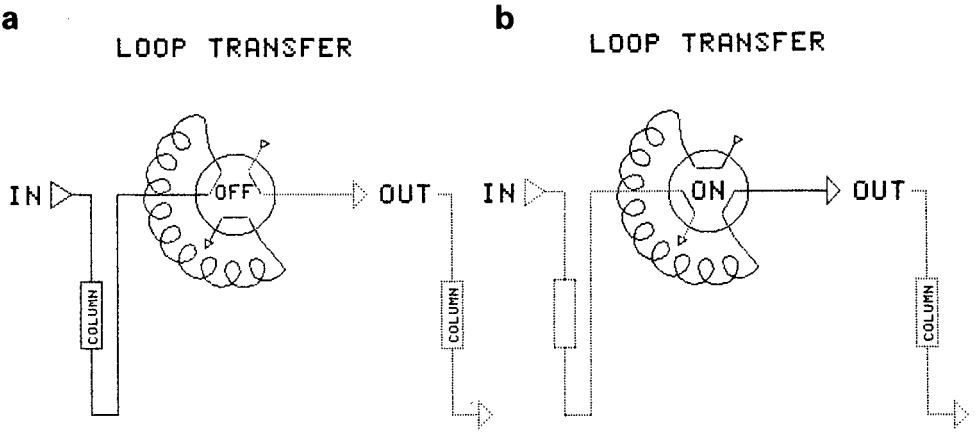

Fig. 5. (a) Elution of the primary column through the transfer loop. (b) Re-injection of the loop volume on to the secondary column with the secondary eluent.

3.5. Column backflushing

The effluent from the primary column is normally vented to waste for bypassing the secondary column and for preventing its contamination by early or late eluting components. Backflushing the primary column removes samples components that are strongly[1] retained. After the fraction of interest has eluted from the primary column and has been transferred for further separation on to the secondary column, this technique reverses the flow of the primary column to waste (Fig. 6a and b).

A more powerful cleaning eluent may replace the mobile phase (Fig. 7a and b). Backflushing speeds up the analysis of complex mixtures without the use of gradient elution.

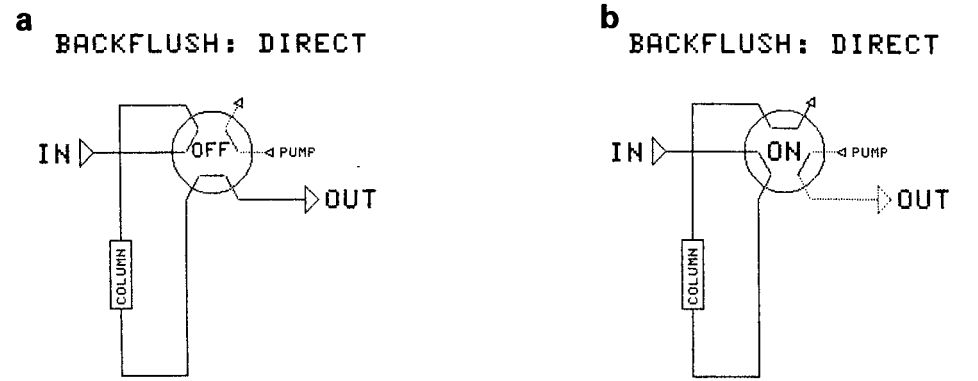

Fig. 6. (a) Elution of the primary column. (b) Backflushing of the primary column with the primary eluent waste.

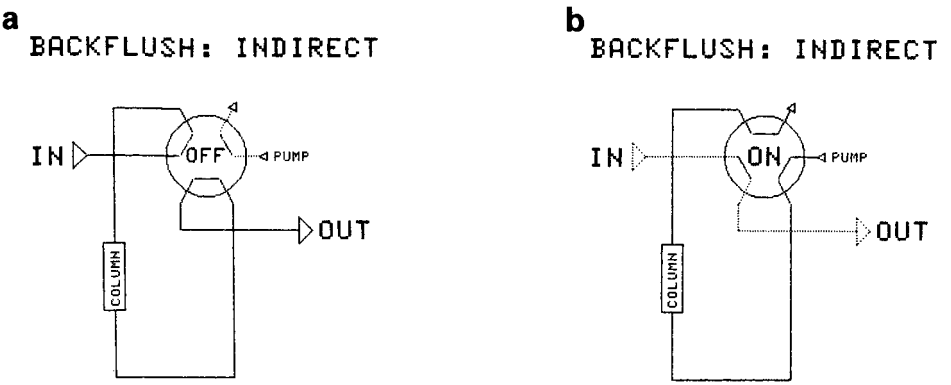

Fig. 7. (a) Elution of the primary column. (b) Backflushing of the primary column with a rinsing eluent to waste. PUMP = rinsing eluent pump.

3.6. Column selection

Column switching systems[6–8], designed to reduce long analysis times, use two or more (different) columns to perform the separation of analytes with very different capacities rather than a single, long column. Late eluting components are directed after the primary (short) column to the detector, whereas the fast eluting components are separated on the secondary (long) column before they are detected (Figs. 8–10).

Usually all columns are run with the same eluent (Fig. 8a). During the elution of components of high capacity from the primary column (Fig. 8b), the secondary column is in the standby position with no eluent flow. Normally no band broadening occurs once the flow of the column is restored, as longitudinal diffusion is very slow in packed columns.

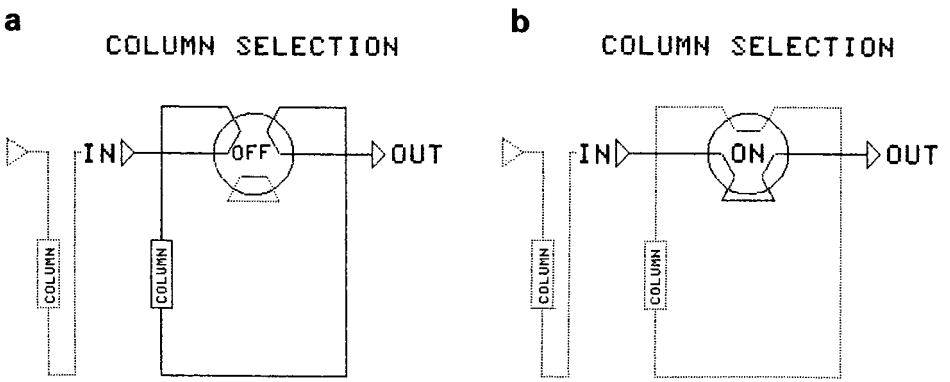

Fig. 8. (a) Elution of both columns with the same eluent. (b) The second column with the early eluting components in the bypass position.

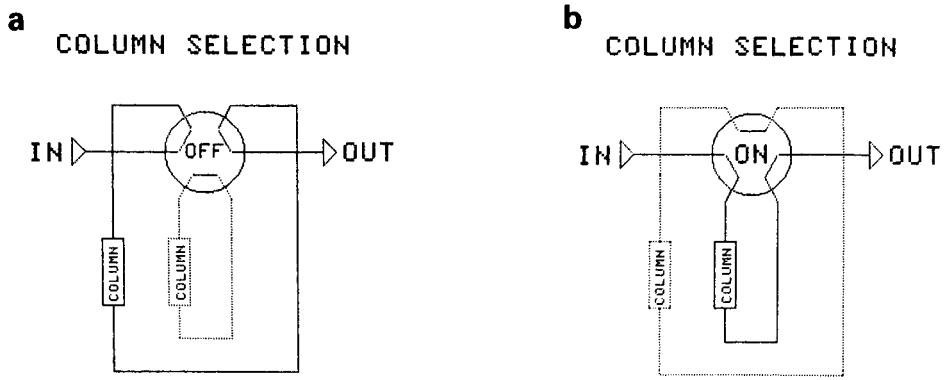

Fig. 9. Either column is to be switched into the eluent stream.

Column selection offers an alternative approach to chromatographic adjustment by solvent switching or gradient elution and it avoids the problem of slow column equilibration.

Fig. 9a and b show a system for selecting two different columns run with the same mobile phase. This arrangement is well suited to select between two different column lengths.

3.7. Recycling chromatography

Recycling chromatographic systems use the same column by re-injecting a portion of the chromatographic effluent from the column. The repeated use of the same column increases the number of theoretical plates. Recycling can be carried out by using an alternative column principle (Fig. 10), where two columns are used in sequence[9,10].

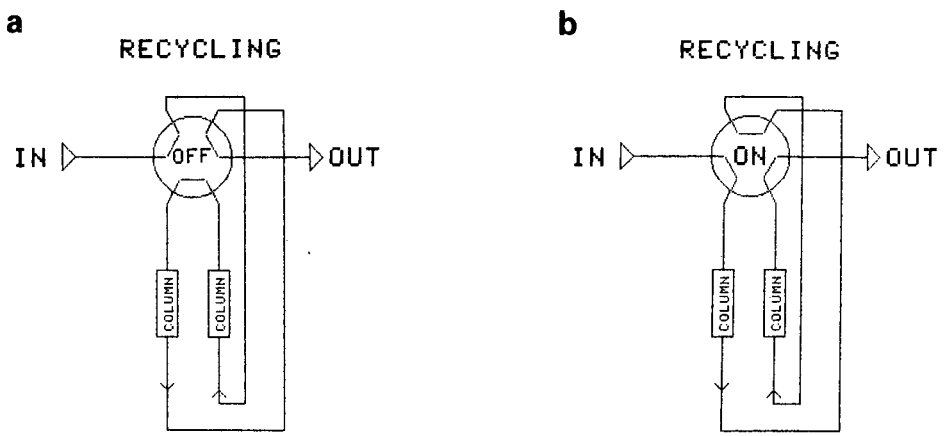

Fig. 10. The sequence of columns is changed periodically, which increases the total column length. (a) Flow from the left column is directed to the right column. (b) Flow of the right column is redirected to the left column.

The recycling technique offers, in comparison with the column selection technique, the possibility of increasing the column length by repeated use of the same columns (Fig. 10a and b).

4. TRANSFER TECHNIQUES AND THE CHROMATOGRAPHIC MODES

4.1. Change of the chromatographic modes by column switching

Highly selective chromatographic separations are achieved by changing the mode of separation during the chromatographic procedure. In particular, mode control or mode sequencing in chromatography involves several changes of the composition of the mobile phase and/or stationary phase. Systematic changes of mode during the chromatographic separation process contribute to a cooperative unlimited and overall increase in the separation capability and selectivity of the system[1,11–15]. These sequential changes of the chromatographic modes are known as multi-dimensional column chromatography, stationary phase programming and selectivity switching, among others.

The term "multi-dimensional" currently encompasses other on-line switching modes, such as backflushing, detector or column bypassing, system setups of two or more columns in parallel, etc., which are switching functions and are not based on a multi-dimensional chromatographic mode.

4.2. Fractional transfers

Wide or narrow cuts of the chromatographic effluents from primary columns are transferred to the secondary column by flow switching and the mobile phase flow is thereby diverted or reversed. The fraction of interest to be transferred on to the secondary column may elute at the front (first eluting zone) or in the middle of the chromatographic effluent (heart) or at the end (last eluting part) of the chromatogram of the primary column. Obviously always one or more fractions or zones of the whole chromatographic effluent are transferred from the primary on to the secondary column. The notations used to specify these fractional parts[16,17] of the chromatograms or to name the switching technique according to the transfer fraction as a front, heart or end cut express neither the column to column transfer technique nor the chromatographic objective intended to be achieved by the switching technique. Therefore, the transfer techniques presented in Section 3 should be used exclusively to describe a column switching system.

4.3. Peak compression after band spreading

The band broadening during chromatographic separations based on diffusion effects necessitates the transfer of wide fractions of the effluent from the primary to the secondary column[7,18]. The separation and sensitivity of the chromatographic system may be improved if sample dilution is minimized throughout the analysis. This involves reconcentration of the transferred fractions in each successive step. The elution strength of the mobile phase and the retention capability of the stationary phase should increase to reconcentrate the analyte at the top of the secondary column to reduce band broadening. This reconcentration effect is referred to as on-column concentration. Reconcentration at the top of the secondary column is also increased by diluting the effluent from the primary column with a solvent of low elution strength before directing the flow on to the secondary column.

4.4. Precolumns and guard columns

Precolumns in the widest sense in the literature are used as primary columns. They are tailored for special purposes (trace enrichment, sample cleanup, etc.) and normally they are not intended to separate samples by chromatographic means. Guard columns are short columns connected in-line with the analytical column. They are strictly used to prevent the analytical column from rapid deterioration. The primary column of column switching systems acts as a guard column for all secondary columns.

5. APPLICATIONS OF COLUMN SWITCHING

Analytical samples are often so complex that one or several of the target components must be determined within a matrix of a very large number of other components that are present at higher or lower concentrations. Multi-step methods are necessary with several purification steps before the final chromatographic determination. Column switching systems should permit the multi-step methods to be transformed into single-step procedures by on-line purification. These systems allow firstly the injection of a large volume of sample to increase the sensitivity and secondly the optimization of the separation by gradual adjustment of the resolution parameters.

Probably the most important applications of column switching are trace enrichment, sample cleanup, group separation and chromatographic adjustment.

5.1. Trace enrichment

The analysis of a single or a few components in trace amounts in biological matrices is a general problem. Trace enrichment or preconcentration by on-line chromatographic techniques are based on the fact that the components will be retained in a narrow zone on the top of the column when a large volume of sample is pumped through the column[19–21]. Good reproducibility is obtained if the column is not overloaded and if the capacity of the column is not exceeded. Overloading can be overcome by diluting the sample before injection. If trace enrichment has to be effected for less strongly adsorbed components, the sample volumes must be smaller or the column volume must be increased to prevent breakthrough. Trace enrichment is performed when relatively non-polar components from aqueous solutions are injected on to a reversed-phase column. Similar phenomena can be exploited with adsorption chromatographic systems using suitable solvent polarities. Subsequent elution with a stronger eluent by indirect transfer (Fig. 2) or by reversed transfer (Figs. 3 and 4) will reconcentrate the sample zone and start the separation procedure on the secondary column. Unfortunately, trace enrichment also concentrates sample components other than the analyte. As a consequence, cleanup may well be insufficient and the subsequent separation of the components of interest from interfering substances may necessitate further on-line cleanup steps.

5.2. Sample cleanup

The principle of on-line sample cleanup is to analyse one fraction and to discard all others[2,21,22]. The degree of improvement of a separation is based on the reduction of the amount of interfering components relative to the amount of analyte. This objective is achieved by selecting the size of the fraction to be transferred from the

primary column to the secondary column in such a way that the transferred fraction contains the analyte and as little as possible of the overlapping interferences. By on-line multi-step fractionation (Fig. 1, direct transfer; Fig. 5, loop transfer; or Fig. 8, column selection), the analyte is gradually enriched relative to the interfering components. Therefore, the degree of separation is improved compared with a single-step operation with the same chromatographic resolution.

5.3. Group separation

In a preliminary fractionation the sample is divided into groups of components sharing some chromatographic characteristics defined by the fractionation method. This chromatographic fractionation selects groups of components with, *e.g.*, similar molecular size[16,18,23] or similar retention characteristics (anion, cation, etc.)[24,25], reflecting a comparable type and strength of interaction with the stationary phase. The choice of a primary chromatographic system, which selects some characteristics unique to the components of analytical interest, reduces the number of components to be transferred on to the secondary column. This results in fewer peaks and increased resolution of the secondary analytical system compared with the direct separation of the whole sample.

Nielen *et al.*[25] used small columns packed with different stationary phases for the on-line group separation and trace enrichment of industrial waste water. They divided the sample into three main groups, a fraction containing non-polar components adsorbed on C_{18} stationary phase, a fraction of medium polarity components adsorbed on PRP-1 (a polystyrene–divinylbenzene phase) and a fraction of polar bases adsorbed on cation-exchange phases. Each fraction was subsequently chromatographed on a C_{18} reversed-phase analytical column using a column switching network built up according to the column selection mode in Fig. 8.

Gel permeation or size exclusion chromatography[16,18,23] selects only those components with comparable molecular sizes. For complex samples a narrow molecular size fraction contains many components with a wide variety of functional groups. These components elute over a wide capacity range in the secondary column. Ogan and Katz[23] described a multi-function system for the determination of polycyclic aromatic hydrocarbons in coal liquid and oils by using the loop transfer technique (Fig. 5) from or on to gel permeation or size exclusion columns.

5.4. Chromatographic adjustment

Selection of a suitable column length for each analyte of the sample or selection of a suitable stationary phase for each group of analytes by the column selection technique are two of various possibilities of the adjustment of chromatographic parameters (Figs. 8–10).

Backflushing of the primary column offers another possibility of adjusting the run time, as late eluting interferents are backflushed to waste, while the analytes are separated on the secondary column (Figs. 6 and 7).

Chromatographic adjustment by column switching offers an effective alternative to solvent adjustment by gradient elution or solvent switching followed by reconditioning of the column.

6. COLUMN NETWORKS

6.1. Example of a two-valve network (three columns)

Fig. 11a–e show a two switching valve network with reversed transfer from the first to the second column and with direct transfer from the second to the third column. This configuration corresponds to a combination of Figs. 3 and 1. The network is used for trace enrichment and cleanup on the first column (Fig. 11a). The first column is washed with mobile phase. After this wash period the valve "transfer reversed" is rotated to position ON (Fig. 11b) and the flow of the first column is reversed and directed to the second column. The analyte is transferred in the reverse flow direction by the stronger eluent of the second column. The original position of the valve (Fig. 11c) is restored (OFF position). The first column is reconditioned, while the analyte is separated on the second column. When the analyte fraction of interest starts to elute from the second column, the valve "transfer direct" is rotated to position ON (Fig. 11d). After the entire analyte fraction has eluted from the second column, the valve is reset (OFF position) and the separation on the third column starts (Fig. 11e).

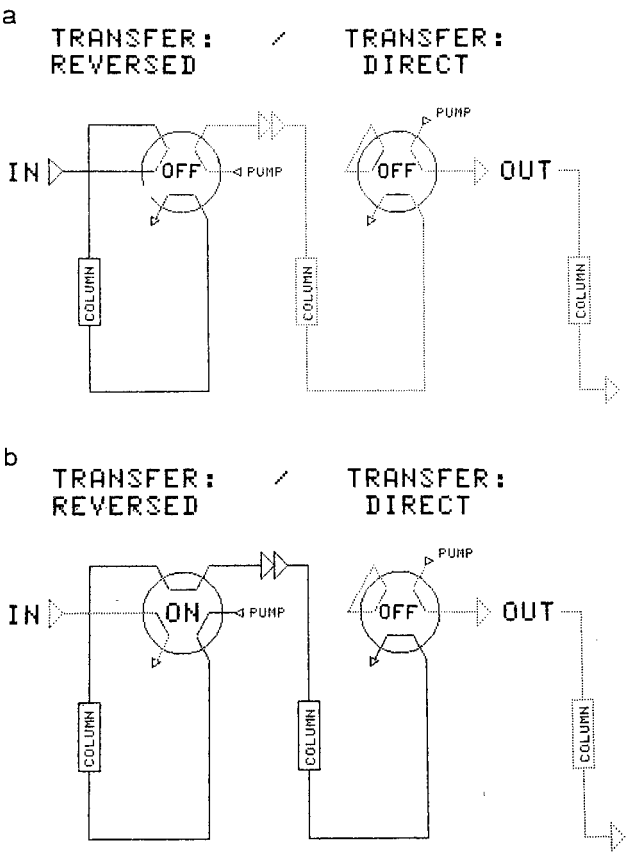

Fig. 11.

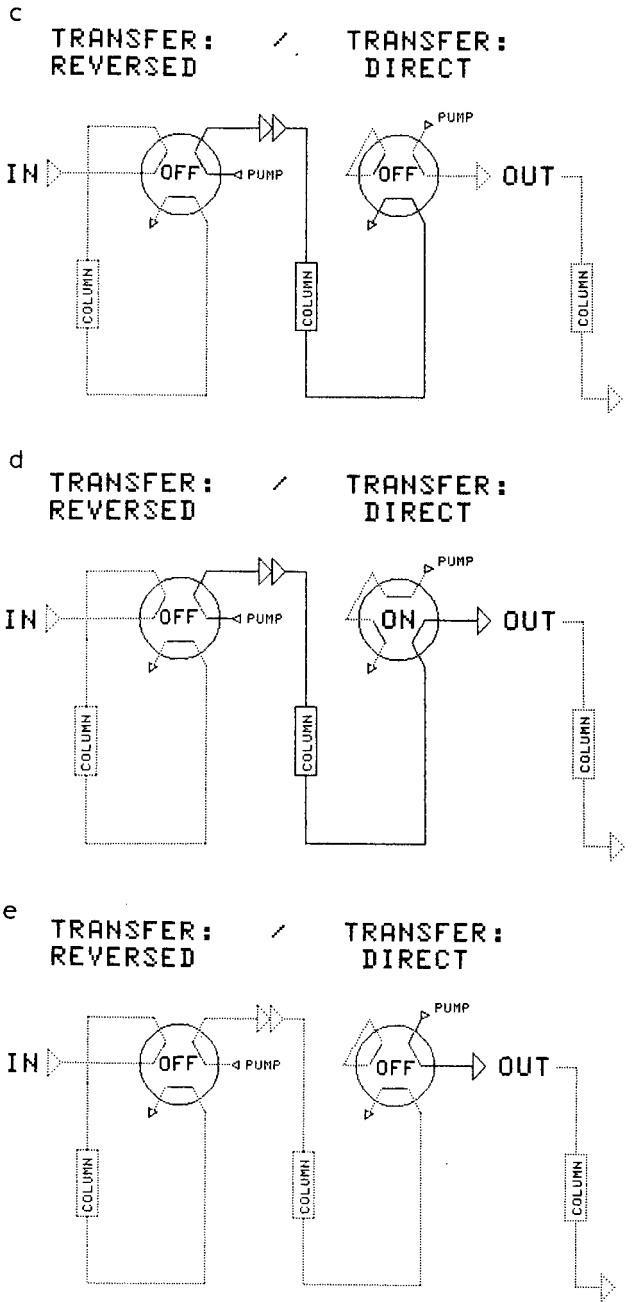

Fig. 11. Column network combination of reversed transfer (Fig. 3) and direct transfer (Fig. 2); column I = left, column II = middle and column III = right column. (a) Elution of the primary column I. (b) Transfer by reversed flow direction on to the secondary column II with the eluent from column II. (c) Elution of column II; the column now becomes the primary column for the next transfer. (d) Transfer of the primary column II on to the secondary column III with the eluent from column II. (e) Elution of the secondary column III.

6.2. Networks with column backflushing capability

Backflushing capability is included in any system configuration when the original column is replaced by the backflushing configuration's IN and OUT ports, respectively. Figs. 12–14 show the direct, indirect and reversed transfer techniques with the additional backflushing valve. The dotted centre column shows the original position of the primary column. The dashed lines in Figs. 12–14 show the flow of the mobile phase for backflushing the primary column. In the direct transfer technique, the mobile phase used to elute and to transfer the analyte is also used to backflush the primary column. In the indirect and reversed transfer technique, a separate pump with normally a stronger solvent than the eluent is used to backflush the column.

7. CONCLUSION

The technique of transfer of effluent fractions governs the application of column switching. Four different column to column transfer techniques together with three additional switching functions meet the needs of most of the on-line chromatographic separation modes described in the literature such as trace enrichment, sample cleanup, chromatographic fractionation and group separation.

By combining the valve configurations described for the specified transfer techniques, the setup of complex column networks is simplified with standard six-port high-pressure switching valves. The networks are described clearly by the transfer technique from column to column and/or the switching function but not by the chromatographic effect achieved by the system.

Special setups for liquid–gas chromatography transfer or chromatographic mode changes (adsorption to reversed or *vice versa*) are not considered in this paper.

8. SUMMARY

The term column switching in liquid chromatography is used if two or more columns are connected to form a network. The aim of column switching is to increase the chromatographic resolution and selectivity without losing sensitivity.

Many terms are used in the literature to characterize different column networks. Generally the resulting chromatographic effects are used to describe the switching system. Only rarely do authors define the technique used to achieve the transfer of a fraction of the eluent from one column to the next. This paper describes four basic techniques for transfer sample fractions: (1) direct transfer, (2) indirect transfer, (3) reversed transfer and (4) loop transfer. To optimize the chromatographic separation by column switching, additional commonly used switching functions are defined: (5) backflushing technique, (6) column selection or column bypass and (7) recycling chromatography. By linear combinations of different transfer techniques and/or switching functions, multifunctional column networks are designed in a simple way. They are used for trace enrichment, sample cleanup, chromatographic fractionation and chromatographic adjustments.

This paper is intended to provide a contribution to the standardization of nomenclature in column switching. Drawings of the particular column and switching valve arrangements are given together with examples of networks built up by combinations of transfer techniques and switching functions.

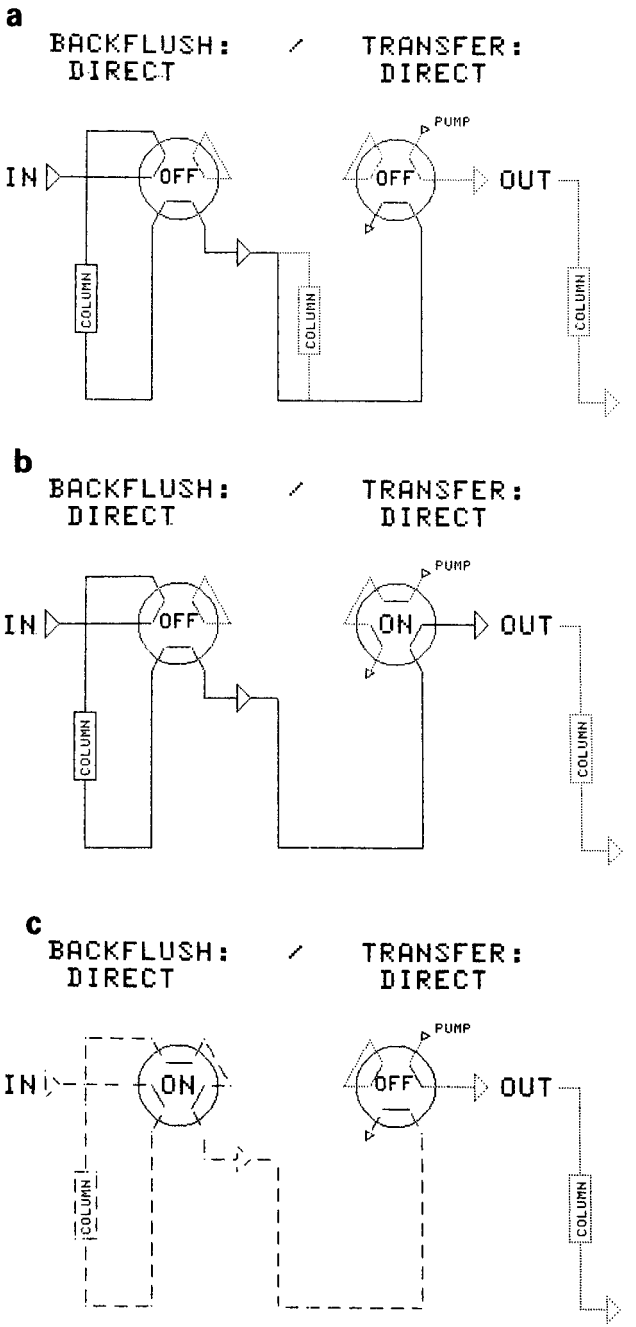

Fig. 12. Column network combination of direct backflushing of the primary column (Fig. 6) with direct transfer on to the secondary column (Fig. 1); dotted column (middle) shows the primary column position substituted by the backflushing valve. (a) Elution of the primary column. (b) Direct transfer on to the secondary column. (c) Broken line shows the backflushing flow of the primary eluent during elution of the secondary column with the secondary eluent.

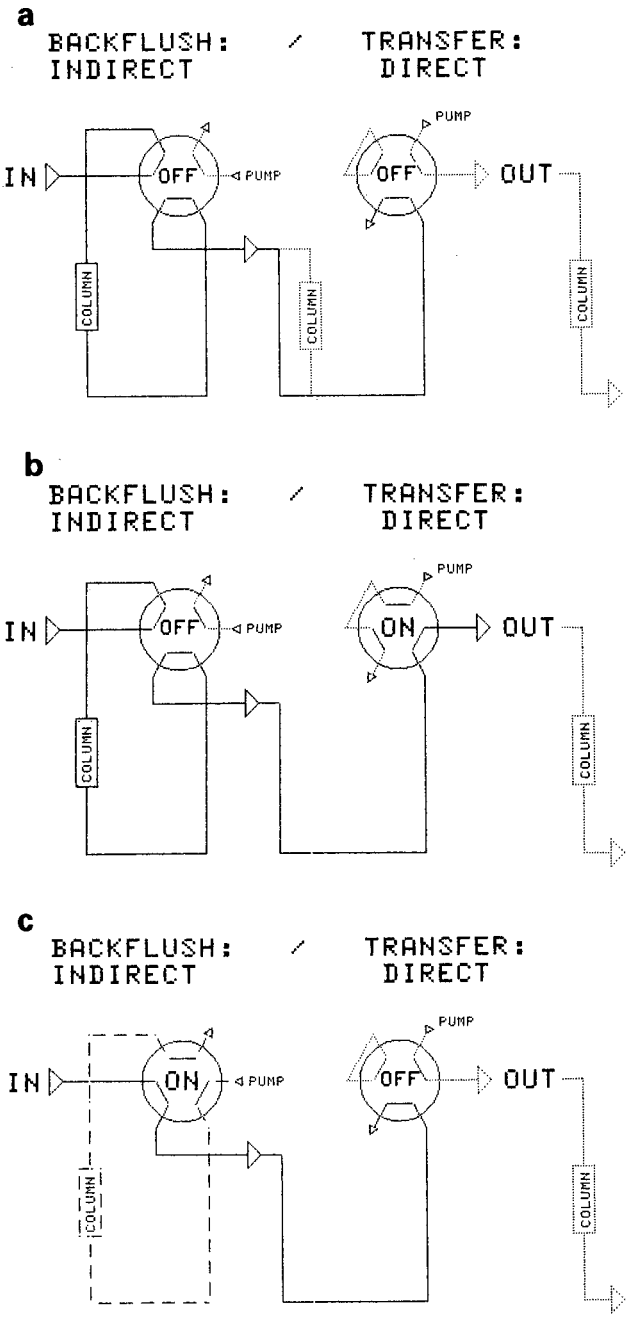

Fig. 13. Column network combination of indirect backflushing of the primary column (Fig. 7) with direct transfer on to the secondary column (Fig. 1); dotted column (middle) shows the primary column position substituted by the backflushing valve. (a) Elution of the primary column. (b) Direct transfer on to the secondary column. (c) Broken line shows the backflushing flow with a rinsing mobile phase not used for elution of the columns, during elution of the secondary column.

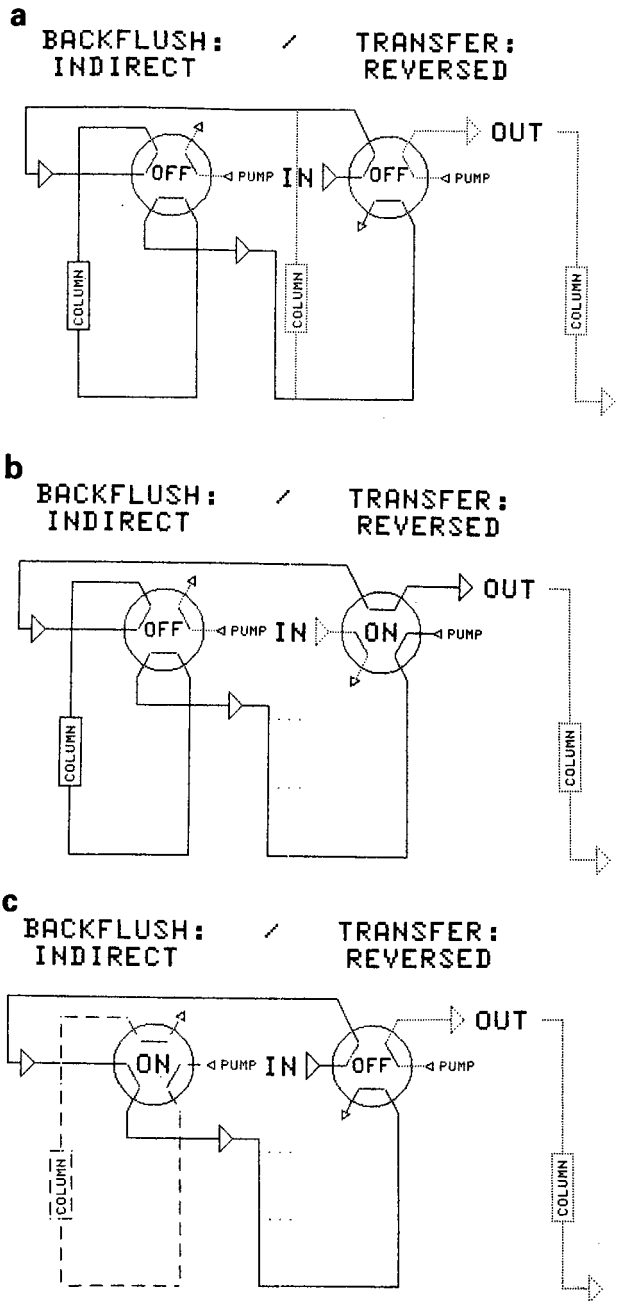

Fig. 14. Column network combination of indirect backflushing of the primary column (Fig. 7) with reversed transfer on to the secondary column (Fig. 3); dotted column (middle) shows the primary column position substituted by the backflushing valve. (a) Elution of the primary column. (b) Transfer by reversed flow direction of the primary column with the eluent from the secondary column. (c) Broken line shows the backflushing flow with a rinsing mobile phase not used for elution of the columns, during elution of secondary column.

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DETERMINATION OF A NEW NON-BENZODIAZEPINE ANXIOLYTIC AND ITS O-DEMETHYL METABOLITE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AUTOMATED COLUMN-SWITCHING

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SUMMARY

An highly sensitive and fully automated high-performance liquid chromatographic assay was developed for the determination of a novel non-benzodiazepine anxiolytic (I) {(*R*)-2-(methoxymethyl)-1-[(7-oxo-8-phenyl-7H-thieno[2,3-*a*]quinolizin-10-yl)carbonyl]pyrrolidine} and its O-demethyl metabolite (II) in plasma, using column-switching for direct injection of plasma samples. After dilution in internal standard solution, the sample was injected onto a pre-column (17 mm × 4.6 mm) dry-packed with pellicular C_{18} reversed-phase material. Polar plasma components were removed by flushing the pre-column with water–acetonitrile (90:10, v/v). Retained substances, including I and II, were backflushed onto an analytical column, separated by gradient elution and detected by means of fluorescence detection (excitation, 304 nm; emission, 475 nm). After washing the analytical column and re-equilibrating the pre-column, the system was ready for the next injection. The limit of quantification for I and II was 0.25 and 0.5 ng/ml, respectively, using a 350-μl specimen of plasma. The practicability of the new method was demonstrated by analysis of more than 300 plasma samples from a tolerance study performed with human volunteers. Owing to its high sensitivity, the method can be used to calculate pharmacokinetic parameters of compounds I and II in man after a single oral dose of about 1 mg of I.

INTRODUCTION

(*R*)-2-(Methoxymethyl)-1-[(7-oxo-8-phenyl-7H-thieno[2,3-*a*]quinolizin-10-yl)-carbonyl]pyrrolidine, I (Ro 19-5686; F. Hoffmann-La Roche, Basle, Switzerland) (see Fig. 1), is a novel substituted thienoquinolizinone acting as a partial agonist at the benzodiazepine receptor complex. This property suggests good anxiolytic action, together with a reduced side-effect profile such as reduced motor impairment, decreased ethanol potentiation and less physical dependence liability[1]. Metabolic investigations

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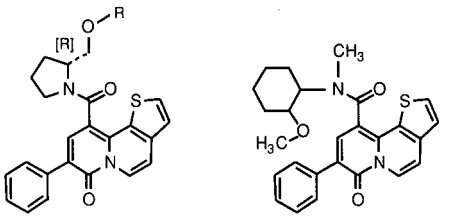

I : Parent compound (R= CH_3) III: Internal standard
II: O-Demethyl metabolite (R= H)

Fig. 1. Chemical structures for the compounds referred to in the text.

have shown that the O-demethyl derivative (II) is the major metabolite in plasma of rat, dog and man, with a similar pharmacological profile to that of the parent compound[2].

For pharmacokinetic studies, a precise method for the determination of plasma concentrations of compounds I and II was needed, taking account of the marked light sensitivity of the thienoquinolizinones. Fully automated high-performance liquid chromatographic (HPLC) column-switching techniques offer certain advantages for the analysis of photolabile compounds[3,4]. Compared with other techniques, minimum sample handling is involved where the samples have to be protected from light, while the on-line clean-up procedure can be carried out under normal laboratory light conditions.

In this report a highly sensitive HPLC column-switching method with fluorimetric detection is described for the determination of compounds I and II in plasma.

EXPERIMENTAL

Laboratory precautions

All handling of reference compounds and solutions was performed in a darkened laboratory. Stock solutions of compounds I–III were prepared in amber-glass volumetric flasks and kept in the dark at 4°C under an helium atmosphere. Working solutions were always freshly prepared prior to use. Plasma standards were prepared in amberized glassware. Pre-treatment of biological samples was carried out in a darkened laboratory. Plasma standards and biological samples were protected from light during storage in the freezer by wrapping the racks in aluminium foil.

Reagents and solvents

Acetonitrile (HPLC grade S) was obtained from Rathburn (Walkerburn, U.K.), and tetrahydrofuran (unstabilized, HPLC grade) from Fisons (Loughborough, U.K.). Methanol (HPLC grade) was obtained from Fluka (Buchs, Switzerland) and water (HPLC grade) from Baker (Deventer, The Netherlands). Plasma standards were prepared using pre-tested fresh frozen plasma from sodium citrated human blood, which was obtained from a blood bank (Blutspendezentrum SRK, Basle, Switzerland).

Solutions and plasma standards

A stock solution of the parent compound and its O-demethyl metabolite was prepared by dissolving 10 mg of compound I and 10 mg of compound II in 10 ml of

methanol. Aliquots of the stock solution were diluted in methanol, providing the working solutions. The plasma standards were obtained by spiking blank plasma (20 ml) with 100 μl of the corresponding working solution, providing concentrations between 0.25 and 2500 ng/ml of the two compounds. The standards were divided into aliquots of 1.5 ml and stored deep frozen (−20°C) until required for analysis.

Instrumentation

A schematic representation of the column-switching system is given in Fig. 2. The single-piston pump P1 (Model 414; Kontron, Zürich, Switzerland) delivered the purge solvent mixture M1 at a flow-rate of 2 ml/min. Aliquots (100–500 μl) of diluted plasma samples were injected by means of an automatic sample injector I1 [Model WISP 710B assembled with a cooling module (10°C); Waters, Milford, MA, U.S.A.] onto the pre-column PC. Injection of plasma samples larger than 200 μl was achieved by using a 1-ml syringe and the built-in auxiliary loop, and by decreasing the syringe motor rate to 1.85 μl/s. An UV detector D1 (Model Spectroflow 773; Kratos, Westwood, NJ, U.S.A.), operating at 254 nm, together with a recorder R (Model W+W

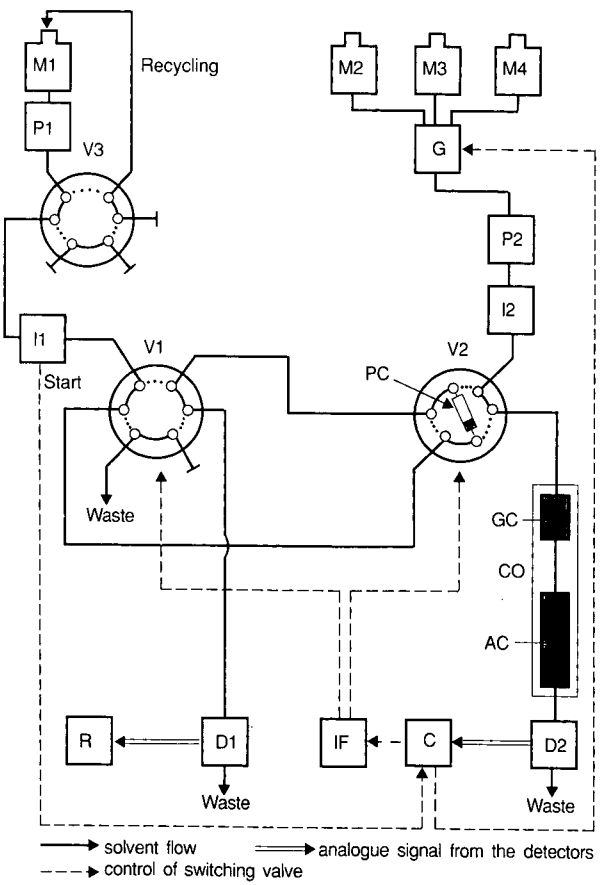

Fig. 2. Schematic representation of the column-switching system. The valves are shown in position 0.

600; Kontron) was used to monitor the removal of plasma components from PC during the purge step. A low-pressure gradient system G (Model Spectroflow 430, Kratos) together with a double-piston pump P2 (Model Spectroflow 400; Kratos) delivered the gradient mobile phases M2–M4 at a flow-rate of 1.5 ml/min for chromatography of the retained substances on the analytical column AC. A column oven CO (Model 7930; Jones, U.K.) allowed the analytical separation to be performed at increased temperature (35°C). A manual injector I2 (Model 7125) equipped with a 100-μl loop (Rheodyne, Cotati, CA, U.S.A.) was used for injection of control solutions directly onto the analytical column. Detection of the eluted compounds was carried out by means of a fluorescence detector D2 (Merck; excitation, 304 nm; emission, 475 nm; sensitivity 1–5). Data handling was carried out by means of a computing integrator C (Model SP 4200; Spectra Physics, San José, CA, U.S.A.) working with a special BASIC program, originally developed for the integrator SP 4100[5]. The gradient former G and the three air-actuated switching valves V1—V3 (Model 7000 assembled with a Model 7001 pneumatic actuator and a Model 7163 solenoid valve; Rheodyne) were controlled by the external time events of the computing integrator. To achieve compatibility, a laboratory-made interface IF was placed between the integrator output and the solenoid valve input[6].

Columns and mobile phases

The pre-column PC (17 mm × 4.6 mm; Bischoff-Analysentechnik, Leonberg, F.R.G.) was dry-packed with Bondapak C_{18} Corasil, 37–50 μm (Waters). In order to prevent blockages caused by viscous or solid plasma constituents, PC was used only with metal sieves (3 μm, two pieces on each side), without fibre-glass filters[7]. The packing was first rinsed with methanol (removal of impurities, activation of the reversed-phase material) and then conditioned with M1 prior to use.

The analytical column AC (250 mm × 4 mm, E. Merck) was packed with μBondapak C_{18}, 10 μm (Waters) by means of the usual slurry technique. A small guard column GC (17 mm × 4.6 mm; Bischoff), slurry-packed with the same material, was used to extend the lifetime of the analytical column.

The pre-column was replaced daily, or after 50 injections. The guard column was exchanged weekly, or after 250 samples. The analytical column had a lifetime of about 750 injections.

Four different mobile phases were used: M1, water–acetonitrile (90:10, v/v); M2, water–acetonitrile–tetrahydrofuran (62:36:2, v/v/v); M3, water–acetonitrile–tetrahydrofuran (50:40:10, v/v/v); M4, water–acetonitrile (15:85, v/v). All mobile phases were degassed with helium prior to use. The gradient system is described under *Analytical procedure*.

Pre-treatment of samples

The samples were thawed at room temperature and homogenized by ultrasonication (2 min). An aliquot of 350 μl plasma was diluted in an equal volume of a solution containing an appropriate amount of internal standard in water–acetonitrile (80:20, v/v). After vortex-mixing for 15 s, the resulting mixture was centrifuged at 1700 *g* for 5 min. The clear supernatant was transferred to the injection vial and stored at 10°C in the autosampler cabinet prior to injection.

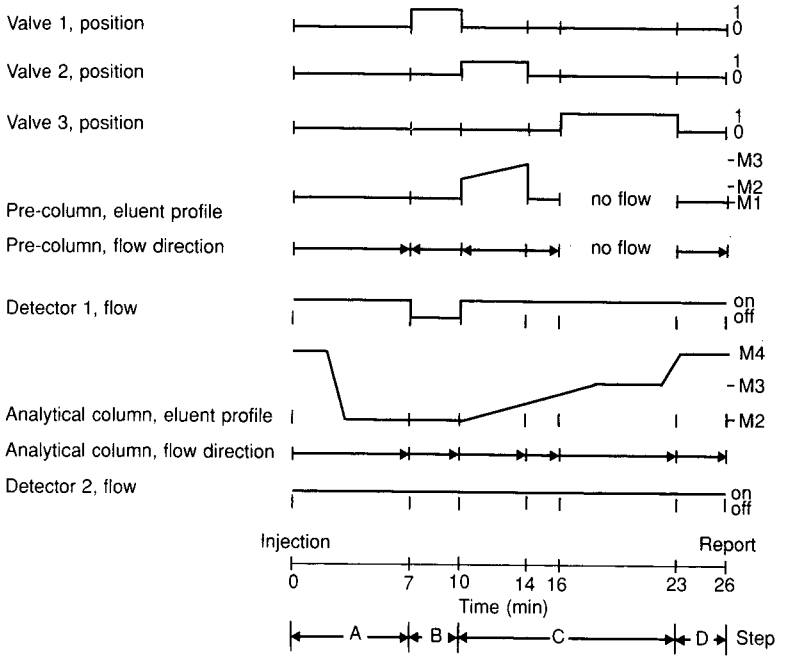

Fig. 3. Flow diagrams of the column-switching procedure (see text for further details).

Analytical procedure

A complete automated sample analysis lasted 26 min, and included the following four column-switching steps (see also flow diagrams in Fig. 3).

Step A (0–7 min; V1 = 0, V2 = 0, V3 = 0). An aliquot of a diluted and centrifuged plasma sample was injected onto PC. Proteins and other hydrophilic compounds were flushed with M1 to waste, while lipophilic compounds (including I–III) were retained on the top of PC. Progress of the clean-up process could be monitored by means of D1. Parallel to this process, GC and AC were washed for 2 min with M4 in order to remove strongly retained plasma components from the previous run. After changing within 1 min from 100% M4 to 100% M2, GC and AC were then reconditioned for 4 min with M2.

Step B (7–10 min; V1 = 1, V2 = 0, V3 = 0). PC was backflushed with M1 in order to remove trapped solid material (abrasion, precipiated proteins, etc.) without washing out the analyte. In order to prevent blockages, the purged fraction was led directly to waste without passing through the detector cell. The reconditioning process of GC and AC with M2 was continued in parallel.

Step C (10–14 min; V1 = 0, V2 = 1, V3 = 0; 14–16 min, V1 = 0, V2 = 0, V3 = 0; 16–23 min, V1 = 0, V2 = 0, V3 = 1). M1 was passed directly to waste. Enriched components were desorbed and transferred from PC to GC/AC in the backflush mode by means of the analytical mobile phase. After 4 min, PC was separated from GC/AC, purged for 2 min with M1 and then left without flow for 7 min. The transferred material was chromatographed on GC/AC using the following gradient system: from 100% M2 to 40% M2–60% M3 (10–16 min), to 100% M3 (16–19 min) and 100% M3 (19–23 min). The separated compounds were quantified by means of D2.

Step D (23–26 min; V1 = 0, V2 = 0, V3 = 0). PC was reconditioned with M1. At the same time, strongly retained components were removed from GC/AC by the following gradient system: from 100% M3 to 100% M4 (23–24 min), and 100% M4 (24–26 min). At the end of Step D the report was generated by the computing integrator and the system was ready for the next injection.

Calibration and calculation

Along with the unknown samples, five to seven plasma standards with appropriate drug concentrations were carried through the procedure. The calibration graph for the parent compound was obtained by weighted linear least-squares regression (weighting factor = $1/y^2$) of the measured peak height ratios of I/III *versus* the concentrations of compound I added to the plasma. In the same way, the calibration graph for the metabolite was established using the peak height ratios of II/III. These calibration graphs were then used to interpolate the concentrations of compounds I and II in biological samples from the measured peak height ratios I/III and II/III, respectively.

RESULTS AND DISCUSSION

Pre-treatment of samples

The fully automated column-switching method required only a minimum of sample pre-treatment: the plasma was diluted in 20% acetonitrile in water (containing compound III as the internal standard), centrifuged and stored in the dark at decreased temperature prior to injection.

Diluting the plasma in water decreased the viscosity of the samples, which had a positive effect on the lifetime of PC. Acetonitrile was added to the samples to improve the recovery of compounds I–III from plasma. Use of the internal standard improved the reliability and accuracy of the method, especially when long sample sequences had to be processed. The centrifugation step was necessary to remove solid material from the sample, thereby preventing premature blockage of PC. Finally, use of a cooled autosampler improved the stability of the compounds in plasma during overnight storage in the automatic sample injector[3].

A detailed study on the photochemical behaviour of compound I had shown that, in the presence of oxygen (air), the compounds were sensitive to light. Not only daylight but also light from neon tubes was absorbed by the yellow compounds, which, consequently, lead to at least twelve photodegradation products[8]. (Under the chromatographic conditions described in this report, these degradation products were not detected.) For this reason, plasma samples had to be carefully protected from light during collection, storage and analysis.

As already pointed out[3,4], column-switching offers certain advantages during the analysis of photo-labile substances. Compared with other techniques, *i.e.*, liquid–liquid extraction, only a few pre-treatment steps are involved in which the samples have to be protected from light. In the case of the method described here, only thawing, dilution and centrifuging of the samples needed to be performed in the darkened laboratory, while the on-line clean-up procedure and chromatography could be carried out under normal laboratory conditions, since the samples are not exposed to light.

Optimization of the on-line clean-up procedure

Various reversed-phase materials with a particle size between 30 and 50 μm were tested as potential sorbents in the precolumn, such as Corasil C_{18}, Phenyl-Corasil (Waters), Nucleosil C_{18} (Macherey-Nagel), Vydac RP (Separations Group), Sepralyte C_{18}, C_2, PH and 2 PH (Analytichem). All these materials were able to retain compounds I–III sufficiently from plasma. Sorbents of the porous layer bead (PLB) type could be cleared more easily from plasma constituents than completely porous materials. Packing of PC was easier with PLB than with porous sorbents. The Sepralyte materials were mechanically less stable than Corasil and Nucleosil, which have been especially designed for high-pressure applications. For these reasons, Corasil C_{18} was selected as the most suitable material for routine applications.

In most applications, water is used (without any additives) as a purge solvent M1 in column-switching systems. However, it has been shown that better recoveries of strongly protein-bound pharmaceuticals can be obtained by adding a small percentage of acetonitrile to the water[4,9]. For this reason, and also to facilitate the removal of undesired plasma components from PC, a mixture of 10% acetonitrile in water was used as M1 during the enrichment step.

The quality of the water used in M1 was a critical factor during the assay. Organic impurities dissolved in the water were trapped on PC during the reconditioning and the enrichment phase, giving rise to interference problems after transfer to GC/AC. For this reason, only special water of HPLC-grade quality was used when low-concentration samples had to be measured. Also the volume of M1 passing through PC during the reconditioning step was limited to a minimum of 10 ml. This was achieved by introducing an additional valve (V1) into the system which enabled PC to be taken out of the stream of M1 for a certain time (7 min) (see diagrams in Fig. 3).

The optimum purge time for complete removal of proteins and other polar plasma constituents from PC was determined by monitoring the purge process by means of D1. By injecting 500-μl samples and adjusting the purge flow-rate to 2 ml/min, the clean-up process was completed in less than 7 min.

Under routine conditions, problematic material such as particulate matter from seal abrasion and precipitated proteins settled during the purge process at the inlet of PC. Since the subsequent transfer step was performed in the backflush mode this material was partly mobilized and then accumulated at the top of GC, thereby adversely affecting the lifetime of the packing. To circumvent this problem, an additional purge step, B, was inserted between the enrichment and transfer phases, in which the *loaded* PC was backflushed with M1 for 3 min. It was demonstrated that only deposited solid material was directed to waste, while the zone of retained components remained unaffected on PC.

Internal standard

HPLC column-switching methods which almost completely avoid conventional sample work-up often do not need an internal standard to improve precision of the measurements. However, as already pointed out elsewhere[6], the introduction of an internal standard can be very useful in compensating for slight changes in enrichment behaviour and chromatographic performance of PC, especially when long sample sequences are processed. For this reason, as well as to have a better control of the

sophisticated three-valve column-switching process, compound III was used as an internal standard in the method.

Limit of detection, limit of quantification

The two thienoquinolizinones I and II showed a strong native fluorescence (λ_{em} = 475 nm) when excited with UV light at 304 nm. Operating with the Merck-Hitachi fluorescence detector at sensitivity range 5, injection of 2.5 ng I and II in 100 μl mobile phase M2 directly onto GC/AC gave peaks of approximately 95 and 85% full-scale deflection (input 8 mV), respectively. The detection limit, defined by a signal-to-noise ratio of *ca.* 3:1, was about 0.1 ng/ml (equivalent to an absolute injected amount of 25 pg) for both compounds. However, the practical limit of quantification defined here as the minimum concentration that could be measured routinely with acceptable precision and accuracy for compounds I and II was 0.25 and 0.5 ng/ml, respectively (see Table I).

Recovery

Recovery was measured on blank plasma samples spiked with compounds I and II at different concentrations. These spiked samples were analyzed in replicate as described and compared with spiked aqueous solutions, directly injected onto GC/AC via port I2, providing the 100% values. For both compounds, high recoveries (>88%) from human plasma were obtained. No species dependence was detected when recovery from human, dog and rat plasma was compared.

First experiments with spiked plasma samples, diluted 1:1 in water and using twice distilled water as the purge solvent M1, resulted in low recoveries, especially for the internal standard (<50%). It appeared that the interaction of the compounds with the plasma proteins was more pronounced than the affinity towards the PC

TABLE I

PRECISION OF THE METHOD IN THE LOW CALIBRATION RANGE (n = 5)

Compound	*Concentration (ng/ml)*		*C.V. (%)*	*Difference between found and added concentration (%)*
	Added	*Found*		
I	0.25*	0.245	7.4	−2.0
	0.50	0.490	6.8	−2.0
	1.00	0.995	1.7	−0.5
	2.50	2.52	4.2	+0.8
	5.00	4.92	1.4	−1.6
	10.0	10.3	3.0	+3.0
	25.0	24.4	1.2	−2.4
II	0.25**	0.275	9.3	+10.0
	0.50*	0.510	16.1	+2.0
	1.00	1.02	5.0	+2.0
	2.50	2.51	4.1	+0.4
	5.00	4.95	1.9	−1.0
	10.0	10.3	2.6	+3.0
	25.0	24.5	1.5	−2.0

* Limit of quantification.

** Below limit of quantification.

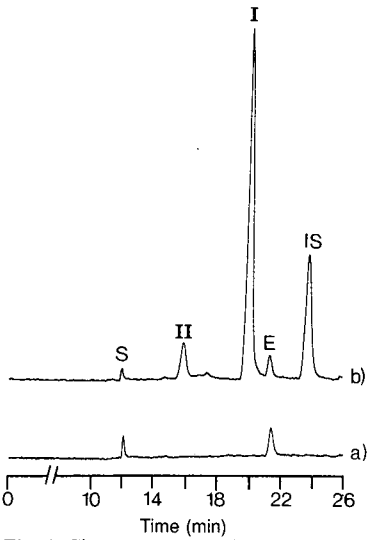

Fig. 4. Chromatograms of human plasma samples: (a) before dose; (b) collected 30 min after a single oral dose of 1 mg of compound I; measured concentrations 21.4 ng I/ml and 2.25 ng II/ml. Peaks: I = parent compound; II = metabolite; IS = internal standard; S = system peak; E = endogenous plasma component. Detector D2, sensitivity 5, attenuation 32.

packing material. This was confirmed by protein binding studies which showed that the parent compound was extensively bound to the plasma proteins with values of $\geqslant 99\%$ for man, $\geqslant 96\%$ for rat and $>91\%$ for dog[10]. In order to "loosen" the protein drug binding to some extent it was necessary to add small quantities of acetonitrile to the plasma samples as well as to the purge solvent M1[4,9]. The percentage of acetonitrile had to be limited to about 10% to prevent any precipitation of plasma proteins which would be deleterious in a column-switching system.

Selectivity

Simple column-switching methods with UV detection often suffer from decreased selectivity in comparison to extraction methods[11]. Using common packing materials such as C_{18} or C_8, only proteins, salts and other highly polar plasma constituents are flushed to waste, while the majority of endogenous compounds are retained on PC and transferred to AC, together with the substances of interest. The application of fluorimetric detectors considerably improves the overall selectivity of column-switching methods, mainly because two analytical wavelengths are used and relatively few compounds have a native fluorescence in the UV region.

More than 50 blank plasma samples from different human volunteers have been analyzed up to the present time. No interfering plasma components were detected co-eluted with compound I, II or III. Fig. 4a shows a typical blank plasma extract.

Precision

The precision of the method was evaluated for the parent compound and the main metabolite over the concentration range 0.25–25 ng/ml by replicate analysis of each concentration over a period of several weeks. The data shown in Table I demonstrate the good precision of the fully automated method.

Linearity

The linear dynamic range of fluorescence detectors is generally less than that of UV detectors. In order to follow plasma concentrations of the parent compound from 2500 ng/ml down to the limit of quantification, the calibration range had to be divided into two linear sub-ranges. For both compounds in each sub-range, excellent linearity and a negligible y-intercept were found routinely.

According to Table I, the coefficient of variation (C.V.) of replicate measurements was nearly independent of concentration in each sub-range, indicating that the variance was roughly proportional to the square of concentration. For this reason, the calibration graphs were calculated by means of weighted least-squares regression, using $1/y^2$ as a weighting factor[12]. The standard software of the computing integrator provided only conventional linear regression and had, therefore, to be modified by means of additional programs[5].

Stability

The anxiolytic and its O-demethyl metabolite were added to human plasma at four different concentrations (1, 10, 100, 1000 ng/ml) and stored for different time intervals (2, 6, 24 h) at room temperature prior to analysis. During storage and analysis the samples were carefully protected from light.

The statistical interpretation of the data followed the procedure recently developed[13]. All measured concentrations indicated that compounds I and II were stable in human plasma at room temperature for at least 24 h. In Table II the stability of the two compounds at the concentration 1 ng/ml is shown.

Practicability

The use of column-switching for on-line clean-up of plasma samples led to a considerable saving of both chemicals and time. A skilled technician was able to analyze more than 40 samples per day and still had sufficient time for laboratory management work, such as documentation of the results, preparation of new pre-columns etc.

TABLE II

STABILITY IN HUMAN PLASMA AT 25°C

Compound	*Exposure time (h)*	*Concn. found (ng/ml)*	*Change of concn. after storage (%)*	*90% Confidence interval (%)*
I	0	1		
	2	1.00	+0.1	−2.6 to +2.9
	6	0.98	−2.3	−5.4 to +1.0
	24	0.97	−2.6	−5.6 to +0.6
II	0	1		
	2	1.06	+5.6	+1.7 to +9.6
	6	1.02	+1.9	−2.6 to +6.7
	24	0.99	−0.8	−8.7 to +7.8

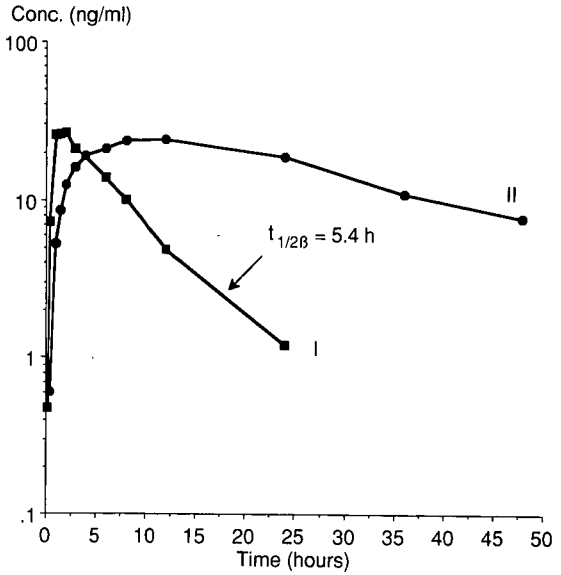

Fig. 5. Plasma concentration–time course of compounds I and II following a single oral dose of 1 mg of I to an healthy male volunteer. $t_{1/2\beta}$ = terminal elimination half-life.

Application to clinical samples

The new method has been applied successfully to the analysis of more than 300 plasma samples from a tolerance study performed on human volunteers. Fig. 4b shows a representative chromatogram. The method was sensitive enough to measure precisely the low concentrations of compound I in plasma for up to 36 h (corresponding to a period of approximately 5 elimination half-lives) after a single intravenous dose of 1 mg to human volunteers (Fig. 5).

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DETERMINATION OF HIGHLY PROTEIN BOUND DRUGS IN PLASMA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND COLUMN SWITCHING, EXEMPLIFIED BY THE RETINOIDS

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SUMMARY

During method development for the determination of either isotretinoin, tretinoin and their 4-oxo-metabolites, or etretinate, acitretin and 13-*cis*-acitretin in plasma using high-performance liquid chromatography and column switching, recovery problems arose, when undiluted plasma samples were injected directly onto the precolumn. These recovery problems may be due to the strong binding of the retinoids to different plasma proteins. Measures to overcome this strong protein binding, such as variation of the injection solution composition and the purge mobile phase, were systematically investigated. Best recoveries were obtained by diluting of plasma with 9 m*M* sodium hydroxide–acetonitrile (8:2, v/v) and protein precipitation with ethanol for the isotretinoin and etretinate series, respectively, in combination with the use of a purge mobile phase containing ammonium acetate and 10–20% acetonitrile. Less effective was the use of a longer precolumn or heating of the precolumn.

INTRODUCTION

The term retinoids is a generic name for a large group of compounds which is structurally related to vitamin A (retinol). According to the definition of Sporn and Roberts[1], a retinoid is a substance that can elicit specific biological responses by binding to and activating a specific receptor or set of receptors. The classical ligands are retinol and retinoic acid, but synthetic ligands may have a better molecular fit to these receptors. Isotretinoin (13-*cis*-retinoic acid, Roaccutan®, 3, Fig. 1) and tretinoin (all-*trans*-retinoic acid, Airol®, 4) are used in the treatment of severe cystic acne[2,3], whereas etretinate (Tigason®, 7) and acitretin (Neotigason®, 6) are effective against psoriasis and other keratinizing disorders[4,5]. However, retinoids also show interesting selective activities in other fields of medicine, such as oncology, inflammation, rheumatism or immune reactions[6].

Among the many analytical methods for retinoids which have been developed within the last ten years, nearly all involve high-performance liquid chromatography (HPLC), including two methods from the authors' laboratory with automated column switching (refs. 7 and 8 and references cited therein). This technique, which allows the

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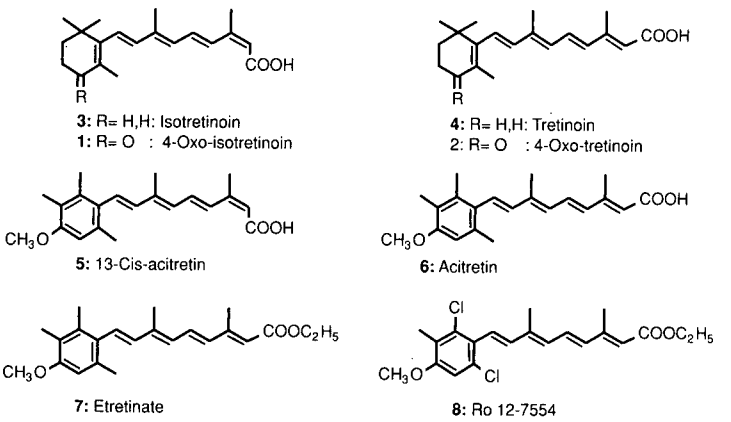

Fig. 1. Chemical structures of the compounds.

direct injection of biological fluids, appeared to be especially suitable for retinoids because of their sensitivity to light and their lipophilicity. Thus, cumbersome extraction in a darkened room can be avoided and preconcentration on a hydrophobic stationary phase should be no problem.

Recently, Westerlund[9] reviewed the possibilities of direct injection of plasma into HPLC systems. Apart from micellar mobile phases, different precolumns can be used for direct injection, including internal surface reversed-phase supports, protein-coated silica and polymer or silica gel based phases for size-exclusion, ion-exchange or affinity chromatography. However, silica bonded hydrophobic phases (C_{18}, C_8, C_2, etc.) are most often used today[9]. In particular, the backflush technique introduced by Roth *et al.*[10] allows the injection of large volumes of plasma without decrease of efficiency of the analytical column. As observed by several authors, polar drugs are the most difficult to handle because of low adsorption on the precolumn, which can lead to breakthrough during the purge step[11,12]. Strategies using different packing materials for precolumns and analytical columns to obtain reconcentration on the second column, depending on the polarity of the drug, have been described[12].

During the development of methods for the determination of either isotretinoin (3), tretinoin (4) and their 4-oxo-metabolites (2 and 1, see Fig. 1)[7], or etretinate (7), acitretin (6) and 13-*cis*-acitretin (5)[8], special measures had to be taken to solve the recovery problems of these highly protein bound drugs (>98%[13]). These problems showed that, after the injection of plasma, not only polar drugs are difficult to pre-concentrate using column switching, but also very lipophilic and highly protein bound drugs. This finding may not be new, but was underestimated by the pioneers of this technique[10,14]. Lecaillon *et al.*[12] recommended C_2 or even CN material in the precolumn for very lipophilic compounds such as retinoids, although he too had not considered the problem of highly protein bound lipophilic drugs. Several measures to overcome strong protein binding in the extraction process, using off-line column techniques, have been described in the literature and comprise dilution[15], change of pH, addition of buffers or salts, addition of ion-pairing agents, heating[16], addition of displacers or change of the chemical structure of the protein or the drug[15]. This paper describes a systematic investigation of some of these measures on the recovery of the retinoid drugs and metabolites 1–8.

EXPERIMENTAL

Materials and reagents

Tetrahydrofuran (HPLC grade), methanol, 2-propanol, acetic acid and ammonium acetate (all puriss. p.a.) were obtained from Fluka (Buchs, Switzerland). Ethanol (HPLC grade) and sodium hydroxide (Titrisol) were obtained from E. Merck (Darmstadt, F.R.G.) and acetonitrile (HPLC grade S) from Rathburn (Walkerburn, U.K.). Water was distilled twice from an all glass apparatus. Argon and helium were obtained from Pan Gas (Lucerne, Switzerland). Substances 1–8 were provided by F. Hoffmann-La Roche (Basle, Switzerland) and were kept under argon at −20°C. Plasma standards were prepared using fresh frozen plasma from sodium citrated human blood, which was obtained from a blood bank (Blutspendezentrum SRK, Basle, Switzerland).

Solutions and standards

The preparation of plasma standards and the dilution or deproteination of the samples were performed in a darkened room. Stock solutions of compounds 1–4 were prepared in amberized volumetric flasks by dissolving 10 mg in 10 ml of methanol. Stock solutions of compounds 5–8 were prepared in the same manner by dissolving 10 mg in 1 ml of tetrahydrofuran and making up to 10 ml with 2-propanol. A 0.1-ml volume of each stock solution was taken and diluted in ethanol to 10 ml. This solution was diluted 1:100 in blank plasma, yielding a plasma standard containing 100 ng/ml each of compounds 1–8.

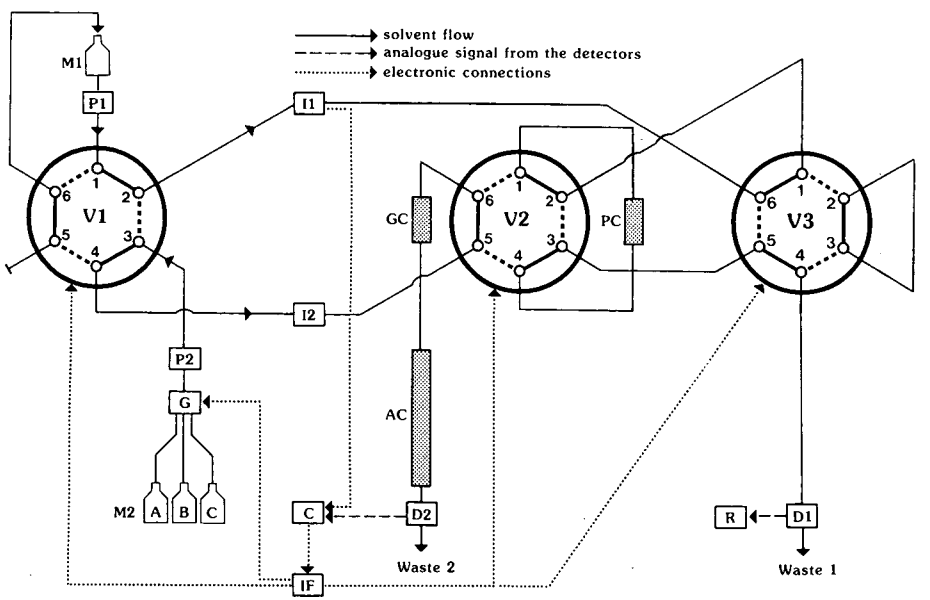

Fig. 2. Schematic representation of the column switching system. The valves V1–V3 are shown in position 0 (see text for further details).

Chromatographic system

The column switching system was similar to that already described[7,8], consisting, in addition, of a third valve for purging the precolumn in the forwardflush and the backflush mode. A schematic representation is given in Fig. 2.

Pump P1 [Model 414 (Kontron, Zurich, Switzerland) with pulse damper (Orlita, Giessen, F.R.G.)] delivered mobile phase M1, which was used as the purge solvent at a flow-rate of 1.5 ml/min. Aliquots (0.5 ml) were injected by a WISP 712 automatic sample injector with cooling module (I1; Waters, Milford, MA, U.S.A.) onto the precolumn (PC). In order to inject sample volumes larger than 200 μl, the autoinjector was used with a 1-ml syringe, the auxiliary sample loop and a syringe motor rate of 1.85 μl/s. For heating of the precolumn, an HPLC column block heater (Model 7930; Jones Chromatography, Hengoed, U.K.) was used. The UV detector (D1) (Spectroflow 773; Kratos, Westwood, NJ, U.S.A.), operating at 240 nm, together with a W + W recorder 320 (R) (Kontron; sensitivity 10 mV, chart speed 0.5 cm/min), were used to monitor the removal of plasma components from the precolumn during the purge step. Pump P2 with a low-pressure gradient system (G) (Spectroflow 400 solvent delivery system and 430 gradient former, Kratos) delivered the gradient mobile phase M2 (flow-rate 1 ml/min) for the elution of the retained components from the precolumn in the backflush mode onto the analytical column (AC).

A guard column (GC) was used to protect the analytical column. A manual injector (I2) (Model 7125 with a 500-μl loop; Rheodyne, Cotati, CA, U.S.A.), situated between pump 2 and valve V2, was used for direct injection onto the analytical column. Detection of the eluted compounds was carried out at 360 nm with an UV detector (D2) (Spectroflow 783, Kratos; rise time 1 s, range 0.02 a.u.f.s.) and integration was performed by means of a computing integrator (C) (Model SP 4200 with Kerr minifile 4100D; Spectra-Physics, San José, CA, U.S.A.; sensitivity 8 mV, chart speed 0.5 cm/min).

The gradient former (G) and the three air-actuated switching valves (V1–V3) (Model 7000A, Rheodyne; shown in position 0 in Fig. 2), the latter connected to three solenoid valves (Model 7163, Rheodyne), were controlled by the external time events of the computing integrator C. To achieve compatibility, a laboratory-made interface (IF) was placed between the integrator output and the solenoid valve input.

Columns and mobile phases

The precolumn (PC) (17 or 40 mm × 4.6 mm I.D.; Bischoff-Analysentechnik, Leonberg, F.R.G.) was dry-packed with Bondapak C_{18} Corasil, 37–53 μm (Waters) and used with sieves (3 μm) without fibre-glass filters to avoid column blocking. The analytical column (AC) consisted of two columns (125 mm × 4 mm I.D.; Hibar type; Merck), linked by a sleeve-nut (Merck) and packed with Spherisorb ODS-1, 5 μm (Phase Separations, Queensferry, U.K.). The guard column (GC) (30 mm × 4 mm I.D.; Merck) contained the same material and was also linked to the analytical column using another sleeve-nut. All columns were packed by a slurry technique. To obtain higher plate numbers, two linked columns, each 125 mm long, were preferred to a single 250-mm column.

Five different variants for mobile phase 1 were used: (a) water, (b) 1% ammonium acetate, (c) 1% ammonium acetate–acetonitrile (9:1, v/v), (d) 1% ammonium acetate and acetic acid–acetonitrile (9:1, v/v) and (e) 1% ammonium

acetate and acetic acid–acetonitrile (8:2, v/v). Mobile phase 2 consisted of three components: (A) 400 ml of 0.1% ammonium acetate, 600 ml of acetonitrile and 30 ml of acetic acid; (B) 150 ml of 0.27% ammonium acetate, 850 ml of acetonitrile and 10 ml of acetic acid; (C) 10 ml of water, 980 ml of acetonitrile and 10 ml of acetic acid. All mobile phases were degassed with helium prior to use.

Analytical procedure

Plasma (0.5 ml) was either injected directly or diluted in 0.75 ml of 7.2 m*M* sodium hydroxide or 9 m*M* sodium hydroxide–acetonitrile (8:2, v/v), or deproteinated by adding 1 ml of ethanol or 2-propanol, then left to stand for 15 min in a refrigerator at 4°C. After centrifugation (5 min at 1800 *g*), 0.8 ml of the supernatant were transferred to the autosampler vial (microtubes 3810; Eppendorf Gerätebau, Hamburg, F.R.G.), and 0.5 ml were injected. The samples were kept at 10°C in the autosampler before injection.

The complete sequence of automated sample analysis took 44 min and included the following five steps.

Step A (0–7 min, V1 = 0, V2 = 0, V3 = 0). Injection of the sample onto PC. Polar components were washed out to waste 1. GC and AC were equilibrated with M2 (100% A).

Step B (7–10 min, V1 = 0, V2 = 0, V3 = 1). PC was purged in the backflush mode by M1.

Step C (10–37 min, V1 = 0, V2 = 1, V3 = 1). M1 passed directly to waste 1. The retained components were transferred from PC to GC/AC in the backflush mode by the gradient M2: from 100% A to 70% A–30% B (10–16 min), 70% A–30% B to 100% B (16–21 min), 100% B (21–32 min), 100% B to 100% C (32–32.1 min) and 100% C (32.1–37 min).

Step D (37–38 min, V1 = 1, V2 = 1, V3 = 1; 38–39.9 min, V1 = 1, V2 = 0, V3 = 0). While M1 was running in a recycling mode, the capillaries between I1 and D1 were purged with M2 (100% C) to prevent any memory effects during the next injection. There was no flow through GC and AC during this period.

Step E (39.9–44 min, V1 = 0, V2 = 0, V3 = 0). M2 was changed from 100% C to 100% A in 0.2 min, and GC/AC and PC were re-equilibrated with M2 and M1, respectively.

Determination of recoveries

Spiked plasma (100 ng/ml of compounds 1–8), treated in different ways as described below, was injected and analysed using five different variants of mobile phase 1. The percentage recoveries were determined by comparing the mean of the peak heights of replicate analyses under the same conditions (n = 2–4) with the mean of the peak heights of compounds 1–8 in 50% ethanol, injected directly onto GC/AC (using I2). A 200-μl volume of 50% ethanol, containing the same amount of substance, was injected, representing the 100% reference value.

RESULTS AND DISCUSSION

Column-switching system

In principle, one switching valve is sufficient for direct injection of plasma onto

a precolumn and subsequent separation on an analytical column[14]. However, in the two methods developed for the determination of compounds 1–4 and 5–7[7,8], two valves have been used, enabling the purge of the steel capillaries between the automatic sample injector and detector 1. The installation of a third valve allows forward- and backflush purging of the precolumn[17]. Proteins and solid particles, which would be partly adsorbed on the sieves on the top of the precolumn, are transferred to waste instead of to the analytical column. This technique proved to be very effective after the injection of tissue samples, which were homogenized with ethanol–water and directly injected after centrifugation. Many more samples could be injected than when using forwardflush purging only[18]. However, more experience is needed to assess this technique regarding its general application, especially with more polar compounds.

Variation of injection solution composition and mobile phase 1

Variation of the injection solution composition and mobile phase 1 appeared to have the greatest influence on the recoveries of compounds 1–8 from plasma in preliminary experiments. In the first investigation, five different mobile phases 1 were

TABLE I

INFLUENCE OF THE COMPOSITION OF THE INJECTION SOLUTION AND MOBILE PHASE 1 ON THE RECOVERIES OF COMPOUNDS 1–8

Injection solution (0.5 ml)	*Mobile phase 1*	*% Recovery of 100 ng/ml plasma standards*							
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Plasma	Water	88	86	50	40	22	57	2	0
	1% CH_3COONH_4	100	100	59	42	29	62	2	1
	1% CH_3COONH_4–CH_3CN (9:1)	97	94	62	47	56	66	3	1
	1% CH_3COONH_4/CH_3COOH–CH_3CN (9:1)	97	93	44	25	62	50	4	2
	1% CH_3COONH_4/CH_3COOH–CH_3CN (8:2)	93	92	56	38	75	65	11	6
Plasma (2 vol.) + 7.2 m*M* NaOH (3 vol.)	Water	81	81	51	42	7	57	4	0
	1% CH_3COONH_4	100	100	63	48	13	65	3	0
	1% CH_3COONH_4–CH_3CN (9:1)	95	93	66	54	35	70	5	2
	1% CH_3COONH_4/CH_3COOH–CH_3CN (9:1)	94	90	47	28	62	53	5	3
	1% CH_3COONH_4/CH_3COOH–CH_3CN (8:2) (8:2)	88	88	57	39	72	67	12	7
Plasma (2 vol.) + 9 m*M* NaOH–CH_3CN (8:2) (3 vol.)	Water	91	89	82	84	76	91	11	0
	1% CH_3COONH_4	100	100	91	88	93	96	12	4
	1% CH_3COONH_4–CH_3CN (9:1)	97	96	91	88	93	92	15	5
	1% CH_3COONH_4/CH_3COOH–CH_3CN (9:1)	94	95	83	71	88	86	17	6
	1% CH_3COONH_4/CH_3COOH–CH_3CN (8:2)	86	89	86	78	89	90	25	13
Plasma (1 vol.) + ethanol (2 vol.)	Water	26	24	50	53	40	41	57	58
	1% CH_3COONH_4	34	32	53	55	42	43	70	62
	1% CH_3COONH_4–CH_3CN (9:1)	23	22	41	43	35	36	66	59
	1% CH_3COONH_4/CH_3COOH–CH_3CN (9:1)	35	35	77	81	66	71	73	68
	1% CH_3COONH_4/CH_3COOH–CH_3CN (8:2)	32	31	76	81	62	68	71	63
Plasma (1 vol.) + 2-propanol (2 vol.)	Water	18	16	26	27	24	24	58	66
	1% CH_3COONH_4	24	23	25	26	24	24	76	78
	1% CH_3COONH_4–CH_3CN (9:1)	17	16	25	25	24	24	61	64
	1% CH_3COONH_4/CH_3COOH–CH_3CN (9:1)	26	25	40	45	34	38	65	71
	1% CH_3COONH_4/CH_3COOH–CH_3CN (8:2)	22	21	39	42	34	36	55	59

compared by replicate injection of five different injection solutions. The results are compiled in Table I.

The simplest approach, injection of undiluted plasma and use of water as M1, was tried first when developing a method for compounds 1–4[7]. Whereas the recovery was sufficient for 1 and 2 (>80%), only 50% could be obtained for 3 and 40% for 4. For compounds 5–8, the recoveries were even lower, as shown in Table I. Improved recoveries for oxazepam metabolites were reported by Roth and Beschke[11], when 1% ammonium acetate was used as M1. This was also observed in this investigation, but the results were still only acceptable for compounds 1 and 2. Dilution of the plasma sample (0.2 ml) with 7.2 m*M* sodium hydroxide (0.3 ml) did not improve the recoveries significantly. Sodium hydroxide was used instead of water, because a basic pH (8–9, depending on the plasma sample) improved the stability of the samples in the autosampler during overnight injection. The big difference between compounds 5 and 6 (7 and 57% recovery using water, 13 and 65% recovery using 1% ammonium acetate as M1, respectively) is surprising, and may be explained by different protein binding of the *cis* and *trans* isomers of acitretin.

The addition of acetonitrile (or an alcohol) to the injection solution resulted in a significant improvement, as observed by other authors[17,19,20]. However, the maximum amount was limited to 30%, otherwise protein precipitation occurred. Good recoveries for compounds 1–6 were obtained, when 0.2 ml of plasma were diluted in 0.3 ml of 9 m*M* sodium hydroxide–acetonitrile (8:2, v/v). Addition of sodium hydroxide was preferred to a buffer such as sodium acetate, since use of the latter

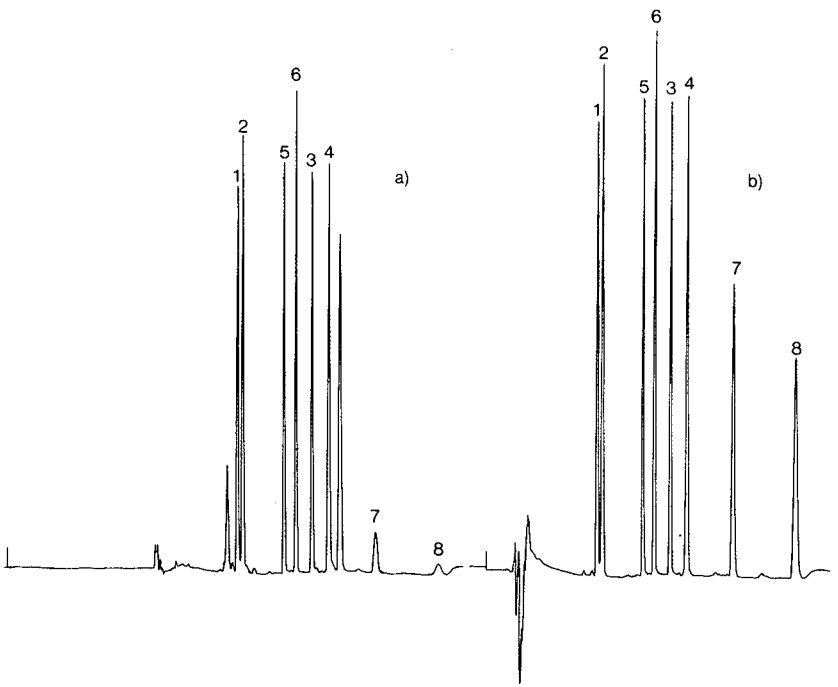

Fig. 3. Chromatograms of compounds 1–8. (a) Plasma standard 100 ng/ml; (b) 100% reference value in 50% ethanol, directly injected onto the analytical column.

resulted in protein precipitation when the autosampler was cooled to 10°C during overnight injection. This injection solution and 1% ammonium acetate–acetonitrile (9:1, v/v) as M1 were the conditions used in a method developed for the determination of compounds 1–4[7]. Compounds 5 and 6 could also be determined under these conditions; the latter served as an internal standard in the above mentioned method[7]. However, when 7 and 8 were injected, the recoveries were still very low (15 and 5%, respectively). Chromatograms of a plasma standard containing compounds 1–8, injected under these conditions, as well as the corresponding 100% reference value are shown in Fig. 3. Addition of acetic acid, and increasing the acetonitrile content of M1 to 20%, resulted in a improvement, which was however still insufficient.

The recoveries of compounds 7 and 8 were significantly improved only when plasma proteins were precipitated with an organic solvent before injection. However, this was not an ideal solution for all eight compounds, as the more polar 1–4 were already partly eluted from the precolumn because of the relatively high content of organic solvent in the injection solution. Ammonium acetate (1%) and acetic acid–acetonitrile (8:2, v/v) as M1 and protein precipitation with ethanol were the conditions chosen for the determination of compounds 5–7 in plasma (using 3 and 8 as internal standards)[8]. Protein precipitation with 2-propanol (see Table I) or methanol (data not shown) did not further improve the recoveries of the compounds investigated.

Variation of precolumn length and temperature

Neither decreasing the flow-rate nor the use of porous ODS materials in the precolumn, as observed by several authors[21,22], had any effect on the recoveries of compounds 1–8. At first, we used a 5 mm long cartridge, packed with C_{18} Nucleosil, 30 μm. Longer precolumns (10 or 20 mm) improved the recovery. However, C_{18} Corasil, 37–53 μm (in a 17 mm long precolumn), gave the same results as C_{18} Nucleosil regarding the recovery, but resulted in less clogging problems, and was therefore chosen for further investigations. In view of the very difficult recovery problems with compound 7 (and 8), a 40 mm long precolumn (packed with C_{18} Corasil) as well as heating of the precolumn was tried, as described for lipoprotein bound steroids, which are known to be difficult to recover[16]. The results of these investigations are summarized in Table II.

The 40-mm column, used at room temperature, did not significantly improve the recoveries of compounds 1–8. Heating of the 17-mm precolumn gave positive results above 60°C only. At 80°C, recoveries from diluted plasma were improved, but the effect was less distinct than with protein precipitation. This latter measure in combination with heating resulted in no further improvement. This is reasonable, because the separation of the drug from plasma proteins has already been effected before injection and heating. Heating of the precolumn to 80°C together with the injection of sodium hydroxide containing solutions resulted in deterioration of the analytical column after only a few injections, probably due to degradation of the silica gel, and was, therefore, not suitable for routine determinations.

Possible explanation of the recovery problems

The retinoids investigated are very lipophilic drugs, with decreasing polarity from compound 1 to 8. When injected in an aqueous solution into a chromatographic

TABLE II
INFLUENCE OF THE PRECOLUMN LENGTH AND TEMPERATURE ON THE RECOVERIES OF COMPOUNDS 1–8

Injection solution (0.5 ml)	*Mobile phase 1*	*Column*		*% Recovery of 100 ng/ml plasma standards*							
		Length (mm)	*Temperature (°C)*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Plasma	Water	17	22	88	86	50	40	22	57	2	0
		40	22	81	78	55	42	24	62	2	1
		17	80	82	79	76	69	29	77	4	2
Plasma (2 vol.) + 9 m*M* NaOH–CH_3CN (8:2) (3 vol.)	1% CH_3COONH_4–CH_3CN (9:1)	17	22	97	96	91	88	93	92	15	5
		40	22	84	85	87	84	88	90	17	4
		17	80	81	87	79	89	83	87	36	18
Plasma (1 vol.) + ethanol (2 vol.)	1% CH_3COONH_4/CH_3COOH–CH_3CN (8:2)	17	22	32	31	76	81	62	68	71	63
		40	22	32	33	71	72	62	68	78	70
		17	80	25	25	62	71	45	51	68	61

system consisting of an aqueous mobile phase and a C_{18} column, the highest adsorption is expected for 8, and the lowest for 1 and 2. In the experiments described the opposite was observed. The greater the lipophilicity of the drug, the lower was the adsorption on the precolumn. This can be explained only by the strength of the binding of the drug to plasma proteins, or the difficulty of lipophilic drug transfer from protein to hydrophobic stationary phase through an hydrophilic mobile phase.

Retinoid drugs are known to be almost completely bound to plasma proteins (99.9 and >98% for compounds 3 and 7, respectively[13]). However, it seems to be not only the extent, but also the strength of protein binding which may be responsible for the observed recovery problems. Retinol is transported in blood bound to retinol binding protein (RBP)[23]. Retinoic acids (compounds 3 and 4) and acitretin (6) are bound to albumin, whereas etretinate (7) is bound to lipoproteins[13]. It is well known that lipoprotein bound drugs are difficult to extract with column techniques[16]. However, it appears that so far these types of drugs have not been analysed using on-line solid-phase extraction with automated column switching.

CONCLUSION

During method development for the determination of compounds 1–4 and 5–7 in plasma using HPLC with column switching, recovery problems arose, which may probably be correlated to binding to different plasma proteins. Injection of undiluted plasma samples and the use of a purge mobile phase of water or 1% ammonium acetate, the "classical" approach[10,11,14], resulted in very low recoveries for most of the retinoids investigated. Recovery problems were overcome by dilution of plasma in 9 m*M* sodium hydroxide–acetonitrile (8:2, v/v) and protein precipitation with ethanol for compounds 1–4 and 5–7, respectively, and use of a purge mobile phase containing ammonium acetate and 10 or 20% acetonitrile.

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FULLY AUTOMATED ANALYTICAL SYSTEM USING LIQUID–SOLID EXTRACTION AND LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF CGP 6140 IN PLASMA

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SUMMARY

Liquid–solid extraction on disposable extraction columns (DECs) and liquid chromatography can be combined in a completely automated analyser. The Gilson ASPEC system was used to develop a procedure for the determination of CGP 6140 in plasma. Both sample preparation via C_8 Bond-Elut DECs and injection were fully automatic.

The fully automated system prepared the samples by performing the same operations as for a manual procedure. The DEC was first wetted with methanol, then with water. A 400-μl volume of plasma and 40 μl of the internal standard solution, diluted with 1 ml of water, were applied to the DEC, rinsed with 10^{-2} mol/l dipotassium hydrogenphosphate and eluted from the DEC with 300 μl of acetonitrile–methanol (50:50, v/v). The eluting strength of the eluate was reduced by dispensing 1 ml of water into each vial prior to direct injection into a Spherisorb ODS column via a 1-ml loop. This allowed the reconcentration of the extracted compounds on the top of the column, as they were injected in a large volume of solvent of lower eluting strength than the mobile phase [acetonitrile–methanol–4 · 10^{-3} mol/l ammonia solution (54.5:5:40.5, v/v/v)]. Reproducibility results are presented.

INTRODUCTION

Liquid–solid extraction (LSE) via disposable extraction columns (DECs)[1] or by column switching[2,3] is being increasingly used in combination with high-performance liquid chromatography (HPLC) as an alternative to time-consuming liquid–liquid extraction[4]. Semi- or fully automated systems for LSE and HPLC have recently been introduced. The advanced automated sample processor (AASP) allows semi-automatic extraction on a DEC and on-line elution into the analytical column[5]. The Zymate Laboratory Automation System permits full automation of LSE and HPLC[6], but the high cost of this instrument limits its application. The PROSPEKT

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system, still under development, should permit on-line liquid–solid extraction and on-line elution into the analytical column[7].

This paper describes a fully automated procedure for the determination in plasma of a compound with antiparasitic properties, CGP 6140. Both sample preparation via C_8 Bond-Elut DECs and injection were fully automated using the Gilson ASPEC system. The procedure described is a modification of a method previously reported using the AASP system for sample preparation[8].

EXPERIMENTAL

Materials and reagents

CGP 6140 [4-nitro-4′-(N-methylpiperazinylthiocarbonylamido)diphenylamine] and CGP 10 631 (4-nitro-4′-acetylaminodiphenylamine) (internal standard) were provided by Ciba-Geigy (Basle, Switzerland). Their structures are shown in Fig. 1. Internal standard and calibration solutions were prepared by dissolving the compounds in methanol and by further dilution with water–methanol (80:20, v/v).

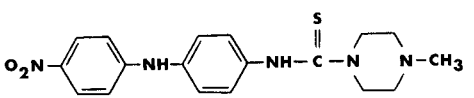
Dipotassium hydrogenphosphate and 25% ammonia solution were purchased from E. Merck (Darmstadt, F.R.G.). Acetonitrile was of HPLC quality (Carlo Erba France, Puteaux, France) and methanol was of RPE-ACS quality (Carlo Erba France).

Apparatus

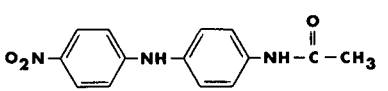
The chromatographic system consisted of a Model 303 pump (Gilson, Villiers-le-Bel, France), an ASPEC (Automatic Sample Preparation with Extraction Columns) system (Gilson) and a Model 773 UV detector (Kratos, Paris, France) set at 405 nm. A Model 4100 computing integrator (Spectra-Physics, Les Ulis, France) was used for data acquisition.

The ASPEC system combines three components (Fig. 2): an automatic sampling injector module, a Model 401 dilutor/pipettor and a set of racks and accessories, necessary for handling DECs and solvents.

LSE has been totally automated as a result of the development of a specific rack. This rack consists of three parts: DEC holder, drain cuvette and collection rack (Fig. 3). The DEC holder is mobile, so that each DEC can be automatically located



CGP 6140



CGP 10 631 (internal standard)

Fig. 1. Structures of CGP 6140 and of the internal standard CGP 10 631.

Fig. 2. Gilson ASPEC system.

either above the drain cuvette (Fig. 3A) or above a collection tube (Fig. 3B), according to the extraction step being performed. After extraction, the collected fraction can be injected in a Rheodyne injection valve for on-line HPLC analysis (Fig. 3C). The solvents required during the extraction process can be aspirated from four different bottles located on a solvent bottle rack.

With the ASPEC, the flow of the solvents through the DEC is carried out under a positive pressure. Precise volumes are delivered by the 401 dilutor, and forced

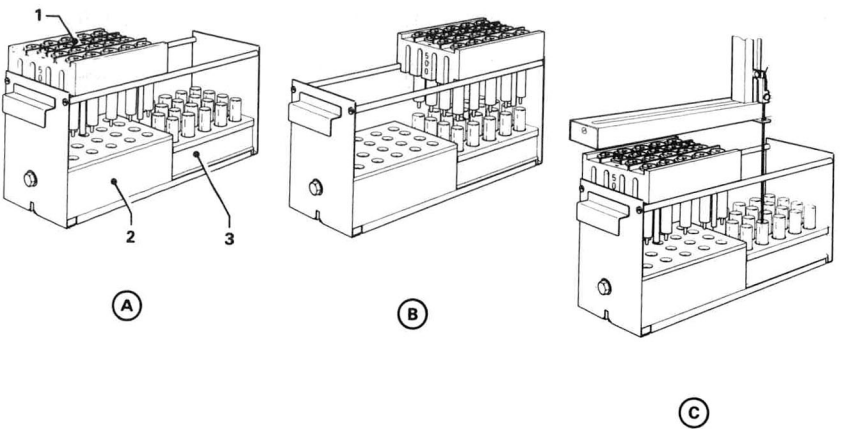


Fig. 3. Liquid–solid extraction rack of the Gilson ASPEC system. 1, DEC holder; 2, drain cuvette; 3, collection rack.

through the packing by air. A special cap fitted on each DEC assures air-tightness when the needle is dispensing liquid or air.

Column

The analytical column was a stainless-steel tube (25 cm × 4.7 mm I.D.) packed with Spherisorb ODS 1, particle size 5 μm (SFCC, Gagny, France).

Disposable extraction columns

C_8 Bond-Elut DECs (100 mg) of capacity 1 ml were used. They were manufactured by Analytichem International (Harbor City, CA, U.S.A.) and supplied by Prolabo (Paris, France).

Sample preparation

A 400-μl volume of plasma, 1 ml of water and 40 μl of the internal standard or calibration solution were transferred manually into a vial and shaken on a Vortex mixer for a few seconds. The vial was then placed on the appropriate rack of the Gilson ASPEC system.

All of the following operations on the samples were performed automatically by the ASPEC system. The automatic sequences were:

(1) DEC conditioning. Draw up 2 ml of methanol from bottle 1 and dispense into the DEC.

Draw up 2 ml of water from bottle 2 and dispense into the DEC.

(2) Liquid–solid extraction. Draw up 1 ml of dilute plasma sample from a tube and dispense into the DEC.

Draw up 2 ml of 10^{-2} mol/l dipotassium hydrogenphosphate solution from bottle 3 and dispense into the DEC.

Shift the rack containing the DECs on top of the part of the rack containing the collection tubes.

Draw up 300 μl of methanol–acetonitrile (50:50, v/v) from bottle 4 and dispense into the DEC. The eluate is collected in the tube positioned under the DEC.

Pull back the rack containing the DECs.

(3) Injection. Draw up 1 ml of water from bottle 2 and dispense into the tube containing the eluate.

Draw up 1 ml of air and dispense into the tube, the needle being lowered into the tube to mix the liquids by bubbling.

Draw up 1.2 ml of mixture from the tube and dispense through the 1-ml injection loop.

Inject into the analytical column.

Each plasma sample was prepared separately during the chromatography of the previous sample. In all instances the needle was rinsed with 2 ml of water and a 40-μl segment of air was created before pipetting the liquid to be transferred, in order to avoid cross-contamination.

Chromatography

The chromatography was carried out at ambient temperature. The mobile phase was acetonitrile–methanol–$4 \cdot 10^{-3}$ mol/l ammonia solution (54.5:5:40.5, v/v/v). The flow-rate of the mobile phase was 1.2 ml/min.

RESULTS AND DISCUSSION

Automatic procedure

The ASPEC system prepared plasma samples by performing the same operations as in a manual procedure: the DEC was first conditioned, dilute plasma was then applied to the DEC and washed with an aqueous solution of dipotassium hydrogenphosphate before elution of the retained compounds with an organic solvent.

CGP 6140 was strongly retained on the DEC sorbent and 300 μl of methanol–acetonitrile (50:50, v/v) were necessary to elute it from the DEC. Generally, in manual procedures combining LSE and HPLC, either the sample collected from the DEC is evaporated to dryness and the residue is dissolved in the mobile phase for injection, or an aliquot of eluate is directly injected on to the column. However, it has been shown that at least 1 ml of sample can be injected without peak broadening if the drug is dissolved in a solvent whose eluting strength is less than that of the mobile phase[9]. The sample is then sorbed on the bonded phase at the head of the column until the injection is finished. Therefore, in the present procedure, the sample collected from the DEC was diluted by the ASPEC system with 1 ml water. The proportions of acetonitrile and methanol in the injected sample were then 11.5% each, whereas they were 54.5 and 5%, respectively, in the mobile phase. When 1 ml of this dilute sample was injected, no peak broadening was observed compared with the injection of 100 μl of CGP 6140 and CGP 10 631 in water. Almost all of the CGP 6140 extracted from plasma could then be injected on to the analytical column, thereby affording good sensitivity.

C_2 Bond-Elut DECs of capacity 2.8 ml and containing 500 mg of sorbent were also used. A high volume (1.5 ml) of methanol–acetonitrile (50:50, v/v) was necessary to elute CGP 6140 and CGP 10 631 from the DEC. It would have been necessary to inject several millilitres of dilute sample on to the column to obtain good sensitivity. Therefore, as no improvement of the separation between CGP 6140 or CGP 10 631 and endogenous plasma compounds was observed with these 500-mg DECs, the smaller 100-mg DECs were preferred.

The time required for sample preparation and injection (10 min) was less than the duration of the chromatography (18 min). Therefore, no attempt was made to shorten the time of the robotic procedure, but this should be feasible by increasing the flow-rate of sampling in some of the automated operations.

Reproducibility and accuracy

Examples of chromatograms are given in Fig. 4. The ratio of the peak areas of CGP 6140 and CGP 10 631 (internal standard) was plotted against the CGP 6140 concentration in plasma. An example of a calibration graph is given in Fig. 5. The equation of the graph was calculated by the least-squares method using weighted linear regression with a weighting factor of $1/(\text{concentration})^2$ (ref. 10).

The within-day reproducibility and accuracy of the method were calculated by analysing, on the same day, replicate plasma samples spiked with different concentrations of CGP 6140 (Table I). The limit of quantitation was 54 nmol/l of plasma (about 20 ng/ml).

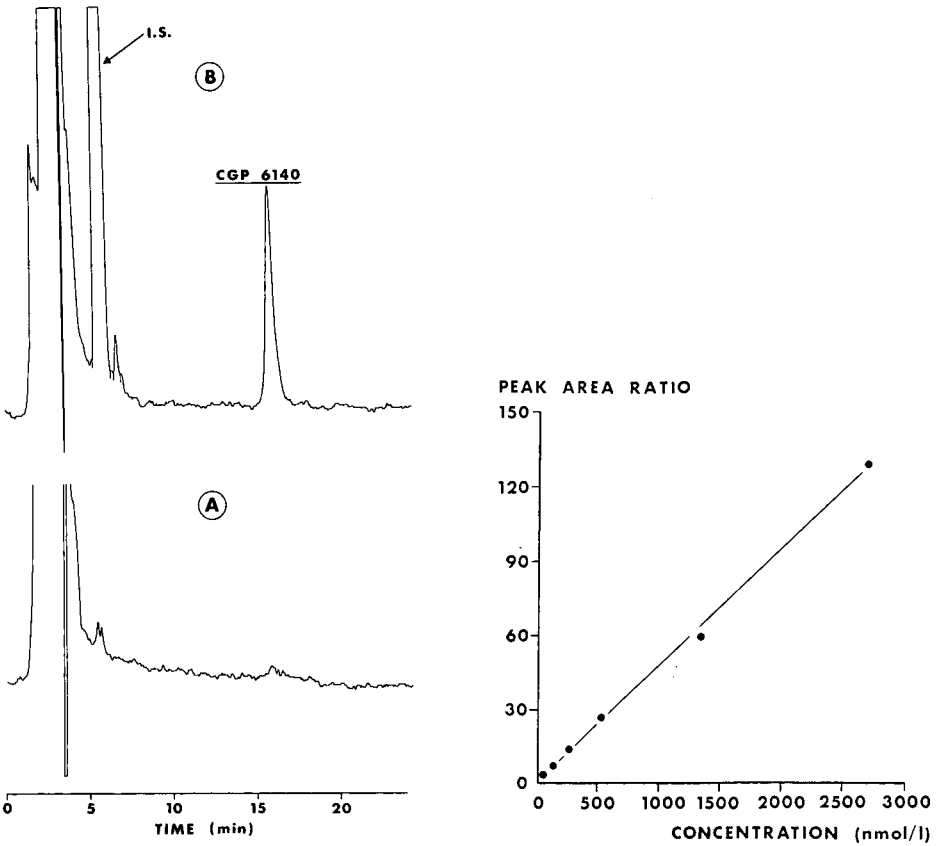


Fig. 4. Chromatograms of extracts of (A) blank plasma and (B) plasma containing 134.6 nmol/l of CGP 6140 and 5.92 μmol/l of CGP 10 631 (internal standard).

TABLE I

WITHIN-DAY ACCURACY AND REPRODUCIBILITY OF THE ASSAY OF CGP 6140 IN SPIKED PLASMA SAMPLES

To convert the results into ng/ml, multiply by 0.3715.

Introduced (nmol/l)	*No. of assays*	*Mean recovery ± S.D. (%)*	*Overall recovery* ± S.D. (%)
53.8	6	94 ± 9	
137	6	101 ± 9	
269	6	104 ± 8	100 ± 8
539	5	104 ± 5	
2693	5	98 ± 5	

Comparison with the AASP system

The procedure was compared with that previously reported for the determination of CGP 6140 in plasma using the AASP system for sample preparation[8]. With the ASPEC system, DECs are automatically and separately processed up to and including injection; elution is carried out at a constant flow-rate in each DEC. With the AASP system, ten DECs are first manually and simultaneously processed by elution at a constant pressure, then automatically eluted on to the column.

The reproducibility and the limit of quantitation of the procedure using the AASP and the Gilson ASPEC system for sample preparation were similar.

With the ASPEC system, CGP 6140 was stored in dilute plasma before injection and was stable for at least 10 h (mean recovery $\pm$ S.D. = 104 $\pm$ 8%, n = 6). Refrigerated racks can be installed on the ASPEC system, thereby increasing the sample throughput for this system. With the AASP system, CGP 6140 was stored in the DEC at ambient temperature before injection and slight degradation of the drug was observed after 8 h of storage.

CONCLUSION

Liquid–solid extraction on Bond-Elut DECs and HPLC can be combined in a completely automated analyser for the determination of CGP 6140 in plasma. The previously described method could be rapidly adapted. The major part of the CGP 6140 plasma extract could be injected on to the analytical column in a large volume of non-eluting solvent, thereby allowing good sensitivity.

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AUTOMATED SEQUENTIAL TRACE ENRICHMENT OF DIALYSATES AND ROBOTICS

A TECHNIQUE FOR THE PREPARATION OF BIOLOGICAL SAMPLES PRIOR TO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The development of the sample preparation process, the automated sequential trace enrichment of dialysates, in association with a cartesian robotic sampler is described. The system has been applied to the total automation of the preparation of biological samples and high-performance liquid chromatographic analysis. Concepts of the technique are reported together with an examination of its application to free and total analyte estimation. Examples of chromatographic separations obtained from the preparation of a variety of different analytes and sample materials are given.

INTRODUCTION

Total automation of one chromatography cycle may be defined as the preparation of untreated samples and chromatographic analysis without operator intervention. Total automation of chromatography systems is beneficial to capitalise on equipment investment, reduce operator costs and increase the throughput of sample analysis.

Theoretically the automation of sample preparation for high-performance liquid chromatography (HPLC) is generally easier than other separation techniques such as gas chromatography, since liquid samples can be introduced onto the analytical column. However, difficulties arise when analytes of small molecular size need to be quantified in samples with complex matrices such as those of biological origin. Sample preparation is required to remove interfering micro- and macromolecular compounds such as proteins, polymers and low-molecular-weight compounds present at very high concentrations. In particular, the injection of protein onto columns can not only create high column back pressures but will also alter the nature of the stationary phase. The ultimate goal of sample preparation is to protect and prolong the lifetime of the analytical column so producing robust chromatographic

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separations that are free from interferences and will yield the same duration and quality of analytical performance as that obtained when chromatographing pure solutions of analytes. If this goal is achieved the benefits accrued apply both to the experienced and inexperienced HPLC user and the cost effectiveness of HPLC is much improved.

Most methods for sample preparation have involved batch processing, where every sample is treated for one operational step before the next operation is performed. Such techniques include protein precipitation[1], liquid–liquid and solid phase extractions[2–4]. These batch processing techniques do not make efficient use of the HPLC system during sample preparation. Total automation has been achieved using anthropomorphic and cylindrical robots[5,6], to simulate the manually performed procedures such as liquid–liquid extractions but the equipment is expensive. Only partial automation of solid phase extraction has been achieved. This is primarily due to the size of the extraction column employed and manual manipulations for the addition of sample and reagents are required. Of the sequential processing methods, where every operational step is performed on a sample before proceeding to the next sample, direct sample injection[7] and column switching techniques[8] have been totally automated. However operating costs are very high due to rapid deterioration in analytical column and pre-columns employed. Furthermore when analysing proteinaceous samples the techniques impose restrictions on the type of HPLC solvents used and the incorporation of guard columns is essential.

A new, "on-line", technique for sample preparation, the automated sequential trace enrichment of dialysates (ASTED[9]) has been shown to be capable of addressing many of the problems that exist in the total automation of sample preparation[10–13]. Although previous reports have been published on the application and validation of ASTED by reference to the estimation of various analytes in biological fluids, little has been discussed on the theoretical concepts of the process. This paper reports on the development of the commercially available ASTED system in association with a Gilson cartesian robotic sampler, that can be applied at present to the analysis of small organic molecules. It discusses the nature of the process and describes examples of HPLC separations obtained from a variety of samples with complex matrices.

EXPERIMENTAL

Instrumentation

Development of the ASTED component parts and the system process control was made in association with Gilson Medical Electronics, Villiers-le-Bel, France. Unless otherwise stated all other equipment was also obtained from Gilson Medical Electronics.

HPLC unit

The HPLC system consisted of an isocratic and gradient unit. This entailed the use of either one, two or three 303/5SC pumps. Several detectors were used depending upon the application. These included a 116 UV detector, 121 fluorimeter and 141 electrochemical detector. Control of the gradient or isocratic HPLC system and integration of chromatographic peaks was made using a 714 system controller (IBM AT with hard disc, EGA graphic card, mouse, MS DOS 3.1 and Windows software).

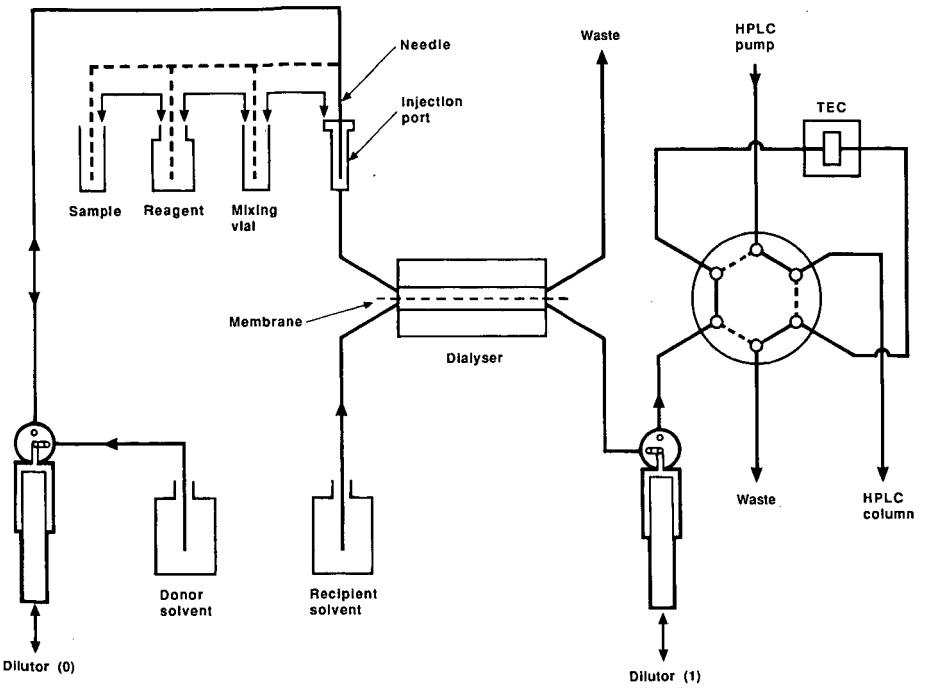

Fig. 1. A schematic diagram of the ASTED system.

The HPLC columns were either 100 mm or 150 mm × 4.6 mm I.D. containing Spherisorb 5 μm ODS 2 (Phase Separations, Queensferry, U.K.). No guard columns were used.

Sample preparation (ASTED) unit

This consisted of a 231 sample injector module incorporating a 7010 Rheodyne injection valve, two 401 dilutors (identified as 401 dilutor 0 and 1) with 1.0-ml syringes for liquid transfer, a polymethyl methacrylate flat plate dialyser fitted with a Cuprophan membrane, and a stainless-steel trace enrichment cartridge fitted in place of the loop on the injection valve. A schematic diagram of the ASTED system is shown in Fig. 1.

Development of the ASTED system

Dialyser unit

A flat plate dialyser was adopted since, with this design replacement of the membrane was simple and had good filling and purge characteristics. For mechanical strength and ease of dismantling the donor and recipient halves, a rectangular shape was chosen, clamped together with bolts. Two sizes of units were developed with donor volumes of 100 μl and 370 μl having path lengths of 186 and 677 mm respectively. By connecting combinations of units in series a range of donor volumes can be achieved.

The channel through which the donor and recipient liquids flowed was a "U" design allowing adjacent inlet and outlet connections. The width and depth of the channels were optimised with respect to a 20-μm thick (wet) Cuprophan membrane. This membrane has a tensile strength of 1050/220 (longitudinal/transversal) cN/15 mm. The membrane has a molecular weight cut-off of approximately 15 kilodalton (kD). The donor channel was 2 mm wide and 0.2 mm in depth, whilst the recipient channel was 0.75 mm in depth. This permitted some flexing of the membrane, problem-free filling and purging of the dialyser.

Trace enrichment cartridge (TEC)

The internal dimensions of the TEC were 1.6 mm (height) × 4.6 mm (diameter). This retained 20 mg of 10 μm reversed-phase spherical silica between stainless-steel meshes held in place by Kel-F end seals. It was necessary to use material with 10 μm (or less) in diameter and materials were slurry packed under vacuum. With these quantities rapid regeneration of the TEC retaining conditions were obtained with small volumes of solvent. The TEC holder was constructed in two threaded halves that could be screwed together and finger-tightened to withstand pressure drops of 48 MPa. This enables rapid interchange of TEC's whilst the holder remains in place connected to the Rheodyne injection valve. The holder and associated 1/16 in. attachment tubing to the Rheodyne injection valve was 316L stainless steel.

Process control

Software on ROM within the 231 controls preparation and clean-up of samples using ASTED, communications with the HPLC system and selects the ASTED process option required.

Preparation and sample clean-up. The ASTED process consists of pre-column sample treatment of the sample by the 231 (up to 5 different reagents may be added, mixed and incubated with the sample, if required). The sample (or sample–reagent mixture) is loaded into the donor channel of the dialyser via the loop filler port of the 231 using the 401 dilutor 0. Larger sample volumes can be dialysed by "pulsing" the sample through the dialyser at pre-determined times. The low-molecular-weight analytes diffuse across the membrane and are continously swept to the TEC in the recipient solvent using the 401 dilutor 1. The retained analytes on the TEC are eluted onto the HPLC column by the HPLC solvent after switching the Rheodyne injection valve to the inject position. After purging the system of excess sample and dialysate, the injection valve is returned to the load position and the TEC retaining conditions regenerated with a small volume of recipient solvent.

Communications with the HPLC unit. After the first sample has been processed the system operates with the HPLC in a constant cycle. Samples can be analysed in a sequential mode, *i.e.* one sample is treated and the analytes separated before the next sample is prepared, or in a concurrent sequential mode[14] when a sample is prepared while the previous one is being chromatographed. When derivatised analytes are unstable, samples must be analysed in a sequential mode.

Selection of process option. ASTED uses two, combined processes to effect sample clean-up. Together these create three different options, dialysis and trace enrichment (process 1), dialysis alone (process 2) and trace enrichment alone (process 3). Fig. 2 shows which option should be selected depending upon the sample complexity and analyte dilution.

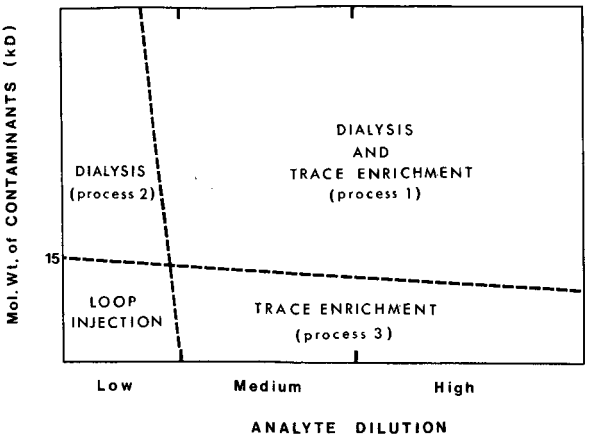

Fig. 2. The areas of application of three alternative ASTED procedures in terms of analyte dilution and molecular weight of contaminants.

Reagents

All HPLC solvents were of analytical grade, obtained from BDH, Poole, U.K. Unless otherwise stated all other chemicals were obtained from Sigma (Poole, U.K.). The water used for all reagent preparations was purified by passing through activated carbon and an ion-exchange resin (Spectrum C system, Elga, High Wycombe, U.K.). Unless otherwise stated the TEC contained 10-μm Hypersil ODS material (Shandon Southern Products, Runcorn, U.K.).

Methods

Most investigations of the ASTED system (using process 1) were carried out on dilute solutions of some anticonvulsant and xanthine drugs. The HPLC conditions for the separation of these analytes have been described previously[12]. Unless otherwise stated, all the methods used a dialyser block with a donor volume of 100 μl.

The performance of dialysis and trace enrichment

(i) The effect of varying flow-rates on dialysis rates was investigated by attaching the dialyser recipient outlet to the UV detector and aspirating recipient solvent continuously through the flow cell using a 401 dilutor (incorporating a 5-ml syringe), located after the detector. The detector response (wavelength 220 nm) was monitored for the dialysis of a static aqueous phenobarbitone solution (800 mg/l) in the donor channel.

(ii) The system was compared with and without incorporating trace enrichment. To do this the TEC was replaced by a 50-μl injection loop and the donor channel was filled with an aqueous phenytoin solution (20 mg/l). After dispensing recipient solvent continuously (1.1 ml/min) through the loop, a 50-μl volume of the dialysate was injected onto the column, at various timed intervals. The same phenytoin solution was processed, replacing the loop with a TEC, and the procedure repeated. The performance of the TEC, when operated in conjunction with dialysis, was also assessed by analysing aqueous solutions (20 mg/l) of theophylline, phenobarbitone and

phenytoin. Various recipient volumes were dispensed through the TEC by altering the flow-rate using the 401 dilutor 1 to maintain a constant 3-min dialysis time. At each flow-rate the amount of analyte loaded onto the HPLC column was measured.

Application of the ASTED system

Free analyte estimation. The ASTED system was examined for its application to the estimation of free analyte concentrations. The drugs phenobarbitone, phenytoin and phenylbutazone were used as models. Protein binding isotherms were determined by adding 15 mg/l of each drug to a range of human serum albumin (HSA) concentrations (0–35 g/l). The HSA was dissolved in sodium phosphate buffered saline (PBS). A volume of 100 μl of each solution was loaded into the donor channel of the dialyser and 2000 μl of PBS aspirated through the recipient channel in a 2-min period. Also a series of different serum samples (free of phenytoin) were obtained from patients with a variety of pathological disorders known to affect protein binding. Each sample was supplemented with 15 mg/l of phenytoin and analysed with the same ASTED conditions as those used for the protein binding isotherms. The isocratic HPLC solvent conditions for the separation of phenylbutazone used a solvent of acetonitrile–20 m*M* ammonium phosphate buffer, pH 5.0 (40:60). The phenylbutazone peak was detected at a wavelength of 263 nm.

Total analyte estimation. For total analyte estimates in serum samples containing protein binders, the effects of competing or releasing reagents on protein-bound theophylline and phenytoin were investigated. Firstly the effect of pH by the addition of varying concentrations of monochloracetic acid (MCA) to serum on the release of theophylline from protein and secondly by the addition of buffered trichloroacetic acid (TCA), pH 7.0, on the release of the anticonvulsant drug phenytoin from serum protein. Amounts of 15 mg/l of each drug were added to pooled human sera. For estimating the efficiency of a releasing agent to minimise matrix effects on the recovery of analytes, 30 different serum samples (free of phenytoin) were obtained from patients with chronic renal failure. Each sample was supplemented with 15 mg/l of phenytoin and the imprecision of the method determined with and without the addition of buffered TCA. For these investigations, 100 μl of sample–reagent mixture (4:1, v/v) was loaded into the donor channel of the dialyser and 2000 μl of a 20 m*M* ammonium phosphate buffer, pH 7.0, was aspirated through the recipient channel of the dialyser for 3 min.

Practical examples. To validate ASTED for the preparation of different types of sample materials and analytes, a range of various materials, were prepared by ASTED, using the various process options described (Fig. 2). Samples were loaded, untreated, directly onto the rack of the 231 and after sample preparation, chromatographed with the HPLC conditions of the publications cited. For ASTED process 1: UV detection of serum total glucocorticoids[13], postmortem blood warfarin[15] and a yeast extract food product for water soluble vitamins[16]. For glucocorticoids, a dialyser block with 370-μl donor channel volume was used. For ASTED process 2: fluorescence detection of amino acids using pre-column derivatisation with *o*-phthalaldehyde–2-mercaptoethanol (OPA–MCE)[17]. Sample types examined were homogenised cerebral cortex brain tissue and red cell lysates. For ASTED process 3: electrochemical detection of urinary free catecholamines[18]. The TEC was packed with hydroxyethyl methacrylate (HEMA) sulphobutyl (Anachem, Luton, U.K.).

RESULTS AND DISCUSSION

Prototype systems implementing the ASTED process have been described[14,19]. However, to automate totally the analytical system it was necessary to incorporate some form of robotics, which have the requisite flexibility[20,21] for the development of total automation applied to sample preparation and HPLC analysis. The ASTED system permits association with any type of HPLC/integrator units, and possible use for other types of analysis. ASTED is a combination of two separate processes, and, on occasion dialysis or trace enrichment alone, can be sufficient to prepare samples depending upon the sample composition. Fig. 2 shows the areas of application for the three alternatives that can be pre-selected in the system. Dialysis combined with trace enrichment has the widest application.

Dialysis

Dialysis is a separation process that depends on the differential transport of solutes of different sizes across a semi-permeable barrier separating two liquids and operates according to Fick's law of diffusion:

$$\frac{dm}{dt} = -DA\frac{dc}{dx}$$

where D = diffusion coefficient, A = membrane surface area, dm/dt = mass flow diffusion rate, and dc/dx = concentration gradient. Whilst dialysis is a highly efficient molecular filter it is inefficient in terms of solute diffusion rates. To increase the diffusion rate the dialyser was designed with a maximum ratio of membrane surface

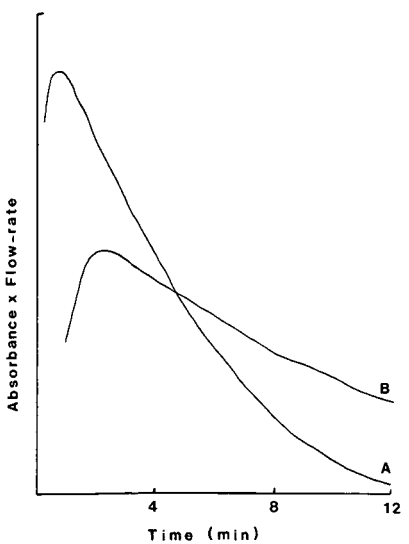

Fig. 3. Effect of flow-rate on dialysis rate. Rate of dialysis of a 800 mg/l phenobarbitone solution exhaustively dialysed at (A) 1.7 ml/min and (B) 0.3 ml/min. Absorbance × flow-rate corrects for the dilution of phenobarbitone.

area/sample volume. To minimise the volume of sample required it was held static in the donor channel of the dialyser whilst continually aspirating solvent through the recipient channel. This form of exhaustive dialysis serves to produce a high solute concentration gradient across the membrane at all times, increasing the rate of diffusion. This is shown in Fig. 3, where a greater diffusion rate for the analyte, phenobarbitone, is obtained at the higher recipient flow-rate. The amount of analyte passing into the recipient decreases exponentially with the volume of recipient passed through the dialyser (Fig. 3). This is due to the falling concentration of analyte in the donor channel of the dialyser resulting in a decrease in the dialysis rate in accordance with Fick's law. For this reason, complete removal of the analyte from the donor channel will require an infinite volume of recipient solvent to be aspirated through the dialyser. The curves in Fig. 3 have been corrected for the dilutions incurred at the two different flow-rates and approximately 68% of the analyte in the donor channel was recovered in 12 min at a recipient flow-rate of 1.7 ml/min. The amount of analyte removed from the sample in the donor channel per unit time will vary with the flow rate of the recipient solvent. The proportion of the analyte transfered into the recipient solvent during a fixed time period is independent of the solute concentration in the donor channel and the time required to obtain a fixed proportion of the analyte transfered is inversely related to the flow-rate of recipient solvent aspirated through the dialyser. Quantitative diffusion rates were always obtained due to the precise aspiration rates obtained using the 401 dilutor.

The body of the dialyser was polymethyl methacrylate so that visual monitoring of the liquid flow in the donor channel could be made. However, if it is necessary to use high concentrations of organic solvents for the donor or recipient solvents the body of the dialyser can be constructed from more solvent resistant polymers. Organic solvents do not appear to adversely affect the performance of the Cuprophan membrane. The choice of membrane with a molecular weight cut-off of 15 kD was made on the basis that ASTED was initially designed to analyse small molecules with molecular weights less than 2 kD. Membranes with higher-molecular-weight cut-off's are available that could extend the analytical range of ASTED. Dialysis, using Cuprophan, has been applied successfully for many years and problems that may be recognised, such as protein binding to the surface of the membrane, can be overcome by increasing the variable purge volume available in the system and occasionally washing the donor channel with weak alkali solution. The sample–sample interaction with ASTED applications has been shown to be less than 1%[10–13].

Trace enrichment

Trace enrichment is a procedure for concentrating solutes from dilute solutions and generally cannot be used for the treatment of samples with complex matrices. Many different types of material can be used for trace enrichment which can provide a range of selectivities.

For the on-line process of ASTED, the height of the trace enrichment bed was kept relatively small so that, packing was simplified and regeneration of retaining conditions could be achieved rapidly. An important consideration was the particle size of the sorbent used in the TEC. In agreement with a previous report[22], sorbents of 10 μm (or less) in diameter were used to suppress band broadening and enable good loading characteristics when analytes are eluted from the TEC onto the HPLC column.

Rigid packing materials must be used with on-line trace enrichment techniques such as ASTED. For analytical techniques, it is unlikely that, using the TEC dimensions and material mass described, the TEC loading capacity[23], *i.e.* the maximum capacity of the stationary phase for the solute when the amount of solute retained is proportional to the mass of stationary phase, will be exceeded. In practice, it is necessary to consider only the TEC working capacity. The TEC working capacity is defined as the amount of solute retained on the TEC when the solute retention rate equals the elution rate. This state exists when the volume of mobile phase passed through the TEC is greater than the solute breakthrough volume and the concentration of solute in the mobile phase is not so high that the loading capacity is exceeded. For a constant mobile composition and mass of stationary phase, the working capacity is proportional to the solute concentration in the mobile phase. Solute breakthrough volume has been clearly defined in an earlier report and a method for its determination described[23]. Solute breakthrough volume is dependent on the nature of the mobile phase and nature and mass of the stationary phase. It is independent of solute concentration in the mobile phase and is the volume of mobile phase passed through the TEC at which elution of solute commences. For quantitative analysis the TEC can be operated at its working capacity provided that the breakthrough volume of the analyte(s) under investigation remains constant.

Dialysis and trace enrichment combined

Trace enrichment combined with dialysis enables dilute solutes in the recipient solvent to be enriched resulting in high analyte recoveries that could not be obtained using dialysis alone. This is demonstrated in Fig. 4 which compares the recovery (detector response) of phenytoin in the dialysate obtained with and without using trace

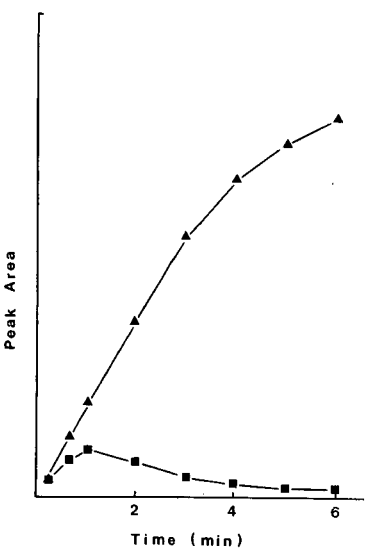

Fig. 4. Recovery of analyte from the dialysate with and without trace enrichment. The peak area (recovery) of phenobarbitone from a 20 mg/l solution exhaustively dialysed for various times and then loaded onto the HPLC using a 50-μl loop (■), and a TEC (▲). The dialysate flow-rate was 1.1 ml/min.

enrichment. When a loop was substituted for the TEC on the injection valve the dialysis kinetics observed (lower curve on Fig. 4) were identical to those observed when the analyte concentration was directly monitored in the dialysate (Fig. 3). To obtain sufficient analyte recovery for detection and quantification it is necessary to enrich the dilute solute(s) in the recipient solvent (Fig. 4). At the constant recipient flow-rate used (1.1 ml/min) approximately 58% of the analyte in the aqueous donor solution was recovered after 5 min dialysis time. The decrease in rate of analyte recovery in time, observed on the upper curve shown in Fig. 4, is a function of the exponential fall of dialysis rate of the analyte, knowing that the breakthrough volume for the analyte under investigation (phenytoin) has not been exceeded. Under these conditions it is inefficient to increase the dialysis time beyond a point where this does not produce a directly proportional increase in the amount of analyte retained on the TEC. When, due to poor assay sensitivity, it is necessary to load larger amounts of analyte onto the HPLC, this should be done by introducing more sample into the dialyser and the enrichment repeated.

Classically, trace enrichment has been used for concentrating minute quantities of hydrophobic compounds such as polynuclear aromatic hydrocarbons in water analysis[22,23]. These are compounds with very large capacity factors and breakthrough volumes. For analytical purposes ASTED must also be capable of dealing with molecules having low capacity factor, and consequently low breakthrough volumes even when the TEC bed height is relatively small. Fig. 5 shows the relationship between the volume of dialysate enriched, and the amount of solute retained by the TEC for three drugs with widely differing polarities. While maintaining a constant dialysis time,

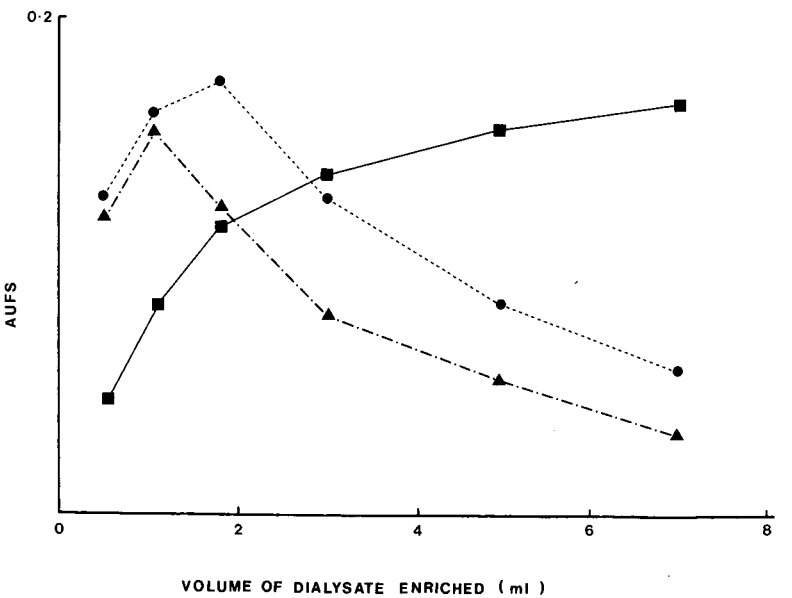

Fig. 5. Recovery of analytes on the TEC with various enrichment volumes. The peak absorbance by three drugs when different volumes of dialysates were enriched and then loaded onto the HPLC column is given. A constant 3-min dialysis time was used. Key: (▲) theophylline, (●) phenobarbitone, (■) phenytoin.

the volume of dialysate enriched was varied by altering the recipient flow rates. As dialysis of the sample in the donor channel proceeds the solute concentration falls causing a concomitant reduction in solute concentration in the recipient solvent. When the breakthrough volume is exceeded the recipient solvent, containing a now much reduced concentration of solute causes solute to be eluted off the TEC. This can be seen by comparing the traces for theophylline (having a low breakthrough volume of approximately 1 ml) and phenytoin (having a high breakthrough volume not identifiable) in Fig. 5. By measuring analyte recovery at different recipient flow-rates and constant dialysis times approximately breakthrough volumes can be established for those analytes having low capacity factor.

For quantitative analysis using ASTED it is important to operate below the breakthrough volume of the analyte under investigation, since the breakthrough volume can be affected by changes in the material bed due to ageing or compression after many regeneration cycles. The heterogeneity of the mobile phase being enriched may also have some effect on solute breakthrough volume. This is especially the case when using process 3 for samples, which, by virtue of their complexity, may contain molecules that exhibit competitions for the TEC material with the analytes under investigation. Modifications made to the recipient solvent can alter the avidity of the analyte for the sorbent in the TEC and therefore the analyte breakthrough volume. Provided that the breakthrough volume of the analyte(s) under investigation is not exceeded then the final recovery of the analyte(s) on the TEC is proportional to the time of dialysis and the volume of recipient solution passed through the TEC. These parameters are controlled by the volume and flow-rate of the recipient solvent dispensed by the 401 dilutor 1. Therefore under specified ASTED conditions where membrane permeability, sample volume, area of membrane in contact with the sample, dialysis time and volume of recipient solvent passed through the TEC are all constant, the dialysis diffusion rate is proportional to the concentration of analyte in the donor channel of the dialyser (Fick's law of diffusion), and quantitative analyte recoveries on the TEC are obtained. Thus the amount of analyte retained on the TEC and loaded onto the HPLC is directly proportional to the concentration of the analyte in the original sample. The use of high recipient flow-rates maintains high concentration gradients and enables shorter sample preparation times with increased analyte recoveries.

Dialysis combined with trace enrichment are two separate processes that complement and overcome the limitations of each other. The low-molecular-weight cut-off of the Cuprophan membrane protects the TEC from compounds that would otherwise reduce its performance. Up to 1000 samples can be handled by a single TEC. Since only molecules with masses of less than 15 kD pass onto the HPLC column the service life of the analytical column is greatly increased.

ASTED applications

Free analytes. Frequently analytes in biological liquids are present in two states: in combination (or bound) with another molecules and as the unbound (or free) compound. In the case of analytes, such as drugs, which bind to tissue and blood proteins it is often necessary to estimate the free analyte concentration since this is the physiologically active fraction[24]. Binding of a drug with a protein is usually a reversible reaction obeying the law of mass action and the affinity between a drug and

its binding site is expressed as the concentration ratio of the drug in the bound form to the product of the unbound drug and protein:

$$P + D \underset{K_2}{\overset{K_1}{\rightleftharpoons}} PD$$

with

$$\frac{[PD]}{[P] \cdot [D]} = \frac{K_1}{K_2} = K_a$$

where [P] is the free protein concentration, [D] is the free drug concentration, [PD] is the drug–protein complex concentration, K_1 and K_2 are the rate constants for the forward and reverse reactions, and K_a is the association constant expressed in mol/l. The greater the affinity between drug and protein the larger the K_a. Its reciprocal value is the dissociation constant K_d. The processes of association and dissociation of drug and protein have half-times of a few milliseconds. Many factors can affect the equilibrium *e.g.* pH, protein concentration, competition of other molecules for the binding sites and temperature. Previous methods that have been used to estimate free analyte concentrations include equilibrium dialysis[25], ultrafiltration[26] and antibody extraction[27]. In spite of the fact that equilibrium dialysis has been recognised as the definitive method, criticisms of the technique have been published[28]. Automation of such procedures at present are impracticable and long dialysis times required to achieve steady state. With the ASTED technique, in common with all procedures incorporating membrane technology, diffusion of only free drug from the donor channel into recipient solvent occurs. As this transfer proceeds, some of the drug–protein complex will dissociate to maintain the equilibrium. It has been shown that the percentage binding of highly-protein-bound drugs in human serum is fairly constant within the therapeutic range of the analytes[29]. Since the rates of diffusion are governed by Fick's law and are proportional to the diffusible solute concentration gradient across the membrane, it should be theoretically possible to estimate a measure of free drug concentrations using ASTED even when the concentration gradients are changing due to the continual movement of the recipient solvent. This would be accomplished by comparing the rates of diffusion (measured as the amount of analyte retained on the TEC) of a drug in protein solution with that obtained with a solution containing 100% free drug (*i.e.* an aqueous solution). As the diffusible free drug passes into the recipient solvent, bound drug dissociates to maintain the equilibrium. This maintains the diffusible drug concentration in the sample at a higher level than the aqueous solution, in proportion to its degree of binding. Therefore to obtain an index of free drug concentration using ASTED the dialysis time must be limited so that deviations in the initial dialysis rates of diffusible drug between the aqueous and protein solutions are negligible.

Some evidence to support these arguments are shown in the protein binding isotherms (Fig. 6a) obtained using ASTED for the three drugs, phenylbutazone, phenytoin and phenobarbitone. These drugs were chosen, as models, since their affinities for albumin differ markedly. The results in Fig. 6a are expressed as

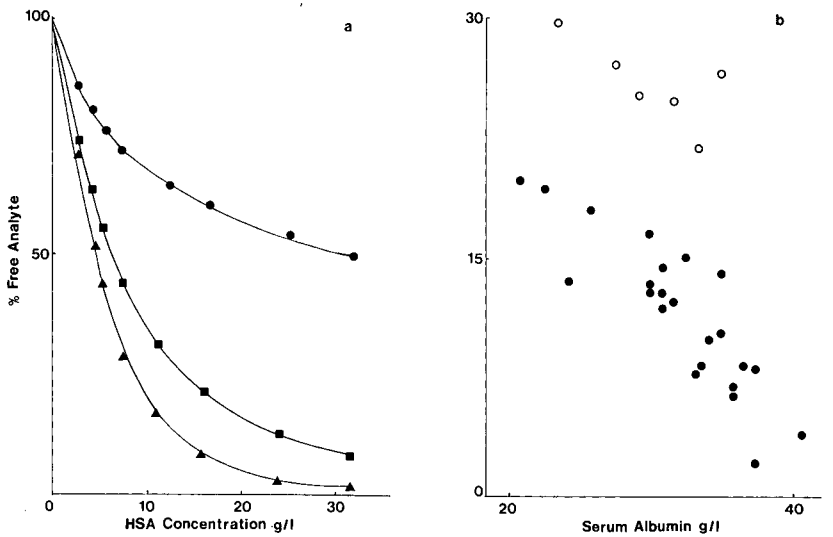

Fig. 6. (a) The binding isotherms for phenobarbitone (●), phenytoin (■), and phenylbutazone (▲) to human serum albumin (HSA). The % free analyte is the amount of solute recovered compared with that from an aqueous solution. (b) The % free phenytoin of various patients' sera each supplemented with 15 mg/l phenytoin compared with their albumin concentration. (○) Patients suffering from chronic renal failure, (●) patients suffering from diseases other than chronic renal failure.

a percentage of free drug calculated by the percentage ratio of the peak area of the drug in protein/peak area of the drug in zero protein solution. At the most concentrated albumin solution the percentage free analyte values were 97.5, 88, and 56 for phenylbutazone, phenytoin and phenobarbitone respectively. These values were in general agreement with the established equilibrium dialysis values of 99, 93, 50% for phenylbutazone, phenytoin and phenobarbitone respectively[30]. A further example using ASTED to measure an estimate of free analyte concentrations is shown in Fig. 6b. Here a range of different patient sera supplemented with the same amount of phenytoin were assayed. The concentration of free phenytoin was found to be inversely proportional to albumin concentration. However, two distinct populations were apparent, the sera from patients suffering from chronic renal failure, all having higher free phenytoin levels than other patients with comparable albumin concentrations. This is due to the disturbed protein binding that occurs in disorders of this type, which results from accumulated serum compounds competing for the protein binding sites[31]. The percentage phenytoin binding ranges observed using ASTED for renal and non-renal patients sera agrees with previous published material[29].

The accurate measurement of free analyte concentrations is recognised as a difficult problem due to the many limitations of methods used. Rate dialysis methods for estimating free analyte concentrations have been proposed[32] but suffered from analyte detection due to the dilute solute in the dialysate. The incorporation of trace enrichment in ASTED has to some extent overcome this limitation although for highly protein bound analytes where solute concentrations are extremely low detection will still be a problem. The automation and speed of analysis using ASTED for free analyte

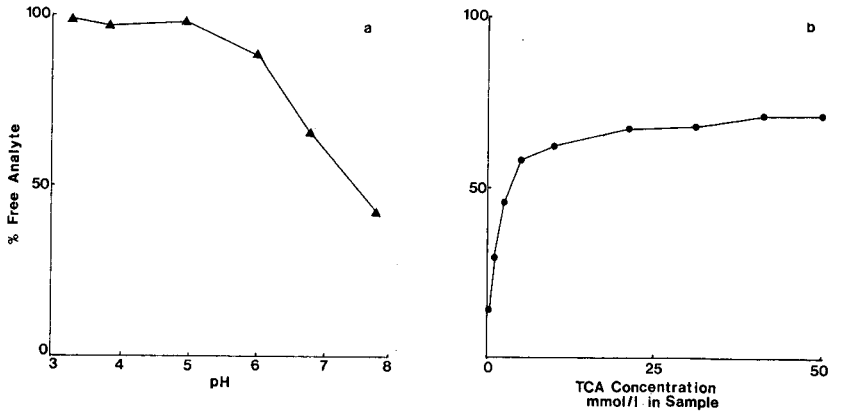

Fig. 7. (a) The effect of sample pH on the release of theophylline from serum proteins expressed as % free analyte. (b) The effect of TCA, at pH 7.0, on the release of phenytoin from serum proteins expressed as % free analyte.

examination is an obvious advantage compared with other methods using membrane separation. However it would only be realistic at this time to suggest that ASTED may be capable of measuring an estimate of free analyte concentrations and much work remains to be completed on this assay potential.

Total analytes. Sample matrix interferences are common in a wide variety of analytical techniques and ASTED is no exception. For the assay of total analytes, it is necessary to overcome any molecular binding effects. Theoretically this can be achieved in a number of ways: either by diluting the binder (*e.g.* protein) in the sample, altering the structure of the binder, adding competing reagents for the binding sites, using calibrants containing an identical binder, or by altering the chemical nature of the analyte.

If the sample dilution is high then analyte detection becomes difficult. Altering the structure of binders such as proteins can be successfully accomplished simply by changing the pH as shown with theophylline where most of the bound drug could be released from serum protein by the addition of MCA (Fig. 7a). MCA was used since a small volume of concentrated reagent generates a lowering of pH without excessive dilution of the sample or risk of precipitating serum proteins. The binding of other xanthines *e.g.* caffeine, and the glucocorticoid cortisol have also been shown to behave in a similar manner[12,13]. The competing effect of reagents for binding sites was demonstrated by the release of phenytoin from serum proteins (Fig. 7b) using buffered TCA[12]. Although phenytoin is not completely released, the competing effects of TCA are sufficient to reduce the between-sample imprecision from 25.2 to 5.1% (C.V.). Altering the chemical nature of the analyte has recently been described to eliminate protein binding of the amino acid cystine prior to its analysis[33].

The analytical performance of ASTED for a number of analytes in different biological samples has been reported previously and in accordance with Fick's law of diffusion, recovery of analyte on the TEC has been shown to vary linearly with solute concentration in the donor channel[10–13]. For total analyte estimations the analytical recovery has been shown to be approximately 100% when using protein based

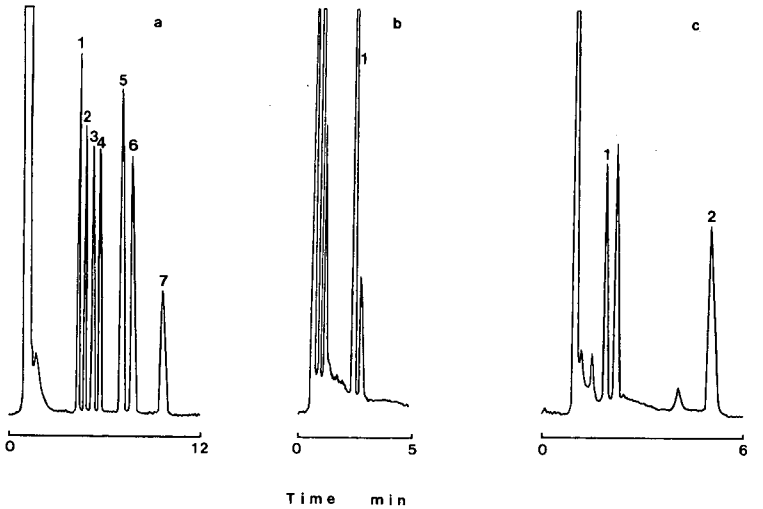

Fig. 8. Chromatograms showing: (a) Serum total glucocorticoids in human serum supplemented with glucocorticoids. Peaks: 1 = prednisone; 2 = cortisone; 3 = prednisolone; 4 = cortisol; 5 = fludrocortisone internal standard (I.S.); 6 = corticosterone; 7 = 11-deoxycortisol. (b) Grossly elevated warfarin level in a sample of *post mortem* blood. Peak 1 = warfarin. (c) Water-soluble vitamins in a food yeast product. Peaks: 1 = thiamine; 2 = riboflavin.

calibrants and compounds to minimise protein matrix effects. The absolute recovery achieved using the ASTED procedure (*i.e.* the amount of analyte recovered from an aqueous solution in the donor channel) is about 50% within a 3-min time interval[12]. Future developments in membrane technology and dialyser design could improve this analytical recovery.

ASTED advantages:

ASTED has been applied to the preparation of a range of different complex homogeneous liquid materials[10–13,34,35] (Figs. 8 and 9) using one of the three different processes. The peaks in the chromatograms represent analyte concentrations found under normal conditions of therapy and metabolism or, in the case of vitamins in the yeast product, concentrations recommended by the manufacturer. The examples used in this report to identify the potential of ASTED have been limited to analytes frequently examined in the routine clinical environment and the theoretical considerations given to these analytes should apply to other molecules of a different chemical structure. Furthermore, the efficiency of the sample clean-up described reduces cost in view of the fact that the TEC can be regenerated many times, no guard column is required and robust chromatographic separations are produced that sustain very long analytical column life times. No restrictions are imposed on the types of HPLC columns, detectors, and HPLC solvent conditions required. Moreover, high sample throughput is achieved using concurrent sequential operations of the sample preparation and HPLC analysis. In general the rate limiting stage is the chromatographic separation time. In conclusion, ASTED in association with robotics offers total automation of sample preparation and HPLC analysis, reducing labour,

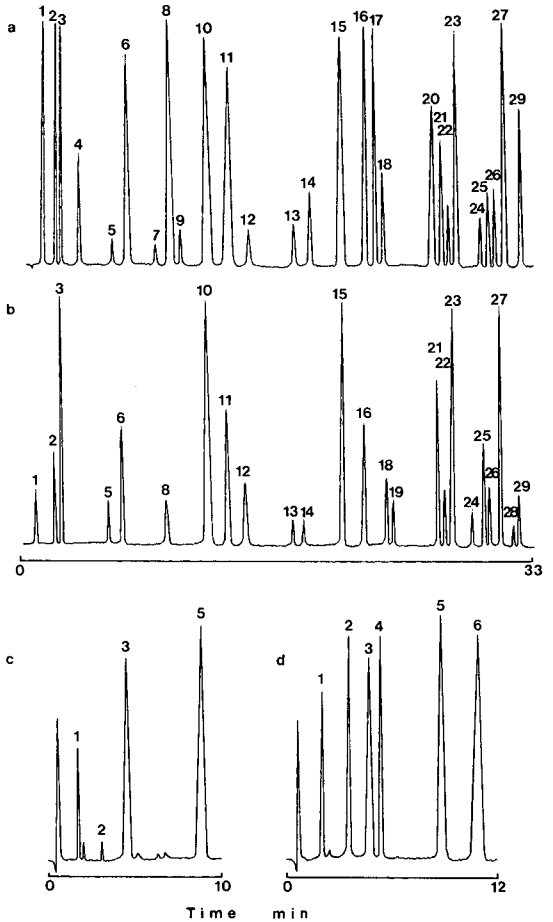

Fig. 9. Chromatograms showing: (a) Amino acids in homogenised brain tissue; (b) in red cell lysate. Peaks: 1 = aspartate; 2 = glutamate; 3 = homocysteic acid (I.S. 1); 4 = cystine; 5 = asparagine; 6 = serine; 7 = histidine; 8 = glutamine; 9 = phosphoethanolamine; 10 = homoserine (I.S. 2); 11 = glycine; 12 = threonine; 13 = citrulline; 14 = arginine; 15 = alanine; 16 = taurine; 17 = γ-aminobutyric acid; 18 = tyrosine; 19 = α-aminobutyric acid; 20 = ethanolamine; 21 = valine; 22 = methionine; 23 = norvaline (I.S. 3); 24 = tryptophan; 25 = phenylalanine; 26 = isoleucine; 27 = leucine; 28 = ornithine; 29 = lysine. (c) Catecholamines in urine from a healthy adult; (d) from a patient with a phaechromocytoma. Peaks: 1 = noradrenaline; 2 = adrenaline; 3 = dihydroxybenzylamine (I.S.); 4 = normetadrenaline; 5 = dopamine; 6 = metadrenaline.

consummable costs and capitalising on equipment investment in a simple but flexible manner.

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IMPROVEMENT OF SELECTIVITY AND SENSITIVITY BY COLUMN SWITCHING IN THE DETERMINATION OF GLYCYRRHIZIN AND GLYCYRRHETIC ACID IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive method is described for the simultaneous determination of glycyrrhizin and glycyrrhetic acid in human plasma. Quantitation is by high-performance liquid chromatography using ion-pair chromatography on a reversed-phase column. Variable-wavelength ultraviolet detection is employed. For the extraction of both compounds from plasma, a new solid-phase ion-pair extraction procedure using octadecylsilane columns was developed. Because of the strong forces involved in the protein binding of glycyrrhizin, quantitative extraction of this compound from plasma was possible only after an alkaline pH shift. A considerable improvement in selectivity and sensitivity was obtained by automated column switching involving on-line preseparation of the solid-phase extract on a short precolumn and chromatographic analysis of a heart-cut from the precolumn eluate. The limit of detection of both glycyrrhizin and glycyrrhetic acid was 0.1 mg/l in 0.5 ml of plasma. From a preliminary study in human volunteers, it was concluded that glycyrrhetic acid rather than glycyrrhizin is preferred in a study in human volunteers to assess the zero effect level of glycyrrhizin.

INTRODUCTION

Glycyrrhizin, the glycoside of glycyrrhetic acid, is a natural compound with an intensely sweet taste. It is extracted from the roots of *Glycyrrhiza glabra* L. which contains 6–14% of glycyrrhizin. In addition to its use as a sweetening agent in many food and luxury products such as liquorice, chocolates, beer, liquor and chewing

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tobacco, glycyrrhizin has been used as a medicine in the treatment of ulcus ventriculi. However, serious side effects including headache, oedema, body weight increase and serious disturbances of body-electrolyte balances related to glycyrrhizin ingestion were observed either after daily consumption of amounts as low as 40 g of liquorice or in clinical use. Therefore, in our Institute a study was started in human volunteers in order to assess the zero effect level of glycyrrhizin. In parallel with this study, the human pharmacokinetics was studied of glycyrrhizin and glycyrrhetic acid, which is the main metabolite of glycyrrhizin and which is rapidly formed from the parent drug.

For this purpose a method was developed for the simultaneous determination of glycyrrhizin and glycyrrhetic acid in human plasma, which was suitable for application to the large series of samples which are commonly involved in pharmacokinetic studies. Few methods have been published on the determination of glycyrrhizin and glycyrrhetic acid in plasma. A very sensitive method involves gas chromatography–mass spectrometry with selected-ion monitoring after formation of the corresponding methyl and trimethylsilyl esters of the two compounds[1]. An insensitive high-performance liquid chromatographic (HPLC) method was reported by which the two compounds were determined during 1–36 h in plasma of rats to which 500 mg glycyrrhizin/kg body weight had orally been administered[2]. Pretreatment of the samples was by protein precipitation with ammonium hydroxide–ethanol. Separation was by reversed-phase chromatography and gradient elution. An HPLC method using anion-exchange chromatography with gradient elution after protein precipitation with methanol was reported which was sensitive enough to determine the concentrations of glycyrrhizin and glycyrrhetic acid in plasma during 10 to 240 min after oral administration of 500 mg glycyrrhizin/kg body weight[3]. A second HPLC method was reported by this group using adsorption chromatography after protein precipitation of plasma or tissue homogenates which could determine glycyrrhizin and glycyrrhetic acid in body fluid and tissues of rats during 1–3 h after oral administration of 100 mg glycyrrhizin/kg body weight[4]. In these three HPLC methods, UV detection at 245–254 nm was employed. None of the HPLC methods was sensitive enough for application in our study in which glycyrrhizin was to be administered in oral doses of up to 800 mg glycyrrhizin daily (10–15 mg/kg body weight). Therefore, a new method was developed with special emphasis on the sample pretreatment.

For the extraction of both compounds from plasma an ion-pair solid-phase extraction procedure was developed using octadecylsilane columns. Quantitation was performed by HPLC with UV detection at 258 nm using ion-pair chromatography on a reversed-phase column. Automated column switching was applied to analyze only a heart-cut from the eluate obtained from a short precolumn on which the solid-phase extract was preseparated.

MATERIALS AND METHODS*

Apparatus

The HPLC analyses were performed by a fully automated system comprised of

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* Reference to a company and/or product is for purposes of information and identification only and does not imply approval or recommendation of the company and/or the product by the National Institute of Public Health and Environmental Protection to the exclusion of others which may also be suitable.

a simple solvent delivery system (Pump 1, Model 9208; Kipp Analytica, Emmen, The Netherlands), from which the damping units were removed to decrease the dead volume for faster solvent switching, and a high-quality solvent delivery system (Pump 2, Spectroflow SF 400; Kratos Analytical Instruments, Ramsey, NJ, U.S.A.) in combination with a sample processor (WISP 710B; Waters Associates, Milford, MA, U.S.A.) and a column thermostat (Model SpH 99; Spark Holland, Emmen, The Netherlands). Solvent-stream switching was controlled and carried out by a time-programmable multiport stream-switch unit including two high-pressure two-position six-port valves and one low-pressure six-position solvent selection valve (MUST-IET, Spark Holland). Detection was performed with a variable-wavelength UV detector (Spectroflow 773, Kratos Analytical Instruments) operated at 258 nm. The detector signal was recorded and integrated via an external-input board by the integrator part of a dual-channel gas chromatograph (Model 5880 A; Hewlett-Packard, Waldbronn, F.R.G.). The settings of the detector and integrator corresponded to a sensitivity of 0.01 a.u.f.s. Both the switching unit and integrator were controlled by the autosampler via its external contact closure junctions.

A 15 mm × 3.2 mm I.D. precolumn was used, packed with 7-μm octadecylsilane (NewGuard RP-18; Brownlee Labs., Santa Clara, CA, U.S.A.). A 150 mm × 4.6 mm I.D. analytical column was used which was slurry-packed in our laboratory with 5-μm Hypersil ODS (Shandon Southern, Runcorn, U.K.). Two 75 mm × 2.1 mm I.D. columns packed with 40-μm octadecylsilane (Chrompack International, Middelburg, The Netherlands) were used respectively as an HPLC-solvent saturation column and as a guard column for the analytical column. All columns were mounted in the column oven, the temperature of which was maintained at 50°C.

The HPLC eluent (mobile phase) was a mixture of methanol and 0.02 *M* phosphate buffer pH 7.2 (0.77 g of sodium dihydrogenphosphate monohydrate and 5.16 g of disodium hydrogenphosphate dodecahydrate per l of water) (80:20, v/v). Cetrimonium bromide was added to the eluent in a concentration of 0.0033 *M*. The mobile-phase flow-rate was 1.0 ml/min. Solvent 1, which was used to load the precolumn with the sample, consisted of methanol, water and 0.02 *M* phosphate buffer pH 7.2 (55:20:25, v/v/v) with 0.0033 *M* cetrimonium bromide. Solvent 2, which was used to clean the precolumn, consisted of methanol and 0.02 *M* phosphate buffer pH 7.2 (90:10, v/v) with 0.0033 *M* cetrimonium bromide. The flow-rate of either solvent was 1 ml/min.

The solid-phase extractions were performed using 1-ml reversed-phase octadecylsilane (ODS) extraction columns (J. T. Baker Chemicals, Deventer; The Netherlands) fitted to a Baker-10 SPE extraction manifold.

Reagents and reference solutions

Reagents used were methanol (Lichrosolv), ethanol, sodium dihydrogenphosphate monohydrate, disodium hydrogenphosphate dodecahydrate, anhydrous sodium carbonate, sodium hydroxide and cetrimonium bromide (all of analytical grade; E. Merck, Darmstadt, F.R.G.). Water was distilled in glass from alkaline permanganate solution. Glycyrrhizin (glycyrrhizic acid ammonium salt) and glycyrrhetic acid (18-β-glycyrrhetic acid) were obtained from Fluka (Buchs, Switzerland). Stock solutions of glycyrrhizin (1000 mg/l) and glycyrrhetic acid (1000 mg/l) were prepared in glass test-tubes in ethanol. These solutions were stable for several months

at +4°C. From these stock solutions, working solutions of 5 mg/l of both compounds were freshly prepared monthly in polypropylene test-tubes in methanol–water (1:1, v/v). Aliquots of these working solutions were used to prepare calibration standards.

All pipetting and transfer of samples and solutions was with variable pipettes using disposable polypropylene tips (Gilson France, Villiers-le-Bel, France).

Sample pretreatment

An aliquot of 0.5 ml of plasma was transferred to a 1.5-ml polypropylene reaction vial (Eppendorf, Hamburg, F.R.G.). To the sample, 350 μl pf 0.028 *M* cetrimonium bromide in 0.15 *M* sodium hydroxide were added. The mixture was vigorously (2500 rpm) vortexed for 1 min. After 15 min of equilibration, the mixture was vortexed again for 1 min. The total sample was processed as described in solid-phase extraction.

Solid-phase extraction

An ODS extraction column was fitted to the Baker-10 SPE extraction manifold. Preceding the extraction, the column was pretreated consecutively twice with 1 ml of methanol, once with 1 ml of water, twice with 1 ml of 0.0055 *M* cetrimonium bromide in methanol and once with 1 ml of 0.0055 *M* cetrimonium bromide in 0.005 *M* sodium hydroxide. In order to obtain a high and reproducible extraction recovery, all fluids should pass slowly through the column and intrusion of air in the column, *e.g.*, caused by vacuum suction, was strictly prevented.

The pretreated sample was placed on top of the SPE column. The eluate was discarded. Water- and methanol-soluble plasma components were removed by washing the column consecutively with 1 ml of 0.0055 *M* cetrimonium bromide in 0.1 *M* sodium carbonate (with a final pH of 10.9) and 1 ml of water. The major part of the residual water was removed by passing 50 μl of methanol through the column. The drugs were eluted with 500 μl of methanol into a 1.5-ml polypropylene reaction vial (Eppendorf) which was placed under the column. The methanol content in the eluate was evaporated under a gentle stream of nitrogen at 40°C until a final sample volume of about 50–100 μl. The final sample volume was determined by weighing of the reaction vial. An aliquot of 200 μl methanol–water (1:1, v/v) was added to the residue and the mixture was vortexed for 30 s, placed in an ultrasonic bath for 5 min and vortexed for 10 s. The final extract was transferred to a 0.4-ml polypropylene micro insert (reaction vial for the Beckman Ultra Micro System of which the cap and the upper 6 mm was cut off) for the WISP sample vial. An aliquot of 100 μl was taken for HPLC analysis.

Oral administration of glycyrrhizin to human volunteers

The concentrations of glycyrrhizin and glycyrrhetic acid were determined in plasma samples obtained from a selection of five healthy volunteers to which glycyrrhizin was administered in daily oral doses of either 400 or 800 mg. Blood samples were collected at 0.5, 1, 2, 4, 8 and 24 h after the first administration and five times further during the next 4 weeks within which glycyrrhizin was daily administered. Samples were collected just before ingestion of the respective daily dose. In addition, blank blood samples were collected from each volunteer before the first glycyrrhizin administration. The blood samples were collected in heparinized tubes, centrifuged and the plasma separated and stored at −25°C until analysis.

RESULTS

Figs. 1–3 show the positions of the two high-pressure two-position six-port valves A and B and the low-pressure six-position solvent selection valve. The thick lines indicate the respective solvent stream which is relevant during a particular step for the transport of the analytes through the chromatographic system. The thin lines indicate the other solvent streams which are present. The solvent streams during the analytical process are determined by the positions of solvent switch A and the solvent selection valve.

Fig. 1 (step 1) shows the preconditioning of the precolumn during 3 min with solvent 1, a sample-loading solvent of low elution strength, and the loading of the sample onto the precolumn with this solvent during 4 min (time of injection is time 0). Fig. 2 (step 2) shows the transport of a heart-cut from the precolumn to the analytical column. This is effected by passing the HPLC eluent over the precolumn. After 5.5 min from the time of injection, the total amounts of glycyrrhizin and glycyrrhetic acid have passed quantitatively to the analytical column. Fig. 3 (step 3) shows the switch settings during separation of the two compounds from the still present plasma components on the analytical column during 5.5 to 12 min after injection. During this time the precolumn is flushed with solvent 2 of high elution strength.

Switch B is used for a regular check of the performance of the precolumn with respect to the retention of glycyrrhizin and glycyrrhetic acid and to its efficiency. If, in step 1, switch B is in the second position, the eluate of the precolumn is distributed to the detector inlet. In this way the elution profile of the two compounds over the precolumn only can be obtained. Fig. 4 shows a chromatogram of glycyrrhizin and glycyrrhetic acid obtained with these switch positions. The two compounds are not separated on the precolumn, which is convenient for effecting a sharp heart-cut from

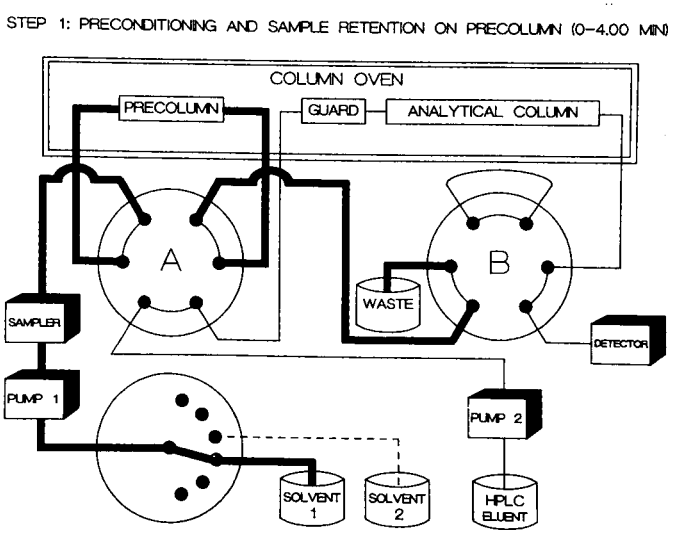

Fig. 1. Column switching scheme for the first step including column preconditioning and loading of the precolumn with the sample. The thick lines show the solvent stream which is relevant to the transport of the analytes through the chromatographic system.

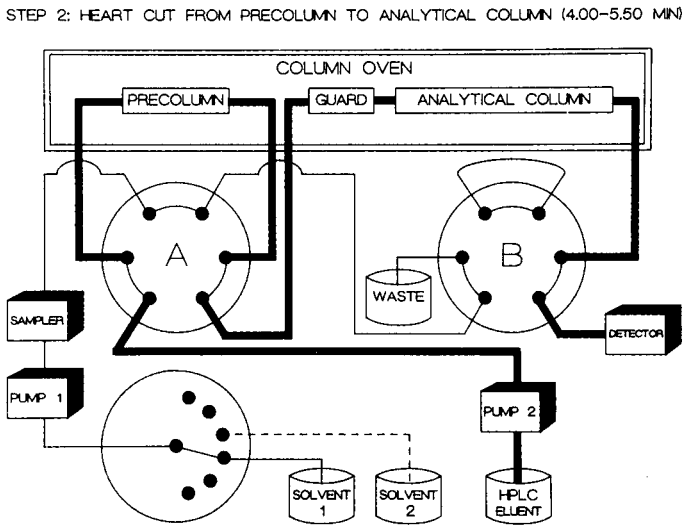

Fig. 2. Column switching scheme for the second step including transport of a heart-cut of the precolumn eluate to the analytical column. The thick lines show the solvent stream which is relevant to the transport of the analytes through the chromatographic system.

the precolumn eluate. The elution of the two compounds from the precolumn starts at about 4.5 min. On the basis of this elution profile, the solvent switch A is switched after 4.00 min.

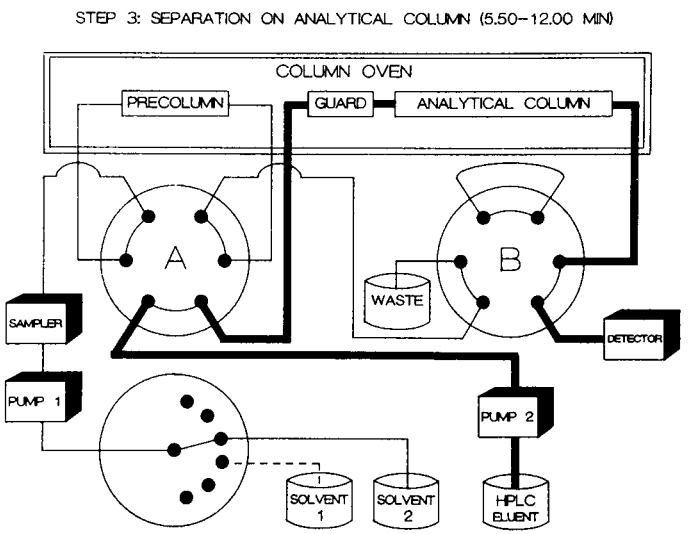

Fig. 3. Column switching scheme for the third step including separation of the analytes on the analytical column and simultaneous flushing of the precolumn. The thick lines show the solvent stream which is relevant for the transport of the analytes through the chromatographic system.

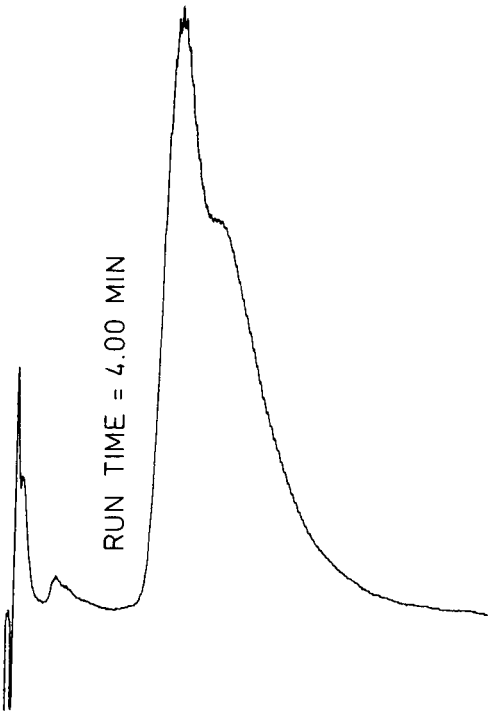

Fig. 4. Elution profile of glycyrrhizin and glycyrrhetic acid from the precolumn. Positions of switch A and solvent selection switch as in Fig. 1. Switch B is in the second position connecting the precolumn eluate to the detector inlet.

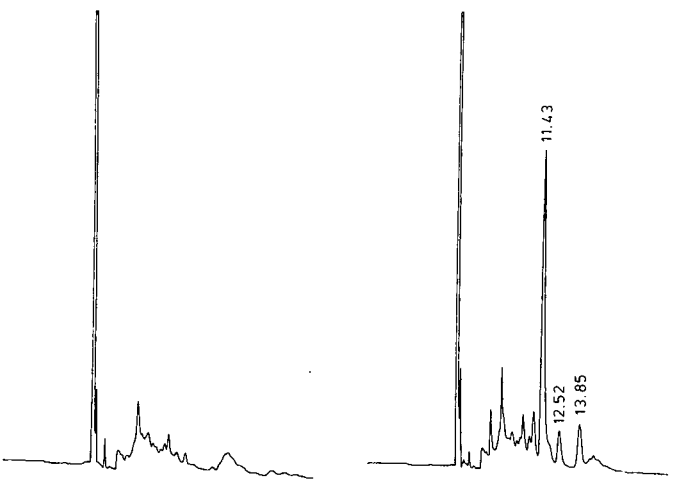

Fig. 5. Chromatogram of an extract of 0.5 ml of blank human plasma (left) and of an extract of 0.5 ml plasma from a volunteer to whom 800 mg of glycyrrhizin (corresponding to 100 g of liquorice) were orally administered (right). Retention times: glycyrrhizin, 9.95 min; glycyrrhetic acid; 11.43 min; the peaks at 12.52 min and 13.85 min are probably due to other (unidentified) glycyrrhizin metabolites.

The elution profiles of glycyrrhizin and glycyrrhetic acid from the used octadecylsilane extraction column were determined by passing aqueous reference solutions through the column. Subsequently, the compounds were eluted in consecutive fractions of 200 μl methanol and the concentrations were determined in each fraction. Based on these elution profiles it was assessed that the washing and elution volumes described provided a maximum elution of the compounds. By comparing the recovered amounts with directly injected reference solutions, the recoveries were calculated as 97% for glycyrrhizin and 89% for glycyrrhetic acid.

The extraction recoveries from plasma were determined from extracts ($n = 3$) of blank human plasma to which glycyrrhizin and glycyrrhetic acid had been added. The samples were carried through the extraction procedure and the extraction recoveries from plasma were determined by comparing the peak areas from the chromatograms obtained with those obtained after direct injection of the respective reference solutions in corresponding concentrations. The extraction recoveries ($\pm$ standard deviations) were 90.0 $\pm$ 6.1% for glycyrrhizin and 85.8 $\pm$ 9.7% for glycyrrhetic acid. The analytical pocedure was calibrated by construction of calibration lines based on series of calibration standards in human plasma, covering relevant concentration ranges.

The lowest detectable concentration of glycyrrhizin or glycyrrhetic acid was 100 μg/l.

Fig. 5 shows typical chromatograms of an extract of blank human plasma (left) and of a plasma extract from a volunteer on a daily oral dose of 800 mg of glycyrrhizin which corresponds to 100 g of liquorice (right). Glycyrrhizin and glycyrrhetic acid were eluted after 9.95 and 11.43 min, respectively. Only glycyrrhetic acid was detected in this plasma sample in a concentration of 1.5 mg/l.

DISCUSSION

The aim of the present study was the development of a method for the determination of glycyrrhizin and its main metabolite glycyrrhetic acid in human plasma. The method had to be applied in a preliminary study in human volunteers with the following objectives. The first objective was the assessment of a glycyrrhizin dose range for a subsequent extended study in human volunteers in which the zero-effect level of glycyrrhizin had to be assessed. The second objective was to obtain insight into the expected biological effects related to glycyrrhizin ingestion, to detect possible effects of gender and to obtain insight into the biological parameters relevant to an adequate registration and interpretation of these effects. The third objective was to obtain insight into the organization and administration of the main study. The final objective was to obtain insight into the pharmacokinetics of glycyrrhizin after single and repeated oral administration and to assess a parameter adequate for monitoring single and repeated glycyrrhizin ingestion. Because of its preliminary character, the study was restricted to eight male and eight female healthy human volunteers. The present report is restricted to the final objective.

Problems encountered during the development of the analytical procedure were in particular related to glycyrrhizin: its strong binding to plasma proteins, its acidic character and its instability above room temperature and at low pH values. These properties of glycyrrhizin excluded the development of a straightforward extraction

procedure including ion suppression at low pH and liquid–liquid or solid-phase extraction of the neutral compounds. The protein binding of glycyrrhizin could be cleaved only at high pH values. Since both glycyrrhizin and glycyrrhetic acid are ionized at this pH, extraction was carried out after formation of ion pairs of the compounds with cetrimonium bromide, a quaternary ammonium compound. Based on our experience with solid-phase extraction procedures[5–8], an ion-pair solid-phase extraction method was developed.

Because of the acidic character of both compounds, gas chromatographic determination was possible only after complex derivatization procedures[1]. For this reason and because of the thermal instability of glycyrrhizin, HPLC was chosen as a quantitation technique. A problem to be solved in the development of an HPLC method was the large difference in polarity between glycyrrhizin and glycyrrhetic acid. Consequently, simultaneous determination of these compounds was possible only by using ion-pair chromatography on a reversed-phase (ODS, octadecylsilane) column using cetrimonium bromide. Sensitive and selective determination of the two compounds by using selective detection techniques was impossible since the two compounds were not sensitive to fluorescence or electrochemical detection and only weakly sensitive to UV detection (absorption maximum at 248 nm).

The method developed in this way included an ODS solid-phase ion-pair extraction procedure and ion-pair chromatography on an ODS column. In both procedures, cetrimonium bromide was used as an ion-pairing agent. UV detection was employed at 258 nm. However, because of a lack of selectivity, both for glycyrrhizin and for glycyrrhetic acid, the procedure was not sensitive enough to detect the compounds in plasma from healthy volunteers after oral ingestion of relevant doses (400 or 800 mg daily) of glycyrrhizin. A considerable improvement in selectivity was obtained by implementation of a stream-switch unit which permitted preseparation of the ion-pair solid-phase extracts on a short (15 mm × 3.2 mm) precolumn filled with 7-μm ODS particles. In contradistinction with the precolumns used in the majority of other published methods, the precolumn we used had an efficiency of 300–750 theoretical plates, which enabled the transport of a sharp "heart-cut", including only the relevant part of the precolumn eluate, to the analytical column. The resulting chromatograms showed a significant clean-up of the extracts resulting in a significant improvement in the selectivity and sensitivity of the procedure. The corresponding reduction in the limit of detection (about 100 μg/l for either compound) permitted the quantitation of glycyrrhizin and glycyrrhetic acid in this study.

A problem was posed by the instability of the precolumn. Although extracts rather than plasma samples were injected, the performance of the precolumn rapidly decreased as evidenced by a rapid decrease in the retention of glycyrrhetic acid and glycyrrhizin after a few sample injections. A total number of about 10–15 samples could be injected on the precolumn before an irreversible loss of retention necessitated its replacement. Obviously, the alkaline chromatographic conditions dictated by the simultaneous analysis of glycyrrhizin and glycyrrhetic acid caused the rapid precolumn deterioration, although the performance of the analytical column was hardly affected by similar chromatographic conditions. In order to check regularly the performance of the precolumn, after every five injections the elution profile of glycyrrhizin and glycyrrhetic acid was recorded. Since the performance of the precolumn is paramount with respect to the overall analytical recovery, the

column-switching configuration with two high-pressure switching valves described, which permits a convenient performance check, is recommended.

Fig. 5 shows a typical chromatogram of a sample extract from a volunteer given an oral dose of 800 mg glycyrrhizin. A similar concentration–time course was observed for glycyrrhizin and glycyrrhetic acid in both 400-mg and 800-mg dose groups: up to 4 h, neither glycyrrhizin nor glycyrrhetic acid was detectable in the plasma samples from either dose group. From 8 h after the first administration, glycyrrhetic acid was detected in all plasma samples (> 100 μg/l). In the next weeks of glycyrrhizin administration a steady state concentration of glycyrrhetic acid in plasma was reached in each volunteer, of 0.5–1.7 mg/l. After terminating the oral glycyrrhizin administration, glycyrrhetic acid was still detectable in plasma during 2–4 days. Glycyrrhizin was not detected (< 100 μg/l) in any of these plasma samples. This is in accordance with results of Sakiya *et al.*[3] who reported, after very high oral doses to rats (500 mg/kg glycyrrhizin), high glycyrrhetic acid concentrations in plasma up to 30 mg/l and only minor concentrations of glycyrrhizin. After bolus administration of glycyrrhizin into the portal vein, glycyrrhetic acid was not detected in plasma. On the basis of these results, they concluded that glycyrrhizin is not metabolized in the body to glycyrrhetic acid, but in the gastro-intestinal tract is converted into glycyrrhetic acid and predominantly absorbed as such.

In addition to the appearance of a glycyrrhetic acid peak at 11.43 min in the chromatograms, two other peaks were recorded at 12.52 and 13.85 min which were absent from the chromatograms of blank plasma from these volunteers and which were related in height to glycyrrhetic acid. Although these peaks were not further identified, it is suggested that they are due to additional metabolites of glycyrrhizin.

Based on our results it can be concluded that glycyrrhetic acid rather than glycyrrhizin is preferred for studies in human volunteers to assess the zero effect level of glycyrrhizin. Beside its potential use in monitoring concentration-related effects, determination of the glycyrrhetic acid concentration in plasma can be used to detect recent (up to 4 days) liquorice (or other glycyrrhizin-containing products) ingestion. Therefore, further optimization of the analytical procedure, especially with respect to chromatographic conditions which are less deleterious for the precolumn, will be directed primarily to the assay of glycyrrhetic acid in plasma.

ACKNOWLEDGEMENTS

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DETERMINATION OF Δ^9-TETRAHYDROCANNABINOL IN PLASMA USING SOLID-PHASE EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A method for the determination of nanogram amounts of Δ^9-tetrahydrocannabinol (THC) in plasma and serum is described. THC was quantitatively isolated by solid-phase extraction after addition of an aqueous solution of urea and methanol to the sample. The extracts were analysed by high-performance liquid chromatography with electrochemical detection in the oxidizing mode. The detection limit of THC is *ca.* 100 pg for a signal-to-noise ratio of 3. With this method, levels of 2 ng/ml of THC in plasma can be measured.

INTRODUCTION

The active constituent of *Cannabis sativa* L., Δ^9-tetrahydrocannabinol (THC) (Fig. 1), can impair the performance of complex coordinated psychomotor skills in, *e.g.*, driving a motor vehicle[1]. Consequently, in pharmacology and forensic toxicology the determination of THC in biological matrices (blood, serum or plama) has already received a great deal of attention and many methods for the determination of THC have been described[2,3].

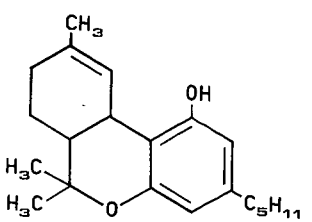

Fig. 1. Structure of Δ^9-tetrahydrocannabinol (THC).

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Very sensitive techniques for the determination of THC are required, on account of the low concentrations of the parent drug usually encountered in body fluids and tissues. Typical concentrations of THC in plasma or blood during intoxication are in the low ng/ml range. For pharmacokinetic studies and screening purposes, fast and cheap methods are preferred and in forensic cases the determination of the inactive metabolite 11-nor-9-carboxy-THC is not sufficient for determining whether a person is actually under the influence of the drug.

Major problems in the determination of THC include extraction from biological matrices with sufficient recovery and obtaining extracts without interfering substances.

Gas chromatographic (GC) methods with electron-capture detection, GC–mass spectrometric (GC–MS) procedures and radioimmunoassay (RIA) have been most frequently[2,3] applied. The GC methods usually require derivatization of the phenolic group of THC; RIA methods do not distinguish completely between THC and its metabolites and degradation products. On the other hand, high-performance liquid chromatographic (HPLC) methods with UV detection are hampered by insufficient sensitivity and selectivity, and fluorescence detection also requires the derivatization of THC[4].

We have developed a simple and sensitive assay for the determination of THC in plasma that can be used in the routine screening of plasma or serum samples and in pharmacokinetic studies. THC is isolated from plasma using solid-phase extraction followed by HPLC with electrochemical detection. The recovery of THC with the described extraction procedure is complete and highly reproducible.

EXPERIMENTAL

Materials

THC (1.00 g mixed with an unknown amount of ethanol in a glass vial) was purchased from Macor (Jerusalem, Israel). The content of the vial was transferred quantitatively into a volumetric flask and diluted to 100 ml with absolute ethanol. The resulting stock solution was stored at −18°C. Standard solutions containing 0.05–10 μg/ml of THC were prepared by diluting portions of the stock solution with methanol and were stored at −18°C until used as standards in chromatography or for spiking plasma. Drug-free citrate plasma was stored at −18°C until used.

Plasma was spiked as follows. The required amount of THC solution in methanol was mixed with the same amount of water in a volumetric flask, then the flask was filled with plasma to the calibration line, mixed and stored for at least 12 h in a refrigerator at 4°C for equilibration before use or storage at −18°C. The volume of the methanolic THC solution mixed with plasma was chosen so that the resulting spiked plasma did not contain more than 1% (v/v) of methanol.

Methanol and diethyl ether were of glass-distilled grade from Rathburn (Walkerburn, U.K.). Tetrahydrofuran (analytical-reagent grade). (Merck, Darmstadt, F.R.G.) was distilled not more than 2 weeks before use and was stored under nitrogen in a brown-glass bottle in a refrigerator. Water was purified using the Milli-Q/Organex-Q system (Millipore, Molsheim, France) and stored in glass containers. Urea was obtained from Sigma (St. Louis, MO, U.S.A.). The urea solution (8 *M* in water) was passed through a Bond-Elut C_{18} column (a 50-ml portion over

a 3-ml activated column containing 500 mg of stationary phase) before use in order to remove organic impurities.

Bond-Elut C_{18} solid-phase extraction columns (1 ml, containing 200 mg of stationary phase) were purchased from Analytichem International (Harbor City, CA, U.S.A.). To these columns a 1-ml polypropylene sample reservoir was attached. Polypropylene tubes (10 ml) were obtained from Greiner (Alphen aan den Rijn, The Netherlands).

Glass centrifuge tubes (10 ml) were silanized by allowing them to dry after rinsing with a 2% (w/v) solution of dimethyldichlorosilane in 1,1,1-trichloroethane (LKB, Bromma, Sweden) and rinsing then with methanol. The tubes were capped with polyethylene cap when in use.

Apparatus

The chromatographic system consisted of a U6K injector and a Model 510 solvent delivery system (both from Waters Assoc., Milford, MA, U.S.A.). A 300 × 4.6 mm I.D. column packed with 5-μm silica was placed between the pump and injector in order to damp pressure pulses. The analytical column was a stainless-steel (100 × 4.6 mm I.D.) Chromsep high-resolution cartridge packed with reversed-phase C_{18}-modified silica of 3 μm particle size (Chrompack, Middelburg, The Netherlands). A standard Chromsep guard column (10 × 2.1 mm I.D.) filled with C_{18}-coated 40-μm pellicular silica (Chrompack) preceded the analytical column.

Isocratic elution was employed with a mobile phase consisting of tetrahydrofuran–methanol–0.005 *M* sodium citrate buffer, pH 7.0 (7.5 : 68 : 24.5, v/v), prepared 24 h before use and degased by sonication for 10 min. Chromatography was performed at ambient temperature at a flow-rate of 1.0 ml/min.

The electrochemical detector used was described by Holthuis[5]. The working electrode was made of glassy carbon (Metrohm, Herisau, Switzerland) with a diameter of 3 mm, and a silver–silver chloride (3 *M* potassium chloride) electrode was used as a reference electrode. The auxiliary electrode was made of stainless steel. The electrochemical cell was connected to a Metrohm 641 VA potentiostat. The glassy carbon electrode was polished daily for 1 min with 0.3 μm aluminium oxide powder (Metrohm, EA 1086).

To speed up the stabilization, the working electrode was polarized in the detector cell for 20 min at +960 mV. The potential was then decreased to the working potential of +760 mV. Chromatograms were recorded with a flat bed BD-40 recorder (Kipp & Zonen, Delft, The Netherlands). Injections were made with a Hamilton (Bonaduz, Switzerland) microlitre syringe.

Sample preparation

Sample preparation was carried out with a Supelco (Supelchem, Leusden, The Netherlands) vacuum manifold. The solid-phase extraction column was fitted to a 10-ml polypropylene sample reservoir. The column was activated by rinsing it with 2 ml of methanol and 2 ml of water.

Plasma (1.00 ml) was transferred into a polypropylene tube and 2.0 ml of 8 *M* urea solution were added. After vortexing for 5 s, 2.0 ml of methanol were added and after vortexing again for 5 s the mixture was transferred into the sample reservoir of the solid-phase extraction column. The fluid passed through the column within 2–3

min. The polypropylene tube was rinsed with 2 ml of water–methanol–8 *M* urea solution (1:2:2) and this fluid was also transferred to the solid-phase column. The reservoir was removed and the column was rinsed subsequently with 2 ml of methanol–water (1:1, w/w), 1 ml 0.2 *M* hydrochloric acid, 1 ml of methanol–water (1:1, w/w), 1 ml 0.01 *M* sodium hydroxide solution and 3 ml methanol–water (1:1, w/w). The column was centrifuged for about 10 min in order to remove remaining fluid. THC was eluted with 0.5 ml of diethyl ether. The eluate was evaporated to dryness at 40°C in a 10 ml silanized tube and the residue was dissolved in 100.0 μl of methanol and vortexed for 5 s. A 10.0-μl volume was injected into the chromatographic system.

Calibration was performed by injecting extracts of plasma samples spiked with THC at concentrations ranging from 0 to 100.0 ng/ml. Peak heights were measured.

The mean extraction yield and inter- and intra-assay variability were determined by spiking 50 ml of blank plasma with THC at a concentration of 20 ng/ml and determining the THC concentration in five 1-ml portions each day on four different days. Peak heights of THC obtained after injection of 10 μl of the plasma extracts were compared with those obtained by injecting 10.0 μl of methanol containing 2.00 ng of THC.

RESULTS AND DISCUSSION

Chromatography and detection

Several stationary phases were tested for the chromatography of THC and plasma extracts, *viz.*, C_2 (5 μm) (250 × 4.6 mm I.D.), cyanopropyl (5 μm) (100 × 3.0 mm I.D.), diol(5 μm) (100 × 3.0 mm I.D.) and C_{18} (5 μm) (100 × 3.0 mm I.D.) bonded phases. However, none of these was suitable for separating THC from plasma components. Methanol as the modifier of first choice resulted in a poor peak shape of THC in some instances. When using a C_2 column this could be improved by using modifiers of lower viscosity, *e.g.*, acetone or acetonitrile. However, in these instances the detector sensitivity decreased rapidly, causing unacceptable baseline drift. With other columns we could not separate THC from plasma components adequately. In order to obtain a greater separation power we tried smaller particle sizes of the stationary phase. C_{18} column material of particle size 3 μm was selected, with which methanol as modifier gave the best detector stability and sensitivity. Tetrahydrofuran gave poor stability and sensitivity but a substantial improvement of chromatographic resolution in an equi-eluotropic concentration with respect to THC. The mobile phase that we used [THF–methanol–buffer (7.5:68:24.5)] was a compromis giving sufficient chromatographic separation and acceptable detector stability and sensitivity.

In order to find the optimal detection potential we constructed a hydrodynamic voltammogram for THC using a THC standard in methanol. Fig. 2 shows the hydrodynamic voltammogram of THC. A plateau is reached at +820 mV, indicating the optimal potential for the detection of THC. A small peak in the chromatogram of some plasma extracts (equivalent to about 2 ng/ml of THC) was not completely separated from the peak of THC. This small peak could be completely eliminated by choosing +760 mV as the detection potential, resulting in a slight loss of sensitivity.

The detection limit (signal-to-noise ratio = 3) is approximately 100 pg, as can be seen in Fig. 3a. The detector signal was linear from 0.5 to 100 ng ($r = 0.9999$), provided that the amounts were injected in the same volume of methanol (*e.g.* 10 μl). Fig.

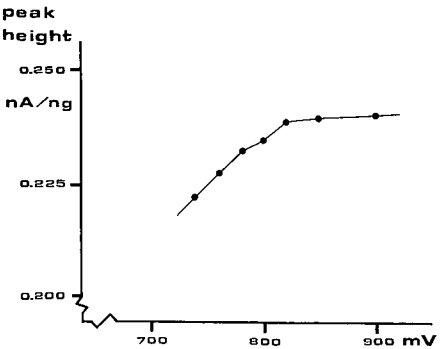

Fig 2. Hydrodynamic voltammogram obtained after repeated injection of 5 ng of THC in 10 μl of methanol.

3 shows chromatograms of 1 ng of THC, extracts of drug-free plasma, plasma spiked with 20 ng/ml of THC and an extract of the serum from a victim of involuntary ingestion of cannabis resin.

Isolation of THC from plasma

Numerous methods for extracting THC from body fluids have been publised, although few of them give exact extraction yields[6–13] (Table I). Most of them give

TABLE I
PUBLISHED EXTRACTION RECOVERIES OF THC

Ref.	*Year*	*Method**	*Sensitivity (ng/ml)*	*Matrix*	*Extraction solvent*	*Recovery (%)*
6	1977	HPLC + GC–ECD**	0.2	Plasma	Heptane–isoamyl alcohol (98.5:1.5)	90.8 ± 2.6
7	1980	TLC-MS	<0.5	Plasma	Extrelut column with diethyl ether or ethyl acetate	81.5–97
8	1981	HPLC + vis.***	50	Plasma, brain tissue	DCM§§–hexane (2:5) after addition of methanol	76.2 ± 9.2
9	1983	GC–MS	0.2	Plasma	Acetonitrile	86
10	1983	GC–MS	5	Serum, blood	Hexane	40
11	1984	TLC + fluor.§	1	Plasma	Methanol, 3% isoamyl alcohol in hexane	95–98
12	1986	GC–MS	0.8	Plasma	Heptane–isoamyl alcohol (98.5:1.5)	64.9
13	1986	GC–ECD, GC–MS	0.3	Plasma	XAD-2 resin after adding 15% (v/v) of acetonitrile	Max. 95%§§§

* ECD = electron-capture detection; TLC = thin-layer chromatography.

** Fractionation by HPLC and determination of THC by GC with ECD after derivatization.

*** Derivatization of THC to a coloured product and determination by HPLC with visible light absorbance detection.

§ Extraction followed by labelling with a fluorescent label and fluorimetry performed on the isolated TLC spot.

§§ DCM = dichloromethane.

§§§ Dependent on contact time between resin and plasma.

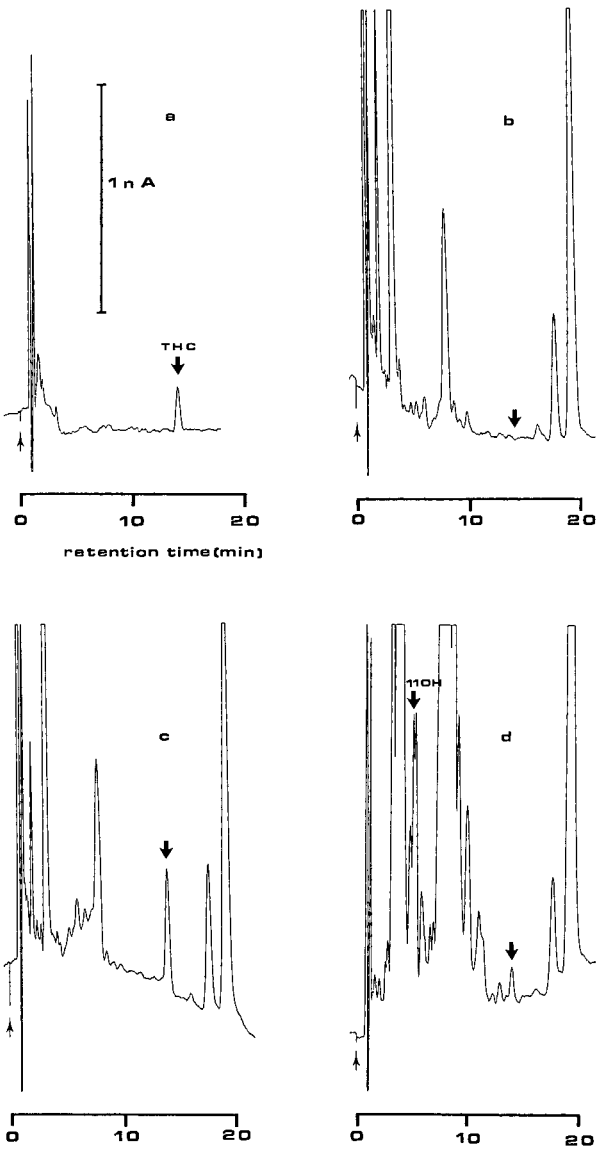

Fig. 3. (a) Chromatogram obtained after injection of 1.0 ng of THC in 10.0 μl of methanol. (b) Chromatogram of blank plasma. (c) Chromatogram of an extract of plasma spiked with 20.0 ng/ml of THC. (d) Chromatogram of an extract of serum from a victim of involutary ingestion of cannabis resin, which appeared to contain 5 ng/ml of THC. The peak marked 11-OH coincides with 11-hydroxy-THC.

incomplete extraction recoveries and poor reproducibility. When studying the solid-phase extraction behaviour of THC from plasma and from spiked aqueous phosphate buffers (0.1 *M*, pH 7.4), we found that the recovery was poor. Retention on and elution from the bonded-phase column could not be the problem, because when THC was placed directly on the packing of the activated bonded-phase column in

a very small volume of methanol (10 μl) the THC could be eluted quantitatively with 0.75 ml of methanol even after washing with 5 ml of methanol–water (1:1, v/v). The recovery from spiked buffers could be improved by adding the same volume of methanol to these aqueous solutions. In this way the solubility of the very hydrophobic THC was increased sufficiently to prevent adsorption on the walls of the vessel in which the solutions were prepared. When plasma is spiked with THC, the THC is solubilized by binding to proteins and lipoproteins. When spiked plasma was placed on a bonded-phase column the recovery was poor (about 40%). Obviously the diffusion of THC from the binding sites to the C_{18} layer through the unmodified aqueous phase has to be facilitated. However, this could not be applied to plasma samples because addition of methanol resulted in precipitation of proteins and with this significant coprecipitation of THC occurred, resulting in clogging of the bonded-phase column, low extraction yields and poor reproducibility.

Obviously protein binding of THC is still a problem after precipitation of the proteins. Therefore, we searched for means of denaturing plasma proteins without precipitation even when methanol was to be added to the solution. It is well known that urea is capable of denaturing proteins by influencing their tertiary structure. The protein molecules are deconvoluted and in this way binding sites for smaller molecules are lost, although sometimes new ones are created[14]. The denaturation by urea is believed to be based on the following two effects[15]. First, the interaction between water molecules is altered in concentrated urea solution, facilitating the dissolution of hydrophobic parts of the protein. This, of course, will also have an effect on the dissolution of other hydrophobic compounds, *e.g.*, THC. Second, urea interacts with the peptide groups of the protein, resulting in the loss of the tertiary structure.

The above mechanisms of protein denaturation might give the impression that urea alone is capable of increasing THC recoveries, but it was evident that the addition of methanol resulting in a final methanol concentration of at least 25–30% (v/v) was also necessary to give maximum extraction yields in bonded-phase extraction. This was in good agreement with the results of Rosenfeld *et al.*[13], who found that extraction of THC with XAD-2 resin was more complete and effected in a shorter time when 15% (v/v) of acetonitrile was added to the plasma sample.

Of course, after reconstitution of the dried extract with 100 μl of methanol evaporation should be avoided and measurement of the injected volume must be

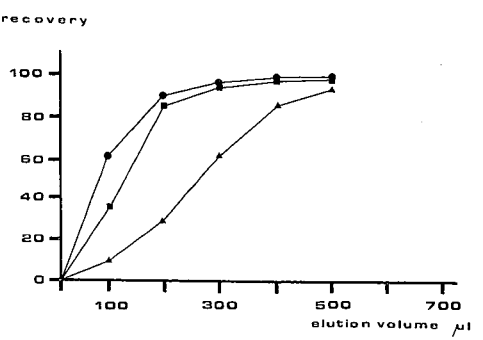

Fig. 4. Cumulative recoveries of 1 μg of THC placed on a solid-phase extraction column and eluted with 100-μl portions of different solvents: ●, diethyl ether; ■, methanol; ▲, hexane.

TABLE II
MEAN EXTRACTION RECOVERY FOR THC AT A CONCENTRATION LEVEL OF 20 ng/ml DETERMINED FIVE TIMES ON FOUR DIFFERENT DAYS

Day No.	*Recovery (%)*	*S.D. (%) (n=5)*
1	99.0	1.79
2	99.7	1.74
3	99.5	1.77
4	99.4	2.05
Mean	99.4	1.84

accurate. When stored well capped at −18°C the extracts were stable for at least 4 days.

THC was eluted from the bonded-phase column with diethyl ether, 0.5 ml of wich was enough for complete elution. The ether fraction dried in about 10 min. Using methanol a larger volume (0.75 ml) was needed and also about 40 min were required for evaporation, the chromatograms being essentially the same. Fig. 4 shows the elution profile of THC from the column with different solvents.

The calibration line measured using samples spiked with concentrations from 6.25 to 50 ng/ml was linear ($r=0.9999$, $n=8$).

To validate this relatively uncommon procedure without the use of an internal standard we determined the intra- and inter-assay variability as described. There was no significant difference between the within-day recovery and the standard deviation of these recoveries and the between-day values at the 20 ng/ml concentration level, as can be seen from Table II. Owing to the complete recovery of THC and the high reproducibility, no internal standard was necessary.

The chromatogram for plasma from a victim of cannabis resin intoxication (Fig. 3d) shows more peaks than that for spiked plasma. This could be due to other cannabinoids present in the cannabis resin and metabolites of THC. We noticed that the peak in Fig. 3d marked 11-OH coincides with the peak of the 11-hydroxy metabolite of THC.

CONCLUSIONS

The recovery of THC from plasma with bonded-phase extraction columns is complete if the transfer of THC from binding sites (proteins, lipids) to the bonded phase is facilitated. In the method described this is achieved by the addition of urea and methanol. The extraction method combined with HPLC and electrochemical detection results in a sensitive method for measuring THC levels in plasma at concentrations of pharmacological interest. Investigations on the applicability of the procedure to the determination of THC metabolites in plasma, serum and urine are in progress.

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ON-LINE SAMPLE PROCESSING AND ANALYSIS OF DIOL COMPOUNDS IN BIOLOGICAL FLUIDS

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SUMMARY

We developed a coupled dual column system with an optional post-column derivatization for on-line sample processing, trace enrichment and analysis of aromatic 1,2-diol and aliphatic *cis*-diol biomolecules (*e.g.* catecholamines, ribonucleosides). The fully automated high-performance liquid chromatography analyzer tolerates the direct injection of proteinaceous fluids by use of a unique bonded-phase precolumn material which allows the simultaneous performance of covalent affinity and size-exclusion chromatography.

INTRODUCTION

Despite its high resolution power, sensitivity, precision and practicability, high-performance liquid chromatographic (HPLC) analysis of biological samples like blood, serum, plasma, urine, milk, lymphatic fluids, liquor, faeces or tissue homogenates is restricted by the pretreatment and processing of such highly complex matrices. Often the sample preparation represents the weakest chain segment in an analytical HPLC procedure as it still involves many elaborate, manual and thus error-prone and time consuming work-up steps. Thus, the goal in bioanalytical sample processing should be a rapid and if possible an automated, HPLC integrated procedure which exhibits a high selectivity for the analyte and which tolerates the direct injection of a biological sample. In many cases, analytes are present in too small amounts and biological samples are too complex or incompatible with conventional HPLC phase systems to permit an analysis by direct injection into an analytical column. Thus, simplification of such multicomponent mixtures as well as analyte enrichment is needed prior to analysis. In general, this is achieved by prefractionation or class separation and preconcentration steps. For this purpose the classical liquid–liquid extraction methods are increasingly substituted by liquid–solid extrac-

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tion procedures, which use silica- or polymer-bonded phase materials packed in disposable cartridges or small, conventional columns. This successfully applied strategy allows sample processing in a manual or semi-automatic off-line mode under low pressure conditions or in an on-line mode under HPLC conditions. The application of electrically or pneumatically driven column switching valves even leads to fully automated on-line sample processing with subsequent analysis. The selectivity for a given analyte can be further enhanced by combining the pre-column technology with a post-column reaction system. Such a total system approach was demanded by Frei[1] and has recently been reviewed by Westerlund[2] for the direct injection of biological fluids, like plasma.

The most serious problem, however, encountered in the development of such bioanalytical HPLC systems is the quantitative removal of the protein matrix, as residual proteins limit the lifetime of the precolumn or deteriorate the efficiency of the analytical column.

Size-exclusion chromatography (SEC) is a very mild, effective and simple method for the quantitative removal of proteins, and unwanted sample constituents usually do not contaminate such columns even when used repetitively.

Thus, our strategy for on-line sample processing of proteinaceous fluids is the use of polymer-based size-exclusion materials as stationary supports. By additional chemical modification of these precolumn materials, we try to introduce the selectivity needed for extraction and trace enrichment of the analytes of interest.

The selectivity of a given material is determined by the different affinities of the sorbent with respect to the compound of interest and the residual matrix constituents. This is expressed by the different distribution coefficients or capacity factors, k', of the analyte *versus* a chemically bonded phase and a mobile phase. Dependent on the relative interaction energies, in the corresponding phase, the analyte will be retained at the stationary phase of the precolumn or move with the mobile phase. Taking into account the relative energies of the different interactions, *e.g.*, ionic, hydrogen bonding, dipole–dipole, Van der Waals, the ideal interaction with the bonded phase would be a covalent one. A prerequisite for such an ideal sample processing, however, would be only a temporary covalent fixation. This means that a total retention ($k' > 10^3$) of the analyte is followed by a total elution ($k' < 10^{-3}$). This "all-or-nothing" or "on-off" principle for extractive processing represents a special kind of affinity chromatography and can be described as covalent or "digital" chromatography.

In this paper two examples are given for on-line sample processing, trace enrichment and analysis of aromatic 1,2-diol and aliphatic *cis*-diol biomolecules (catecholamines, ribonucleosides) by a fully automated, commercially available HPLC system.

EXPERIMENTAL

Detailed conditions have been described for catecholamine analysis[3] and for ribonucleoside analysis[4–6].

Instrumentation

The HPLC analyzer comprises modular units from E. Merck (Darmstadt, F.R.G.) (Fig. 1): an LC controller Model L 5000 (LC-C); two pumps Model 655A-12

(P_1, P_2); an autosampler Model 655A-40 (AS); an automatic valve switching system Model ELV 7000 (AVS); a fluorescence detector Model F-1100 (FD) for catecholamine analysis; an UV detector Model 655A-22 (UV) for ribonucleoside analysis; an integrator Model D 2000 (I); a precolumn (PC) and an analytical column (AC). For the optional use of post-column derivatization of the catecholamines norepinephrine and epinephrine to the corresponding trihydroxyindole derivatives, a reaction system Model 655A-13 (RS) was integrated.

Sampling

For catecholamine analysis, native human urine was acidified to pH 3.0–3.5 with 10 *M* hydrochloric acid and frozen at −20°C. 24-h Urine was collected in a receiver filled with 20 ml hydrochloric acid (25%), adjusted to pH 3.0–3.5 with 10 *M* sodium hydroxide and frozen at −20°C. The frozen urine samples were slowly thawed, centrifuged for 5 min in an Eppendorf desk centrifuge and 1 ml of the supernatant was transferred to an autosampler vial.

For plasma analysis of norepinephrine and epinephrine, 20 µl of a preservative solution (0.16 *M* EGTA, 0.3 *M* reduced glutathione, adjusted to pH 7.0 with sodium hydroxide), and for serum analysis 10 µl of a preservative solution (0.3 *M* reduced glutathione, adjusted to pH 7.0 with sodium hydroxide), were added per ml of freshly

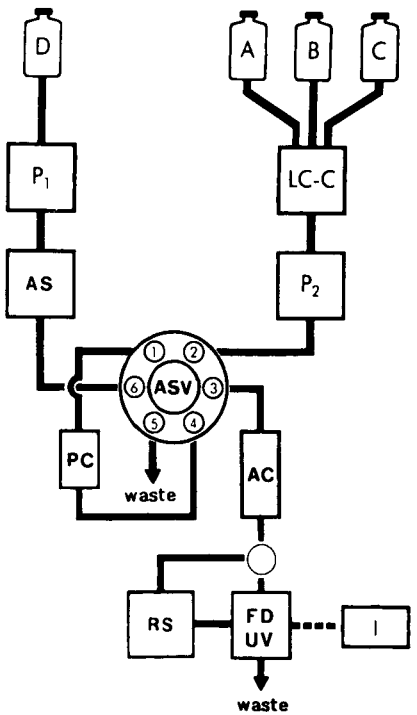

Fig. 1. Apparatus flow-diagram. LC-C = Liquid chromatograph controller; P_1, P_2 = pumps; AS = autosampler;ASV = automatic switching valve; PC = precolumn; AC = analytical column; FD = fluorescence detector; UV = ultraviolet detector; RS = reaction system; I = integrator; A = analytical buffer; B = methanol; C = doubly distilled water; D = precolumn buffer.

withdrawn blood sample. After the preparation of the plasma or serum fraction by centrifugation, the samples were stored at −20°C. These sampling procedures guarantee stability of the biogenic amines for at least 12 h at 4°C on the autosampler tray.

For ribonucleoside analysis in urine, serum and milk, samples were adjusted to pH 4.0 with concentrated formic acid and stored at −20°C until investigation. Prior to analysis, thawed samples were centrifuged at 3000 *g* for 3 min and an aliquot (100 µl urine, milk; 500 µl serum) was applied to the HPLC system.

RESULTS AND DISCUSSION

Figs. 2 and 3 show the biomolecules investigated (unmodified, modified and hypermodified ribonucleosides as well as the parent catecholamines), the quantitation of which in body fluids is of importance in pathobiochemistry and clinical chemistry as these compounds serve as diagnostic marker molecules for a variety of distinct metabolic disorders.

For on-line sample processing, *i.e.*, covalent affinity chromatography the aromatic 1,2-diol and the aliphatic *cis*-diol compounds were chosen as a selectivity

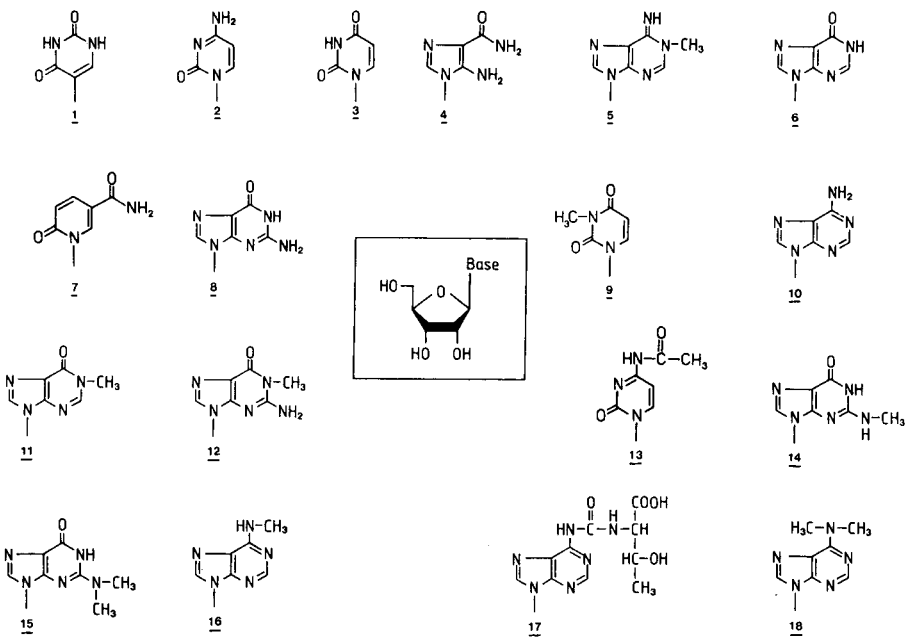

Fig. 2. Structural formulae of ribonucleosides and catecholamines investigated. 1 = Pseudouridine (ψ); 2 = cytidine (Cyd); 3 = uridine (Urd); 4 = 5-aminoimidazole-4-carboxamido-N-ribofuranoside (AICAR); 5 = N^1-methyladenosine (m^1 Ado); 6 = inosine (Ino); 7 = 2-pyridone-5-carboxamino-N-ribofuranoside (PCNR) = 1,6-dihydro-6-oxo-1-(β-D-ribofuranosyl)-3-pyridinecarboxylic amide; 8 = guanosine (Guo); 9 = N^3-methyluridine (m^3 Urd); 10 = adenosine (Ado); 11 = N^1-methylinosine (m^1 Ino); 12 = N^1-methylguanosine (m^1 Guo); 13 = N^4-acetylcytidine (ac^4 Cyd); 14 = N^2-methylguanosine (m^2 Guo); 15 = N^2-dimethylguanosine (m^2_2 Guo); 16 = N^6-methyladenosine (m^6 Ado); 17 = N-carbamoyl-threonyadenosine (t^6 Ado); 18 = N^6-dimethyladenosine (m^6_2 Ado).

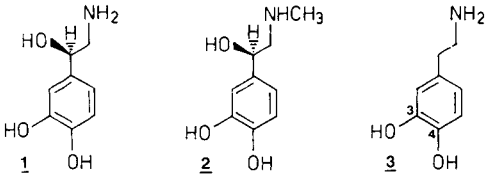

Fig. 3. Structural formulae of catecholamines investigated. 1 = *R*-(−)-1-(3,4-Dihydroxyphenyl)-2-aminoethanol (norepinephrine, NE); 2 = *R*-(−)-1-(3,4-dihydroxyphenyl)-2-(methylamino)ethanol (epinephrine, E); 3 = 2-(3,4-dihydroxyphenyl)ethylamine (dopamine, DA).

criterion, as these structural moieties reversibly form a cyclic diester with tetrahedral boronic acid under alkaline conditions[7]. This affinity ligand has been immobilized via its 3-aminophenyl derivative to various gel supports, *e.g.*, agarose, cellulose, polyacrylamide, and used for the manual or partially automated[8] clean-up of ribonucleosides[9–18] and catecholamines[19] in an off-line mode. For on-line analysis of catecholamines in urine, the use of silica-based boronic acid affinity supports in coupled-column HPLC systems has been described[20–22]. For direct injection and on-line processing of plasma samples, Edlund and Westerlund[22] developed a liquid chromatographic system comprised of three coupled columns, two of which are precolumns for selective isolation (phenylboronic acid modified polyacrylamide) and subsequent enrichment (silica RP C_{18}). In contrast to the system described here, this method requires time consuming reconditioning steps for the additional enrichment column, complex, partially laboratory-made instrumentation and, due to the low recovery, the addition of an internal standard.

An analogous approach, *i.e.*, a pH-dependent on-off mechanism for catecholamine sample processing, was studied by Frei[1] in a model system based on ligand-exchange chromatography on a copper(II)-loaded iminodiacetate-modified silica.

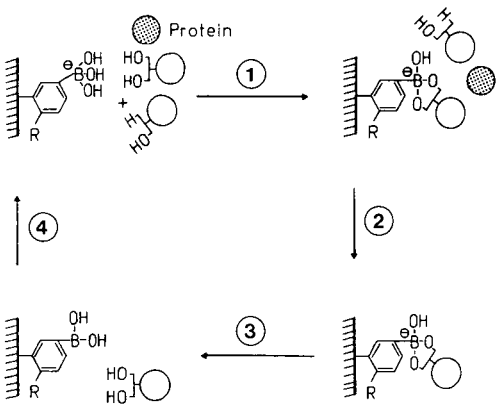

Fig. 4. Chromatographic properties of the sample processing precolumn. (1) pH-dependent covalent affinity chromatography of aromatic 1,2-diol and aliphatic *cis*-diol compounds, pH > 8.0; (2) SEC of residual matrix constituents; (3) on-line transfer elution (pH 3.0) of diol compounds to analytical column; (4) pH-controlled regeneration of precolumn.

For direct injection and on-line processing of ribonucleosides in urine, we developed a coupled dual-column system using a laboratory prepared phenylboronic acid modified silica[23] as precolumn packing material[24–27]. By preparing a new bonded-phase material[28] we succeeded in the development of the first dual-column HPLC analyzer for ribonucleosides[4–6] and catecholamines[3] in proteinaceous body fluids.

Fig. 4 schematically shows the chromatographic properties of the precolumn material. The stationary phase is a chemically modified co-polymer which tolerates pH values from 1 to 12 and a back pressure up to 2000 p.s.i.

The column material allows the simultaneous performance of two different modes of liquid chromatography. First, by virtue of its size-exclusion properties, residual matrix constituents, *e.g.*, proteins can be separated quantitatively from the solution[3]. Secondly, by immobilizing a specifically modified phenylboronic acid to the gel support, high-performance affinity chromatography (HPAC) can be performed. The lifetime of the precolumn material exceeds more than 3000 urine analyses (100-μl aliquots) and more than 500 plasma (500-μl aliquots) or milk analyses (100-μl aliquots) without reduction of the chromatographic efficiency.

In principle, the desired group-selective prefractionation, *i.e.*, on-line sample processing of ribonucleosides and catecholamines is carried out by a simple pH-step elution, followed by the analytical resolution under reversed-phase conditions. The overall on-line analysis cycle is characterized by five discrete steps:

(1) Sample application (10–500 μl) via the autosampler (*cf.*, Fig. 1). Chemoselective binding as well as enrichment of the diol compounds on the affinity ligand of the precolumn (*cf.*, Fig. 4) under slightly alkaline, *i.e.*, buffer D conditions ("HPAC step").

(2) Simultaneous, quantitative elution of the residual matrix constituents from the precolumn into the waste ("SEC step"; *cf.*, Fig. 4).

(3) Microprocessor controlled valve switching (*cf.*, Fig. 1). Quantitative, group-selective elution of the diol compounds from the precolumn (*cf.*, Fig. 4) by acidification (buffer A) of the immobilized cyclic boronate ester and simultaneous on-line transfer in a single, narrow elution band through positions 2, 1, 4, 3 of the valve (*cf.*, Fig. 1) to the top of the series-connected analytical column ("transfer step"; *cf.*, Fig. 4).

(4) Automated valve switching (*cf.*, Fig. 1) and separation of the diol compounds on the analytical column under reversed-phase chromatographic conditions ("separation step"; *cf.*, Fig. 4).

(5) Reconditioning of the tetrahedral trihydroxyboronyl functionality (*cf.*, Fig. 4) for a new extraction cycle during the analytical step with the initial eluent D ("regeneration step").

Figs. 5–11 represent typical chromatograms, which demonstrate that the compounds investigated can easily be identified and quantitated using the analytical approach described.

For method validation, a comparison of on-line with purely analytical column (off-line) chromatography of standard mixtures was performed. It revealed correlation coefficients between 0.96 and 1.00 for ribonucleosides and catecholamines, respectively. Fig. 12 shows typical chromatograms obtained for catecholamine analysis with natural fluorescence detection.

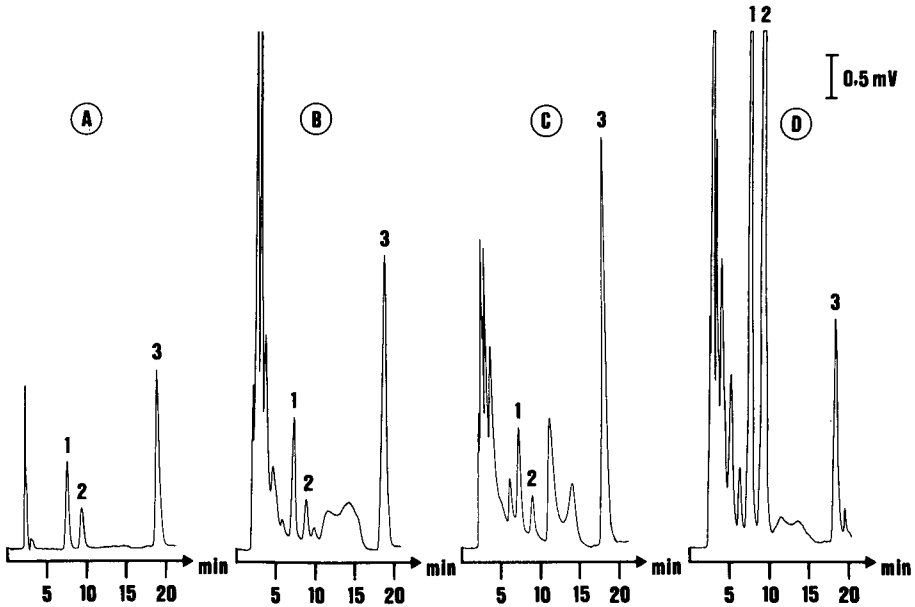

Fig. 5. Automated on-line analysis of free urinary catecholamines with natural fluorescence detection. (A) Standard (pmol), sample volume 100 μl: 1 = norepinephrine, 50; 2 = epinephrine, 20; 3 = dopamine, 130. (B) Native urine (pmol), sample volume 100 μl: 1, 60; 2, 20; 3, 250. (C) 24-h urine (pmol), sample volume 100 μl: 1, 60; 2, 20; 3, 340. (D) Pathological urine (phaeochromocytoma) (pmol), sample volume 100 μl: 1, 260; 2, 290; 3, 160.

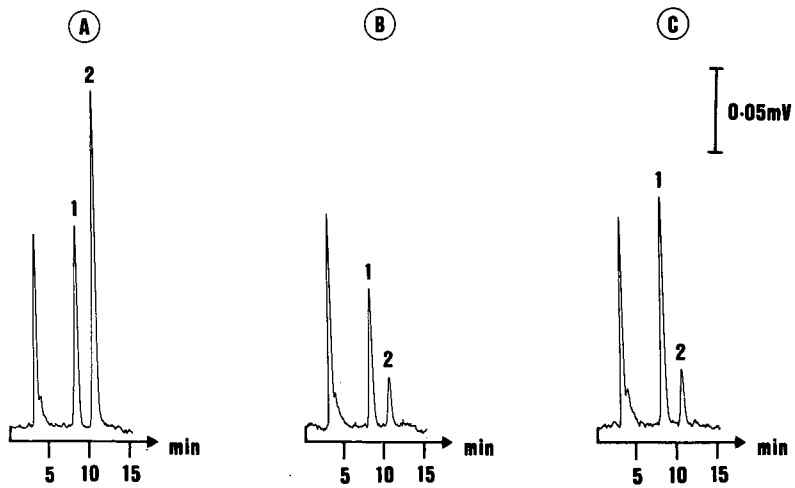

Fig. 6. Automated on-line analysis of free catecholamines in plasma and serum with trihydroxyindole fluorescence detection. (A) Standard (pmol), sample volume 100 μl: 1 = norepinephrine, 3.4; 2 = epinephrine, 2.8. (B) Plasma (pmol), sample volume 500 μl: 1, 2.4; 2, 0.3. (C) Serum (pmol), sample volume 500 μl: 1, 3.6; 2, 0.3.

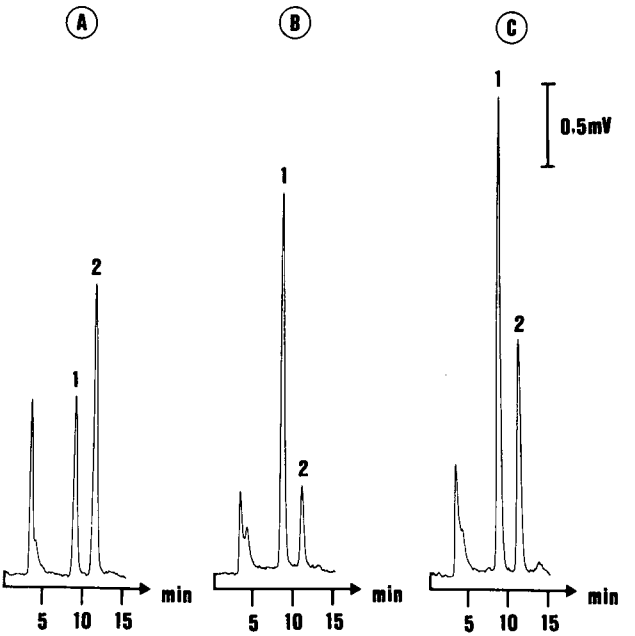

Fig. 7. Automated on-line analysis of free urinary catecholamines with trihydroxyindole fluorescence detection. (A) Standard (pmol), sample volume 200 μl: 1 = norepinephrine, 6.4; 2 = epinephrine, 5.6. (B) 24-h urine (pmol), sample volume 50 μl: 1, 13.6; 2, 1.3. (C) Native urine (pmol), sample volume 50 μl: 1, 17.6; 2, 3.9.

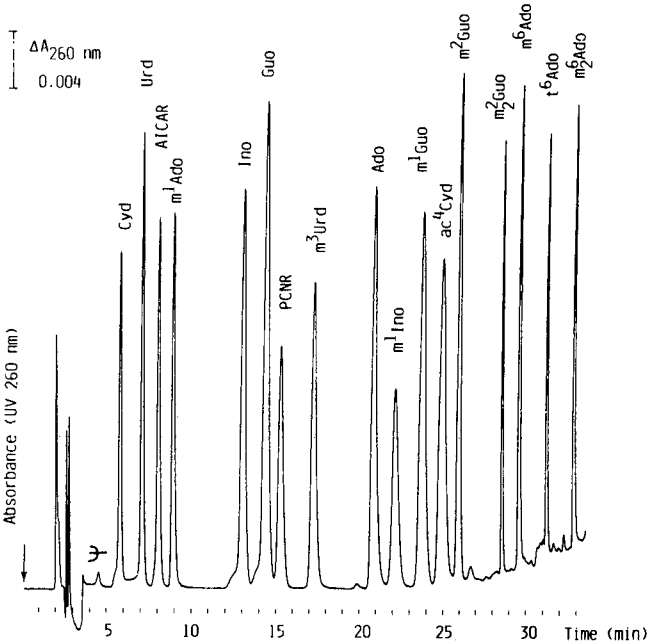

Fig. 8. Automated on-line analysis of a synthetic mixture of ribonucleosides. Sample volume: 100 μl. Compounds (nmol): AICAR (0.35); m^1 Ado (0.81); Ino (2.06); Guo (1.83); PCNR (1.49); Ado (1.30); m^1 Ino (1.88); m^1 Guo (2.11); ac^4 Cyd (1.05); m^2 Guo (1.03); m_2^2 Guo (0.47); m^6 Ado (0.54); t^6 Ado (0.53); m_2^6 Ado (0.62).

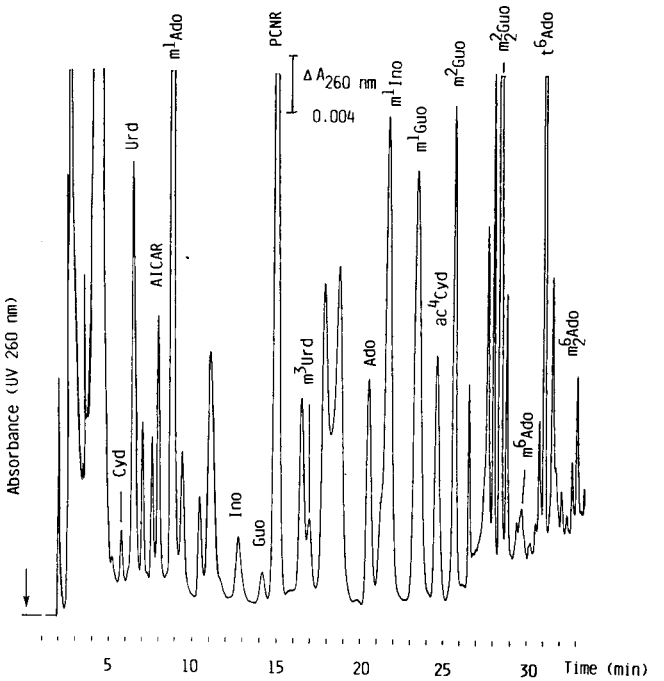

Fig. 9. Automated on-line analysis of ribonucleosides in 100 μl normal human urine. Compounds (nmol): AICAR (0.27); m^1 Ado (4.17); Ino (0.42); Guo (0.09); PCNR (4.87); Ado (0.71); m^1 Ino (5.43); m^1 Guo (2.88); ac^4 Cyd (0.59); m^2 Guo (1.10); m^2_2 Guo (4.21); t^6 Ado (1.98); m^6_2 Ado (0.09).

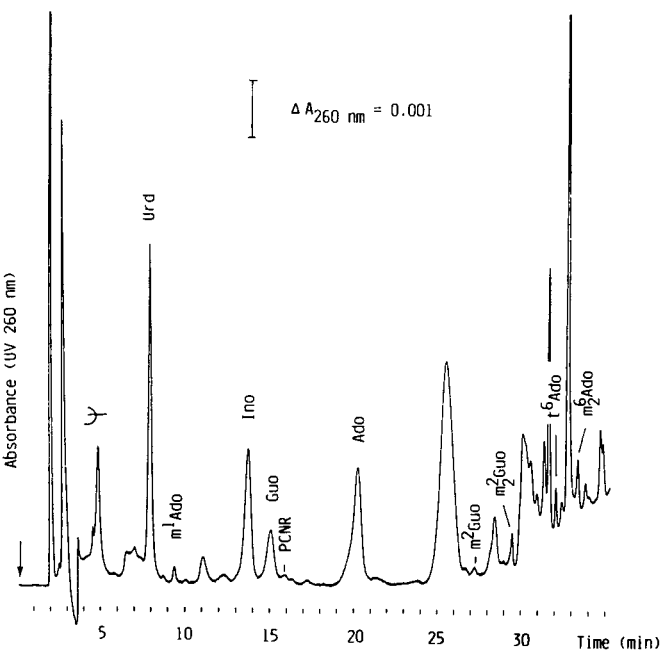

Fig.10. Automated on-line analysis of ribonucleosides in 500 μl normal human serum. Compounds (pmol): m^1 Ado (6); Ino (199); Guo (72); PCNR (17); Ado (174); m^2 Guo (7); m^2_2 Guo (14); t^6 Ado (57); m^6_2 Ado (31).

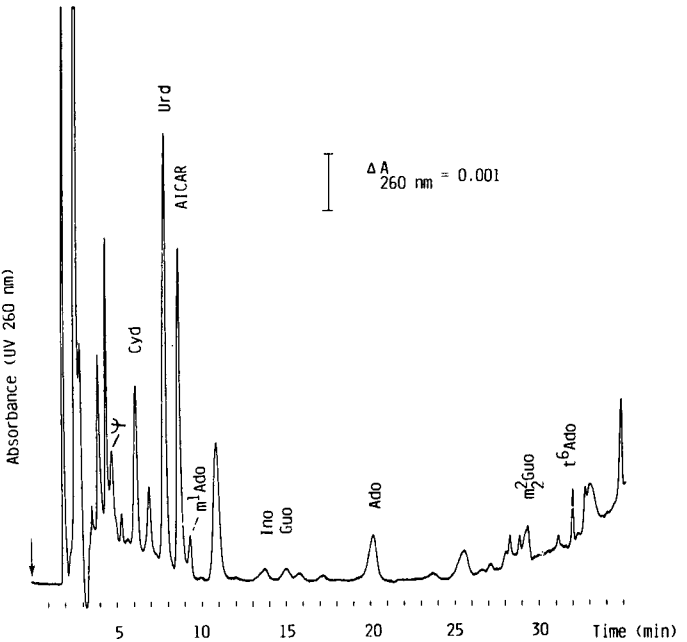

Fig. 11. Automated on-line analysis of ribonucleosides in 100 μl breast milk. Compounds (pmol); AICAR (84); m^1 Ado (32); Ino (35); Guo (22); Ado (72); m_2^2 Guo (17); t^6 Ado (18).

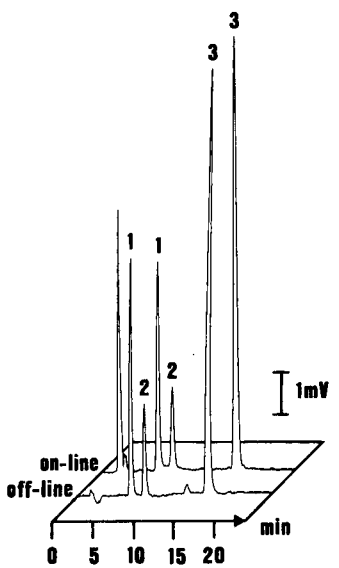

Fig. 12. Comparison between a catecholamine analysis of a standard sample with physiological concentrations in the off-line and the on-line mode. (sample volume 100 μl). 1 = Norepinephrine, 50; 2 = epinephrine, 20; 3 = dopamine, 210 pmol.

TABLE I

RELIABILITY

	Catecholamines	*Ribonucleosides*
Recovery	96–103%	95–104%
Imprecision*	3.9–6.3%	2.7–5.6%
Inaccuracy*	0.1 pmol (3.7%)	n.d.
Specificity	Chemoselective	Chemoselective
Detection limit**	20 fmol	10 pmol
Linearity	50 fmol–12 pmol***	0.25–25 nmol
	100 pmol–3 nmol§	

* For physiological concentrations and day-to-day analysis.

** For 1-ml sample volume; $\underline{x} = x + 3\sigma$ where $\underline{x}$ = detection unit, x = mean value of peak area of blank sample matrix at the appropriate retention times, and σ = standard deviation.

*** Trihydroxyindole fluorescence.

§ Natural fluorescence.

The recovery of the analytes after direct sample injection is quantitative, independent of the amount of analytes and independent of the biological matrix investigated. Thus, this method does not require the addition of an internal standard and calibration can be performed simply with reference samples. The data in Table I document the reliability of the coupled dual column system.

CONCLUSIONS

The use of a coupled dual column system for on-line sample processing, trace enrichment and analysis of aromatic 1,2-diol and aliphatic *cis*-diol biomolecules (catecholamines, ribonucleosides) has led to the development of a fully automated HPLC analyzer which tolerates the direct and repetitive injection of proteinaceous body fluids. The precolumn technology is based on a newly developed and unique bonded-phase material which allows the simultaneous performance of covalent affinity and size-exclusion chromatography.

The high accuracy, based primarily on the quantitative and matrix-independent recovery of the analytes investigated, the practicability and the commercial availability of the HPLC analyzer render the system attractive for analytical investigations in the biochemical research field as well as in clinical laboratories.

Further applications of this method will be: (1) trace enrichment for structural characterization of diol-containing compounds in biological fluids; (2) small scale preparation of natural diol-containing compounds; (3) investigation of disorders in catecholamine, ribonucleoside, ribonucleotide and/or RNA metabolism; (4) a non-invasive screening test (urinary modified ribonucleosides) for cancer diseases in humans; (5) investigation of renal reutilization processes; (6) therapeutic drug monitoring during nucleoside or catecholamine chemotherapy; (7) protocols for sample processing and on-line analysis of glycosylated proteins, *e.g.*, haemoglobin A_1, coenzymes, *e.g.*, reduced nicotinamide-adenine dinucleotides, ribonucleotides, *e.g.*, ATP and dinucleotides, *e.g.*, Ap_4A.

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DETERMINATION OF RESIDUES OF CARBADOX AND SOME OF ITS METABOLITES IN SWINE TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING ON-LINE PRE-COLUMN ENRICHMENT AND POST-COLUMN DERIVATIZATION WITH UV–VIS DETECTION

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SUMMARY

A high-performance liquid chromatographic (HPLC) method that uses UV–VIS detection and post-column derivatization with sodium hydroxide was developed for the determination of the growth-promoting antibiotic carbadox and three of its metabolites in swine muscle, liver and kidney tissues. Sample pre-treatment involves extraction with methanol–acetonitrile, purification over an alumina–Florisil column and partition with isooctane. A 2-ml volume of the final aqueous extract is injected into a column-switching HPLC system; detection is performed at 420 nm. The limits of determination are in the range 1–5 μg/kg. Preliminary experiments show a good precision with mean recoveries of 81–87% and a coefficient of variation of 4–10%. The method is highly selective and can be used in routine monitoring programmes.

INTRODUCTION

Carbadox (CBX) methyl 3-(2-quinoxalinylmethylene)carbazate-N^1, N^4-dioxide, is used extensively in veterinary practice as a growth-promoting and chemotherapeutic agent for young swine. The structures of the parent drug and some of its metabolites are given in Fig. 1. Carbadox is administered through the feed at levels of 50–150 mg/kg. To ensure the absence of residues of CBX and its metabolites from edible products, a withdrawal period of 4 weeks has been established. Further, CBX should not be administered to swine over 4 months old.[1].

Metabolism studies have shown that CBX is rapidly converted into monooxy and desoxy metabolites. Quinoxaline-2-carboxylic acid (QCA) is considered to be the last remaining major residue and may serve as a marker substance[2]. However, CBX and desoxy-CBX are considered to be carcinogenic and mutagenic[2,3] and methods should be available to monitor even very low concentrations in edible tissues.

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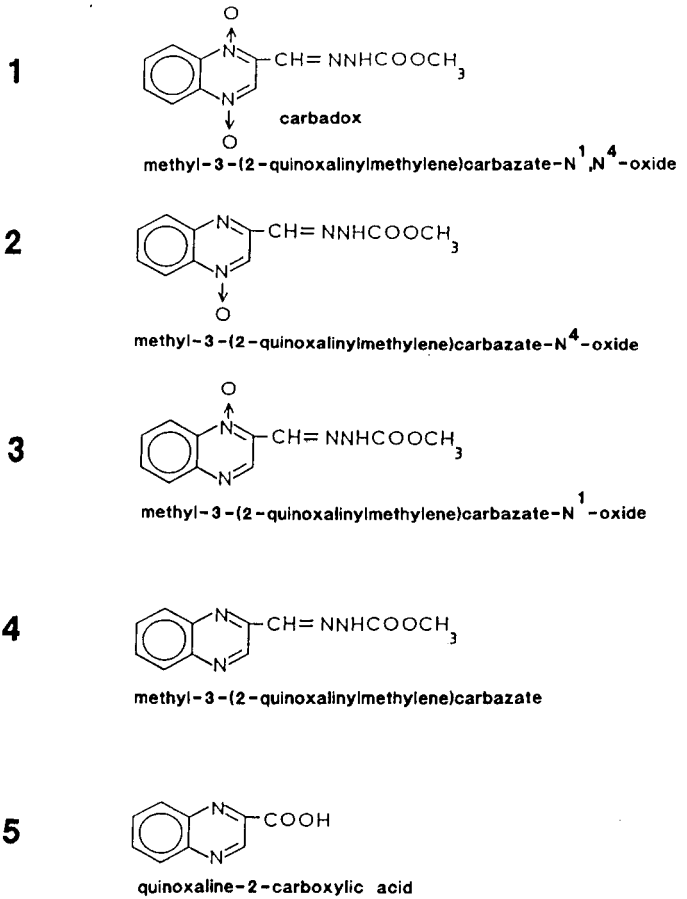

Fig. 1. Molecular structures of carbadox (1) and its monoxy (2 and 3), desoxy (4) and quinoxaline acid (5) metabolites.

High-performance liquid chromatographic (HPLC)[4–9], polarographic[10–12], thin-layer chromatographic (TLC)[13] and gas chromatographic (GC)[14] methods have been described for the determination of residues of CBX and/or its metabolites in various matrices, including feeds[8,9]. CBX has been detected electrochemically[10–12,15] and using fluorescence[4,5,16] or UV–VIS absorption spectrometry[5–9,17–21]. In some studies no chromatographic separation was performed before the actual determination[10–12,15,19–21]. A few workers have also studied the analysis of some metabolites. The supposed final metabolite QCA can be detected electrochemically[10–12] or, after derivatization, with electron-capture detection or mass spectrometry in the selected ion mode[14]. The monoxy- and desoxy-CBX metabolites can be detected by UV–VIS spectrometry at 280 or 350 nm[5,6], but our preliminary experiments showed no active fluorescence.

All of the relevant residue methods mentioned require extensive sample clean-up to remove interfering components and/or to hydrolyse CBX-related residues to QCA[6,11–14]. Often a number of liquid–liquid extraction and off-line column puri-

fication steps are performed, resulting in the analysis of only a few samples per day. The limits of detection lie in the range 5–40 μg/kg for CBX and desoxy-CBX and 30–100 μg/kg for QCA.

Previous methods do not provide the desired limits of detection and are not always practical or suitable for metabolite analysis. Therefore, in this work a routine method was developed for the determination of CBX and a number of relevant metabolites in swine tissues at levels of 1–5 μg/kg.

EXPERIMENTAL

Chemicals and reagents

All chemicals were of analytical-reagent grade (Merck, Darmstadt, F.R.G.). Standard carbadox was obtained from Pfizer (Groton, CT, U.S.A.) and standard solutions of methyl 3-(2-quinoxalinylmethylene)carbazate (desoxy-CBX), methyl 3-(2-quinoxalinylmethylene)carbazate N^1-oxide (N^1-monooxy-CBX) and methyl 3-(2-quinoxalinylmethylene)carbazate N^4-oxide (N^4-monooxy-CBX) were a gift from the Central Veterinary Institute (Lelystad, The Netherlands).

Florisil (0.075–0.150 mm) was purchased from Merck and alumina (Woelm neutral, activity 1) (Art. No. 02090) from Woelm Pharma (Eschwege, F.R.G.). The extraction solution was acetonitrile–methanol (1:1). The HPLC eluent was prepared by mixing 850 ml of 0.01 *M* sodium acetate buffer (adjusted to pH 6 with acetic acid) with 150 ml of acetonitrile. Water was purified with a Milli-Q purification system (Millipore, Milford, MA, U.S.A.). Stock standard solutions contained 100 μg/ml in acetonitrile–methanol (1:1) and working standard solutions had concentrations of 0.001, 0.005 or 0.01 μg/ml in water.

Instrumentation and chromatographic conditions

The column-switching HPLC system depicted in Fig. 2 consisted of two liquid chromatographic pumps capable of maintaining a constant pulseless flow-rate of

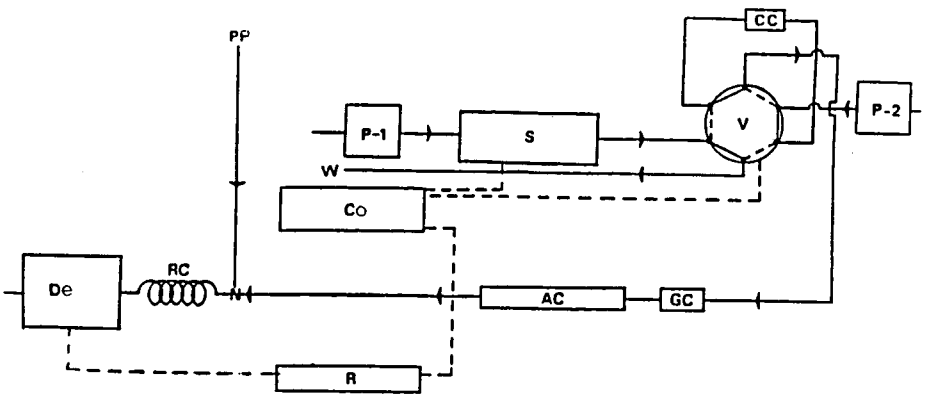

Fig. 2. Schematic representation of the HPLC system used. The sample is introduced with pump P-1 and sampler S to the column-switching sample enrichment module with the six-port valve V and enrichment column CC. After the HPLC separation using pump P-2, guard column GC and analytical column AC, the sample passes the post-column derivatization unit with the peristaltic pump PP and the reaction coil RC and is detected by the UV–VIS detector De. Peaks are recorded by recorder R. All timed events are handled by a microprocessor Co.

0.2–2 ml/min [Model 6000 A (Millipore) and Spectroflow 400 (ABI-Kratos, Ramsey, NY, U.S.A.)]. A Model 7125 fixed-loop (2 ml) injector (Rheodyne, Berkeley, CA, U.S.A.), an automatic six-port valve (Rheodyne 7010), a Model 450 controller (Kratos), a UV–VIS spectroflow 783 absorbance detector (Kratos), an M 2002 peristaltic tubing pump (Skalar, Breda, The Netherlands), a 1/16 in. × 0.75 mm tee (Valco, Houston, FL, U.S.A.) and a 2 m × 0.5 mm I.D. knitted PTFE reaction coil were used. The analytical column was a 200 × 3 mm I.D. cartridge containing 5-μm ChromSpher C_{18} (Chrompack, Middelburg, The Netherlands) and the guard column was a 10 × 2.1 mm I.D. cartridge containing 37–50-μm Bondapak C_{18}/Corasil (Millipore). The enrichment column used in the final procedure was a 60 × 4.6 mm I.D. stainless-steel column containing 37–50-μm Bondapak C_{18}/Corasil.

During method development, 60 × 4.6 mm I.D. or 10 × 2.1 mm I.D. columns containing 53-μm Partisil ODS-3 (Whatman, Clifton, CA, U.S.A.) or the polymeric materials 50–100-μm XAD-4 (Serva, Heidelberg, F.R.G.) or 15–25-μm PRP-1 (Polymer Labs., Amherst, MA, U.S.A.) were tested. Also, a 150 × 4.6 mm I.D. analytical column containing 5-μm Supelcosil LC-8 DB (Supelco, Bellefonte, PA, U.S.A.) was tested. A Model 400 stomacher laboratory blender (Lameris, The Netherlands), a Pierce (Rockford, IL, U.S.A.) evaporator and an MSE-coolspin centrifuge (MSE Crawley, Sussex, U.K.) were used. The eluent flow-rate was 0.6 ml/min, the sample enrichment flow-rate was 0.5 ml/min and the derivatization reagent flow-rate was 0.23 ml/min. The timed events sequence used for enrichment and separation was as follows: 0 min, start sampling and flushing; 20 min, activate six-port valve for back-flushing and set detector at auto-zero; 25 min, reset six-port valve; 35 min, new sample.

Sample preparation

Caution: solutions of CBX and its metabolites are light-sensitive and therefore all handling should be performed under artificial yellow light and amber glassware should be used.

Accurately weigh *ca.* 10 g of homogenized sample (muscle, liver, kidney) in a 1000-ml stomacher bag. Add 40.0 ml of acetonitrile–methanol (1:1) and blend for 3 min. Transfer the crude extract into a centrifuge tube and centrifuge for 5 min at 2000 *g*. Apply the supernatant to an alumina–Florisil column prepared by pouring first 8 g of alumina and then 2 g of Florisil into a 400 × 10 mm I.D. glass column, and collect the first 10 ml of eluate in a calibrated tube. Evaporate the solvent with a gentle stream of nitrogen at 40–50°C to a volume of 1–1.5 ml. Dilute to 4.0 ml with water and mix. Extract this solution with 2 ml of isooctane, centrifuge for 5 min at 2000 *g* and inject 2.0 ml of the aqueous phase on to the HPLC column.

RESULTS AND DISCUSSION

General

In multi-component residue analysis in complex matrices, the selective detection of the analytes is one of the major problems. This also applies to the determination of carbadox and its metabolites in swine tissues. Therefore, after the HPLC separation conditions have been established, this aspect will be discussed before the sections dealing with extraction, purification and sample enrichment. As selective

UV–VIS detection of the more persistent, but toxicologically not very relevant, metabolite QCA proved to be impossible, as is shown in the post-column derivatization section, this metabolite was not evaluated further in the extraction, purification and enrichment experiments.

Chromatography

In the literature, most eluent systems used for the reversed-phase separation of CBX and related compounds are composed of a mixture of acetonitrile and water or an acidic (pH 3–4) buffer[4,5,8,16,18]. De Graaf *et al.*[5] used a linear acetonitrile–water gradient to separate CBX and desoxy-CBX from gastrointestinal tract matrix interferences. MacIntosh and Neville[6] were able to separate CBX, desoxy-CBX and N^1- and N^4-monooxy-CBX with an acetonitrile–ammonium acetate buffer–ethanol (pH 6.8) eluent on a C_8-bonded phase. With the system used in our laboratory for the determination of CBX in feeds, the complete resolution of CBX, N^1- and N^4-monooxy-CBX and desoxy-CBX was achieved on a C_{18}-bonded phase with an even simpler eluent, acetonitrile–0.01 *M* sodium acetate buffer (pH 6) (15:85) (Fig. 3). This system was used in this present study.

Two types of analytical columns were tested: a 150 × 4.6 mm I.D. column containing Supelcosil LC-8 DB and a 200 × 3 mm I.D. cartridge column containing Chromsep C_{18}. The columns showed similar resolution but, probably owing to the smaller inner diameter of the cartridge column, the peak broadening was less in the

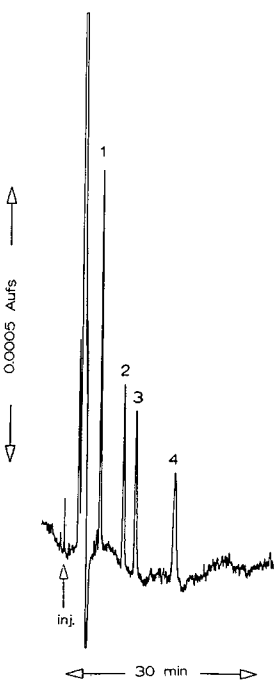

Fig. 3. HPLC trace obtained after injection of 50 µl of a standard solution containing 2.5 ng of each carbadox and its monooxy and desoxy metabolites. For experimental conditions, see text. Peaks: 1 = carbadox; 2 = N^4-monooxy; 3 = N^1-monooxy; 4 = desoxy.

latter instance, resulting in a 50% increase in the peak response. This phenomenon, originating from technical features rather than characteristics of the packing material, was observed earlier in another residue method development study[22].

Post-column derivatization

Carbadox and its metabolites show UV–VIS absorption maxima at wavelengths ranging from 275 to 380 nm. In Fig. 4 the UV–VIS spectra of CBX, desoxy-CBX and QCA are shown. For N^1- and N^4-monooxy-CBX, an insufficient amount of material was available to measure their UV–VIS spectra. As shown by De Graaf *et al.*[5] and MacIntosh and co-workers[6,7], extensive sample clean-up is necessary to remove UV-absorbing interferences when CBX or its metabolites are determined in biological matrices and detected at 280–380 nm. Therefore, a more selective detection method was necessary.

In feed analysis, a number of direct UV–VIS spectrometric methods have been developed for the determination of high levels of CBX (50–150 mg/kg). The final step in these procedures is the reaction of CBX with 0.1 *M* sodium hydroxide[19–21]. The yellow reaction product is detected at 420 nm, thereby strongly reducing feed matrix interferences. The compound formed is not very stable and has not been characterized. The sodium hydroxide reaction is, however, fast and reproducible. This opened up the possibility of a post-column reaction with which a well defined, short reaction time and a reproducible time interval between reaction and detection can be achieved. In Fig. 4, the UV–VIS spectra of CBX and its metabolites after the addition of 0.5 *M* sodium hydroxide are also shown. Both CBX and desoxy-CBX show a shift of their absorption maxima to higher wavelengths; QCA apparently does not give a similar

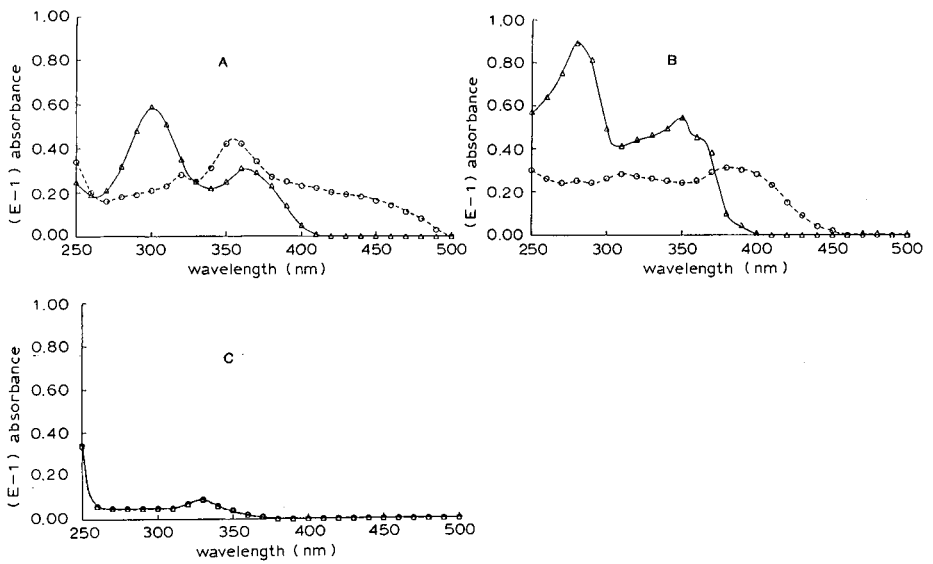

Fig. 4. UV–VIS spectra of (A) carbadox, (B) desoxy-carbadox and (C) quinoxaline-2-carboxylic acid dissolved in the mobile phase both before (△) and after (○) the addition of 0.5 *M* sodium hydroxide. Conditions: path length, 1 cm; analyte concentration, 100 μg/ml.

reaction with sodium hydroxide. This indicates that the carbazate moiety is essential for the reaction, or presumably both monooxy-CBX metabolites will also react.

As the UV maximum of QCA is near 245 nm and the compound is very polar, it will be extremely difficult to detect QCA selectively in the presence of tissue matrix components using HPLC with UV–VIS absorption detection. Therefore, QCA was not evaluated further in this study. The choice of 420 nm as the detection wavelength was a compromise between maximal adsorbance of the analytes and minimal interferences by matrix components. Addition of sodium hydroxide increases the molar absorptivity at 420 nm from less than 10^2 to $1.5 \cdot 10^4$ for CBX and from less than 10^2 to $2.2 \cdot 10^4$ for desoxy-CBX. Usually, CBX is detected at its second UV maximum at 365 nm and not at its higher maximum at 280 nm, because there are fewer interferences at 365 nm.

As can be seen from Fig. 4, the absorptivity at 420 nm after sodium hydroxide addition is essentially the same as that obtained at 365 nm without sodium hydroxide addition. In other words, the intrinsic sensitivity remains the same, but the selectivity is improved.

The sodium hydroxide reaction was tested in the post-column derivatization system by mixing the reagent with the column effluent and letting them react in a knitted PTFE coil at room temperature. The sodium hydroxide concentration was varied from 0.1 to 1.0 *M* to optimize the response and noise. A 0.5 *M* reagent concentration proved to be optimal (Fig. 5A), giving a compromise between noise and response. To test the time dependence of the reaction, coils of different lengths, *i.e.*, with different residence times, were tested (Fig. 5B). The optimal response was obtained with a 2 m × 0.5 mm I.D. coil or a 28-s residence time.

Extraction and sample clean-up

In most of the extraction and purification experiments, CBX was used as a model compound. Because CBX is more polar than its metabolites, it can be considered to be a good indicator substance for the monooxy and desoxy-metabolites.

Preliminary experiments using a very simple aqueous extraction of animal tissue, followed by on-line dialysis and HPLC with pre-column enrichment and post-column sodium hydroxide derivatization, in analogy with a method developed for sulphonamides in biological samples[23], showed that, in principle, the fully automated determination of CBX was possible but that the limit of detection was high (about 25 μg/kg). This was probably due to the limited efficiency of the on-line dialysis and not to poor extraction from the sample. Therefore, another approach was followed, using an organic sample extraction. Carbadox is readily soluble in various organic solvents, and reported extractants include dimethylformamide[5,20], ethanol[6], chloroform–methanol (3:1)[19], dichloromethane[4], acetonitrile–methanol (1:1)[17,24] and 2% ammonia in acetone[8]. In our department acetonitrile–methanol (1:1) had been used for several years in feed analysis, where it has shown to give 100% recovery[24]. Further, acetonitrile and methanol are efficient deproteination reagents that yield well separated phases after extraction. Therefore, acetonitrile–methanol (1:1) was used further in this study.

With a practical sample size of 10 g, 40 ml of acetonitrile–methanol (1:1) gave over 95% recovery for spiked (1–100 μg/kg tissue) CBX and desoxy-CBX samples. The extraction was performed with a stomacher blending apparatus. Stomacher

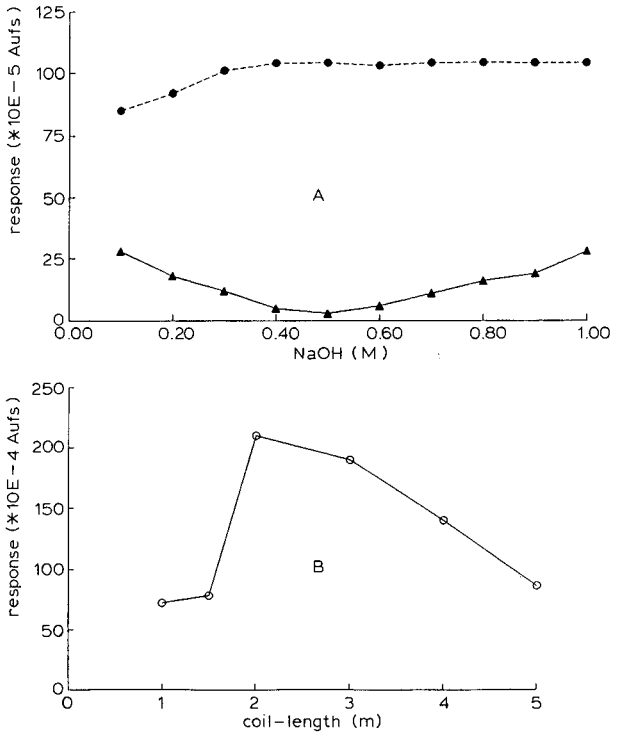

Fig. 5. (A) Plot of the response (●) and system noise (▲) obtained at 420 nm after injection of 20 ng of CBX into the column-switching HPLC system *versus* the concentration of the sodium hydroxide solution added post-column to the analytical column effluent. Reagent flow-rate, 0.23 ml/min. (B) Plot of the response obtained at 420 nm after injection of a carbadox standard solution into the HPLC system *versus* the length of the 0.5 mm I.D. knitted PTFE coil used in the post-column derivatization with 0.5 *M* sodium hydroxide.

blending if often used in microbiological analysis. The sample is introduced in a polypropylene bag and blended with the solvent without direct physical contact with instrument parts. Also, the bags are used only once. Hence, compared with blending with, for instance, an Ultra-Turrax, the risk of cross-contamination is strongly reduced.

After centrifugation of the crude extract, the supernatant (about 30 ml) was purified over an alumina–Florisil column to remove fat and colouring agents. CBX and its metabolites were not adsorbed on the column because of the water present in the extract, originating from the tissue sample. Different amounts of alumina (0–8 g) were tested with, always, 2 g of Florisil. Although the extracts became cleaner when the amount of alumina was increased, an amount of 8 g was chosen as a practical upper limit because otherwise too much extraction liquid was sorbed on to the column material. When 8 g of alumina were used, a maximum of 20 ml of eluate could be collected. Of these 20 ml, the last 10 ml eluted much more slowly than the first 10 ml. Fractionation of the eluate showed that the CBX concentration was slightly (10%) higher in the first 2 ml, and also that this first fraction produced cleaner chromatograms (see below). However, in order to achieve the intended low detection

levels, it was necessary to collect a minimum of 10 ml of eluate. This corresponded to about 2.5 g of tissue sample or to 2.5 ng of CBX when the tissue sample contained 1 μg/kg of CBX. Collection of more eluate increased the amount of CBX, but on the other hand resulted in a more time-consuming purification and subsequent evaporation of the eluate. When the eluate fraction was evaporated to dryness, dissolution of CBX became irreproducible. Therefore, the 10 ml of eluate were gently evaporated until essentially all organic solvent had been removed and about 1.5 ml of aqueous phase remained.

After evaporation, the aqueous residue was diluted to 4 ml with water. This allowed duplicate injection in the HPLC analysis and improved the reproducibility of the procedure.

When 2 ml of the final 4 ml of solution were injected into the final column-switching HPLC system, irreproducibly eluting components, interfering with CBX or its metabolites, were sometimes observed. In a later stage of this study, these interferences could be effectively removed by a simple extraction with 2 ml of isooctane. Isooctane is almost immiscible with water and CBX and its metabolites do not dissolve in it. Fig. 6 shows the effect of the isooctane extraction on the chromatograms obtained from different eluate fractions of blank liver samples. It allows the use of a five-fold increased sample amount without an increase in the matrix interferences.

The use of an internal standard was not considered practical, because it could interfere with the detection of unknown metabolites or other veterinary drugs (see also under *Characteristics and applications of the analytical method*).

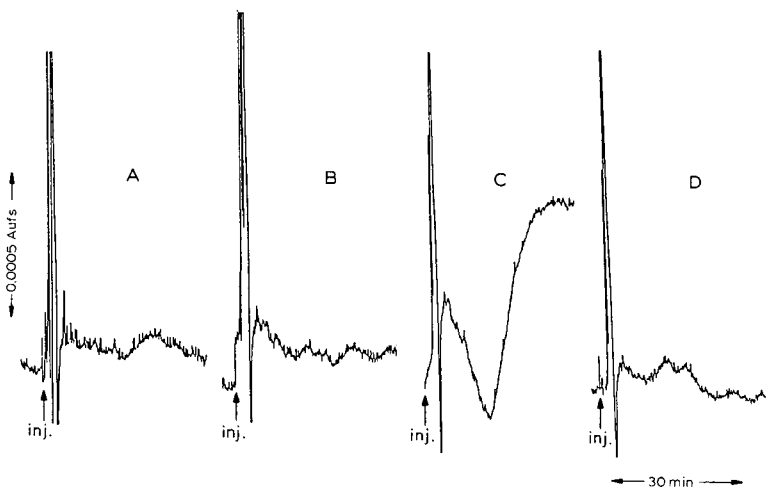

Fig. 6. Chromatograms obtained with blank swine liver samples using the final extraction and purification procedure, except for variations in the alumina–Florisil eluate fraction and isooctane extraction. (A) First 2 ml of eluate; without isooctane extraction. (B) First 2 ml of eluate; with isooctane extraction. (C) First 10 ml of eluate; without isooctane extraction. (D) First 10 ml of eluate; with isooctane extraction. In each instance the eluate was evaporated and diluted to 4 ml with water before injecting 2 ml into the HPLC system.

On-line sample enrichment and purification

Normally, a sample volume of only up to about 200 μl can be injected directly on to a conventional 4.6 mm I.D. HPLC column without too much additional peak broadening. Therefore in residue analysis, in general, trace enrichment is necessary during sample pre-treatment. In our study, an off-line sample enrichment factor of only about 2.5 was achieved on going from the crude extract mixture to the final aqueous HPLC injection solution, as discussed above. Additional off-line enrichment would have required time-consuming and recovery-lowering procedures. Therefore, an alternative approach was chosen, using on-line HPLC purification and enrichment with short pre-columns, similar to a recently described study[23].

Two types of stainless-steel enrichment columns were tested: 10 × 2.1 mm I.D. and 60 × 4.6 mm I.D. The columns were packed with chemically bonded silica material such as 53-μm Partisil ODS-3 and 37–50-μm Bondapak C_{18}/Corasil and with polystyrene–divinylbenzene copolymers such as 50–100-μm XAD-4 and 15–25-μm PRP-1. Material with relatively large particle sizes was chosen to prevent column clogging due to residual matrix components present in the final extract, and to permit the use of a low-pressure pump to introduce the samples because a second HPLC delivery pump will not always be available.

The enrichment characteristics of the various materials were tested with CBX and desoxy-CBX as model compounds, these being the most polar and apolar compound, respectively.

Carbadox enrichment. Aliquots of 2 ml of a standard solution containing 20 ng were injected on to the enrichment column. After injection, the column was flushed with a variable volume (2–16 ml) of water. Next, the column was backflushed to the analytical column with eluent for 5 min, and the CBX response (as peak height) was determined after post-column derivatization. On each column tested, a series of 2-ml injections was applied using increasing flushing volumes. A decrease in the response

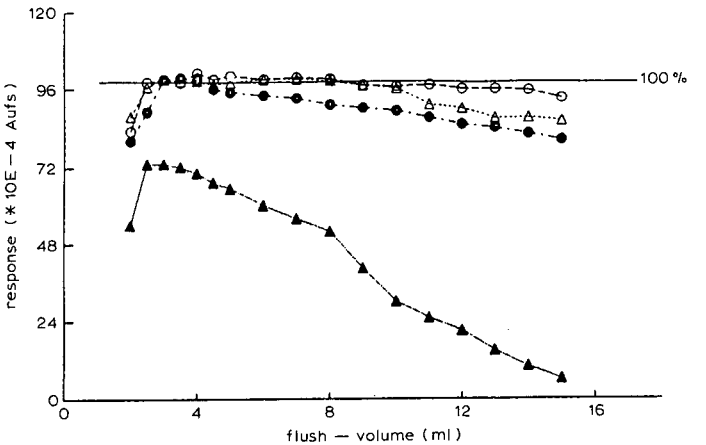

Fig. 7. Plot of the response at 420 nm obtained when 2-ml injections of a standard solution of carbadox (containing 20 ng) were applied to 60 × 4.6 mm I.D. enrichment columns filled with various packing materials *versus* the aqueous flushing volume applied before CBX was back-flushed to the analytical column. The straight line represents the response obtained after a direct 50-μl injection of 20 ng of CBX. For experimental conditions, see text. (○) Partisil ODS-3; (△) μBondapak C_{18}; (●) PRP-1; (▲) XAD-4.

at higher flushing volumes indicated that CBX was no longer fully retained on the column and/or that peak broadening occurred. Fig. 7 shows examples of response curves obtained according to this procedure for CBX on 60 × 4.6 mm I.D. columns. For comparison, the response obtained when 20 ng of CBX were introduced directly on to the analytical column as a 50-μl injection, without pre-column enrichment, is also shown. As can be seen, only the coarse polymerix XAD-4 material failed to retain CBX fully, and it also gave some additional peak broadening as indicated by the peak width at half-height, which increased by 15% on going from a flushing volume of 4 ml to 15 ml. The C_{18}-bonded phases all show good retention. This may be partly due to hydrogen bond interaction with the silanol groups (see below).

Desoxy-CBX enrichment. The retention behaviour of desoxy-CBX was tested in a similar way, but with fewer data points. In Table I the responses obtained after pre-column enrichment with three different aqeous flushing volumes are presented as a percentage of the maximum response obtained with a 50-μl direct injection. The desoxy-CBX results are comparable to those for CBX with regard to the behaviour on the C_{18} materials. However, with both polymer materials low responses are found, which indicates that polar interactions play an important role in the retention process.

TABLE I

EVALUATION OF ENRICHMENT PROCEDURE FOR DESOXY-CBX

Conditions: 20 ng of desoxy-CBX injected as a 2-ml injection on various 60 × 4.6 mm I.D. enrichment columns. Response compared with direct injection of 20 ng in 50 μl on to the analytical column.

Aqueous flushing volume (ml)	*Relative response (%) for column packed with*			
	XAD-4	*PRP-1*	*Bondapak-C_{18}*	Partisil ODS-3
2.5	50	50	100	100
10.0	55	65	100	100
15.0	55	65	100	100

The results imply that the N^1- and N^4-monooxy-CBX metabolites, with intermediate polarity, will probaly also be fully retained on the C_{18} bonded phases.

The Bondapak/Corasil C_{18} material was selected for further study because it is spherical and therefore generates less back-pressure than does Partisil, which is irregularly shaped.

Enrichment-column dimensions. Essentially, the retention characteristics of the 10- and 60-mm cartridges were similar under the experimental conditions used when standard solutions were injected. However, when spiked tissue extracts were injected, the 10-mm columns retained only about 30% of the CBX. Probably, surface modification of the small column due to sorption of residual matrix components partly blocks adsorption sites. The 60-mm columns, which contain 30 times more packing material, did not present this problem, as indicated by the 97–100% response obtained when CBX, N^4- and N^1-monooxy-CBX and desoxy-CBX were added to kid-

ney extracts (in the final 4 ml of aqueous phase) and analysed according to the pre-column enrichment HPLC procedure. A summary of the final analytical procedure is presented in Table II.

TABLE II

SUMMARY OF THE FINAL ANALYTICAL PROCEDURE

Parameter/step	*Value/conditions*
Sample size	10 g
Extraction	40 ml of acetonitrile–methanol (1:1)
Alumina–Florisil clean-up	Collect first 10 ml of eluate
Evaporate and dilute to	4 ml with water
Partition with	2 ml of isooctane
Inject	2 ml into the HPLC system (Fig. 2)
Enrichment-flush water flow-rate	0.5 ml/min
Enrichment column	60 × 4.6 mm I.D. 37–50-μm C_{18}-Bondapak/Corasil
Flushing time	20 min
Back-flushing time	5 min
Eluent	Acetonitrile–0.01 *M* sodium acetate, pH 6 (15:85)
Eluent flow-rate	0.6 ml/min
Analytical column	200 × 3 mm I.D., 5-μm Chromspher-C_{18}
Derivatization reagent	0.5 *M* sodium hydroxide
Derivatization flow-rate	0.23 ml/min
Reaction coil	2 m × 0.5 mm I.D. knitted PTFE
Detection	420 nm, 0.001 a.u.f.s.

Characteristics and applications of the analytical method

Preliminary precision and selectivity experiments were performed using the final method. Swine muscle, kidney and liver samples were spiked by injection of an aliquot (<0.5 ml) of a standard solution to the homogenized tissue sample and equilibration for 15 min. The samples were fortified with 1, 5 and 10 μg/kg of each CBX and desoxy-CBX. The amount of monooxy metabolites available during the study was not sufficient to include them in these spiking experiments. The results of the recovery experiments were pooled because no significant differences between matrices were observed. For CBX a mean recovery of 81% (coefficient of variation, C.V. = 10.3%; n = 12) was found for the 1, 5 and 10 μg/kg experiments. For desoxy-CBX a mean recovery of 87% (C.V. = 4.1%; n = 8) was found for the 5 and 10 μg/kg levels. A small positive bias, resulting from the slightly higher CBX concentration in the first 2 ml of alumina–Florisil eluate, is included in the observed recoveries. The high recoveries that were obtained indicate that no substantial analyte losses occur during the spiking and sample clean-up procedures (*i.e.*, enzymatic conversion in liver). However, future experiments will include stability studies for biological matrices. For CBX, 1 μg/kg could be determined in all matrices; for desoxy-CBX, however, 1 μg/kg was below the limit of detection. As the number of experiments was limited, an accurate calculation of the limit of detection was not possible. On the basis of the chromatograms obtained and with the equipment used, the estimated limits of detec-

tion are 0.5–1 μg/kg for CBX and 2–3 μg/kg for desoxy-CBX in liver, muscle and kidney. Preliminary experiments indicate that the limits of detection of the N^1- and N^4-monooxy-CBX metabolites are 1–2 μg/kg.

Fig. 8 shows typical chromatograms for blank and spiked muscle, liver and kidney samples. Very clean chromatograms were obtained even at the very sensitive detector setting of 0.001 a.u.f.s. Obviously, the combination of detection at 420 nm and off-line plus on-line sample purification removes interferences due to lipids, proteins, colouring agents or other animal tissue components very effectively. The method also seems applicable to other biological matrices.

About 20 samples can be analysed per day, which makes the method suitable for monitoring, surveillance and pharmacokinetic studies. In order to test the selectivity of the method, a number of veterinary drugs were injected into the HPLC system at concentrations corresponding to about 100 μg/kg of tissue. None of the compounds listed in Table III interfered in the determination of CBX or its N^1- and N^4-monooxy of desoxy metabolites. The nitrofuran drugs furazolidone, nitrofurazone, furaltadone and nitrofurantoine, however, also reacted with sodium hydroxide and eluted between CBX and desoxy-CBX, thus potentially interfering with the determi-

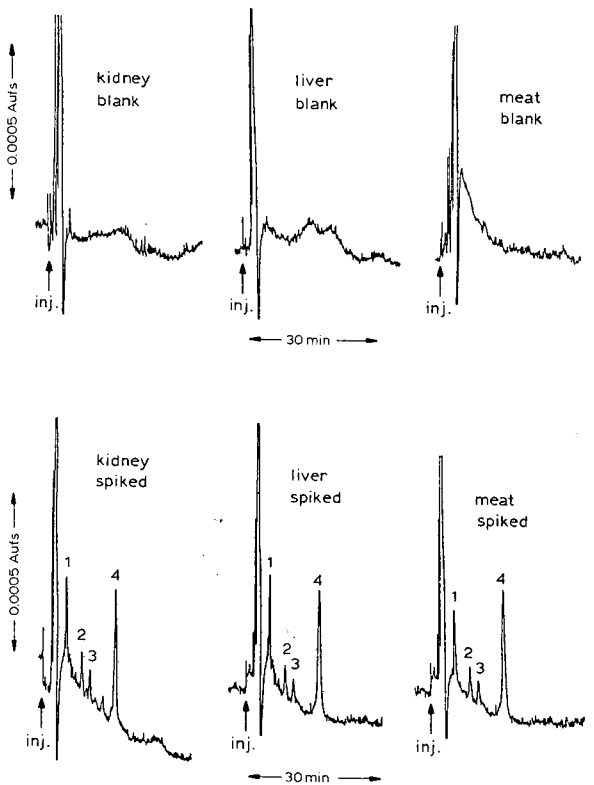

Fig. 8. Chromatograms of blank swine kidney, liver and muscle samples and corresponding samples spiked with 1 μg/kg of (1) CBX, (2) N^4-monooxy-CBX, (3) N^1-monooxy-CBX and (4) 5 μg/kg of desoxy-CBX. Detection at 420 nm, 0.001 a.u.f.s. For other experimental conditions, see text.

TABLE III

VETERINARY DRUGS THAT DID NOT INTERFERE WITH THE DETERMINATION OF CBX, N^1-MONOOXY-CBX, N^4-MONOOXY-CBX AND DESOXY-CBX

Standard solutions corresponding to a tissue concentration of 100 μg/kg were injected into the HPLC-system.

Drug	*Drug*
Chloramphenicol	Nitrovin
Chlorotetracycline	Olaquindox
Clopidol	Oxytetracycline
Dapsone	Pyranteltartrate
Decoquinate	Robenidine
Dimetridazole	Ronidazole
Dinitolmide	Sulphadiazine
Doxycycline	Sulphanilamide
Ethopabate	Sulphadimethoxine
Fenbendazole	Sulphadoxine
Furnicozone	Sulphamerazine
Halofuginone	Sulphamethazine
Ipronidazole	Sulphamethoxazole
Methylbenzoquate	Sulphaquinoxaline
Nicarbazin	Tetracycline
Nifursol	Thiophanate
	Trimetoprim

nation of N^1- and N^4-monooxy-CBX. In further studies, we shall attempt to include the important group of nitrofurans in the method in order to obtain a comprehensive method capable of monitoring the presence of both CBX and its metabolites and nitrofurans in animal tissues.

CONCLUSIONS

A rapid, sensitive and selective method has been developed for the trace-level determination of carbadox and its monooxy and desoxy metabolites in swine muscle, liver and kidney tissue. A liquid–liquid extraction followed by a simple alumina–Florisil clean-up and partitioning with isooctane is combined with column-switching HPLC with the post-column addition of alkali to convert the analytes of interest into yellow compounds that can be detected at 420 nm. The method permits the determination of concentrations down to 1 μg/kg of CBX and its monooxy metabolites and down to 5 μg/kg of desoxy-CBX. Preliminary recovery experiments indicate a good precision with a mean recovery of about 85%. About 20 samples can be analysed per day. The method also seems applicable to other biological matrices.

Further research is planned to study the ruggedness of the method, the incorporation of nitrofuran drugs in the method and the confirmation of positive samples by fluorescence, diode-array UV–VIS and mass spectrometric detection.

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RECOVERY OF ORGANIC COMPOUNDS FROM LARGE-VOLUME AQUEOUS SAMPLES USING ON-LINE LIQUID CHROMATOGRAPHIC PRECONCENTRATION TECHNIQUES

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SUMMARY

It is demonstrated that in order to achieve maximal solute preconcentration on one or two given precolumns, it is necessary to use a sample volume larger than the breakthrough volume, even at the risk of obtaining recoveries lower than 100%. This is an important condition when traces of organic pollutants have to be determined in water samples with relatively low organic contamination.

A theoretical model is proposed for such conditions using a single precolumn or two precolumns in series and the experimental feasibility of such an approach (sample volume > breakthrough volume) is demonstrated. In addition, a critical comparison is made of different experimental methods for measuring amounts of preconcentrated solutes and the corresponding recoveries. A recommended procedure for the measurement of recoveries is proposed, involving the determination of variations of peak areas obtained during the determination of preconcentrated compounds in increasing volumes of water samples. In each sample, the concentration of the solutes is adjusted in order to have a constant amount. This method has the advantage that recoveries are measured under the same chromatographic conditions as those used for the real sample. At the same time breakthrough volumes can be estimated. Several solutes can be studied simultaneously and the efficiency of spiking can also be tested by this approach. A range of organic pollutants of medium to low polarity and two adsorbents, *viz.*, a bonded C_{18} silica and the polymer-based PRP-1, were chosen as a model system.

INTRODUCTION

The low concentrations of organics in relatively pure water (*e.g.*, natural compounds or pollutants in drinking water) necessitate the development of analytical techniques sensitive at the sub-ppb level. At this level, a concentration step is necessary before the analysis proper and the problem of component recoveries becomes paramount. In order to minimize sample loss and the risk of contamination and to

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increase the potential for automation, sample transfer steps, solvent evaporation and any manual manipulation should be eliminated or reduced to a minimum.

Sample concentration based on liquid–liquid sorption techniques has been shown to be a good alternative to liquid–liquid extraction, which requires several sample handling steps and evaporation of large solvent volumes[1]. When using off-line concentration techniques, trace components are concentrated on a convenient sorbent and then eluted by a small volume of suitable solvent; this volume has to be reduced and frequently only an aliquot is injected into the analytical system; in addition to the inherent disadvantage of an increased detection limit, there are still handling steps of the concentrated sample so that losses and contamination risks can still occur. For quantitative analysis one has to verify that there is no loss or degradation during the evaporation step; some studies have reported evaporation losses depending on the solute concentration and on the final volume[2], so that internal standards are often required.

Many of these drawbacks can be avoided by using on-line enrichment on precolumns. Trace compounds are trapped on a precolumn packed with a convenient sorbent; the precolumn is coupled to an analytical column via switching valves and the compounds adsorbed are then eluted directly from the precolumn to the analytical column with a suitable mobile phase. If adsorption and desorption are efficient, one can expect more accurate quantitative results as there is no sample manipulation between preconcentration and analysis.

Pertinent applications of on-line preconcentrations have been reported for the determination of specific compounds in complex aqueous mixtures[3–9]. Selectivity towards specific compounds can be provided with proper detection methods[10–12], but it can also be increased during the sample handling step by coupling different precolumns in series and by using more selective precolumns such as ion-exchange resins[4,13–15] or metal-loaded sorbents[16–19]. There are also examples of more general analyses, the compounds studied being very different in size and polarity; again, for greater selectivity in the sample handling steps the use of more than one precolumn is unavoidable. One example is the determination of organics in waste waters[14]; combination of three precolumns gives a satisfactory group separation prior to chromatographic analysis: a C_{18} bonded phase traps the non-polar compounds, a styrene–divinylbenzene copolymer the moderately polar compounds and a cation exchanger the more polar cations. In these procedures, each precolumn is eluted separately and the eluate analysed.

We applied this technique to drinking water by increasing the sample volume to 500 ml. When many compounds are to be quantified simultaneously, they are not concentrated on only one precolumn but often some solutes are recovered from two precolumns. Hence one has to take into account the recovery from each precolumn, because for some solutes breakthrough occurs so that the recovery is not complete whereas for others the recovery is 100%. When applying a comparison with pure water samples spiked with known amounts of solutes or a comparison with direct loop injection, solute amounts are quantified but the determination of the concentrations in the sample being analysed is linked to a knowledge of the recoveries. The aim of this study was to examine the recoveries in quantitative analyses when applying precolumn techniques to large sample volumes utilizing more than one precolumn. Although it is generally assumed that it is better to work with a 100% recovery, it will

be shown that for trace analysis the most important point is the amount available for detection, which has to be as high as possible for most compounds even if for some of them the recovery is not complete.

GENERAL CONSIDERATIONS FOR RECOVERY CALCULATION

Description of precolumn technique with two precolumns in series

Samples are percolated through two precolumns in series. In order to prevent band broadening in the analysis step, small sizes (about 1 cm × 2 mm I.D.) are chosen[20–23]. The affinity of solutes is stronger for the second than for the first precolumn[24]. After percolation of a known sample volume, the two precolumns are flushed with pure water in order to remove interferents. Each precolumn is then eluted separately on-line by the mobile phase to the analytical column. Extraction of solutes by sorbents is complete if breakthrough of analytes does not occur. Two criteria are responsible for breakthrough during the concentration step, *viz.*, the retention and the capacity.

Breakthrough volume

The most important parameter for trace analysis is the sensitivity of the method (or the minimal detectable concentration of the compound in water), determined by the sensitivity of the detector used, the compounds under study, the adsorption capacity of the sorbent in the precolumn, the sample volume, the desorption procedure and the chromatographic procedure. On percolating water containing organic compounds through a concentration column, a chromatographic process occurs which proceeds as frontal chromatography because the flow-rate of the water and the concentrations of compounds are constant. Compounds are extracted from the water by the sorbent according to their partition coefficients for the water–sorbent system. If sorption occurs, the water leaving the precolumn is free from solutes; after a certain percolated volume, breakthrough occurs and the effluent water contains the compounds again.

Fig. 1 shows frontal curves for two solutes having different affinities for sorbent A (solid line) and for sorbent B (broken line). These frontal curves were obtained by percolating water through only one precolumn at a time. Under ideal conditions, they have a bilogarithmic shape and the inflection point is the retention volume, V_r, of the solute eluted by pure water if the column is not overloaded. At 1% of the sample UV absorbance, we define the breakthrough volume, V_f, which corresponds to the sample volume that can be percolated without any elution of analyte; at 99% of the UV absorbance we define the maximal percolated volume, V_m. Theoretical studies of on-line preconcentration have been presented by Werkhoven-Goewie *et al.*[3,25] and Nondek and Chvalovsky[22,23]. We have the relationships

$$V_f = V_r - 2\sigma_v \quad (1)$$

and

$$V_m = V_r + 2\sigma_v \quad (2)$$

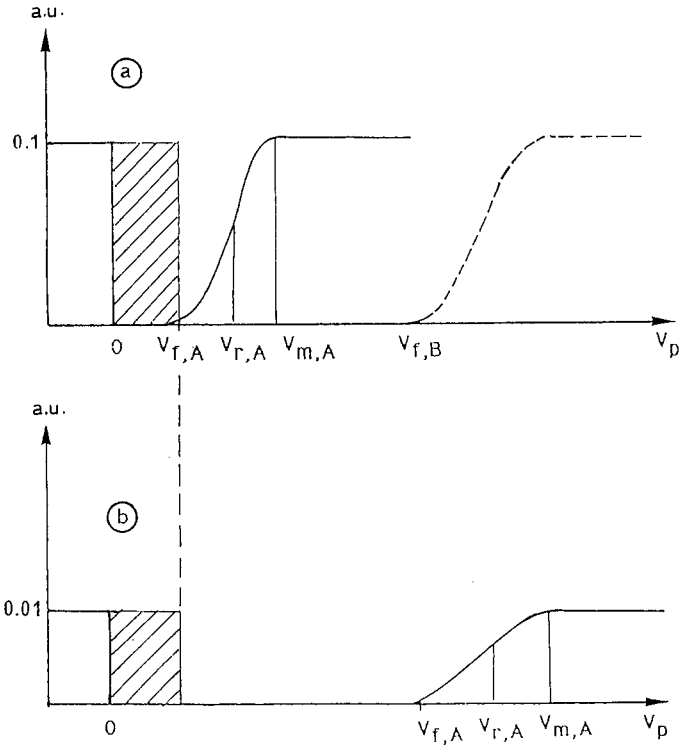

Fig. 1. Determination of breakthrough volumes from the recording of frontal chromatograms for two water samples spiked with (a) solute 1 and (b) solute 2. The sample UV response (total absorbance) is measured by coupling directly the spiked sample with the detector. At $V_p = 0$, sample is percolated through the precolumn; V_f is measured at 1%, V_r at 50% and V_m at 100% of the total absorbance. The solid line and broken line represent frontal chromatograms recorded with precolumn packed with sorbent A alone and with precolumn packed with sorbent B alone, respectively.

where σ_v is the standard deviation which is dependent on the axial dispersion of analyte along the bed of particles in the precolumn and can be expressed in terms of precolumn length (L), particle diameter (d_p) and reduced plate height (h) according to

$$\sigma_v = V_r (hd_p/L)^{\frac{1}{2}} \tag{3}$$

For trace analysis, the enrichment factor (f) is of prime importance; this term, defined by the ratio between the final concentration and the sample concentration can be expressed in terms of sample volume (V_p) desorption volume (V_d) and recovery (R) by

$$f = (V_p/V_d)R \tag{4}$$

Increasing f means increasing both the sample volume and recovery and reducing the desorption volume, so that preconcentrated analytes have to be eluted from the precolumn by the smallest volume equal at least to the void volume of the precol-

umn. Increasing f is at first an optimization of the precolumn geometry, which has been widely studied[21–25]; another method has been considered by Nondek and Chvalovsky[23], which consists in percolating a higher volume than V_m through the precolumn in order to extract a maximal amount of solute. This is particularly advantageous for analytes which have low V_r values. The maximal extracted amount is $V_r C_p$ and is obtained for a percolated volume V_p equal or higher than V_m owing to the symmetrical form of the frontal curve.

Recovery, defined by the ratio (amount extracted)/(amount percolated), is 100% only for $V_p < V_f$; the maximal amount extracted does not correspond to a 100% recovery but is reached by percolating a volume higher than V_m, in spite of a lower recovery. From a practical point of view, quantitative analysis is easier when the recovery is 100%. However, in addition to the case of poorly retained solutes as pointed out by Nondek and Chvalovsky[23], when several solutes are to be quantified simultaneously, Fig. 1 shows that it can be necessary to carry out analyses with partial recoveries; if solutes 1 and 2 are together, a 100% recovery for both solutes is reached for a maximal percolated volume equal to $V_{f,A}$ and the amount of solute 1 and 2 extracted by precolumn A correspond to the hatched areas in Fig. 1. If solute 2 has poor UV absorbance properties, the amount extracted is not enough to be detected, so the percolated volume has to be increased; the amount of solute 1 adsorbed remains constant but its recovery is then below 100%. When solutes are numerous it is impossible to adjust the sample volume in order to have 100% recovery for each one and the above situation will be encountered.

Let us now consider the second precolumn B coupled after A. For solute 1, breakthrough occurs for $V_{f,A}$ for precolumn A and for $V_{f,B}$ if precolumn B is alone; when A and B are coupled, breakthrough occurs for $V_{f,A} + V_{f,B}$; in fact, it is a first approximation because, for the second precolumn, conditions for frontal analysis are not required as the concentration of compound entering the second precolumn is not constant and increases depending on the shape of the breakthrough front from the first precolumn. Solute 1 will be extracted by both A and B for a percolated volume higher than $V_{f,A}$ and a knowledge of $V_{f,A}$ and $V_{f,B}$ is necessary in order to determine the respective recoveries.

Retention data are important because V_f can be estimated to a first approximation from V_r values. These retention values are often time consuming to measure in pure water. In reversed-phase chromatography, retention data can be extrapolated to pure water conditions from data known at two percentages of organic modifier because of the logarithmic relationship between capacity factor and methanol content[3,5]. There is also a very useful logarithmic relationship between capacity factors in water and the water–octanol partition coefficients of solutes[26,27].

Capacity

Breakthrough can occur if the precolumn capacity is exceeded. In overloading conditions, the volume denoted V_r does not correspond to the retention volume of solutes. The capacity of the sorbent depends on the type of stationary phase, on the bed volume of the precolumn and on the nature of the solute. One has to verify that the concentrations of analytes and interferences are low and that breakthrough due to overloading of the precolumn does not occur, otherwise the proposed theory is not valid.

TABLE I

EQUATIONS FOR EXTRACTED AMOUNTS, Q, AND RECOVERIES, R, ON C18 AND PRP1 DEPENDING ON PERCOLATED SAMPLE VOLUMES, V_p

See Fig. 1 for definition of characteristic volumes measured on breakthrough curves.

V_p	Q_{C18}	Q_{PRP1}	R_{C18}	R_{PRP1}
$< V_{f,C18}$	C_pV_p	0	1	0
$> V_{f,C18}$ $< V_{m,C18}$	$C_pV_{f,C18} +$ $+ \int[C_p - y(V_p)]dV_p$	$\int y(V_p)dV_p$	$1 - \int[y(V_p)/C_pV_p]dV_p$	$\int[y(V_p)/C_pV_p]dV_p$
$> V_{m,C18}$ $< V_{f,PRP1}$	$C_pV_{r,C18}$	$C_p(V_p - V_{r,C18})$	$V_{r,18}/V_p$	$1 - V_{r,C18}/V_p$
$> V_{f,PRP1}$ $< V_{m,PRP1}$	$C_pV_{r,C18}$	$C_p(V_{f,PRP1} - V_{r,C18}) +$ $+ \int[C_p - Z(V_p)]dV_p$	$V_{r,C18}/V_p$	$1 - (V_{r,C18}/V_p)$ $- \int[z(V_p)/C_pV_p]dV_p$
$> V_{m,PRP1}$	$C_pV_{r,C18}$	$C_p(V_{r,PRP1} - V_{r,C18})$	$V_{r,C18}/V_p$	$(V_{r,PRP1} - V_{r,C18})/V_p$

Theoretical recovery calculations from breakthrough curves

In this study the two coupled precolumns were packed with C_{18} bonded silica and with the styrene–divinylbenzene copolymer PRP-1 and will be denoted C18 and PRP1, respectively. The characteristic volumes in Fig. 1 were measured separately on each precolumn. When the water sample is percolated through the two precolumns, $V_{f,C18}$, $V_{r,C18}$ and $V_{m,C18}$ correspond to the volumes obtained when C18 is need alone, whereas $V_{f,PRP1}$, $V_{r,PRP1}$ and $V_{m,PRP1}$ represent the volumes obtained with PRP1 alone plus the values obtained for C18 alone. For most of the neutral solutes, these volumes increase in the following order:

$$V_{f,C18} < V_{r,C18} < V_{m,C18} < V_{f,PRP1} < V_{r,PRP1} < V_{m,PRP1} \quad (5)$$

The curves representing the front will not be mathematically expressed and are denoted $y(V_p)$ for C18 and $z(V_p)$ for PRP1. The amounts extracted on C18, PRP1 and on both (total) are Q_{C18}, Q_{PRP1} and Q_T, respectively, and the corresponding recoveries are R_{C18}, R_{PRP1} and R_T. The equations and corresponding data are reported in Table I and Fig. 2 shows the variations of (a) extracted amounts and (b) recoveries with the percolated sample volume. Variations of the extracted amounts are simple: for a sample volume smaller than the breakthrough volume of the first precolumn, $V_{f,C18}$, the extracted amount and percolated amount are equal and the recovery is 100%. For a percolated volume greater than $V_{f,C18}$, the amount extracted by C18 increases to a maximal value but is different from the percolated amount; the fraction eluted from C18 by the water sample is then extracted by the second precolumn PRP1 until $V_{f,PRP1}$ is reached. The recovery from C18 decreases whereas that from PRP1 increases, but the total recovery is always 100%. The maximal extracted amount is then obtained for a sample volume $V_{m,PRP1}$ but corresponds to a total recovery below

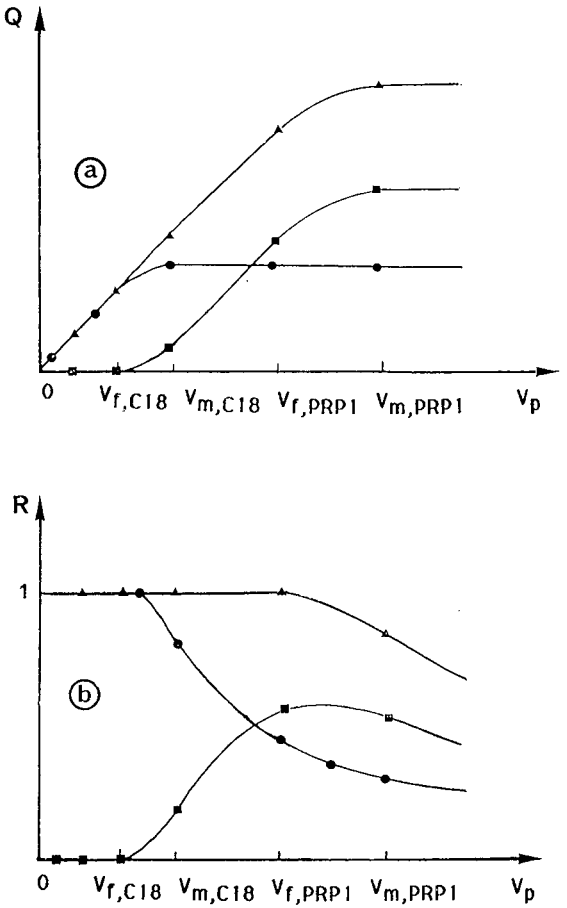

Fig. 2. Variations of (a) extracted amounts and (b) recoveries with percolated volume from C18 (●), from PRP1 (■) and total (▲) when the two precolumns are coupled together.

100%. We also note that when V_p is between V_f and V_m, the recovery and extracted amounts cannot be exactly calculated as the front curve has to be known.

Variations of extracted amounts and recoveries with percolated volumes are useful. In trace analysis, detection limits are the main interest. From Fig. 2a and b, the best condition for detection is to work with a sample volume higher than $V_{m,PRP1}$ because the amount extracted by PRP1 is higher than that extracted by C18 even if the corresponding recovery is then only 50%. This point is particularly important for solutes having poor detection properties.

Two procedures are carried out to determine solute concentrations in samples. The first consists in determining solute amounts by injecting directly a known amount of solute into the analytical column via a conventional loop injection; hence the amount in the percolated sample is determined by calibration graphs for the detector and of course recovery data are necessary in order to calculate the concentration. A second method, more widely used, consists in spiking water samples with known amounts of solutes and then measuring the corresponding extracted amount. This

method does not necessitate knowing recoveries if spiked samples have exactly the same volume as unknown samples and if the solubilization of the solutes on spiking is complete. If the sample volumes are changed, other spikings have to be performed; there are many examples where the sample volume has to be adapted for the same analysis (*e.g.*, determination of pollutants in waste waters and drinking waters).

It is therefore of interest to be able to optimize and adapt the sample volume to the analytical problem at hand by considering possible the initial concentrations and the detection mode. Recovery determinations are therefore necessary.

Theoretical calculations can be effectively made from breakthrough curves but we shall also show that from spiked samples it is possible, under certain conditions, to determine both recoveries and extracted amounts. In this first part we have shown a theoretical determination of recovery from the elution front, but we can also measure these recoveries directly with the two precolumns in series.

Experimental recovery calculation from peak areas

Percolation of several sample volumes is carried out with constant percolated amounts of solutes; hence, when the percolated sample volume increases, the concentrations (C_p) are adjusted in order to have a constant amount (C_pV_p). Depending on V_p and the breakthrough values, the solutes being determined are extracted only on the first precolumn or on both. On elution to the analytical column and analysis, peak areas can be measured and are proportional to the amount extracted. Fig. 3 shows two chromatograms obtained for 10 ml of water spiked with three herbicides at 120 μg/l and for 150 ml of water spiked with the same compounds at 8 μg/l. With the 10-ml sample, the three herbicides are extracted only by C18 whereas with the 150-ml sample they are recovered on both C18 and PRP1.

Let us consider first that only one precolumn is used (*e.g.*, C18). If V_p is smaller than $V_{f,C18}$, Fig. 2 shows that extracted and percolated amounts are equal: as the percolated amount is constant from one percolation to another, the peak area is constant and for one given solute we have the condition

$$A(V_p) = kC_pV_p \tag{6}$$

When V_p is higher than $V_{m,C18}$, the extracted amount becomes equal to $C_pV_{r,C18}$, so that

$$A(V_p) = kC_pV_{r,C18} \tag{7}$$

Because the concentrations of the solutes in the sample are adjusted in order to have a constant percolated amount, C_p can be expressed in terms of C_{p1} and V_{p1}, for the first concentration and the first sample volume, respectively, and we then have

$$C_p = (C_{p1}V_{p1})/V_p \tag{8}$$

Denoting by A_1 the first percolation ($A_1 = kC_{p1}V_{p1}$) for which it is verified that V_{p1} is smaller than $V_{f,C18}$, eqn. 7 is expressed by

$$A(V_p) = A_1(V_{r,C18}/V_p) \tag{9}$$

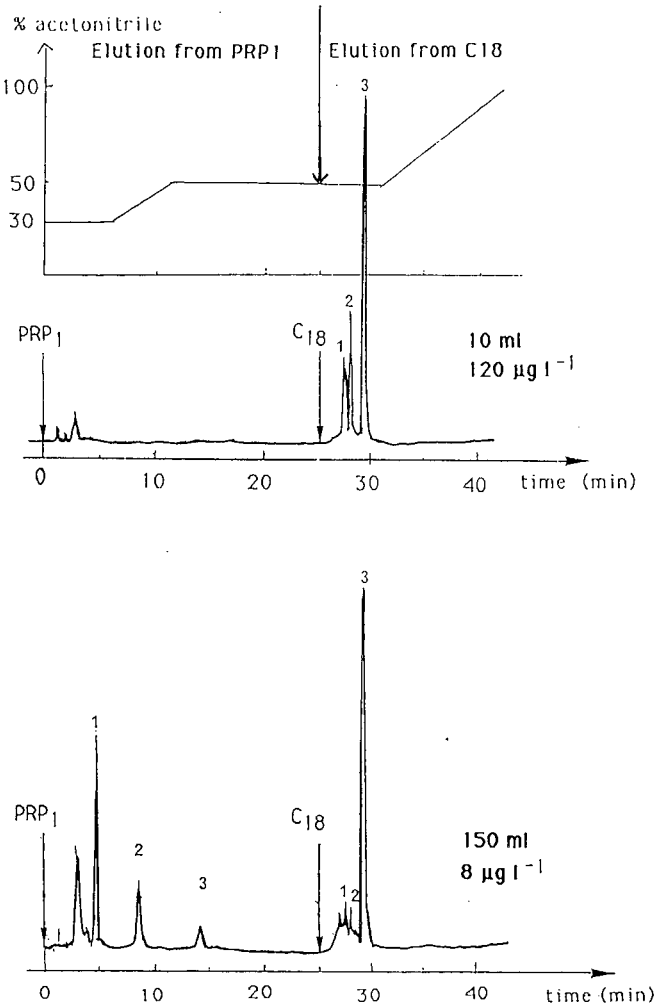

Fig. 3. Analysis of two water samples spiked with the same amounts of herbicides: (a) preconcentration of 10 ml of water spiked at 120 µg/l for each solute; (b) preconcentration of 150 ml of water spiked at 8 µg/l for each solute. Solutes: 1 = simazine; 2 = atrazine; 3 = linuron. Preconcentration through two precolumns (1 × 0.21 cm I.D.) in series packed with RP-18 silica and PRP-1 copolymer at a flow-rate of 3 ml/min; elution to analytical column (ODS-2; 15 × 0.46 cm I.D.) of PRP-1 first by a acetonitrile gradient from 30 to 50% and then RP-18 from 50 to 100% at a flow-rate of 1.5 ml/min.

If peak areas are normalized by areas obtained for a first percolation under A_1 conditions, we have the following relationships:

$$V_p < V_{f,C18}: \qquad A(V_p)/A_1 = 1 \tag{10}$$

and

$$V_p > V_{m,C18}: \qquad A(V_p)/A_1 = V_{r,C18}/V_p \tag{11}$$

Comparison of these with those in Table I indicates that they represent recoveries obtained when C18 is used alone.

When the two precolumns are used in series, there is a decrease of peak areas with V_p obtained on C18 elution which is accompanied by an increase of peak areas from PRP1. With the same notations for the characteristic volumes as those defined for breakthrough studies, the values of the normalized areas obtained on C18 and PRP1 on elution and analysis of extracted compounds are as follows:

$V_p < V_{f,C18}$:

$$A(V_{p,C18})/A_1 = 1 \quad (12)$$

$$A(V_{p,PRP1})/A_1 = 0 \quad (13)$$

$V_{m,C18} < V_p < V_{f,PRP1}$:

$$A(V_{p,C18})/A_1 = V_{r,C18}/V_p \quad (14)$$

$$A(V_{p,PRP1})/A_1 = 1 - (V_{r,C18}/V_p) \quad (15)$$

$V_p > V_{m,PRP1}$:

$$A(V_{p,C18})/A_1 = V_{r,C18}/V_p \quad (14)$$

$$A(V_{p,PRP1})/A_1 = (V_{r,PRP1} - V_{r,C18})/V_p \quad (16)$$

Comparing eqns. 12–16 with the calculations in Table I, one can see that the normalized peak areas represent exact recoveries; measurement of peak areas when preconcentrating several sample volumes spiked with a constant amount of solute allows one to determine recoveries and extracted amounts.

EXPERIMENTAL

Apparatus

Percolation of water was performed with a Milton Roy pump (LDC, Riviera Beach, FL, U.S.A.) and precolumn elutions and analyses were carried out with a Model 5060 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with a UV 200 variable-wavelength UV spectrophotometer, a Polychrom 9060 diode-array detector (Varian) and a fluorimetric detector (Kratos, Ramsey, NJ, U.S.A.). Precolumn and analytical column switching was performed with two valves (Rheodyne, Berkeley, CA, U.S.A.). Quantitative measurements of peak areas were made with a CR3A integrator–computer (Shimadzu, Kyoto, Japan).

Stationary phases and columns

Water samples were preconcentrated on 1 cm × 2.1 mm I.D. stainless-steel precolumns available from Chrompack (Middelburg, The Netherlands), which were packed manually with a thick slurry using a microspatula or with a thin slurry using a syringe. The stationary phases were 10-μm octadecylsilica RP-18 (Merck, Darmstadt, F.R.G.) and spherical 10-μm styrene–divinylbenzene copolymer PRP-1 (Hamilton, Reno, NV, U.S.A.). The analytical column was a 15 cm × 4.6 mm I.D. stainless-steel column prepacked with 5-μm octadecylsilica Spherisorb ODS 2 (Whatman, Clifton, NJ, U.S.A.).

Chemicals

HPLC-grade acetonitrile was obtained from Rathburn (Walkerburn, U.K.)

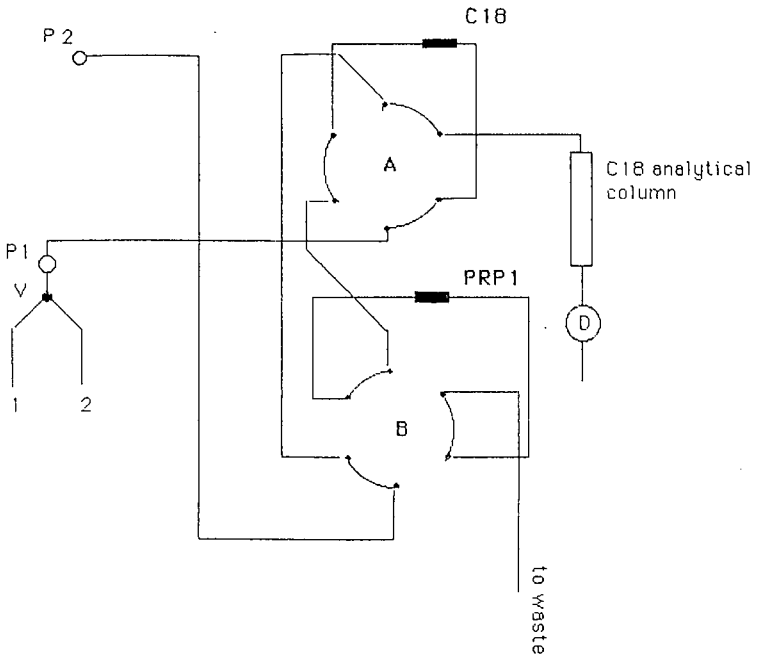

Fig. 4. Experimental set up for the on-line trace enrichment and analysis of water samples. P1 = pre-concentration pump; P2 = high-pressure pump; V = low-pressure valve for sample (1) and flushing with 0.001 *M* perchloric acid (2); A and B = high-pressure six-port switching valve; C18 = precolumn (1 x 0.21 cm I.D.) packed with RP-18 silica; PRP1 = precolumn (1 × 0.21 cm I.D.) packed with PRP-1 copolymer; analytical column, 15 × 0.46 cm I.D. packed with ODS-2 silica.

and methanol from Prolabo (Paris, France). Water was deionized and evaporated in a quartz apparatus from Quartex (Paris, France). The various solutes were supplied by Prolabo, Merck or Fluka (Buchs, Switzerland).

Procedure

Stock solutions of selected solutes were prepared by weighing and dissolving them in methanol. LC-grade water samples were spiked with these solutions at the ppb level and adjusted to pH 3 with perchloric acid. The final standard solutions did not contain more than 0.5% of methanol.

Breakthrough curves of selected analytes were recorded as follows: the standard solution was first directly coupled to the detector to measure the absorbance of the studied solute and then coupled to the precolumn (which has been previously conditioned) at a flow-rate of 2.5 ml min^{-1}.

The experimental set up is shown in Fig. 4 according to ref. 14. A water sample was introduced via pump P1 onto the two precolumns in series; the precolumns were flushed with 4 ml of 10^{-3} *M* perchloric acid. Each precolumn was then separately coupled to the analytical column by switching valve A or B and backflush-eluted by an acetonitrile gradient via pump P2. Precolumns in series were cleaned with pure acetonitrile and regenerated with 25 ml of 10^{-3} *M* perchloric acid.

RESULTS AND DISCUSSION

Breakthrough curves

Fig. 5 shows breakthrough curves recorded for a few solutes such as phthalates, herbicides and phenolic compounds on (a) C18 or (b) PRP1. Neither of these frontal analysis chromatograms has the ideal shape of a bilogarithmic front which would correspond to a gaussian peak in elution chromatography and the fronts are different from one solute to another. One reason is that the geometric volumes of these precolumns are small (35 μl) and, as they are filled with a microspatula, their plate number is not very high. Another reason is that solutes have different exchange kinetics between the sorbent and the aqueous mobile phase. Breakthrough volumes and maximal volumes were measured on the curves at 1% and 99% of the sample absorbance and retention volumes at 50%. With non-symmetrical curves, this determination of retention volumes is an approximation and one should probably use momentum theory to obtain more accurate retention values from breakthrough curves. These values are reported in Table II. The breakthrough volumes are higher with PRP1 than with C18 for all solutes except for trinitrophenol, which is in fact ionized at pH 3 because of the influence of the three nitro groups (pK_a = 0.3); it can be easily verified that apolar compounds are extracted by C18; for instance, when percolating 100 ml with solutes such as linuron and diethyl phthalate of course all other phthalates and less polar solutes (hydrocarbons, etc.) will also be adsorbed. Moderately polar compounds such as phenolics are better extracted by PRP1 than by C18. When percolating 100 ml of 2,4,5-trimethylphenol (solute No. 5) it is impossible to neglect the amount extracted by C18, whereas for 2-nitrophenol (solute No. 2) one can neglect it although these two solutes have similar breakthrough volumes on PRP1. It is also noticeable that there is a great difference beween V_r and V_f; *e.g.*, simazine has a

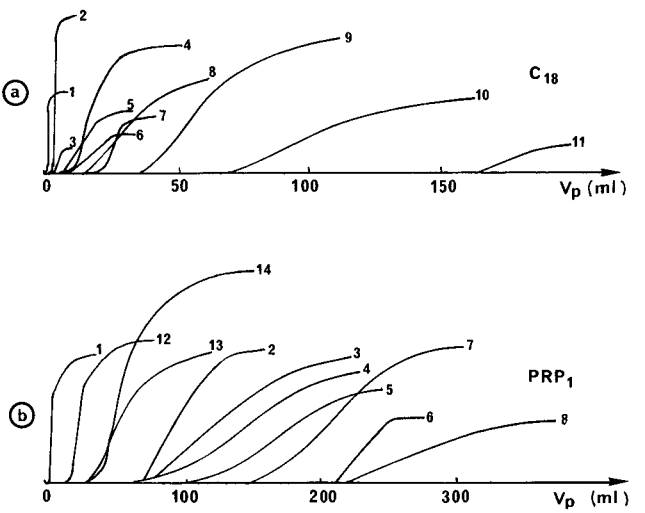

Fig. 5. Breakthrough curves for solutes with (a) C18 packed with RP-18 and (b) PRP1 packed with PRP-1 copolymer. Samples: dilute solutions in water at pH 3 (10^{-3} *M* perchloric acid); flow-rate, 2.5 ml/min; UV detection at variable wavelength. See Table II for solute numbers.

TABLE II

CHARACTERISTIC VOLUMES (ml) FROM BREAKTHROUGH CURVES RECORDED SEPARATELY ON C18 AND PRP1

V_f = Breakthrough volume corresponding to 1% of total front height; V_r = retention volume corresponding to 50% of total height; V_m = maximal volume corresponding to 99% of total height.

No.	*Solute*	*C18*			*PRP1*		
		V_f	V_r	V_m	V_f	V_r	V_m
1	2,4,6-Trinitrophenol	0.1	0.7	5	2	5	30
2	2-Nitrophenol	1.5	2.5	7	70	95	130
3	Toluene	2.5	5	9	65	140	230
4	*m*-Xylene	6	14	27	65	150	250
5	2,4,5-Trimethylphenol	5	15	34	60	145	220
6	Dimethyl phthalate	7	15	28	212	230	270
7	2,4,6-Trichlorophenol	14	31	58	215	283	360
8	Simazine	19	26	36	130	207	280
9	Atrazine	37	60	110	–	–	–
10	Linuron	70	105	165	–	–	–
11	Diethyl phthalate	165	180	195	–	–	–
12	2-Methylphenol	–	–	–	15	23	61
13	2,4-Dinitrophenol	–	–	–	27	50	103
14	2,4-Dimethylphenol	–	–	–	26	52	105

breakthrough volume of 130 ml on PRP1 and a retention volume of 207 ml; therefore, calculation of V_f from V_r, widely used in the literature, is to be considered only as a first approximation.

Precolumn capacity

Fig. 6 shows breakthrough curves recorded for increasing concentrations of dimethyl phthalate in water on C18. For water spiked with 0.3 and 0.9 ppm, breakthrough occurs at the same percolated volumes (and V_r is the same), but for higher concentrations the breakthrough volumes decrease. Assuming a Langmuir adsorption isotherm, overloading occurs when 20 μg of dimethyl phthalate are adsorbed on C18. The capacity depends on the size of the solute and on its steric configuration. Under the same conditions, it was estimated as 50 μg for xylene. Our results correspond to an adsorption of up to 4 mg/g of bonded silica for xylene. The results in the literature vary considerably and of course depend on the solutes and on the types of bonded silicas. Capacity values for bonded silicas up to 15–60 mg/g of packing material have been reported[24]. Although we did not measure the capacity of PRP1, it is considered to be higher than that of C18. A value reported earlier[8] is 186 mg/g for 2,6-dichlorophenol, which is much higher than the capacity of C18. Another study[27] estimated that the capacities of both C18 and PRP1 are greater than 1 mg/g of sorbent.

One has to make sure that no breakthrough occurs due to overloading of sorbent in the precolumns. Although we should consider the total concentration of both solutes and interferences, concentrations in surface water samples typically are at the ng/l to the μg/l level, so that overloading is rather unlikely to occur. The concentrations of standard solutions were adjusted so that the sum of the adsorbed species was always below 15 μg in all our investigations.

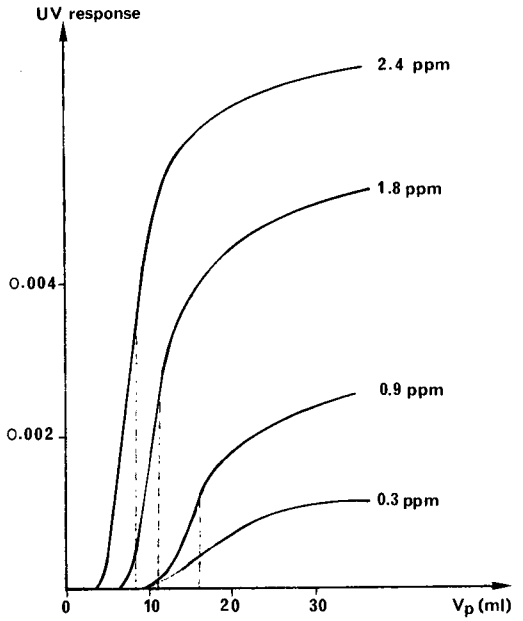

Fig. 6. Estimation of the capacity of C18 by recording breakthrough curves for water samples with increasing concentrations of dimethyl phthalate. Precolumn (1 × 0.21 cm I.D.) packed with RP-18 silica; flow-rate, 2.5 ml/min; UV detection at 260 nm; sensitivity, 0.1 a.u.f.s.

Recoveries from breakthrough volumes

Recoveries are calculated from data reported in Tables I and II. Variations with sample volume are shown in Fig. 7 for six solutes with increasing breakthrough volumes. For a solute such as nitrophenol, having a small $V_{f,C18}$, the recoveries from C18 and from PRP1 are 10% and 90%, respectively, for a 25-ml sample volume, whereas the same values are obtained for dimethyl phthalate with a 150-ml sample volume.

Frontal analysis is therefore a powerful method for rigorously calculating recoveries but requires the measurement of the characteristic volumes from breakthrough curves. The curves in Fig. 7 also show that a knowledge of retention data is not sufficient for an accurate determination of recovery.

Before measuring experimental recoveries by percolations with constant amount of solutes, one must also test the desorption efficiency of the coupled system, as the detected amount is generally determined via the whole system by measuring peak area of peak height.

Comparison of results obtained by direct injection and on-line preconcentration analysis

Comparison of quantitative results obtained by direct injection into the analytical column and by preconcentration of a water sample containing the same amount of solutes is made in order to test the efficiency of coupling a precolumn to an analytical column, that is, the desorption and transfer of solutes from the precolumn to the analytical column. In order to eliminate the problems inherent in spiking samples with hydrophobic compounds, the moderately polar 2-chlorophenol was chosen and

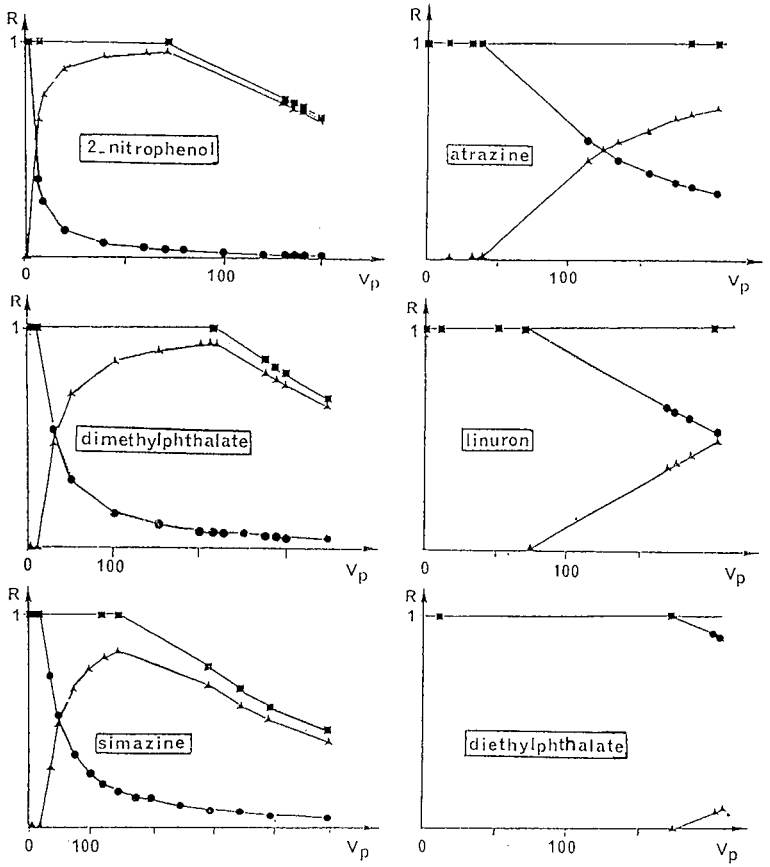

Fig. 7. Variation of recoveries calculated from characteristic volumes determined from experimental breakthrough curves (Table II) with percolated volume of samples. (●) Recovery from C18; (▲) recovery from PRP1; (■) recovery from C18 and PRP1 together (total).

the study was carried out with PRP1 alone (V_f = 32 ml). In order to test also the reproducibility of precolumn packings, a series of four precolumns were packed with a thin or thick slurry. Table III reports the retention time, plate number, peak area and peak height for three replicate analyses of 2-chlorophenol directly injected or preconcentrated from 10-ml water samples on the four different PRP1 columns.

Comparison of efficiencies. Band broadening comes from the transfer of the solute from the precolumn to the analytical column; from a geometric point of view, in order to avoid band broadening it is necessary to use precolumns of small dimensions compared with those of the analytical column, a condition which has been easily realized. The fact that the precolumn and analytical column are packed with different stationary phases can also be a cause of band broadening[24], especially when the retention of the solute by the precolumn sorbent is greater than that by the analytical column sorbent, as for 2-chlorophenol in this study. Band broadening can be suppressed by compressing the relatively broad profile in the precolumn at the top of the analytical column by the choice of a proper mobile phase and stationary phase com-

TABLE III

COMPARISON OF RESULTS OBTAINED BY DIRECT INJECTION OF 20 μl OF A SOLUTION OF 2-CHLOROPHENOL AND BY PRECONCENTRATION OF 10 ml OF WATER SAMPLE SPIKED WITH THE SAME AMOUNT OF 2-CHLOROPHENOL

Reported values are average values of three identical analyses; relative standard deviations (R.S.D.) are also reported; retention volumes are in ml; efficiency was calculated using $N = 5.54\,[t_R/w_{0.5}]^2$, where $w_{0.5}$ is the peak width at half-height; peak areas and heights are in arbitrary units; PRP-1 precolumns packed manually with (a) a thin slurry using a syringe or (b) with a thick slurry using a microspatula.

	Retention volume	*Efficiency (plate number, N)*	*Peak area*	*Peak height*
Direct injection	5.06	6060	2 047 323	9.35
R.S.D. (%)	0.2	3	0.3	2.6
Precolumn A (a)	5.08	6330	1 889 877	9.0
R.S.D. (%)	0.2	2.3	7.2	9
Precolumn B (a)	5.10	6160	1 847 978	8.7
R.S.D. (%)	1	5.6	1.4	4.6
Precolumn C (b)	4.65	6120	1 865 798	9.7
R.S.D. (%)	1.5	4.3	7	4.7
Precolumn D (b)	4.8	6100	1 834 194	9.35
R.S.D. (%)	1.5	1.8	5.5	5.2

bination[16]. In our study, backflush-desorption is used and Table III shows that no band broadening effect occurs; the efficiencies are identical with and without the precolumn. A relative standard deviation (R.S.D.) below 6% is observed when a precolumn is coupled to the analytical column and this is not very different from the R.S.D. of 3% measured with the analytical column alone; hence one can conclude that the introduction of the precolumn does not modify the apparent efficiency of the analytical column for the four precolumns.

Comparison of peak areas and heights. It can be seen (Table III) that the average peak area obtained with direct injection is slightly higher (about 10%) than the average peak areas with all the precolumns tested. This decrease cannot be explained by a possible breakthrough as the sample volume and the flushing volume (10 + 4 ml) before elution are far below the breakthrough volume of 2-chlorophenol (about 30 ml). This discrepancy can therefore be attributed to imprecisions in the percolated volume and in the calibration of the 20-μl injection loop, which has not been verified.

Reproducibility of precolumn packing. For the four packed precolumns, the reproducibility between peak areas and peak heights obtained for three consecutive preconcentrations is good, within an average R.S.D. of 3% for peak areas and 6% for peak heights (see Table III). This indicates that reproducible quantitative analyses are possible, provided that reproducible breakthrough volumes are obtained. Table IV reports V_f values for various solutes with the four precolumns. Up to V_f values of 20 ml the four precolumns are not very different; for 2-chlorophenol, 2-nitroaniline and 2-nitrophenol, A, B and D give similar V_f values but C gives lower values. These results show that differences in packing homogeneity are more critical when high volumes are percolated. This study does not show a clear influence of the packing

TABLE IV

TEST OF REPRODUCIBILITY OF PRECOLUMNS BY MEASURING BREAKTHROUGH VOLUMES OF VARIOUS SOLUTES WITH PRP1

See Fig. 5 for experimental conditions.

Solute	*A*	*B*	*C*	*D*
Phenol	3.5	4	2.5	3.5
4-Chloroaniline	4.5	6	6.5	8
2-Cresol	16.5	19	16	22
2-Naphthylamine	30	30	22	35
2-Chlorophenol	32	30	23	30
2-Nitroaniline	62	75	36	58
2-Nitrophenol	–	115	70	101

procedure (thin or thick slurry). We did not test commercially available precolumns but one can assume that they are packed under more reproducible conditions.

Recovery from peak areas with percolations at constant C_pV_p

Several 10-ml water samples spiked with different solutes are concentrated and peak areas are measured on elution. The recorded values are taken for A_1 values; several percolations with a constant amount of solute but a different volume are performed and, as described above, the recoveries correspond to the ratio of the peak areas measured for a volume V_p and of peak areas for a 10-ml volume. These values are reported in Table V for concentrations on C18 alone or on PRP1 alone. For most of the solutes the variations in recovery with sample volumes correspond to those

TABLE V

NORMALIZED PEAK AREAS WITH PERCOLATED SAMPLES HAVING THE SAME AMOUNT OF SOLUTES

A_1 measured with 10-ml samples; recoveries calculated with each precolumn.

Precolumn for concentration	*Solute*	*Sample volume (ml)*					
		10	*25*	*50*	*100*	*200*	*400*
C18	Dimethyl phthalate	1.00	0.84	0.51	0.25	0.13	0.11
	Diethyl phthalate	1.00	0.88	0.84	0.83	0.83	0.63
	Dibutyl phthalate	1.00	0.86	0.79	0.86	0.95	1.07
	Pyrene	1.00	0.92	0.82	1.16	1.51	1.90
PRP1	2-Methylphenol	1.00	0.90	0.62	0.35	–	–
	2,4-Dimethylphenol	1.00	0.93	0.92	0.88	–	–
	2,4,5-Trimethylphenol	1.00	0.92	0.91	0.91	–	–
	2-Chlorophenol	1.00	0.94	0.84	0.57	–	–
	2,4-Dichlorophenol	1.00	0.91	0.97	0.85	–	–
	2,4,6-Trichlorophenol	1.00	0.96	0.94	1.03	–	–
	2-Nitrophenol	1.00	0.96	0.94	0.90	–	–
	2,4-Dinitrophenol	1.00	0.98	0.94	0.89	–	–
	2,4,6-Trinitrophenol	1.00	0.63	0.44	0.33	–	–

calculated from breakthrough curves, *viz.*, a decrease in recovery for sample volumes above V_f.

The efficiency of spiking is clearly shown in Table V when only one precolumn was used; it is difficult to ensure that a recovery below 100% is due to breakthrough of the solute and not to inefficient solubilization of the test compounds or possible adsorption on the vessels and connection tubes. For a sample volume smaller than V_f, the recovery should be 100% and, taking account of the reproducibility of peak area measurements and of spiking, a value between 95% and 105% is acceptable. This is the case for methyl-, nitro- and substituted phenols as these compounds are sufficiently soluble in water; however, for butyl phthalate and pyrene, which are more hydrophobic compounds and difficult to solubilize in water, we see that for a volume higher than 100 ml the recovery increases to 190%; increasing the sample volume and decreasing the concentration (as C_pV_p remains constant) allows a more efficient solubilization so that the peak areas increase. For these solutes the lowest percolation volume to normalize peak areas is not 10 ml but at least 400 ml.

Fig. 8 shows the results for three herbicides concentrated on the two precolumns in series and can be compared with Fig. 7b, c and d. The sum of peak areas recovered from C18 and PRP1 is constant within a 4% deviation so that up to 200 ml there is no breakthrough from PRP1 for any of the three compounds. Thus, from a 100-ml sample, only 45% of simazine, about 95% of atrazine and 100% of linuron are recovered from C18, whereas simazine is concentrated to 55% and atrazine 5% on PRP1. One can say that PRP1 alone would, of course, concentrate the three compounds to 100%, but this is a typical example demonstrating the advantage of coupling precolumns. If only PRP1 is used, all other non-polar compounds are trapped together with the analytes and interfere during the analysis. However, with C18 coupled before PRP1 some non-polar interferents are trapped, hence rendering analysis of the PRP1 fraction more selective.

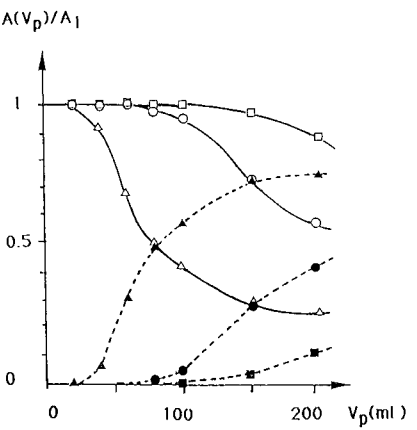

Fig. 8. Experimental variations of normalized peak areas with percolated water samples having a constant amount of herbicides. Preconcentration of samples at pH 3 (10^{-3} *M* perchloric acid) through the two precolumns in series; see Fig. 3 for analytical gradient and other experimental conditions. Solutes: (△) simazine; (○) atrazine; (□) linuron; peak areas measured during elution of C18; (▲) simazine; (●) atrazine; (■) linuron; peak areas measured during elution of PRP1.

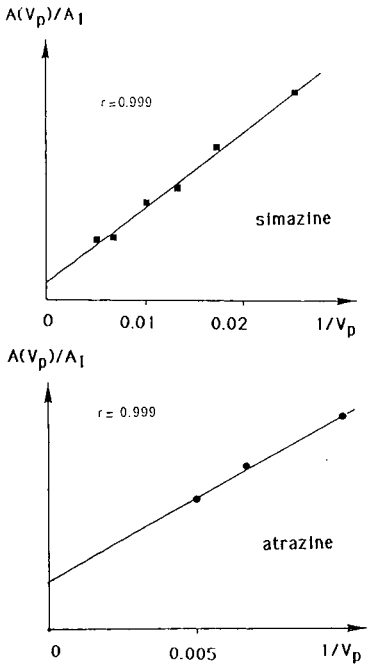

Fig. 9. Determination of breakthrough for (■) simazine and (●) atrazine by extrapolating the variations of normalized peak areas with the inverse of the percolated volume. Peak areas measured during elution of C18. Experimental conditions as in Fig. 8.

Another application of peak-area variations is an extrapolation to breakthrough values. $V_{f,C18}$ corresponds to the volume from which recovery from C18 begins to decrease and $V_{f,PRP1}$ to the volume from which the total recovery begins to decrease. From Fig. 8, the breakthrough volumes of simazine, atrazine and linuron can be roughly estimated to be 20, 50 and 75 ml on C18 whereas the real values determined from breakthrough curves are 19, 37 and 70 ml, respectively.

Fig. 9 shows the variations of $A(V_{p,C18})/A_1$ with the inverse of the percolated volume for simazine and atrazine when V_p is higher than $V_{f,C18}$. As seen from eqn. 14, these variations should be linear for V_p higher than $V_{m,C18}$, whereas for V_p between $V_{f,C18}$ and $V_{m,C18}$ there is no simple theoretical expression for the recovery as it is related to the shape of the front. For the two herbicides, excellent linear relationships are obtained (Fig. 9), hence showing the validity of eqn. 14 and the validity of measuring recoveries from peak areas. The last points of the straight line correspond to extrapolated V_m values and are about 40 ml for simazine and 100 ml for atrazine. Values taken from the breakthrough curves are 36 and 110 ml, respectively.

Table VI shows the differences between recoveries measured from variations in peak areas with various percolated volumes and those calculated from breakthrough curves. The latter are always slighty higher (about 15%). These differences are linked to the procedures in the two methods. The breakthrough method is based on calculations from experimental values carried out with only one precolumn and recovery determinations depend on the accuracy of the recorded breakthrough curves and

TABLE VI

COMPARISON OF RECOVERIES FROM C18 CALCULATED FROM BREAKTHROUGH CURVES AND FROM VARIATIONS OF PEAK AREAS WITH SAMPLE VOLUME (V_p, ml) WHEN PERCOLATING SAMPLES AT CONSTANT C_pV_p THROUGH THE TWO PRECOLUMNS IN SERIES

Solute	V_p	*Calculated recoveries from breakthrough curves*	*Calculated recoveries from peak areas*
Simazine	50	0.52	0.80
	100	0.26	0.43
	150	0.17	0.28
Atrazine	50	0.95	1.00
	100	0.60	0.96
	150	0.40	0.72
Linuron	50	1.00	1.00
	100	0.90	1.00
	150	0.70	0.96

especially on the reading of characteristic volumes. In order to not overload the precolumn, frontal analysis chromatograms have to be obtained with very dilute solutions and are recorded at a low UV range (0.005 to 0.01 a.u.), as shown in Fig. 5; hence for compounds having poor UV absorbance properties, measurement of V_f at 1% of the total front height is not always accurate, especially if the front is spread over a large volume range. In addition, uncertainties from spiking affect these calculations.

Recoveries determined from peak-area measurements, on the other hand, are certainly more accurate as it is always easier to measure peak areas than fronts and if the first two or three percolations with small volumes (assumed to be smaller than the breakthrough volume on the first precolumn) give the same areas. Determinations are rapid because several solutes can be studied simultaneously even if several percolations are necessary. However, the most important point is that these determinations are performed via the whole on-line system and the same operating conditions as used for actual analysis. Errors due to inefficient spiking can occur but, as shown above, they can easily be detected.

From a practical point of view, it seems interesting and more accurate to determine quantitative results by this latter method. As alreadey mentioned, quantitative analyses are carried out by studying spiked samples in order to calibrate detectors and to test the reproducibility of the system. We therefore recommend spiking the samples with a constant amount of solutes and different volumes. There are many advantages to such an approach: recoveries and extracted amounts can be calculated, the efficiency of spiking can be verified, sample volumes can be adjusted to the detection properties of the solutes and breakthrough volumes can be estimated.

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SELECTIVE, ON-COLUMN EXTRACTION OF ORGANOCHLORINE PESTICIDE RESIDUES FROM MILK

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SUMMARY

A rapid procedure has been developed that allows a single-step, selective extraction of organochlorine pesticide (OCP) residues from milk on solid-matrix disposable columns by means of acetonitrile–saturated light petroleum. Recovery experiments were carried out on homogenized milk (3.6% fat content) spiked with an ethanolic solution of nine OCPs, *viz.*, HCB, α-HCH, β-HCH, γ-HCH, heptachlor epoxide, dieldrin, endrin, *p,p'*-DDE and *p,p'*-DDT, at levels ranging from 0.002 mg/kg for α-HCH to 0.008 mg/kg for *p,p'*-DDT. Average recoveries of four replicates were 77% for HCB and almost quantitative (94–113%) for the other pesticides, with relative standard deviations from 2.9 to 7.3%. Coextracted fatty material amounted to about 5 mg/ml of milk before the clean-up. The described procedure also showed a satisfactory performance with milk powder. The extraction procedure requires about 60 min. The main advantages are that emulsions do not occur, several samples can be run in parallel by a single operator, reusable glassware is not needed and simple operations are required.

INTRODUCTION

The determination of organochlorine pesticide (OCP) residues in milk has always presented problems because the most common approach has involved total extraction of fat together with other lipophilic compounds including OCP residues. This approach has been based on the assumption that complete extraction of OCP residues from fatty foodstuffs is not possible unless all of the fat is extracted[1,2]. As a result, complex and lengthy procedures are in use for the extraction of milk fat and lipophilic pesticides. For instance, one of the most commonly used procedures[3] employs triple extraction of fluid milk with light petroleum–diethyl ether (1:1) with addition of sodium or potassium oxalate and ethanol to break up the emulsions, disrupt the fat globule membrane and improve the recovery of both fat and OCP residues. Combined extraction solvents are washed with salt solution, dried by passage through anhydrous sodium sulphate and concentrated to dryness to obtain milk fat. Another popular extraction system employs triple extraction of milk with *n*-hexane–acetone (1:1)[4]. Both procedures have some drawbacks, *e.g.*, the amounts of solvents

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and glassware used, the number of manual operations involved, which strongly affect the throughput of residue laboratories, centrifugation after each extraction and troublesome emulsions, which sometimes are not easily controlled by centrifugation and addition of ethanol, especially with whole milk.

Further, OCP residues need to be separated from the relatively large amount of milk fat so obtained by means of liquid–liquid partition[5,6], size-exclusion chromatography[7–10] or sweep co-distillation[11–15]. Adsorption column chromatography on Florisil[16–18], alumina[2,19] or silica gel[20] has been used as a final clean-up step before determination by gas chromatography (GC) with electron-capture detection (ECD)[21–24]. When a sufficient sensitivity is available in the final GC–ECD determination, it is also possible to clean up only a portion of the extracted fat by means of adsorption chromatography alone using minicolumns of Florisil[25,26], alumina[2,27] or silica gel[28].

In contrast to such conventional extraction procedures, selective extraction of OCP residues from milk has been reported by Suzuki *et al.*[26]. The addition of small amounts of acetonitrile and ethanol to the milk, before extraction with *n*-hexane, allows almost complete recoveries of OCP residues with minimum extraction of fatty substances. This method offers a substantial advantage over classical procedures in that the small weight of fatty extracts so obtained represents a significant fraction of the original sample, but requires only a minicolumn of adsorption material to remove it before GC–ECD. In our hands, the major drawback of this selective extraction procedure is the formation of stable emulsions in the second and third extraction stages, which are not controlled by centrifugation. Particularly in the extraction of whole-milk powder, which is reconstituted with water and extracted like fluid milk, the formation of a semi-solid gel in the organic phase almost completely prevents the recovery of the extraction solvents.

In order to minimize this effect we have developed a single-step, selective method for the extraction of OCP residues from milk and milk powder which is carried out on disposable, ready-to-use, solid-matrix cartridges where the efficiency of extraction is improved and emulsions do not occur.

EXPERIMENTAL

Reagents

Analytical-reagents grade light petroleum ether (b.p. 40–60°C), isooctane, acetonitrile and ethanol were redistilled in glass.

Ready-to-use Chem Elut CE 1010 cartridges were obtained from Analytichem International (Harbor City, CA, U.S.A.).

Organochlorine pesticide reference standards were from the collection in this laboratory.

Apparatus

The GC analyses were carried out on a DANI 6800 gas chromatograph equipped with an electron-capture detector. A glass column (1.8 m × 4 mm I.D.) was packed with OV-17–QF-1 (1.5% + 1.95%) on Chromosorb W HP (100–120 mesh). The temperatures were as follows: oven, 210; inlet block, 230; outlet block, 250; and detector, 250°C. The carrier gas was nitrogen at a flow-rate of 55 ml/min.

Procedure

In an erlenmeyer flask mix 10 ml of milk, 5 ml of acetonitrile and 1 ml of ethanol. Pipette 8 ml of this mixture on to a Chem Elut CE 1010 solid-matrix, ready-to-use column, allow it to drain and wait 10 min to obtain an even distribution. Attach a hypodermic needle to the column outlet as a flow regulator. Add to the column 10 ml of the upper phase (UP) obtained by equilibrating light petroleum–acetonitrile–ethanol (100:25:5). Wait 10 min, then elute with further 40 ml of UP. Collect the eluate and concentrate it to dryness. Dissolve the residue in 1–2 ml of light petroleum and clean up the extract by Florisil minicolumn adsorption chromatography[26]. Analyse the final sample by GC–ECD.

For milk powder, reconstitute 1 g of the powder with pesticide-grade water (1:9), then proceed as for fluid milk.

For recovery experiments, add 1 ml of an ethanolic solution containing OCPs instead of 1 ml of ethanol. Allow the mixture to stand overnight at 4°C. Equilibrate the sample to room temperature before proceeding with the above procedure.

RESULTS AND DISCUSSION

Chem Elut CE 1010 columns are ready-to-use, disposable cartridges filled with a macroporous diatomaceous earth with a nominal volume of 10 ml. In this procedure, the cartridges were used as a solid support to carry out the selective extraction of OCP residues from milk. As observed previously[1], OCP residues are more readily (but not completely) extracted than milk lipids with hydrocarbon solvents. However, the addition of a small amount of acetonitrile to the milk significantly improved the pesticide recoveries without increasing the fatty extracts[26]. Following these indications, a selective extraction of OCP residues from milk supported on a solid matrix was accomplished by eluting the column with acetonitrile-saturated light petroleum. The selective character of the extraction may be attributed to the phase-transfer properties of acetonitrile[29].

To test the performance of the method, milk and a sample of milk powder were analysed. Commercial pasteurized homogenized milk (3.6% fat content) was spiked (see *Procedure*) with nine typical OCPs at levels ranging from 2 to 8 μg/kg of milk and analysed according to the above procedure; the weight of the crude extract was calculated before the clean-up. The results obtained with fluid milk are presented in Table I. Typical chromatograms of spiked milk, unspiked milk and "blank" analyses are shown in Fig. 1. They show that all nine OCPs were satisfactorily recovered from milk with a carryover of fatty substances of only 5 mg/ml. This amount of fatty substances is of the same order as that reported by Suzuki *et al.*[26], thus allowing the use of a Florisil minicolumn for the clean-up. The AOAC[3] and *n*-hexane–acetone[4] extraction procedures would have extracted the nominal fat content and would have required either partition followed by adsorption chromatography or the sole adsorption chromatography of a fraction of the total fatty extract.

The milk powder sample (*ca.* 24% fat content) was prepared from milk to which a solution of nine OCPs in milk fat had been added before the spraying process. The sample was kindly provided by the EEC Community Bureau of Reference (BRC) and analysed under blind conditions according to the present procedure and, for comparison, also according to the AOAC[3] and *n*-hexane–acetone[4] extraction

TABLE I

RECOVERY OF NINE ORGANOCHLORINE PESTICIDES FROM FORTIFIED WHOLE MILK (3.6% FAT CONTENT)

Pesticide	*Spike level (μg/kg)*	*Mean recovery (%) (n = 4)*	*Relative standard deviation (%)*
HCB	4	77	7.3
α-HCH	2	94	6.7
γ-HCH	4	105	4.9
β-HCH	8	113	4.5
Heptachlor epoxide	4	99	6.4
p,p'-DDE	4	99	3.3
Dieldrin	4	106	2.9
Endrin	6	103	2.9
p,p'-DDT	8	98	4.1
Fatty extract (mg/ml milk)*		5.0	4.0

* Weight of extract prior to clean-up.

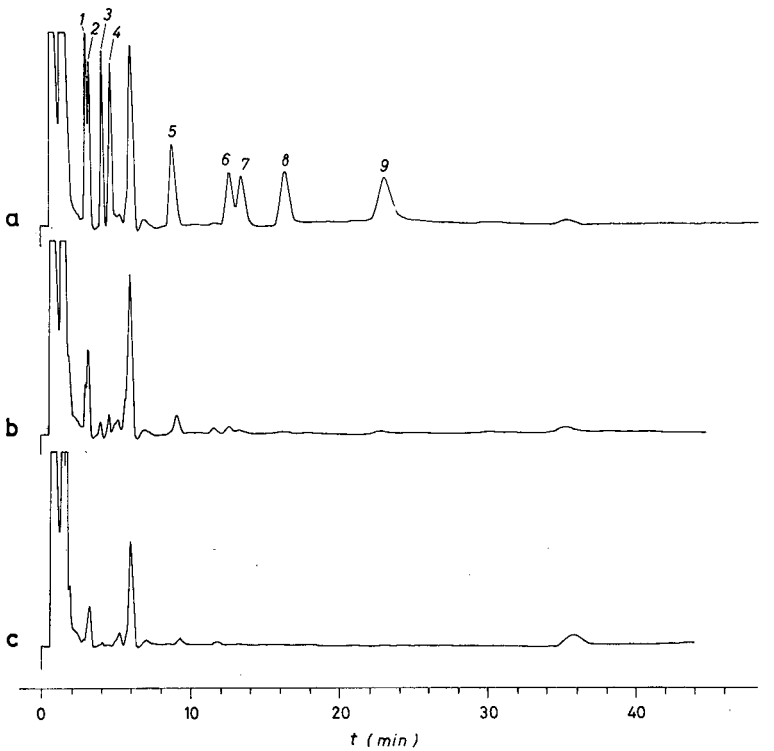

Fig. 1. Gas chromatograms of (a) spiked milk, (b) unspiked milk and (c) "blank", analysed according to the described procedure. Peaks: (1) HCB, 4 ppb; (2) α-HCH, 2 ppb; (3) γ-HCH, 4 ppb; (4) β-HCH, 8 ppb; (5) heptachlor epoxide, 4 ppb; (6) *p,p'*-DDE, 4 ppb; (7) dieldrin, 4 ppb; (8) endrin, 6 ppb; (9) *p,p'*-DDT, 8 ppb.

procedures. In all instances the fatty extracts were cleaned up by Florisil minicolumn chromatography[26] using a ratio of not more than 80 mg of fatty extract per 2.5 g of Florisil.

The results obtained with the milk powder sample are presented in Table II. The results obtained with this procedure appear in general to be slightly higher than the corresponding values obtained with the *n*-hexane–acetone procedure. In contrast, the AOAC procedure gave slightly higher results, at least for certain pesticides, notably γ-HCH, *p,p'*-DDE and *p,p'*-DDT. Although the results are in general satisfactory, the discrepancies may be an indication that, at least for a difficult sample such as milk powder which has a high fat and protein content, the pesticide–matrix interaction has not been completely broken. Hence some further refinements of the extraction conditions may be necessary in the present procedure.

However, it is noteworthy that the extraction of the milk powder according to this procedure did not pose particular problems, whereas it was not possible according to the procedure reported by Suzuki *et al.*[26] because of the formation of a semi-solid gel in the organic phase. Troublesome emulsions with this sample also occurred when the AOAC and *n*-hexane–acetone procedures were used. The results obtained with the present procedure are slightly more scattered (as judged from the relative standard deviation) than those given by the other two methods. Possible explanations for this may include variability of the packing characteristics of the Chem Elut material with possible "channelling" effects and variability of the blank analyses which were subtracted from the sample values. Indeed, blank analyses of the Chem Elut columns showed variable amounts of a peak with the retention time of α-HCH, of another

TABLE II

CONCENTRATION OF NINE ORGANOCHLORINE PESTICIDES IN A MILK POWDER SAMPLE

Results in μg/kg expressed on a dry-mass basis.

Pesticide	*Extraction procedure*					
	This work		*AOAC*[3]		*n-Hexane–acetone*[4]	
	Mean value (n = 4)	*Relative standard deviation (%)*	*Mean value (n = 5)*	*Relative standard deviation (%)*	*Mean value (n = 4)*	*Relative standard deviation (%)*
HCB	41.1	11.8	42.1	10.7	37.0	8.8
α-HCH	23.1	26.7	28.0	7.2	20.0	7.6
γ-HCH	45.5	21.1	52.9	4.4	42.6	13.8
β-HCH	11.8	14.7	13.9	1.4	10.4	6.4
Heptachlor epoxide	39.2	13.1	38.4	3.5	32.3	11.3
p,p'-DDE	49.1	6.6	62.9	10.8	46.7	9.7
Dieldrin	46.4	2.9	45.4	4.3	35.2	12.1
Endrin	6.8	10.9	10.2	2.5	5.1	10.5
p,p'-DDT	64.4	12.7	72.8	10.5	58.1	2.0
Fatty extract on dry-mass (%)*	5.9	42.3	24.5	2.9	22.4	3.7

* Weight of extract prior to clean-up.

major peak that does not interfere with the OCPs investigated and some minor (mostly early eluting) peaks.

In conclusion, we can say that, although the purity of the Chem Elut columns is not at present completely satisfactory as a pesticide-grade product, the results presented indicate that a simple, straightforward extraction of OCP residues from fluid milk and milk powder can be carried out on a solid matrix with satisfactory recoveries. As the extraction procedure requires only disposable items and almost unattended operations, it offers a means of improving the throughput of the residue laboratory with significant savings of reagents, glassware and time.

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Note

Single-step solid-matrix clean-up of vegetable extracts for organophosphorus pesticide residue determination

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In the analysis of organophosphorus pesticide (OP) residues in fruits and vegetables with multi-residue procedures based on water-miscible solvents (acetone, acetonitrile) as extraction solvents, a major problem is that the purification involves multiple stages (performing separate functions) with successive manual operations[1].

For instance, with acetone as extraction solvent, pesticide residues are separated from crude aqueous acetone extracts by a liquid–liquid partition stage followed by a clean-up stage. The first stage typically employs dilution of the aqueous acetone extract with a salt (sodium chloride, sodium sulphate) solution and several successive separating funnel extractions into dichloromethane. Under these conditions a wide range of both polar and non-polar pesticide residues can be recovered[1–9]. The dichloromethane extract is dried by passage through a column of anhydrous sodium sulphate and subjected to clean-up before the final determination. For the clean-up stage, the most commonly used techniques for OP residues are size-exclusion chromatography[5,8–13], sweep co-distillation[14–17] and column chromatography on Florisil[18] or charcoal and its mixtures[4,19,20]. Taking advantage of phosphorus-selective detectors, such as flame photometric detectors (FPD-P) or the alkali-bead thermionic detector (NPD), the dichloromethane extract has also been used without any clean-up for the determination of OP residues[3,6].

The drawbacks of the above and other similar procedures, however, are the amounts of solvents and reagents required, the washing and preparation of glassware, the occurrence of troublesome emulsions in the aqueous acetone–dichloromethane extraction stage with certain vegetable products, the preparation and maintenance of costly apparatus and, most important, the number of handling operations, which strongly affect the throughput of the residue laboratory.

We have therefore developed a rapid, single-step procedure in which solid-matrix, disposable cartridges are used as a support to carry out the extraction and clean-up of OPs from crude aqueous acetone extracts of vegetable products. The resulting solution is suitable for the direct determination of OP residues by gas chromatography (GC) with FPD-P detection.

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EXPERIMENTAL

Reagents

Analytical-reagent-grade light petroleum (b.p. 40–60°C), dichloromethane and acetone were redistilled in glass.

Ready-to-use Extrelut-20 columns, code No. 11737, were obtained from Merck (Darmstadt, F.R.G.).

Organophosphorus pesticide reference standards were from the collection in this laboratory.

Apparatus

The GC analyses were carried out on a Perkin-Elmer Sigma 4-B gas chromatograph equipped with a flame photometric detector operated in the phosphorus mode (FPD-P). A glass column (1.8 m × 4 mm I.D.) was packed with 5% QF-1 on Chromosorb W HP (100–120 mesh). The temperatures were as follows: oven, 180; and inlet and outlet blocks, 225°C. The carrier gas was helium at a flow-rate of 60 ml/min. The hydrogen and air flow-rates to the detector were set according to the manufacturer's instructions. A source of pure nitrogen, capable of delivering gas at a flow-rate of 2 l/min measured with a rotameter, was used.

Procedure

Prepare aqueous acetone extracts of fruits and vegetables according to ref. 2 or 4. Take a 15-ml aliquot of the extract equivalent to *ca.* 5 g of crop and transfer it on to the top of an Extrelut-20 column. Allow the liquid to drain and wait 10 min to obtain an even distribution on the filling material. Pass through the column, from bottom to top, a nitrogen flow of 2 l/min for 30 min. Disconnect the Extrelut-20 column from the gas line, attach to the column outlet a 0.70 × 32 mm Luer-lock needle (supplied with the column) as a flow regulator and elute the column with four 20-ml portions of light petroleum (b.p. 40–60°C), then with four 20-ml portions of dichloromethane–light petroleum (b.p. 40–60°C) (1:3) to elute dimethoate. Concentrate to a small volume using a rotary evaporator (40°C; reduced pressure), then to dryness by manually rotating the collecting flask. Dilute to a suitable volume with acetone and analyse by GC. Determine the OP concentration in the sample extract by comparison of the peak height with that of an external standard of comparable concentration.

For recovery experiments, add suitable amounts of standards to the chopped vegetables in the homogenization jar. Allow the solvent to evaporate, then proceed with the extraction.

RESULTS AND DISCUSSION

Extrelut-20 columns are ready-to-use, disposable cartridges filled with a macroporous Kieselguhr-type material and have found several applications in the extraction of drugs from body fluids[21] and in pesticide residue analysis[22–24]. Although a column can hold *ca.* 20 ml of liquid, we have applied only 15 ml of aqueous acetone extract (corresponding to *ca.* 5 g of crop) in order to avoid possible mechanical displacement of water into the eluate. The removal of the major part of acetone (*ca.* 90% of the limiting weight loss under the described conditions; see Fig. 1) is necessary

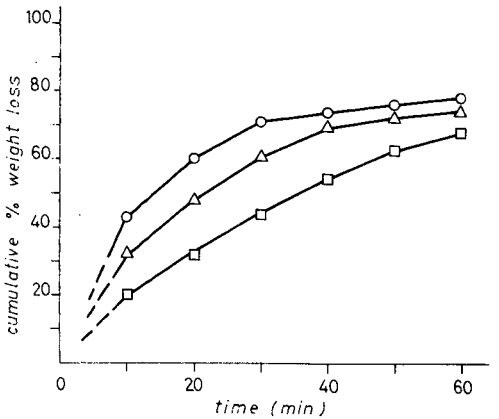

Fig. 1. Rate of removal of acetone from Extrelut-20 columns loaded with 15-ml of sample extract as a function of time and nitrogen flow-rate, expressed as cumulative percentage weight loss. (□) 0.5 ml/min; (△) 1 l/min; (○) 2 l/min.

to prevent the carry-over of the water by the eluting solvent. Further, the small zone of unwetted filling material exerts some adsorptive effect toward the green pigments of vegetables and possibly other coextractives. This effect, combined with the low polarity of the eluent used, leads to a substantial retention of coextractives on the column. In fact, by applying 15 ml of aqueous acetone extracts of different crops, namely lettuce, onion, strawberry, apple, yellow pepper, peach, tomato, broccoli, cauliflower and radish, the amount of coextractives in the eluate ranged from 2 to 10 mg. This weight range is of the same order as that obtained by subjecting the aqueous acetone extracts to the classical, time-consuming sequences of separating funnel partition against dichloromethane[1–9], drying over anhydrous sodium sulphate, solvent exchange and clean-up by Florisil[18] or charcoal column chromatography[4,19,20], size-exclusion chromatography[5,8–13] or sweep co-distillation[14–17]. However, compared with these previous methods, the same performance with our method is obtained in a shorter time (*ca.* 60 min), with very simple operations, and by using only one disposable item and a reduced volume of solvents. With the crops tested, the extracts were almost uncoloured or light yellow.

After this clean-up the extracts are clean enough to be concentrated and analysed by GC without further treatment. Owing to the small amount of coextractives injected, the injection port of the gas chromatograph afforded many injections of sample extracts before cleaning was necessary, and the GC column did not display any loss of performance. Further, owing to the high selectivity of the detector used, the chromatograms of the "blank" crops tested were all free from interfering peaks and were similar to those obtained by injecting solvent alone; the chromatograms of the "spiked" crops, at the levels tested, were indistinguishable from those obtained by injecting the standard compounds. In particular, in contrast to Luke *et al.*'s findings[3], we did not observe interfering peaks in the chromatograms of broccoli, cauliflowers, onions and radishes obtained according to our procedure.

The recovery of pesticides was investigated for 18 OPs representative of a wide range of polarities and water solubilities (diazinon, etrimfos, chlorpyrifos-methyl,

pirimiphos-methyl, chlorpyrifos, bromophos, bromophos-ethyl, malathion, fenitrothion, methacrifos, fonofos, fenchlorphos, dimethoate, parathion-methyl, parathion, methidathion, carbophenothion and ethion). For recovery experiments, blank vegetables (see Table I) were spiked with nine-component OP mixtures containing those compounds which are separated in a single GC run under the described conditions. Most of the pesticides tested were recovered by light petroleum (b.p. 40–60°C) and dimethoate was recovered by further eluting the column with 80 ml of dichloromethane–light petroleum (1:3). However, especially for screening purposes, all of the pesticides tested may be recovered by a single elution with 80 ml of the latter solvent mixture. Also with this solvent mixture, green pigments are retained on the column.

The results of the recovery experiments are presented in Table I. The recovery of pesticides was determined (in triplicate) at different spiking levels. At the levels tested, ranging for the different OP compounds from *ca.* 0.1 to 1.4 mg/kg, the recoveries were between 75 and 110%. These values are satisfactory for residue analysis and are of the same order as those obtained by using more complicated methodologies.

The main feature of the described procedure is that the column appears to perform in a single step several functions, *viz.*, the removal of water and hydrophilic coextractives, the transfer of pesticide residues into a low-boiling solvent and a low-activity adsorption clean-up, giving a solution amenable to direct GC analysis. In classical schemes, the same functions are carried out through separate, time-consuming and labour- and glassware-intensive operations. Unlike the classical separating funnel partitioning[1–9], with the described procedure the extraction is rapid,

TABLE I

RECOVERY OF ORGANOPHOSPHORUS PESTICIDES FROM VEGETABLES

Blank spaces denotate that the specific pesticide–crop combination concerned was not analysed.

Pesticide	*Spike level (mg/kg)*	*Average recovery (%) (n = 3)*					
		Peach	*Broccoli*	*Radish*	*Onion*	*Tomato*	*Cauliflower*
Methacrifos	0.1	83.2	85.4		79.6	82.5	84.9
Fonofos	0.2	90.9	90.3		85.3	82.2	87.0
Fenchlorphos	0.4	101.1	93.3		87.8	84.5	91.8
Dimethoate	0.6				78.0	75.4	77.9
Parathion-methyl	0.7	97.9	90.0		88.9	88.0	92.8
Parathion	1.0	97.1	92.0		88.8	85.3	90.5
Metidathion	1.2	96.4	88.3		87.9	84.5	87.3
Carbofenothion	1.4	100.0	100.0		89.7	88.5	86.7
Ethion	1.0	97.6	90.5		88.0	88.0	89.7
Diazinon	0.2	95.0		94.6	96.7	106.8	100.0
Etrimfos	0.4	98.7		100.0	102.7	108.4	98.6
Chlorpyrifos-methyl	0.4	101.9		100.0	96.8	79.1	103.7
Pirimiphos-methyl	0.3	96.7		96.4	102.6	107.7	100.0
Chlorpyrifos	0.5	97.9		97.7	98.9	89.8	103.9
Bromophos	0.6	102.5		100.0	101.3	108.1	108.6
Bromophos-ethyl	0.6	102.9		98.5	100.0	108.1	103.0
Malathion	1.0	107.0		91.2	89.7	92.4	102.7
Fenitrothion	0.6	104.4		95.2	101.6	110.0	104.0

emulsions do not occur and addition of salt solution and drying of the extraction solvent with anhydrous sodium sulphate are not necessary. Compared with instrumental clean-up techniques (size-exclusion chromatography, sweep co-distillation) the described procedure is very simple, rapid, inexpensive and does not require the preparation or maintenance of costly apparatus or skilled operators.

In conclusion, the main features of this clean-up system compared with previous methods are the good clean-up and recoveries, the wide range of applicability, the minimum of glassware and reagents required, the lack of emulsions, the reduced time for a single clean-up and the simplicity of the operations involved, which allows parallel handling of several sample extracts.

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SELECTIVE ENRICHMENT PROCEDURES FOR THE DETERMINATION OF POLYCHLORINATED BIPHENYLS AND POLYCYCLIC AROMATIC HYDROCARBONS IN ENVIRONMENTAL SAMPLES BY GEL PERMEATION CHROMATOGRAPHY

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SUMMARY

An improved two-step clean up procedure involving alumina–silica column chromatography and gel permeation chromatography (GPC) of air particulate matter (NBS SRM 1648) and river sediment extracts and a GPC clean up procedure for marine biota samples are described for the determination of polycyclic aromatic hydrocarbons with two to five rings and selected polychlorinated biphenyl congeners, respectively. Bio-Beads SX-12 and SX-3 were used as packing materials. The recoveries obtained varied from 52 to 78% depending on the compound. Quantitative data for NBS SRM 1648 were comparable with those described previously for this sample.

INTRODUCTION

Environmental matrices contain many compounds interfering in the determination of xenobiotics. Consequently, extended time consuming clean up procedures, involving laborious fractionation steps, are required. The isolation of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) from environmental samples has been performed by liquid–liquid partitioning, by adsorption chromatography using silica gel, alumina, silica–alumina or Florisil, and by high-performance liquid chromatography[1–3]. Gel permeation chromatography (GPC) is an alternative technique to remove the higher-molecular-weight coextractive lipids which so often interfere in the gas chromatographic (GC) analyses of organophosphorus and organochlorinated pesticides, PCBs and PAHs[4–9].

The separation mechanisms in GPC involve adsorption, partition and size exclusion. The predominance of one mechanism over the others is largely determined by the choice of mobile phase and packing pore size. In the case of GPC packings with large pore sizes (1000–2000 molecular weight exclusion), size exclusion and adsorption occurs in the presence of poorly solvating mobile phases. This will be the case when using Bio-Beads SX-3 for analyzing PCBs with different eluting solvents such as cyclohexane, dichloromethane–hexane, dichloromethane–cyclohexane, toluene–ethyl acetate and ethyl acetate–cyclohexane[5,8,9]. On the other hand, when smaller pore sizes (400 molecular weight exclusion) are used in combination with highly polar solvents,

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such as tetrahydrofuran (THF), dimethylaniline (DMA) or dimethylformamide (DMF), only size exclusion predominates[10]. Other examples in the literature include multistep analytical approaches with silica–alumina column chromatography and GPC with Sephadex LH-20[11] and Bio-Beads SX-12[12] to isolate PAHs from selected environmental samples.

The objective of the present study is to test the validation of GPC for selective trace enrichment of PCBs and PAHs in different environmental matrices: air particulate, river sediment and biota. We report the use of two different GPC packing materials Bio-Beads SX-3 and SX-12 for isolation of seven PCB individual congeners (I.U.P.A.C. Nos. 28, 52, 101, 118, 153, 138 and 180) from biota samples and PAHs and sulphur-containing compounds from air particulate matter and river sediments, respectively. A reference material (NBS SRM 1648) was subjected to the same fractionation procedure for PAHs in order to validate the quantitative data.

EXPERIMENTAL

Materials

The solvents, ethyl acetate and cyclohexane, were pesticide grade (SDS, Peypin, France) and THF was HPLC grade (Shepshed, U.K.). Isooctane, dichloromethane and methanol were distilled from glass before use. Alumina and silica gel (70–230 mesh) were supplied by E. Merck (Darmstadt, F.R.G.). Analytical reagent grade PCB individual components (Promochem, Wesel, F.R.G.) were used: I.U.P.A.C. numbers 28, 2,4,4′-trichlorobiphenyl; 52, 2,2′,5,5′-tetrachlorobiphenyl; 101, 2,2′,4,5,5′-pentachlorobiphenyl; 118, 2,3′,4,4′,5-pentachlorobiphenyl; 138, 2,2′,3,4,4′,5′-hexachlorobiphenyl; 153, 2,2′,4,4′,5,5′-hexachlorobiphenyl and 180, 2,2′,3,4,4′,5,5′-heptachlorobiphenyl.

2,3-Dimethylnaphthalene and benzo[*a*]pyrene were obtained from Fluka (Buchs, Switzerland) and chrysene from Scharlau (Barcelona, Spain). Alkyldibenzothiophene isomers were kindly provided by Professor Milton L. Lee (Brigham Young University, Provo, UT, U.S.A.). Other standards were available in our laboratory.

Sample preparation for PCBs

An homogenized mixture of fish tissue (1–2 g) was mixed with 20–30 g of anhydrous sodium sulphate and extracted for 18 h with ethyl acetate in a soxhlet apparatus. The ethyl acetate extract (100 ml) was evaporated just to dryness and the residue was dissolved in 50–100 μl of ethyl acetate–cyclohexane (1:1). Afterwards the fish extracts were injected onto the GPC column using the same eluent.

Sample preparation for PAHs

Air particulate. A 2-g amount of NBS Substance Reference Material 1648 was soxhlet extracted for 48 h using methanol–benzene (1:3) The extracts were concentrated to near dryness in a rotary evaporator, reconstituted up to 1 ml and adsorbed onto 1 g of alumina. The solvent was evaporated under a gentle stream of nitrogen and the mixture was transferred to the top of a glass column (25 cm × 0.9 cm I.D.), slurry packed with 8 g of silica gel and 7 g of neutral alumina (top), previously deactivated with 5% of water. The PAH fraction (AS-3) was eluted with 40 ml of 20% dichloromethane in hexane as described previously[2].

Sediment. A river sediment sample (Besós) was collected with a Van Veen dredge, wrapped in aluminium foil and frozen at −20°C until analysis. The sampling site was located 2 km upstream from the river mouth. A 20-g amount of freeze-dried sediment was extracted by sonication using dichloromethane–methanol (2:1). Sample fractionation was performed as described for air particulate.

Chromatographic analysis

GPC. Eluent delivery was provided by a 64 high-pressure pump (Knauer, Bad Homburg, F.R.G.) coupled with a Vari-chrom UV–VIS detector (Varian, Sunnyvale, CA, U.S.A.) at 254 nm. Samples were injected via a 50-μl loop from Rheodyne (Cotati, CA, U.S.A.). Stainless-steel columns (450 mm × 10 mm I.D.) (Tracer Analítica, Barcelona, Spain) packed with Bio-Beads SX-3 and SX-12 (mesh size 200–400) (Bio-Rad Labs., Richmond, CA, U.S.A.) were used. For the PCB samples, the column was placed in a jacket and thermostatted at 40°C, The eluting solvents pumped, respectively, at 0.5 and 1.0 ml min^{-1} were ethyl acetate–cyclohexane (1:1) for PCBs and THF for PAHs. Five fractions (G1–G5) were collected for PAHs.

GC. GPC fractions were evaporated to dryness, dissolved in isooctane and injected on to a 6000 Vega series gas chromatograph (Carlo Erba, Milan, Italy) equipped with flame ionization (FID), flame photometric (FPD) and electron-capture (ECD) detection. A 50 m × 0.25 mm I.D. CP-Sil 5 CB (Chrompack, Middelburg, The Netherlands) and a 25 m × 0.32 mm MPMS (Alltech, Deerfield, IL, U.S.A.) fused-silica capillary column were used. Hydrogen was the carrier gas at 50 cm s^{-1}. The injector and detector temperatures were held at 300 and 330°C, respectively. The former column was programmed from 60 to 300°C at 6°C min^{-1}, the latter at 15°C min^{-1} to 150°C and then to 270°C at 4°C min^{-1}, keeping the final temperature for 20 min. Quantitation was performed using phenanthrene, chrysene and benzo[*ghi*]-perylene as external standards.

GC–mass spectrometric (MS) analyses were carried out in 5995 instrument interfaced to a 9825A data system (Hewlett-Packard, Palo Alto, CA, U.S.A.). Helium was used as the carrier gas (30 cm s^{-1}). A 25 m × 0.25 mm I.D. CP-Sil 5 CB (Chrompack) fused-silica column was introduced directly into the ion source. Other chromatographic conditions were identical to those described for the GC analyses. The ion source and the analyzer were held at 200 and 230°C, respectively. Spectra were obtained by elctron impact at 70 eV at a scan speed of 0.86 scans s^{-1}.

RESULTS AND DISCUSSION

GPC optimization

PAHs. PAHs were isolated using Bio-Beads SX-12 as the packing material. The column efficiency was determined at different flow-rates (0.25–2 ml min^{-1}) for coronene, the optimum value being 2990 plates m^{-1} at 0.5 ml min^{-1}. The separation mechanism of PAHs under these conditions can be inferred from the fact that the elution volume increases from coronene to naphthalene, thus indicating a predominance of size exclusion. Further, it should be noticed that coronene is coeluted with chrysene indicating that catacondensed structures are more retained than pericondensed ones (Table I). Klimisch and Reese[13] studied the elution behaviour of PAHs in the THF/styrene–divinylbenzene system (Bio-Beads SX-8). They noted that molecular

TABLE I

CHROMATOGRAPHIC CHARACTERIZATION OF STANDARD COMPOUNDS

Column packing	*Compound*	*Spike level**	*Elution volume** (ml)*	*Recovery (%)****
Bio-Beads SX-12	Naphthalene	18.8	26–30	81
	Anthracene	16.6	24–28	88
	Chrysene	11.5	20–24	72
	Benzo[*a*]pyrene	5.0	20–24	70
	Coronene	50.8	18–22	63
Bio-Beads SX-3	PCB No. 28	2.8	22–34	52
	PCB No. 52	2.5	22–34	54
	PCB No. 101	2.3	22–34	73
	PCB No. 118	2.0	22–34	78
	PCB No. 153	1.7	22–34	78
	PCB No. 138	2.0	22–34	78
	PCB No. 180	1.2	22–34	78

* Spike level was in μg for PAHs and in ng for PCBs.
** Measured at a flow-rate of 1 ml min^{-1}.
*** Mean of two determinations.

sieving effects predominated for alkanes and catacondensed PAHs, while pericondensed PAHs were eluted strictly according to adsorption principles. The different behaviour between this chromatographic system and the one used in this work can be basically ascribed to the difference in pore sizes between the columns, enabling adsorption of the larger pore size material.

Recoveries of PAHs having two to seven aromatic rings are listed in Table I. Apparently they decrease as the molecular weight increases, ranging from 63 to 88% at the μg level. Giger and Shaffner[11] reported different results on Sephadex LH-20, eluting with benzene–methanol (1:1), with recoveries exceeding 100%. At present, there is no explanation for these features.

It should be mentioned that Bio-Beads SX-12 could not be used as GPC column packing for isolating PCBs because of the molecular weight exclusion limit (400 daltons).

PCBs. A standard sample containing seven PCB congeners (I.U.P.A.C. Nos. 28, 52, 101, 118, 153, 138 and 180) was analyzed using Bio-Beads SX-3 as the GPC column packing with a molecular weight exclusion limit of 2000 daltons and the experimental conditions indicated above. The collection time was from 22 to 34 min at 1.0 ml min^{-1}. The mean recovery varied from 52 to 78% while the relative standard deviation (R.S.D.) varied from 12 to 15 ($n = 10$) as shown in Table I. Although PCB congeners 28 and 52 exhibited recovery values between 50 and 60%, these are in the range previously reported in interlaboratory studies[4]. Another factor that needs to be taken into consideration is that previously published recovery values using Florisil or silica–alumina column chromatography were referred to total PCBs as, *i.e.*, Aroclor 1254, with values up to 80%[2] and often being lower than in GPC[8]. In addition, using the same experimental GPC conditions for clean up of PCBs, a mixture of PAHs was injected onto the GPC apparatus and exhibited a collection time between 30 and 40 min which interfered with PCBs, using dichloromethane–cyclohexane as previously

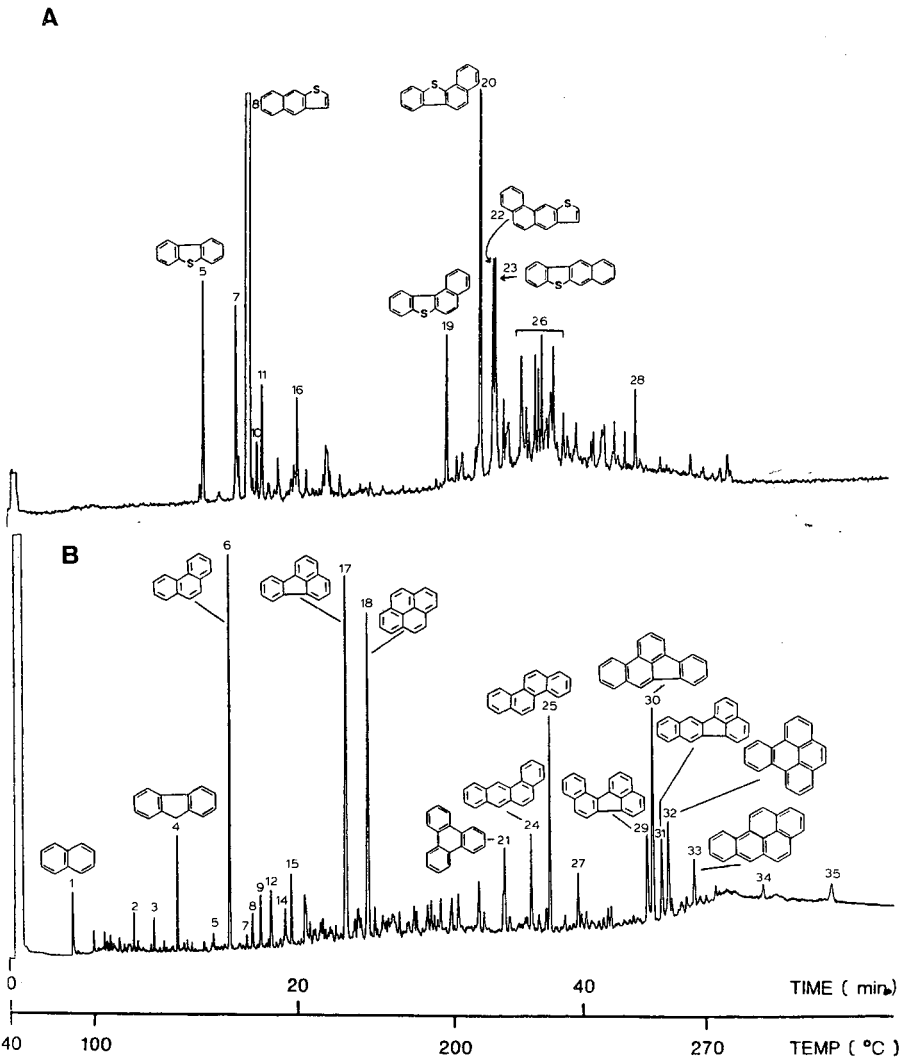

Fig. 1. Gas chromatograms of fraction G-5 from NBS SRM 1648; (A) FPD and (B) FID traces. A 25 m × 0.32 mm I.D. MPMS liquid crystal fused-silica column was used. Compound identification as in Table II.

reported[6]. Therefore the use of Bio-Beads SX-12 offers a better solution for clean up of PAHs, as reported in the present paper.

Environmental matrices

Air particulate sample. Fig. 1 shows the FID and FPD chromatograms of PAH fraction G-5 obtained from an air particulate (NBS SRM 1648). The compound identification and quantitation are given in Table II. Several isomeric pairs that cannot be resolved on isotropic stationary phases were successfully resolved (methyldibenzothiophenes, triphenylene/chrysene, benzofluoranthene isomers). The quantitative data obtained (Table II) are in accord with those reported for this sample[14] except for

TABLE II

PAHs AND S-PACs IDENTIFIED IN NBS SRM 1648

Peak No.	*MW*	*Concentration** ($\mu g\ g^{-1}$)	*Identification*
1	128	0.63	Naphthalene
2	168	0.26	Dibenzofuran
3	172	0.21	C_1-substituted dibenzofuran
4	166	0.75	Fluorene
5	184	0.29	Dibenzothiophene
6	178	4.16	Phenanthrene
7	198	a	4-Dibenzothiophene
8	184	1.40	Napho[2,3-*b*]thiophene
9	192	0.46	9-Methylphenanthrene
10	198	a	2-Methyldibenzothiophene
11	198	a	3-Methyldibenzothiophene
12	192	0.70	1-Methylphenanthrene
13	198	a	1-Methyldibenzothiophene
14	192	0.29	2-Methylphenanthrene
15	204	0.69	2-Phenylnaphthalene
16	208	a	Phenanthro[4,5-*bcd*]thiophene
17	202	5.65	Fluoranthene
18	202	4.63	Pyrene
19	234	a	Benzonaphtho[1,2-*d*]thiophene
20	234	0.54	Benzonaphtho[2,1-*d*]thiophene
21	228	1.20	Triphenylene
22	234	a	Phenanthro[1,2-*b*]thiophene
23	234	a	Benzo[*b*]naphtho[2,3-*d*]thiophene
24	228	1.32	Benz[*a*]anthracene
25	228	3.03	Chrysene
26	248	a	C_1-substituted 234
27	240	0.72	11H-Benz[*bc*]aceantrylene
28	258	a	Chryseno[4,5-*bcd*]thiophene
29	252	1.19	Benzo[*j*]fluoranthene
30	252	3.30	Benz[*e*]acephenanthrylene
31	252	1.14	Benzo[*k*]fluoranthene
32	252	1.32	Benzo[*e*]pyrene
33	252	0.65	Benzo[*a*]pyrene
34	276	a	Indeno[1,2,3-*cd*]pyrene
35	276	a	Benzo[*ghi*]perylene

* Concentrations below 0.15 $\mu g\ g^{-1}$ are indicated with a.

high-molecular-weight components which presented lower values. These results are consistent with the lower recovery data obtained for higher-molecular-weight standard compounds (Table I).

River sediment. The Bio-Beads SX-12 fractionation was evaluated for the corresponding PAH fraction isolated by silica–alumina column chromatography from a sediment river extract (AS-3). This fraction was further fractionated into five subfractions (G-1 to G-5). Fig. 2 shows the reconstructed total ion current of G-2 to G-5 and compound identifications are listed at Table III. Fraction G-1 contained high-molecular-weight components not amenable to recovery under the analysis conditions. In G-2 were identified alkyl and steryl wax esters, with molecular weights

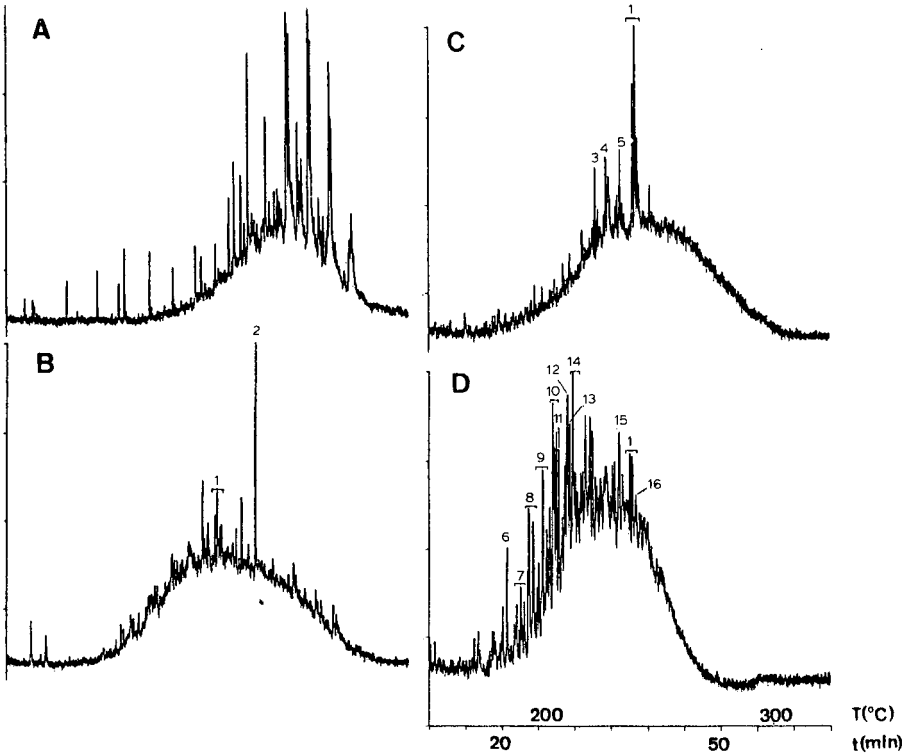

Fig. 2. Total ion current of GPC fractions (A) G-2 (13.2–15.4 ml), (B) G-3 (15.4–17.4 ml), (C) G-4 (17.4–19 ml) and (D) G-5 (19–27.5 ml) eluted with THF at 0.5 ml min^{-1} from fraction AS-3 of a Besós river sediment extract. A 25 m × 0.25 mm I.D. CP-Sil 5 CB fused-silica capillary column was used. Compound identification as in Table III.

TABLE III

COMPOUNDS IDENTIFIED IN A FRACTION FROM BESÓS RIVER SEDIMENT SUBFRACTIONATED BY GPC

Peak No.	*GPC fraction*	*MW*	*Identification*
1	3—5	312	Polystyrene trimer isomers
2	3	410	Squalene
3	4	272	Unknown (*m/z:* 181, 167)
4	4	286	Unknown (*m/z:* 181, 167)
5	4	300	Unknown (*m/z:* 181, 167)
6	5	178	Phenanthrene
7	5	198	C_1-substituted dibenzothiophenes
8	5	192	C_1-substituted phenanthrenes
9	5	212	C_2-substituted dibenzothiophenes
10	5	206	C_2-substituted phenanthrene
11	5	202	Fluoranthene
12	5	226	C_3-substituted dibenzothiophenes
13	5	202	Pyrene
14	5	226	C_3-substituted dibenzothiophenes
15	5	228	Chrysene
16	5	242	C_1-substituted chrysene

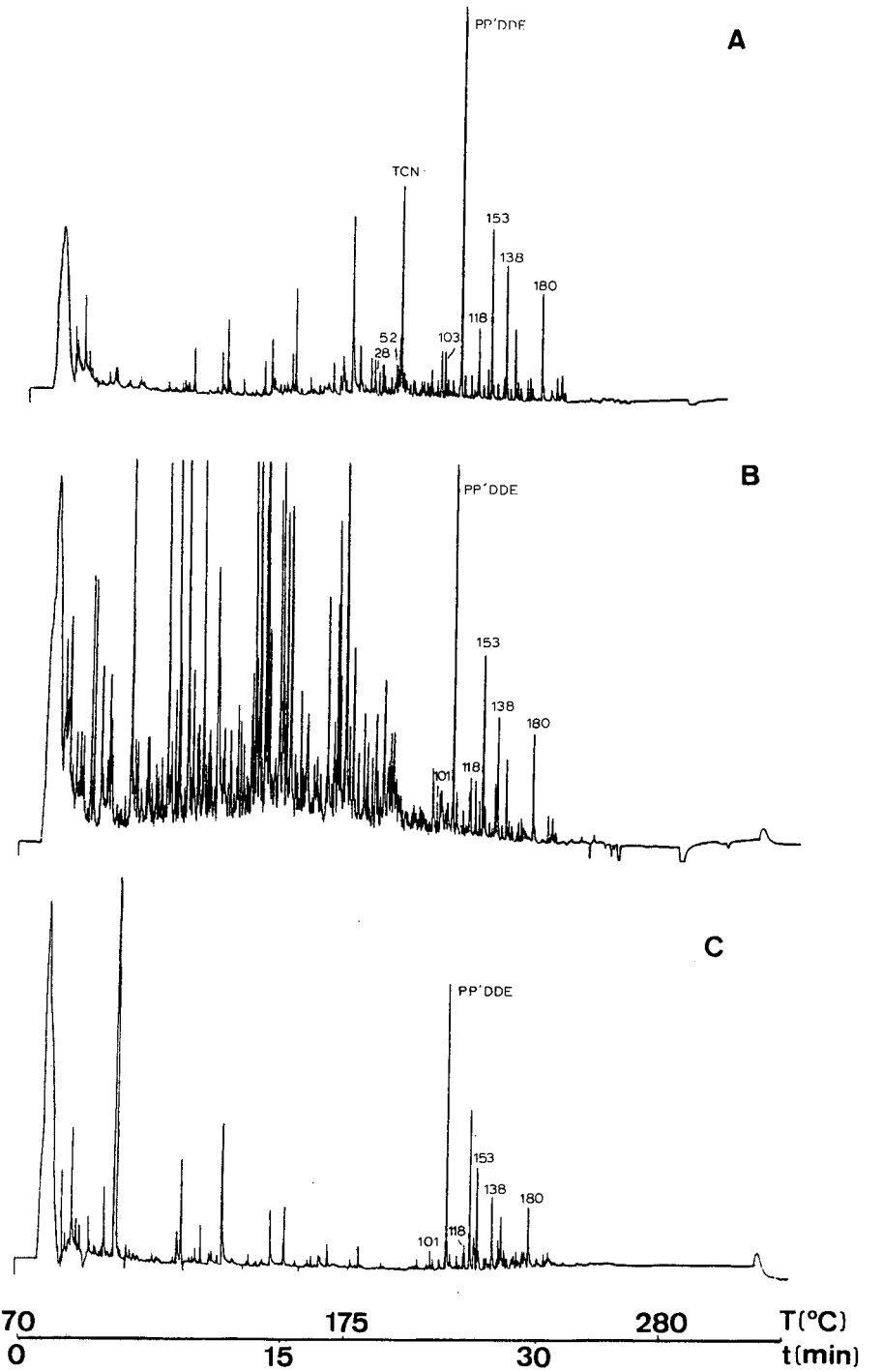

Fig. 3. GC–ECD chromatogram of *Gambussia affinis* extracts after the GPC clean up and indicating the individual PCB congeners. (A) Typical crude extract with the internal standard 1,2,3,4-tetrachloronaphthalene (TCN), (B) Complex crude extract using the same procedure as in (A). (C) Crude extract from (B) with further clean up using sulphuric acid. A 50 m × 0.25 mm I.D. CP-Sil 5 CB column was used.

ranging from m/z 424 to 508, whereas in fractions G-3 and G-4 an isomeric mixture of polystyrene trimer isomers in addition to squalene (major component in fraction G-3) were detected. PAHs were eluted entirely in fraction G-5, exhibiting a characteristic profile of fossil hydrocarbons with a great predominance of alkylated over parent components. Sulphur containing PAHs (S-PAC) (C_1-substituted dibenzothiophene isomers) in a relative high concentration with respect to the neutral PAHs were detected for this sample. It should be noted that no interferences were found in the PAHs containing fraction (G-5), although this chemical class was in relatively low abundance.

Biota. GPC in combination with GC–ECD analysis was applied to different biota samples of the mosquito fish *Gambussia affinis* collected during different periods in 1987 at the rice fields of the Ebro Delta (South Barcelona). A typical chromatogram of a fish extract is shown in Fig. 3A. When crude extracts were injected chromatograms as in Fig. 3B were obtained. These profiles can be attributed either to seasonal changes of the samples or to the size and type of sample, exhibiting in some cases higher lipid contents. For these complex mixtures a further clean up is needed for analyzing PCBs with the use of 1–2 ml of sulphuric acid. However, it should be mentioned that the quantitative results for the individual PCB congeners shown in Fig. 3B and 3C are the same. Any difference will be for the compounds eluted before PCBs which are not stable to the treatment with sulphuric acid.

In conclusion, the determination of two- to five-ring PAHs and PCBs from complex environmental samples was successfully achieved by GPC. In this sense, GPC is a good alternative to classical clean up methods for PAHs (*e.g.* saponification), because no destruction of the more polar PAHs occurs. As regards the determination of PCBs from marine biota samples, it has been demonstrated that the technique used in this paper offers valuable clean up and cannot be underestimated *versus* normal phase liquid chromatography fractionation[15].

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PRECONCENTRATION AND ANALYSIS OF ATMOSPHERIC ISOPRENE AND MONOTERPENES

SYSTEM AUTOMATION

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SUMMARY

As part of a study on terpene hydrocarbons in the biosphere–atmosphere exchange processes, an entirely automatic device has been elaborated for sampling, preconcentration and analysis of isoprene and the principal atmospheric monoterpenes. This device comprises mainly a gas chromatograph for analysis and a programmable controller for the management of the operations. It permits measurement of the concentration and flux rate of terpenes. Automatic and continuous cycles (sampling–analysis) were carried out over several days. They were characterized by a reproducibility lower than 1%; their analytical characteristics were quite acceptable.

INTRODUCTION

In environmental matter, essential problems arise concerning, first, the perturbation, due to human activities, of the biosphere–atmosphere equilibrium, imposed by natural phenomena and then the eventual change in atmospheric air composition. Indeed even if there is no change in proportion of the major atmospheric compounds, there may be one in the composition of the trace compounds. Some of those compounds, CO, hydrocarbons, NO_x, O_3, aerosols, etc., although in low or sometimes very low concentrations, have an important rôle in the planet radiative balance, in atmospheric chemistry, in biomass, in biogeochemical cycles, etc.

The atmospheric vulnerability to external perturbations wherever they come from (solar, natural or human) has consequences, especially for climate, forests, lakes, etc. This inexorable evolution constitutes a problem which is going to increase for the next decades and so retains the attention of the international scientific community.

Aims of research throughout the world are to estimate the actual state on a planetary scale and to comprehend better the emission and evolution of its components, then to predict the future and to propose, if necessary, solutions.

Among the numerous volatile organic compounds present in the atmosphere, emitted by natural sources, isoprene and monoterpenes were the most studied in the

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last few years[1–4] because of their predominance in vegetal emissions and their important chemical reactivity. These compounds seem to be implicated in very important tropospheric processes. Under UV solar irradiation, in the presence of nitrogen oxides, they are oxidized by OH radicals, ozone and may participate in many reactions which are sources of "nuclei", aerosols and carbon monoxide[5–9]. Those hydrocarbons can also react by night with the NO_3 radical according to poorly understood chemical mechanisms[10,11].

Although numerous studies[1,12–14] were made to determine the part played by those biogenic compounds in aerosol formation and in the global ozone balance, the results in rural zones are misstated and not sufficient.

Comprehension of the contributions of isoprene and monoterpenes to atmospheric physicochemistry requires knowledge of the variations in their daily, seasonly and geographically concentrations and emissions. This research can be pursued only if an analytical method is available that can characterize and measure terpenes in natural atmospheres in which their concentrations are at the μl/l level.

We have devised a totally automatic apparatus for measuring isoprene and monoterpenes in atmospheric air, which operates continuously and sequentially.

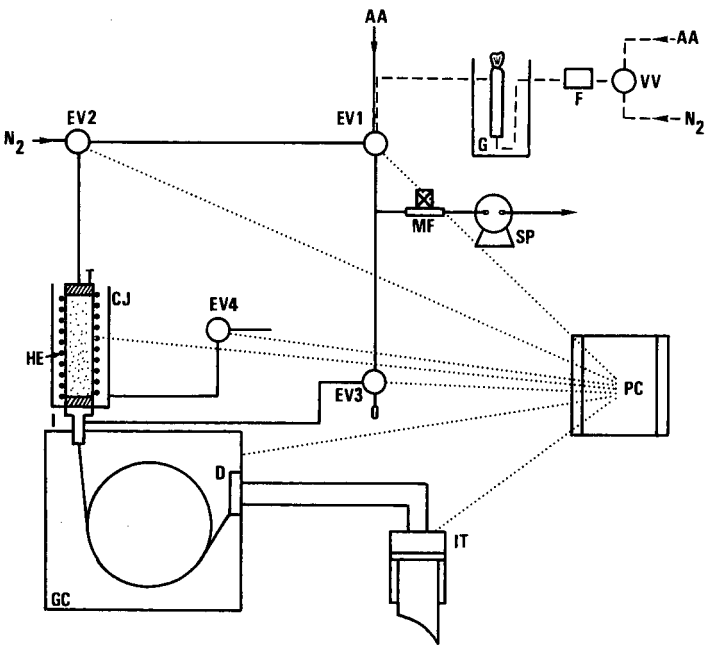

Fig. 1. Automatic device for sampling and analysis — → —, Standard atmospheres sampling circuit. AA = Atmospheric air inlet for natural atmospheric samples. ....., Programmable controller action. PC = Programmable controller; GC = gas chromatograph; CJ = cooling jacket; D = flame ionization detector; MF = mass flow meter; EV_1, EV_2, EV_3 = three-way electrovalves; EV_4 = two-way electrovalve; F = filter; G = generator; I = injector; IT = integrator; T = trap; HE = heating element; SP = suction pump; VV = three-way valve.

EXPERIMENTAL

Instrument design

The automatic sampling and analysis device is shown in Fig. 1. Its principal elements are a gas mixture generator (G), a trap (T), a NO A18 Type KNF membrane suction pump (SP) (Neuberger, Village-Neuf, France), a gas chromatograph (GC) and a programmable controller (PC). The generators used, working by permeation or by diffusion, have been discussed previously[15,16]. The traps consisted of pretreated nickel tubes (15 cm × 4.5 mm I.D.) packed to a depth of 120 mm with approximatively 350 mg of 60–80 mesh Tenax GC (Alltech-Assoc., Deerfield, IL, U.S.A.) were mounted directly on the injector (I); the trap is surrounded by a brass cooling jacket (CJ) in which compressed air can circulate. A heating element HE (Thermocoax, Paris, France) coiled round the tube supplies heat to the adsorbent heating. The trap and its mode of attachment to the injector is shown in Fig. 2.

The chromatographic system consisted of an Hewlett-Packard Model 5890 A equipped with a split–splitless injector, a flame ionization detector (D), a fused-silica semi-capillary column OV-1 (30 m × 0.53 μm) and a Model 3390 integrator (IT) (Hewlett-Packard). The programmable controller is a Syrelec Model SLP 3001 (Bordeaux, France) with electronic technology containing 24 input interfaces and 8 output interfaces.

The dead volumes of the gas circuits after the trap were systematically reduced. For this purpose the trap was directly connected to the chromatographic injector (Fig. 2).

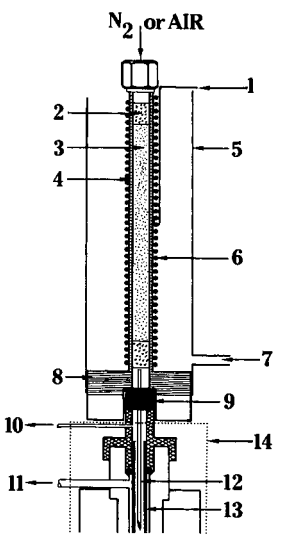

Fig. 2. Trap and its mode of fixing to the injector. 1 = Thermocouple; 2 = glass-wool plug; 3 = adsorbent; 4 = trap; 5 = cooling jacket; 6 = heating element; 7 = compressed air inlet; 8 = cooling jacket fixing nut on injector; 9 = septum; 10 = septum purge; 11 = split vent; 12 = fused-silica needle; 13 = insert; 14 = injector.

System operations

The automation of the whole apparatus was done with an electronic programmable controller which controls, in real time, machines or sequential logic processes. Its role consists in collecting information appropriated by the probes:

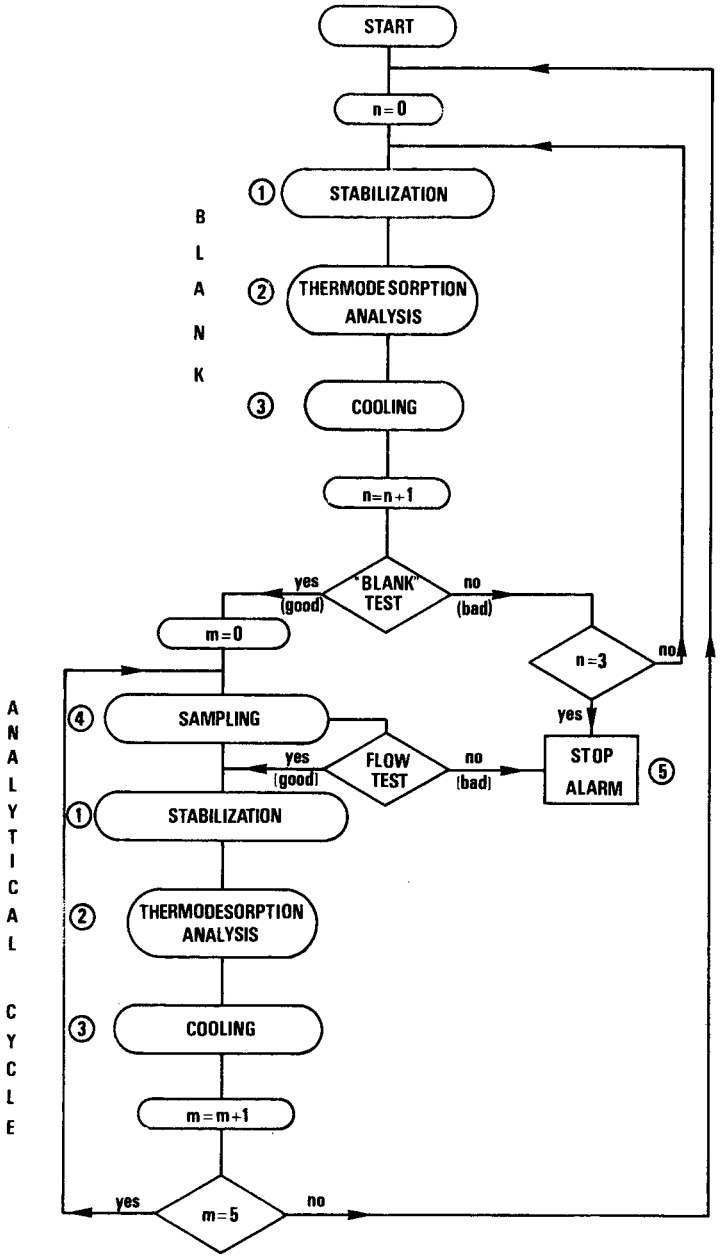

Fig. 3. Flow chart of an automatic sampling–thermodesorption–analysis cycle.

temperature, flow sampling, chromatographic signal; treating that information according to instructions and data it has received previously: management of sampling time, heating time, automatic stop device if the information does not correspond with the data; producing output signals able to be managed by the processor devices: electrovalve activation, trap heating and chromatograph simultaneous release, integrator start.

The controller can also give information on its own behaviour: visualizing the output states; showing the program or active part evolution; visualizing the inner data evolution: counter values, temporizations; ensuring its own control and uttering alarm signals in the case of failure.

To pass from our experimental definition to its programming we prepared a flow chart which specifies: automation characteristics; the function the controller must ensure; the king of information implicated in the working part automation; safety conditions.

The different steps in the flow chart of the working cycle to be automatized are shown in Fig. 3. The analysis cycle of the atmospheric terpenes starts with a "blank" to test the quality of the analytical apparatus. This "blank" contains three steps.

(1) Stabilization. All the dynamic elements: gas flows, chromatographic signal. During this step the controller simply acts as a timer.

(2) Thermodesorption and analysis. Clean trap heating and the absence of a chromatographic signal control. The trap heating was optimized to subject the adsorbed compounds to a rapid thermic-flash to obtain consequently a rapid injection

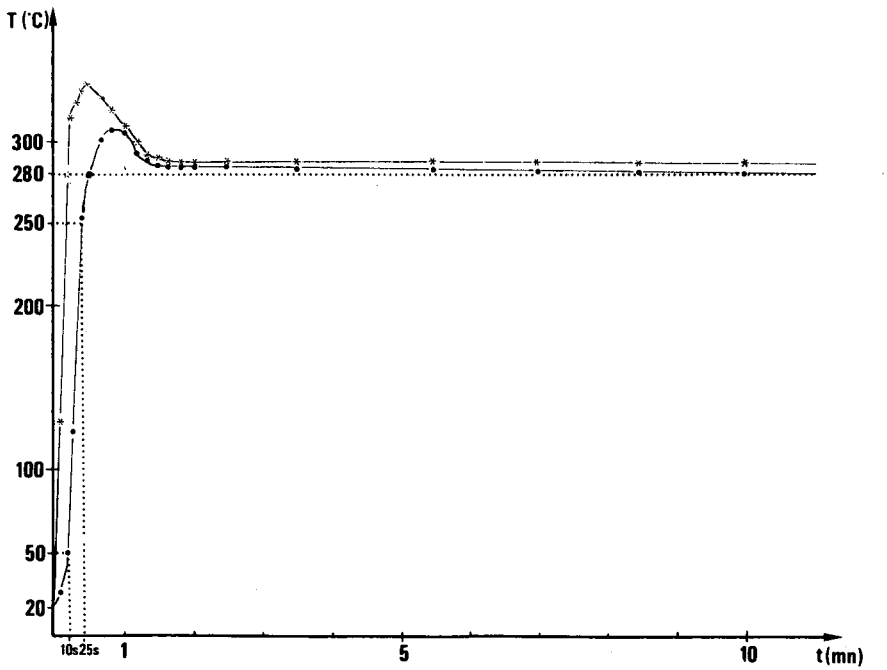

Fig. 4. Trap temperature evolution during thermodesorption. ●—●, Temperature in the adsorbent; *—*, trap external wall temperature.

TABLE I
EXPERIMENTAL CONDITIONS FOR ANALYSIS OF ISOPRENE AND MONOTERPENES

Thermodesorption	Trap temperature	280°C
	Entraining gas	Nitrogen, 10 ml/min
	Desorption time	Time of analysis
Analysis	Column temperature	4 min at 30°C, then programmed at 10°C/min to 90°C during 10 min
	Other temperatures	Injector: 150°C Detector: 300°C
	Carrier gas	Nitrogen, 10 ml/min

in the chromatograph. To avoid damage to the adsorbent (Tenax GC), we were careful to keep its temperature under 310–320°C. Heating was done with an electric element at a maximum initial power (500 W, 80 V) for a few seconds, then under a reduced power (50 W, 25 V). This protocol permits the trap rapidly to attain 280°C and to be maintained at this temperature during the analysis. The passage from one way of heating to another is done automatically by a "home-made" electronic apparatus. Curves of the temperature attained by the trap external wall, and inside the adsorbent, are shown in Fig. 4.

By this step, the controller manages the electrical supply to the heating element, the desorption temperature and their duration. Experimental conditions are given in Table I.

(3) Cooling. By the end of the analysis, the rapid cooling of the trap to ambient temperature is ensured by a compressed air circulation round the trap. At the end of step 3 three ways can be taken: first, if the "blank" test is bad, *i.e.*, the chromatographic baseline has been appreciably perturbed, the test is begun again with three times at most; secondly, if, at the end of the third test the "blank" test is bad again, the whole procedure is stopped and an alarm signal is sounded subject to manual intervention; thirdly, if the "blank" test is good, the analytical cycle is normally done along the steps:

(4) Sampling. Atmospheric air circulation in the trap, in which organic compounds are adsorbed, at ambient temperature. Then, the air is exhausted by the injector "split" circuit.

TABLE II
PROGRAM TO IMPLEMENT AUTOMATED REPETITIVE OPERATION OF A SAMPLING–THERMODESORPTION–ANALYSIS CYCLE

Time (min)	*Command*	*Comments*
0	Valves 1, 2, 3 on	Sampling
10	Valves 1, 2, 3 off	Stabilization
15	HE, GC, IT on	Thermodesorption
30	HE, GC, IT off Valve 4 on	Cooling
35	Valve 4 off Valves 1, 2, 3 on	New sampling

(1) Stabilization

(2) Thermodesorption and analysis. The trap is heated, thermodesorbed compounds are carried by carrier gas to the column head and analysed.

(3) Cooling. By the end of this step, the apparatus is ready for another cycle.

Five successive analytical cycles are done, with the same sequence. At the end of the fifth cycle, a new "blank" test is done.

In case of a breakdown of the electric circuit, or a desired end to the procedure, the whole sequence is reset again at step 1.

Table II gives the model program listing the successive cycles of automatic execution. In this example, sampling and analysis last 10 and 15 min respectively.

SYSTEM PERFORMANCE

In a preconcentration process on a solid adsorbent, a general efficiency of the adsorption–desorption of volatile compounds close to 100% is a necessary condition to obtain a quantitative analysis of each compound adsorbed. In this work, we devised an efficiency determination method, called "gaseous deposit" explained in a previous paper[17] and particularly appropriate to very volatile compounds. For isoprene, for example, the general efficiency of adsorption–desorption is 96.5% at 25 ± 1°C.

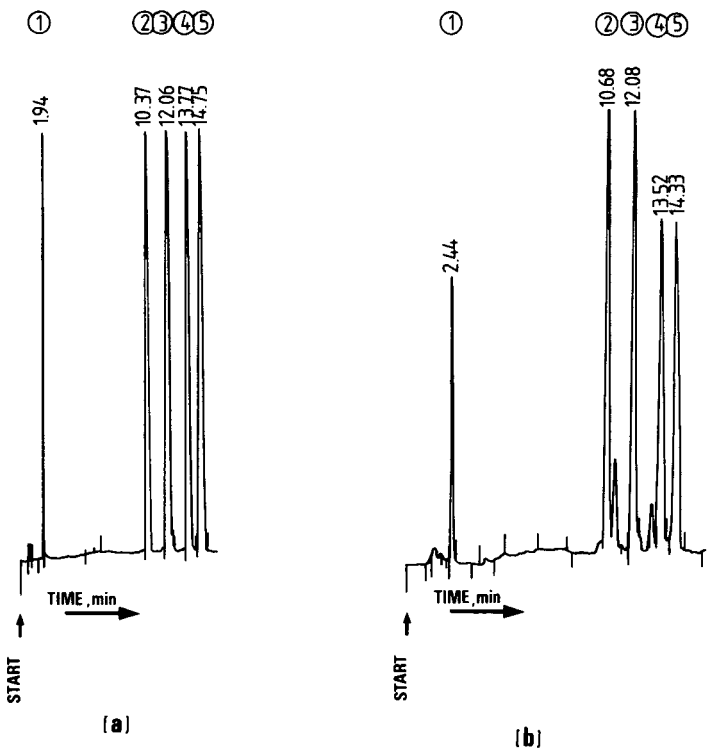

Fig. 5. Chromatograms of mixture of isoprene and monoterpenes: (a) direct injection with a microsyringe; (b) injection after a sampling–thermodesorption–analysis cycle managed by the automatic device. Peaks: 1 = isoprene; 2 = α-pinene; 3 = β-pinene; 4 = Δ_3-carene; 5 = limonene.

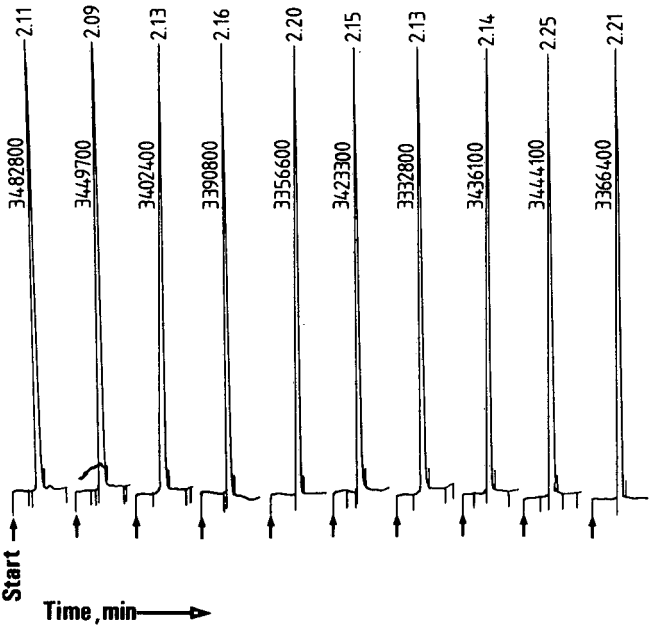

Fig. 6. Repeatability of signals obtained after automatic sampling–thermodesorption–analysis cycles for a mixture of nitrogen gas and traces of isoprene.

The chromatograms shown in Fig. 5 result from the injection of a mixture of isoprene and monoterpenes with a microsyringe (a) and after an automatic preconcentration–thermodesorption–analysis cycle of the same compounds (b). The quantities injected by the two techniques differ. The chromatographic resolution is practically unaffected by the preconcentration–thermodesorption mode. We think this is due to a good injection of the products from the trap. This is a practically instantaneous injection thanks to the rapid thermodesorption resulting from a rapid thermic-flash. The systematic reduction of the dead volumes of the whole trapping and injection circuit leads to a low diffusion of te compounds.

Beside the recovery of the compounds, after adsorption–desorption and the good analytical performance of the apparatus used a most important factor of the automatic apparatus liability is its repeatability over long time periods. This factor is illustrated in Fig. 6 which shows signals obtained from a mixture of nitrogen gas and traces of isoprene, subjected to a complete adsorption–thermodesorption–analysis cycle. We show 10 signals at regular intervals in a series of 60 recording automatically over a 48-h period. The very low coefficient of variation found (0.9%), indicates an excellent repeatability.

In our experimental cycles, the sampling of the compounds on a solid adsorbent and their direct chromatographic analysis after thermodesorption avoids the need for an intermediate cryogenic trapping, while retaining analytical performance. Then, the avoidance of liquid nitrogen makes this device easier to carry out and increases its autonomy.

The apparatus developed permits the measurement of isoprene and monoterpenes present in traces (a few μl/l) in laboratory prepared atmospheres. Its automatic working over long time periods is confirmed by our results. However, it remains to demonstrate its applicability to natural atmospheres in rural or forest zones, non-polluted or polluted at low levels. Those experiments or studies should be achieved during future international campaigns (DECAFE, BIATEX) in which we will participate. There have been only a few previous realizations[18–22] of devices that can ensure simultaneous sampling–thermodesorption–analysis.

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GROUP FRACTIONATION OF FREE AND CONJUGATED STEROIDS BY MEANS OF DISPOSABLE SILICA-BASED ANION-EXCHANGE COLUMNS

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SUMMARY

Adsorption and desorption modes have been elaborated which allow the separation of complex steroid mixtures of biological fluids into an unconjugated, a glucuronic acid conjugated and a sulphuric acid and mixed conjugated fraction. Capillary gas chromatographic analysis of the fractions obtained provides "total steroid profiles" of urine, blood, ovarian follicle fluid and amniotic fluid.

INTRODUCTION

In studies dealing with complex gas chromatographic steroid profiles an important gain in sensitivity and specificity of detection can be obtained by applying fractionation of the steroid extracts according to the conjugate forms. The Sephadex LH-20® column chromatography method[1] using chloroform–methanol containing 0.01 *M* sodium chloride is still frequently used. Apart from a sulphate and a disulphate fraction, a mixed unconjugated and glucuronic acid-conjugated fraction are obtained. Alternative methods use hydrophilic or lipophilic Sephadex-based anion exchangers. Separate glucuronide, monosulphate and disulphate conjugated steroids can be obtained with the anion exchanger diethylaminohydroxypropyl (DEAP)-Sephadex LH-20[2]. It was also possible to separate simultaneously a phenolic and a neutral steroid fraction if a strong anion exchanger triethylaminohydroxypropyl-Sephadex LH-20 was used[3]. Recently strong anion exchangers based on silica have been made available as disposable sample preparation columns. We have determined the best working conditions to allow them to be used for the fractionation of steroids and their conjugated forms.

MATERIALS AND METHODS

Bonded octadecylsilica and quaternary amine strong anion-exchange disposable columns (500 mg dry material), respectively Bond Elut® C_{18} (BE C_{18}) and Bond Elut® SAX (BE SAX), were from Analytichem International (Harbor City, CA, U.S.A.). Also from Analytichem was a ten-place vacuum manifold (Vac Elut) allowing simultaneous elution of up to ten disposable columns. Methanol was of residue-

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free quality. Triethylammonium sulphate (TEAS, 0.5 *M*, pH 5.0) was prepared by titrating triethylamine with 0.5 *M* aqueous sulphuric acid. This solution was used to prepare the 0.25 *M* triethylammonium sulphate in methanol–water (1:1). Lipidex 5000® was obtained from United Technologies Packard. The *Helix pomatia* juice (Industrie Biochimique Francaise, Clichy, France) contains 100 000 F.U. β-glucuronidase and 1 000 000 R.U. sulphatase per ml [one Fishman Unit (F.U.) is the amount of β-glucuronidase liberating 1 μg of phenolphtalein in 1 h at 37°C; one Roy Unit (R.U.) is the amount of sulphatase that hydrolyses 1 μmol *p*-nitrocatechol sulphate per h at pH 5 and 37°C].

Sample processing

Anion exchange. To the biological fluid (serum, urine, amniotic fluid) was added water, 1.5 *M* potassium acetate buffer and 0.5 *M* triethylammonium sulphate solution in water (both pH 5.0) so that final concentrations of respectively 0.15 and 0.125 *M* were obtained[4]. This mixture was applied to the BE C_{18} column (1.5 cm × 0.9 cm I.D.) (primed with 5 ml methanol and 10 ml water) and sucked through at two drops per second. After rinsing with 7.5 ml 0.15 *M* potassium acetate buffer (pH 5.0), the column was sucked dry and removed from the Vac Elut. The BE SAX column (1.5 cm × 0.9 cm I.D.) was washed successively with 4 ml methanol, 5 ml water, 25 ml 0.5 *M* acetic acid in water and again 25 ml water in order to replace the original chloride counter ion by the acetate ion. Then 4 ml methanol were applied and sucked through, except for 1 ml which was left in the anion-exchanger column. The BE C_{18} column upon which the steroids were adsorbed was attached to the BE SAX column. A 4-ml volume of methanol was applied to desorb the free and conjugated steroids from the C_{18} column and simultaneously load them on the SAX column (rate: two drops per second). The non-retained unconjugated steroids were collected using Rotavapor-adapted 40-ml ground-glass tubes. After removing the C_{18} column an additional 1 ml methanol was sucked through the SAX column, which completed the elution of the unconjugated steroids. The glucuronic acid conjugates were eluted with 8.4 ml 0.2 *M* formic acid in methanol–water (1:1). The steroid sulphates were desorbed with 8.4 ml of the 0.25 *M* TEAS solution.

Enzymatic hydrolysis and solvolysis. The steroid glucuronide fraction was taken nearly to dryness using a rotary evaporator. The residue was dissolved in 4.5 ml water, brought to pH 4.8 with 0.5 *M* sodium hydroxide (three drops) and incubated with 40 μl *Helix pomatia* extract at 37°C for 24 h. The incubate was saturated with solid sodium bicarbonate and extracted with 20 ml ethyl acetate. Finally, the extract was washed with 5 ml water.

The steroid sulphate fraction was taken to dryness and redissolved in water (4.5 ml). A 1-g amount of sodium chloride was added and the pH adjusted to $\leqslant 1$ using one drop of concentrated sulphuric acid. A 30-min extraction with 20 ml ethyl acetate (automatic shaking apparatus) was then performed. After centrifugation at 1500 *g*, the water layer was removed and the upper layer incubated during 16 h at 37°C. Finally the ethyl acetate was washed with saturated bicarbonate and water.

Derivatization and gas chromatography. Methoxime-trimethylsilyl ethers were formed, purified using Lipidex 5000® columns and injected into a 25 × 0.32 mm I.D. polydimethylsiloxane capillary column (CP Sil 5 from Chrompack, The Netherlands) using a falling needle injector, as described previously[4,5]. A Varian 3500 in-

strument was used. The hydrogen carrier gas flow-rate was 2 ml/min. The oven temperature was first programmed from 160°C at 20°C/min to 220°C and then at 4°C/min to 285°C. Detection was done by a flame thermionic detector except for compounds without a methoxime function. These were detected using flame ionization.

Recovery estimation. Recovery of steroid glucuronides was assessed by analysing urine samples from adults (containing merely glucuronide-conjugated steroids) and comparing the results with those obtained with a similar procedure not including the BE SAX fractionation[5]. Likewise, steroid sulphate recovery was estimated in serum samples from adults as in this fluid the vast majority of steroids occur as sulphate conjugates.

Measurement of elution curves and recoveries with radioactive steroids and steroid conjugates. Twenty thousand counts of respectively [2,4,6,7-$^3H_4$]oestradiol, [2,4,6,7-$^3H_4$]oestradiol-17β-glucuronide and [7-^{3}H]dehydroepiandrosterone-3β-sulphate were mixed with 1 ml of an urine sample and the conjugates separated as described, except that fractions of 1.4 or 1 ml were collected. Solvents were evaporated with nitrogen, the residues dissolved in scintillation fluid and counted.

Thin-layer chromatographic confirmation of steroid conjugation in different fractions. This was done on high-performance silica gel plates from Merck using propan-2-ol–chloroform–methanol–water (10:10:5:2) as the eluent. Detection of the steroids was made possible by heating at 120°C after spraying with an ethanolic solution of sodium *p*-phenolsulphonate (0.2 g per 100 ml) and phosphoric acid (8.5%).

Quantitation. Appropriate amounts of different internal standards were added: for unconjugated steroids 1.25 μg tetrahydrocortisol were added before the adsorption on BE C_{18}. Testosterone was added after the BE SAX column to both sulphate and glucuronide fractions (for 1 ml urine, 2.5 and 10 μg respectively). Relative response coefficients for each particular steroid were determined in relation to the internal standard and used in the calculation of concentrations.

RESULTS*

As shown in Fig. 1, radioactive steroids and steroid conjugates are completely separated from each other. Quantitative elution is obtained with only small amounts of the indicated eluents. The recovery for unconjugated radioactive oestradiol was 98%. Less than 2% of the total radioactivity was eluted in the formic acid-containing eluate. Of oestradiol glucuronide, less than 1% eluted in both the unconjugated and sulphate fraction. Up to 99% dehydroepiandrosterone (DHA) sulphate was found in the 0.25 *M* TEAS fraction.

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* Abbreviations used in the figures and tables: Andr = androsterone; Etio = etiocholanolone; DHA = dehydroepiandrosterone; KA = 11-oxo-androsterone; T = testosterone (internal standard); HA = 11β-hydroxyandrosterone; HE = 11β-hydroxyetiocholanolone; THE = tetrahydrocortisone; THA = tetrahydro-11-dehydrocorticosterone; THB = tetrahydrocorticosterone; aTHB = 5α-tetrahydrocorticosterone; THF = tetrahydrocortisol; aTHF = 5α-tetrahydrocortisol; E = cortisone; F = cortisol; eAndr = epiandrosterone; 16 DHA = 16α-hydroxydehydroepiandrosterone; 'Pon = pregnenolone; 16β-DHA = 16β-hydroxydehydroepiandrosterone; $\alpha\alpha$Pon = 3α-hydroxy-5α-pregnan-20-one; Pg = progesterone; 16'Pon = 16α-hydroxypregnenolone; B = corticosterone; IS = 20α-dihydroprogesterone (used as the internal standard); androstenediol = 3β,17β-dihydroxy-5-androstene; pregnenediol = 3β,20α-dihydroxy-5-pregnene; pregnenetriol = 3β,17α,20α-trihydroxy-5-pregnene; MeOH = methanol; TEAS = triethylammonium sulphate.

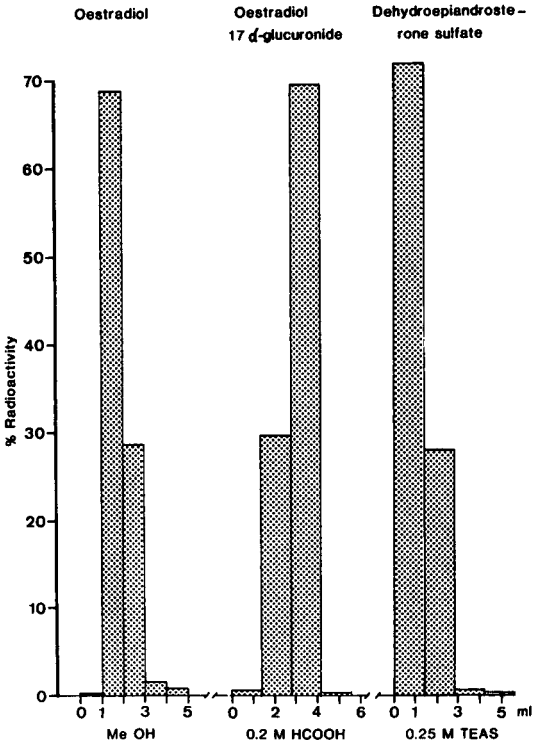

Fig. 1. Separation of radioactive steroid(conjugate)s oestradiol, oestradiol-17α-glucuronide and dehydroepiandrosterone sulphate using a disposable strong anion-exchange column.

TABLE I

RELATIVE RECOVERY OF THE BE SAX METHOD COMPARED WITH A REFERENCE METHOD FOR STEROID GLUCURONIDES (G) IN URINE AND FOR STEROID SULPHATES (S) IN SERUM

Steroid conjugate	*Recovery (%)*	*Coefficient of variation (%)*
Urine		
Androsterone G	106.3	6.4
Etiocholanolone G	99.2	3.1
Tetrahydrocortisone G	107.4	5.0
Tetrahydrocortisol G	104.7	3.7
5α-Tetrahydrocortisol G	106.6	4.0
Serum		
Androsterone S	116.0	5.1
Dehydroepiandrosterone S	95.7	4.3
Epiandrosterone S	100.5	4.7
Pregnenediol S and diS	105.2	6.3
16α-Hydroxydehydroepiandrosterone S	113.3	3.6

TABLE II

REPRODUCIBILITY DATA FOR FIVE ANALYSES OF AN URINE AND OF A SERUM POOL

Steroid conjugate	*Coefficient of variation (%)*
Urine	
Androsterone G	6.5
Etiocholanolone G	7.9
Tetrahydrocortisone G	5.4
Tetrahydrocortisol G	2.8
5α-Tetrahydrocortisol G	6.5
Serum	
Androsterone S	9.0
Dehydroepiandrosterone S	4.3
Androstenediol S and diS	5.0
Pregnenediol S and diS	4.2
Pregnenetriol S and diS	6.3

As we could not obtain a radioactive steroid disulphate conjugate, the elution of disulphates was tested with thin-layer chromatography (TLC). The disulphate of 5-androstene-3β,17β-diol exhibited an R_F value of 0.43 and its monosulphate one of 0.66. The R_F values for glucuronides were below 0.1. All conjugates tested were eluted quantitatively in their appropriate fraction. As expected, glucurosulphate double conjugates are eluted in the sulphate fraction.

Relative recoveries, calculated for urinary steroid glucuronides and steroid sulphates in serum, are given in Table I. As stated above, a method not including anion exchange is used as a reference here. Five different urines and six different serum samples have been analysed.

The reproducibility for determinations in urine and serum is given in Table II. In the urine samples represented in the chromatograms of Fig. 2 the concentrations were: 1.4 mg/l for androsterone glucuronide and 2.3 mg/l for tetrahydrocortisone. The androsterone sulphate concentration (Fig. 2B) was 0.54 mg/l and we calculated the dehydroepiandrosterone sulphate concentration to be 0.70 mg/l.

The serum sample (Fig. 3) contained 151 ng cortisol per ml. The dehydroepiandrosterone sulphate (Fig. 3C) concentration was 1287 ng/ml, whereas tetrahydrocortisone glucuronide (Fig. 3B) amounted to only 182 ng/ml.

DISCUSSION

The preliminary adsorption on octadecylsilica may seem at first glance unnecessary. We however were not able to bind quantitatively the steroid glucuronides when starting with an aqueous biological matrix as diluted urine or serum. Positive ions present in the biological fluids may from ion pairs with the steroid glucuronides, so avoiding sorption to the anion exchanger. An octadecylsilica purification step was found earlier[5] to retain quantitatively free steroids as well as conjugated steroids, if triethylammonium sulphate was used as an ion-pair-forming reagent. Moreover, by including adsorption on BE C_{18}, samples with different biological matrices, *e.g.*, tissue extracts, amniotic fluids with high protein concentrations, are made similar so

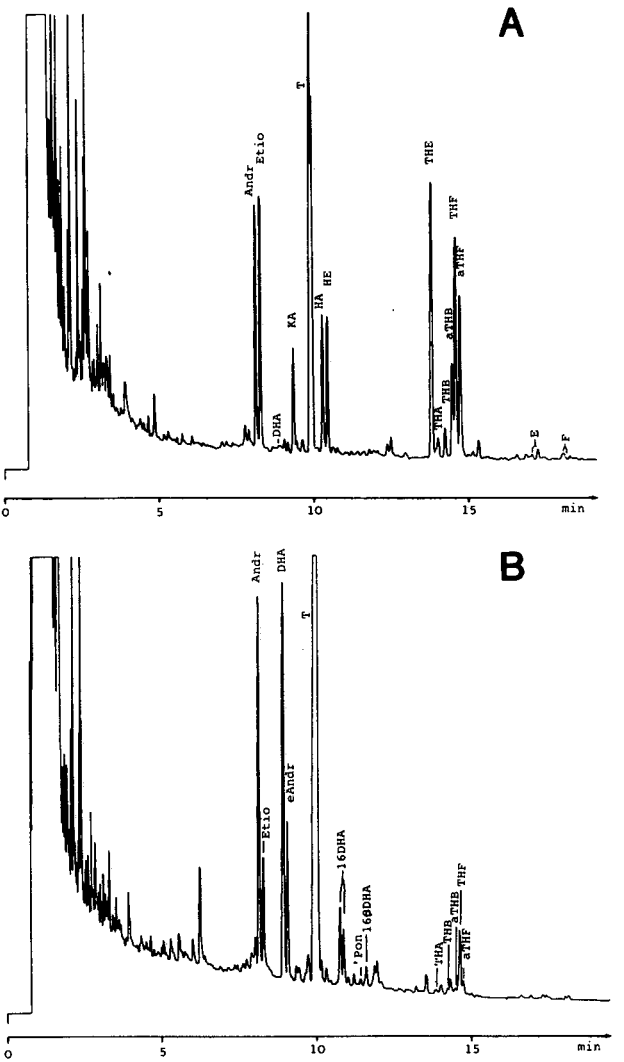

Fig. 2. Gas chromatograms of steroids in urine. (A) Steroid glucuronides; (B) steroid sulphates. For chromatographic conditions, see Materials and methods. Flame thermionic detection was used.

that the BE SAX fractionation is of general applicability. Conversion of the BE SAX into its acetate form is necessary because the results with the original chloride-ion form are strongly influenced by varying residual water concentrations in the methanol eluate from the BE C_{18} column.

The possibility to retain the conjugates on the BE SAX column from a methanol solution allowed direct elution of the C_{18} eluate into the coupled BE SAX column. Time-demanding transfers and the introduction of impurities from glassware are so avoided.

Complete desorption of steroid disulphates could in our hands be obtained only with a triethylammonium sulphate-containing eluent. More selective anions, *e.g.*,

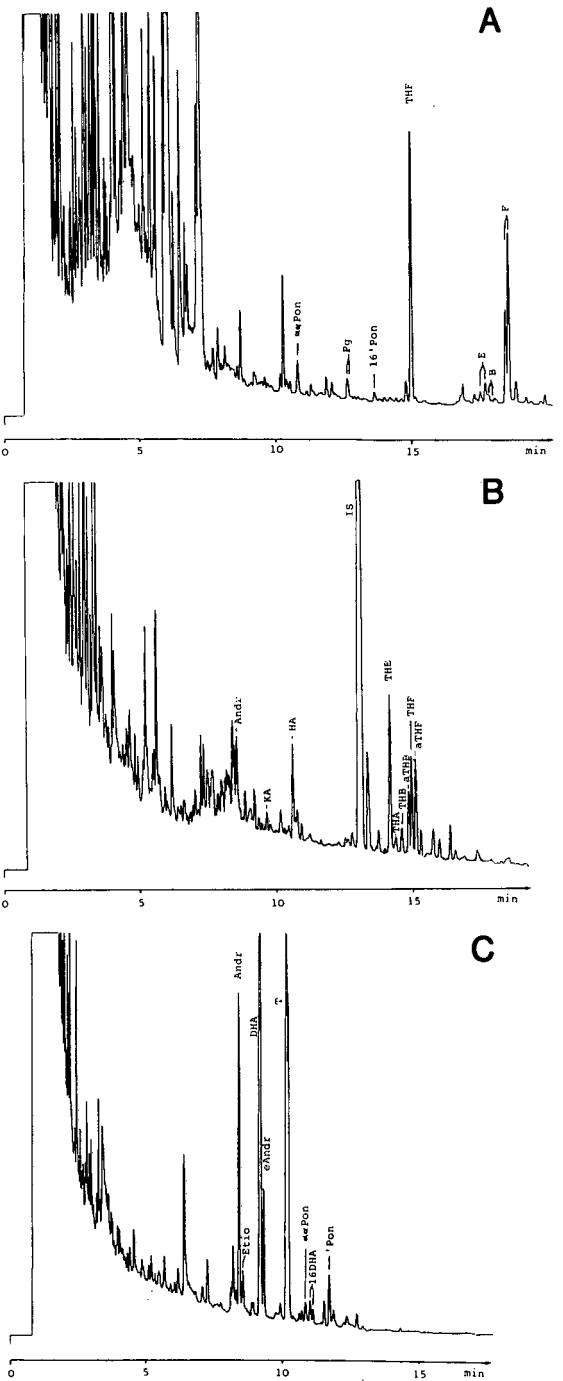

Fig. 3. Gas chromatograms of steroids in serum. (A) Unconjugated steroids; (B) steroid glucuronides; (C) steroid sulphates. For chromatographic conditions, see Materials and methods. Flame thermionic detection was used.

citrate or higher molarity (1 *M*) and higher pH (6.0) acetate buffers in 50% methanol–water did not result in quantitative recovery of, *e.g.*, 5-androstene-3β,17β-disulphate. Perhaps an adsorptive interaction mechanism with residual free silanol groups on the silica material (although the manufacturers claim complete end-capping) plays a role here. Amines are known to block specifically these silanol functions.

Initially, we included a second BE C_{18} adsorption step to remove triethylammonium sulphate prior to the solvolysis or enzymatic hydrolysis (a kind of desalting step). Experiments proved this to be unnecessary.

Steroid concentrations are generally higher (see Table I) after application of the BE SAX group fractionation. This is probably due to the more favourable hydrolysis conditions obtained after purification of the samples. Most urines contain androsterone and also tetrahydrocortisol as a sulphate. The reference method without group fractionation should thus provide higher steroid concentrations. This is not the case, indicating good relative recovery with the BE SAX method.

The solvolysis procedure described is necessary for the quantitative hydrolysis of especially androsterone and epiandrosterone sulphate (both 3α-sulphates of a 5α-steroid) in serum or urine. In urines of newborn infants, enzymatic hydrolysis resulted in only 20% of what was obtained using solvolysis for 16α-hydroxypregnenolone and 3β,16α-dihydroxy-5-androstene-17-one. These urines contain large quantities of mixed conjugates (especially of oestriol and 3β,16α,18-trihydroxy-5-androstene-17-one) for which both solvolysis and enzymatic hydrolysis has to be applied.

CONCLUSIONS

A less time-consuming and less labour-intensive method has been developed to separate steroids according to their conjugate forms. The total steroid profiles obtained after capillary gas chromatography of the different fractions allow quantitations in the presence of much larger amounts of other conjugate forms. Especially the recognition of steroid-synthesizing defects in neonates is made easier: here the high concentrations of sulphates of 5-ene-steroids interfere with the quantitation of gluco- and mineralo-corticosteroid glucuronides. The method has been applied to many different body fluids, *e.g.*, amniotic fluid containing high amounts of proteins and tissue extracts. It is predicted that, also for the analysis of conjugates of pharmaceutical products, the procedure would be of great value.

ACKNOWLEDGEMENT

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RADIO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ^{14}C-LABELLED LF 2-0254, A 1,4-DIHYDROPYRIDINE CALCIUM ANTAGONIST, IN RAT AND DOG PLASMA USING OFF-LINE LIQUID SCINTILLATION COUNTING

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SUMMARY

LF 2-0254 is a 1,4-dihydropyridine calcium antagonist with a slow onset of action. The pharmacokinetics of [^{14}C]LF 2-0254 were studied in rats and dogs. A sensitive high-performance liquid chromatographic method using liquid scintillation counting was developed for the quantitation of labelled LF 2-0254 in plasma. The peak height of the internal standard in the chromatogram was measured by UV detection and the mobile phase containing the chromatographic peak of [^{14}C]LF 2-0254 was collected and counted for radioactivity. The concentration of labelled drug in the plasma was then determined using a calibration graph constructed from the determination of [^{14}C]LF 2-0254 of known specific activities. The limit of determination was dependent on the specific activity of the drug administered. This method permits the measurement of the radioactive drug in biological fluids.

INTRODUCTION

LF 2-0254, [8-aza-8-(4-chlorophenyl)-1,4-dioxaspiro-4,5-decan-2-yl]methyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate (Fig. 1), is a calcium antagonist with a slow onset of action *in vitro*. Unlike nifedipine and nicardipine, LF 2-0254 displays a long duration of action[1]. Dihydropyridine calcium antagonists are a potent group of drugs that exert their desired effects *in vivo* at concentrations of 10^{-9}–10^{-7} M[2]. Sensitive techniques are therefore required to detect and quantitate the levels of these drugs and their metabolic products in body fluids.

The most common techniques employed are packed-column gas chromatography (GC) with electron-capture detection (ECD)[3–6] and capillary GC with either nitrogen-selective detection or ECD[7–11]. Quantification by stable isotope dilution and electron-capture negative-ion chemical ionization has been applied to nitrendipine[12]. Many workers have utilized reversed-phase high-performance liquid chromatography (HPLC) to separate and determine 1,4-dihydropyridine in plasma. With the exception of one electrochemical HPLC method to determine nifedipine[13], the application of UV detection appears to be common[13–20]. The principles of reverse isotope dilution have been applied to quantitate a new 1,4 dihydropyridine[21].

For the determination of LF 2-0254 in plasma, an HPLC method with UV

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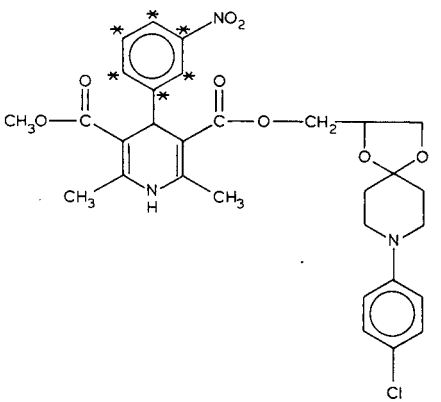
detection at 239 nm has been developed in our laboratory. Its sensitivity (2 ng/ml) did not allow us to analyse plasma from animals administered low doses of the drug (0.1 mg/kg). A more sensitive method using liquid scintillation counting was then set up for the quantitation of labelled LF 2-0254 in plasma by counting the radioactivity of the [^{14}C]LF 2-0254 chromatographic peak. The limit of determination in plasma was 0.24 ng/ml for a 0.1 mg/kg dose (specific activity 136.95 μCi/mg).

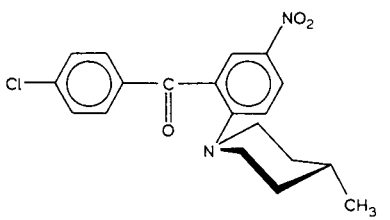
EXPERIMENTAL

Chemicals

[^{14}C]LF 2-0254, specific activity 136.95 μCi/mg (3030.30 MBq/mmol), radiochemical purity >95% by thin-layer chromatography and HPLC, was a custom synthesized by Isotopchim (Mougins, France). LF 2-0254 was labelled at all six carbons of the nitrophenyl ring. The internal standard, LF 1608, synthesized in our Department of Organic Chemistry, was chosen for its good HPLC and extraction properties and for its stability (Fig. 1). Hexane and diethyl ether (RPE, unstabilized) were obtained from Burdick and Jackson (Muskegon, MI, U.S.A.) and Carlo Erba (Milan, Italy). Sodium hydrogencarbonate was of analytical-reagent grade (Fluka, Buchs, Switzerland). Acetonitrile was of HPLC grade S (Rathburn, Walkerburn, U.K.). Other solvents, of the best grade available, were triethylamine (Fluka), perchloric acid (Prolabo, Paris, France) and deionized water (Milli Q system, Millipore, St. Quentin en Yvelines, France).



LF 2-0254



LF 1608

Fig. 1. Structure of LF 2-0254 and LF 1608 (internal standard). ^{14}C labels are indicated by asterisks.

Animals and dosing

Male Wistar rats (body weight 175–232 g) (Iffa Credo, l'Arbratsle, France) were given [^{14}C]LF 2-0254 in polyethylene glycol (PEG 400) and distilled water [1:1 (v/v), dose 0.1 mg/kg, i.v.; or 2:1 (v/v), dose 3 mg/kg, p.o.]. Beagle dogs (body weight 9.7–13.5 kg) (Carriere, Villeneuve/Lot, France) received [^{14}C]LF 2-0254 (0.1 mg/kg, i.v.) in a solution of PEG 400–water (2:1, v/v).

Blood samples were taken at regular intervals after administration into heparinized tubes. Plasma was immediately separated by centrifugation and kept at −20°C until analysis.

Radioanalysis

^{14}C in excreta and solutions was assayed by liquid scintillation spectrometry using a Packard Tri-Carb Instrument Model 2000 CA (Packard, Rungis, France). Aliquots were counted in glass vials with a counting efficiency of greater than 93% using Packard Instagel liquid scintillant. Quench correction was achieved with reference to an external standard using a calibration graph. The quench correction curve was established with a set of manufacturer's quenched standards and stored in the memory of the counter. Subtraction of the background counting rate was performed by the counter's built-in software, using blank vials counted at the start of each batch. Machine performance was verified at frequent intervals with reference to a sealed standard. For radio-HPLC a Gilson Model 201 fraction collector (Gilson, Villiers le Bel, France) was used to collect 0.5- or 1.5-ml fractions of column eluent in minivials, which were counted for ^{14}C following the addition of a scintillant.

Chromatographic procedure

For HPLC a Spectra-Physics Model SP 8780 XR autosampler (Spectra-Physics, La Verpillière, France), Kratos Model 400 pumps, a Kratos Model 783 detector and a Kratos Model 450 solvent programmer (Kratos, Fontenay/Bois, France), were used. The column was Altex C_{18} (5 μm), 150 × 4.6 mm I.D. (Beckman, Gagny, France). This system was linked to a Shimadzu CR3-A integrator (Shimadzu, Kyoto, Japan). The mobile phase was filtered and degassed on a Millipore GVWP 0.45-μm membrane. It consisted of acetonitrile–water (containing 0.02% triethylamine, pH 3.5, adjusted with concentrated perchloric acid (70:30, v/v) during the first 12 min of analysis, followed by a column wash with 100% acetonitrile for 7 min (Fig. 2). This column wash successfully eliminated peaks with long retention times and maintained column performances.

Sample treatment

Plasma samples from five rats in each dose group were pooled by time point. Plasma samples from dogs ($n=6$) were analysed individually. The extraction procedure was performed using amber glassware or tubes wrapped with aluminium foil in order to prevent photo-oxidation of the dihydropyridine ring. A 50-ng amount of LF 1608 (internal standard) dissolved in 50 μl of acetonitrile was added to 1 ml of plasma in Corning tubes with PTFE screw-caps. The solution was made alkaline by addition of 1 ml of 0.5 *M* sodium hydrogencarbonate and the aqueous phase was extracted once with 8 ml of hexane–diethyl ether (75:25, v/v). After centrifugation, the organic phase was evaporated to dryness under a stream of nitrogen. The residues were

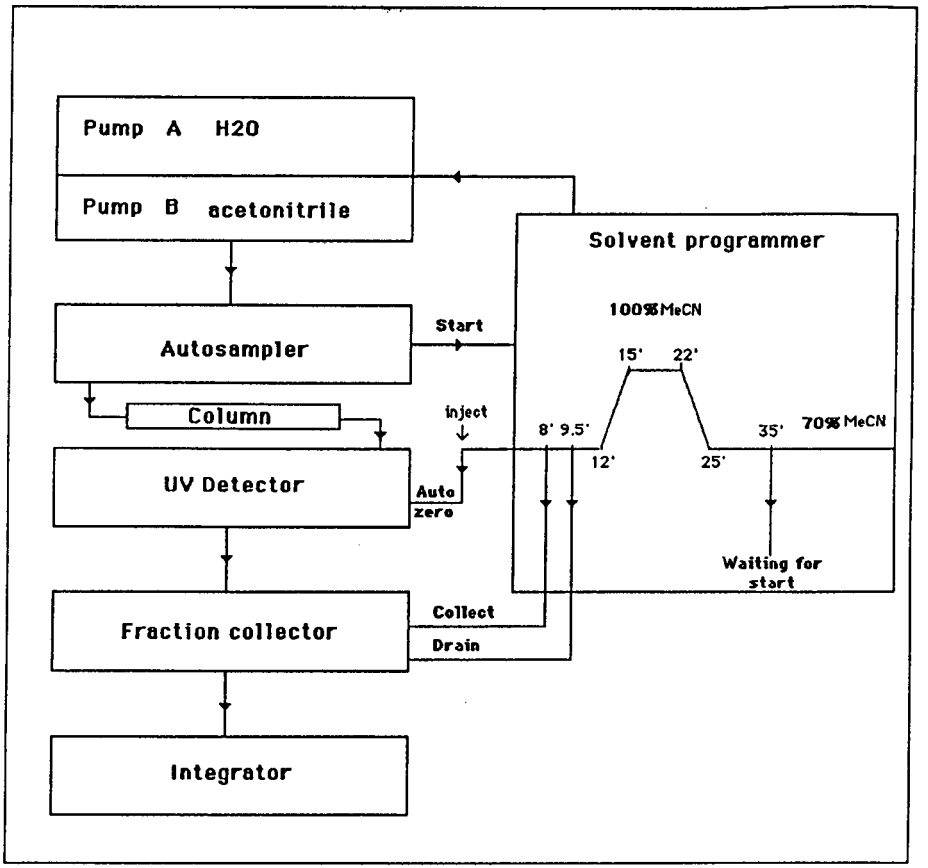


Fig. 2. Scheme of the chromatographic system. After injection of the sample, the programmer, started by the autosampler, begins the solvent gradient and controls the collection of the peak between 8 and 9.5 min. At 12 min into the run, the column wash starts. Acetonitrile (MeCN) begins ramping from its initial value of 70% to 100% in the next 7 min, then ramps back to 70%. A period of 10 min is enough to establish the initial conditions. At 35 min, the programmer is waiting for the next cycle of analysis.

dissolved in 80 μl of acetonitrile and an aliquot of 50 μl was injected on to the HPLC column.

The LF 2-0254 peak, located by its retention time at 239 nm, was collected between 8 and 9.5 min in a glass scintillation vial and counted for 20 min for ^{14}C after the addition of 20 ml of scintillant. It was established by collecting 0.5-ml fractions of mobile phase that no interfering labelled metabolites co-eluted during this interval of time (Fig. 3). As shown with synthetic standards, the pyridine metabolite did not interfere with the [^{14}C]LF 2-0254 assay. This collection of 1.5 ml of eluate was sufficient to obtain the whole peak of [^{14}C]LF 2-0254 in the case of high concentrations of drug. Calibration graphs and quantification were effected by correlating the dpm value obtained after counting the [^{14}C]LF 2-0254 fraction with the peak height of the internal standard detected by UV absorption at 239 nm (Fig. 4).

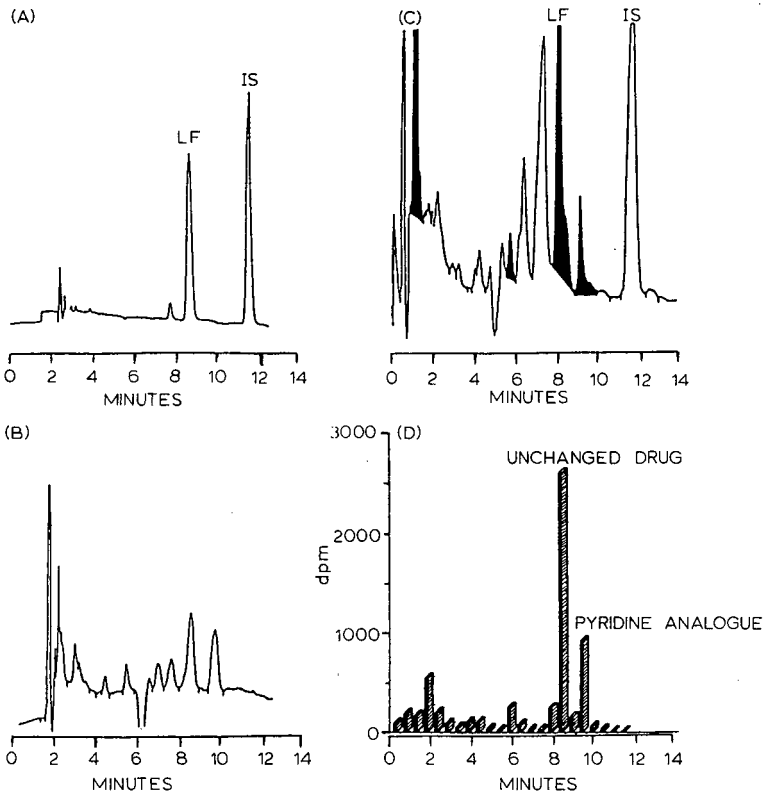


Fig. 3. Chromatograms of rat blank plasma, standards and rat plasma after dosing. (A) HPLC separation of a mixture of LF 2-0254 and LF 1608 (IS) with UV detection at 239 nm ($1.6 \cdot 10^{-3}$ a.u.f.s.). (B) Separation of rat blank plasma with UV detection at 239 nm ($1.6 \cdot 10^{-3}$ a.u.f.s.). (C) HPLC analysis of rat plasma withdrawn, 1.5 h after administration of [^{14}C]LF 2-0254 (3 mg/kg, p.o.) with UV detection at 239 nm ($1.6 \cdot 10^{-3}$ a.u.f.s.). (D) Radiochromatogram of labelled unchanged drug and labelled pyridine analogue of rat plasma withdrawn 1.5 h after administration of [^{14}C]LF 2-0254 (3 mg/kg, p.o.).

RESULTS

Accuracy and precision

Control plasma samples (1 ml) were spiked with 72–172 000 dpm of [^{14}C]LF 2-0254 with a specific activity of 136.95 μCi/mg (0.24–565 ng/ml). The calibration-graphs correlating the dpm counted with the peak height of the internal standard allowed us to determine [^{14}C]LF 2-0254 in plasma knowing the initial specific activity of the dose administered (Fig. 4). The intra-day coefficient of variation was in the range 1.2–11.9% for five extractions per concentration with an accuracy of 0.4–13.3% (Table I). The inter-day coefficient of variation was in the range 5.6–16.3% for 5 days with an accuracy of 0.2–9.6%. For concentrations of LF 2-0254 above 5 ng/ml, a good correlation between the radioactivity calibration graphs and the UV calibration graph was found (data not shown). The limit of determination (0.24 ng/ml) showed a precision of 8% with an accuracy of 17% for twelve extractions.

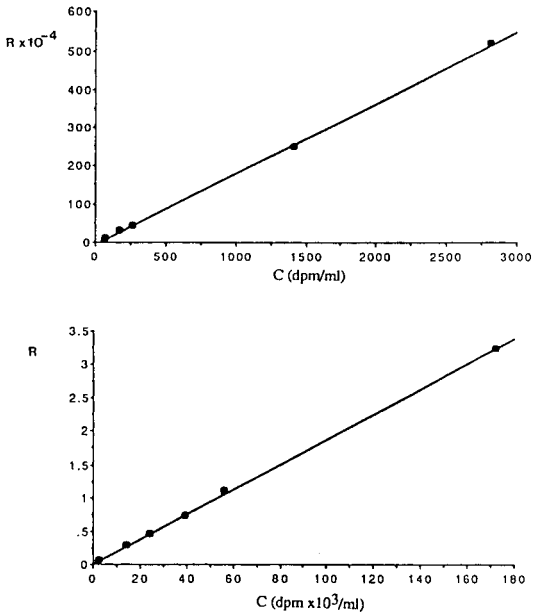


Fig. 4. Intra-day linear regression. Top, calibration graph for the low concentrations; bottom, calibration graph for high concentrations. *R* represents the ratio between the amount of radioactivity in the [^{14}C]LF 2-0254 peak fraction and the peak height of the internal standard detected by UV absorption.

Recovery

Seven extractions at concentrations of 280 dpm/ml (0.9 ng/ml) and 54 600 dpm/ml (180 ng/ml) showed recoveries of 73.7% and 72.6%, respectively (specific activity 136.95 μCi/mg).

TABLE I
INTRA-DAY REPRODUCIBILITY

Concentration added (dpm/ml)	*Detection* (dpm/ml)*	*Precision (%)*	*Accuracy (%)*	*Slope and r***
72.5	82 ± 5	6.6	13.3	Slope = 0.185; r = 0.993
180	200 ± 13	6.4	11.3	
267	265 ± 21	8.2	0.6	
1424	1377 ± 118	9.5	3.3	
2811	2833 ± 268	9.5	0.8	
14217	15960 ± 958	6.0	12.0	Slope = 0.188; r = 0.991
24261	24580 ± 285	1.2	1.3	
39325	38771 ± 2140	5.5	1.4	
56334	54131 ± 3796	7.0	3.9	
172033	172690 ± 20483	11.9	0.4	

* Mean ± S.D. (n=5).
** r = correlation coefficient.

TABLE II

PHARMACOKINETIC PARAMETERS OF LF 2-0254 IN RAT AND DOG PLASMA

Plasma samples from five rats in each dose were pooled by time point. Plasma samples from dogs were analysed individually. Results are expressed as mean ± S.D. Parameters were calculated using mean concentration–time data for five rats, because five rats were killed at a designated time for blood sampling.

Species	*Route*	*Dose (mg/kg)*	*No. of animals and sex*	$t_{\frac{1}{2}}$ *(h)*	C_{max} *(ng/ml)*	t_{max} *(h)*
Rat	I.V.	0.1	5M	2.2	–	–
	Oral	3	5M	3.8	30.8	4
Dog	I.V.	0.1	3M	7.3 ± 1.8	–	–
			3F	11.1 ± 10.8	–	–

Limit of determination

The limit of detection was set at 33 dpm (twice the background). Vials were counted for 20 min for ^{14}C with an efficiency of 93% and a precision of 10%. Following the procedure described above (see *Sample treatment*), this gave a limit of determination in plasma of 72 dpm/ml. The limit of determination in plasma was dependent on the specific activity of the drug administered; it was 0.24, 0.51 and 0.37 ng/ml for the doses 0.1 mg/kg, i.v. (specific activity 136.95 μCi/mg) and 3 mg/kg, p.o. (specific activity 64.17 μCi/mg) in the rat and 0.1 mg/kg, i.v. (specific activity 8.59 μCi/mg) in the dog.

Application

This radio-HPLC method gave reproducible results and was sensitive enough for the determination of [^{14}C]LF 2-0254 in plasma samples. It was used to establish the pharmacokinetics of LF 2-0254 after oral and i.v. administration to rats and dogs (Table II).

DISCUSSION

The method shows high specificity and the assay is sensitive and reproducible. It allowed us to determine the concentration of [^{14}C]LF 2-0254 in rat and dog plasma for pharmacokinetic studies. The limitation of many methodologies is their inability to separate, detect and quantitate the main metabolites of 1,4-dihydropyridines in biological fluids. Most methods measure the parent drug only and a minority can determine major metabolites. This assay could allow the detection of labelled metabolites in a simple chromatographic run. Major metabolites could be collected, identified by mass spectrometry and quantified using synthetic standards. This work is in progress in our laboratory.

The use of radio-HPLC offers several advantages over conventional techniques. With a sensitivity higher than that of UV detection, it is more selective and allows discrimination between co-eluting peaks or peaks with close retention times, which could interfere with UV detection. The chromatographic conditions are easier to set up than with UV detection because labelled drug may co-elute with unlabelled UV-detectable impurities. Compared with UV detection, the time of analysis is shortened. The limitation of this procedure is that a labelled drug with a relatively high

specific activity (>50 μCi/mg) is needed. The use of a ^{3}H-labelled drug with a higher specific activity could be of interest for lowering the limit of determination in plasma.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF VALPROIC ACID IN PLASMA USING A MICELLE-MEDIATED PRE-COLUMN DERIVATIZATION

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SUMMARY

A method for the determination of valproic acid (2-propylpentanoic acid) in plasma by high-performance liquid chromatography (HPLC) after pre-column derivatization is described. The derivatization of valproic acid with a fluorophore and UV label, 4-bromomethyl-7-methoxycoumarin, is performed in plasma diluted with an aqueous micellar system. No extraction or solvent evaporation steps are required. The mechanism of the derivatization of the carboxylic acid is based on phase-transfer catalysis. The sample preparation, including the derivatization step, is rapid and very simple. The proposed HPLC-method was evaluated and compared with a standard immunological assay used for the determination of valproic acid in plasma.

INTRODUCTION

Valproic acid (VPA) (2-propylpentanoic acid) is a commonly used anti-epileptic drug and its therapeutic level in plasma ranges from *ca.* 260 to 840 μM[1,2]. Usually VPA is monitored by gas chromatography (GC) or by an immunological assay[3]. GC analysis is hampered by the fact that it is necessary first to extract the drug with an organic solvent. VPA can be monitored directly using immunoassays. However, these techniques are expensive and it is not possible to determine several anti-epileptic drugs simultaneously, in contrast to, *e.g.*, high-performance liquid chromatography (HPLC)[4].

Only a few HPLC methods have been reported for the analysis of VPA[3,4]. Because of the low detectability, the quantification of VPA in plasma is often performed after the attachment of a suitable chromophore or fluorophore to the carboxylic function[3]. The problems of derivatization procedures prior to HPLC analysis are in general caused by the necessary extraction of the drug with a suitable organic solvent. The volatility of VPA during the solvent evaporation steps causes an extra problem in its determination[3]. In this paper we report an HPLC method for the

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determination of VPA in plasma after a simple labelling procedure with a sensitive fluorophore and UV label[5], 4-bromomethyl-7-methoxycoumarin (BrMMC). This labelling with BrMMC is performed in plasma after the addition of an aqueous micellar system[6]. The mechanism of the micelle-mediated derivatization of carboxylic acids is described in more detail in the accompanying paper[6].

EXPERIMENTAL

Chemicals and solutions

Millipore water (Milford, MA, U.S.A.) was used throughout. Except for Arkopal N-130, the chemicals were of analytical-reagent grade.

The non-ionic surfactant Arkopal N-130 (a condensate of nonylphenol and a polyoxyethylene chain of 13 units on average) was kindly supplied by Hoechst Holland (Amsterdam, The Netherlands). Tetrahexylammonium bromide (THxABr) and 4-bromomethyl-7-methoxycoumarin (BrMMC) were obtained from Fluka (Buchs, Switzerland). Acetonitrile, acetone (distilled before use), methanol, 18-crown-6 ether and nonanoic acid (NA) were obtained from Merck (Darmstadt, F.R.G.), 10-undecenoic acid (UA) from OPG (Utrecht, The Netherlands), valproic acid (VPA) from Sigma (St. Louis, MO, U.S.A.) and sodium valproate (Na-VP) from LaBaz (Brussels, Belgium). The Na-VP was dried at 105°C prior to use; the water content was less than 1%. A 50 m*M* phosphate buffer (pH 7.0) was used throughout.

Nonanoic acid, 10-undecenoic acid and valproic acid were dissolved in acetone and stored at 4°C. Sodium valproate was dissolved in water and stored at 4°C.

Preparation of incubation vials

A 1.65-g amount of Arkopal N-130, 650 mg of THxABr and 60 mg of BrMMC were dissolved in 20 ml of acetone. After the transfer of 240 μl of this solution into a type 3814 reaction vessel (Eppendorf, Hamburg, F.R.G.) the acetone was evaporated at room temperature under a stream of nitrogen. Because of the photosensitivity of the label, the preparation of the incubation vials was performed with exclusion of light. The prepared vials were stored at −20°C and used for the incubation method 2 (see below).

Incubation procedures

Method 1. The preliminary experiments were based on the incubation procedure described previously[6]. A micellar stock solution of 100 m*M* Arkopal N-130 and 72 m*M* THxABr was prepared in 50 m*M* phosphate buffer (pH 7.0). Of this micellar solution 250 μl were added to 250 μl of a mixture of plasma and buffer and 10 μl of an internal standard solution were added. The derivatization was started by the addition of 25 μl of an acetone solution of 8 mg/ml BrMMC. The samples were derivatized at 70°C in a water-bath.

Method 2. In the new derivatization procedure in general 400 μl of buffer and 100 μl of patient's or spiked plasma were placed in a prepared incubation vial and 10 μl of an internal standard were added. After vortexing for 5 s the vials were incubated at 70°C in a water-bath for *ca.* 40 min.

The derivatizations using methods 1 and 2, which were performed with the exclusion of light, were terminated by the addition of 500 μl of acetonitrile. The

plasma samples were centrifuged at 3000 *g* for 5 min after the addition of acetonitrile. Volumes of 20 μl of the samples were injected into the HPLC system.

In addition to the incubations in the aqueous micellar system derivatizations were also performed in acetone. The procedure for incubation in acetone was described previously[6,8]. The carboxylic acid was added to an acetone solution that contained 18-crown-6 ether and potassium carbonate. The reaction was started by the addition of BrMMC (25 μl of an 8 mg/ml solution in acetone). The incubation was performed for 30 min at 60°C.

Chromatographic system

Samples of 20 μl of the diluted incubation mixture were injected into a fully automated HPLC system consisting of a Chromspher C_{18} (5 μm) column (100 × 3.0 mm I.D.) (Chrompack, Middelburg, The Netherlands), a laboratory-made precolumn of LiChroprep RP-8 (5–20 μm) (10 × 3.00 mm I.D.) (Merck), two M 6000 A pumps and an automated gradient controller (all from Waters Assoc., Milford, MA, U.S.A.) controlled by a Promis autosampler (Spark, Emmen, The Netherlands).

After the initial step with methanol–water (80:20, v/v) for 3 min a linear gradient was run to 100% methanol in 6 min, the final conditions being maintained for 4 min. The solvents were deaerated ultrasonically prior to use.

A Model 650 fluorescence detector (Perkin-Elmer/Hitachi, Tokyo, Japan) was used. The optimized exitation and emission wavelengths were 330 and 395 nm, respectively. A Waters 440 absorbance detector was used for UV detection at 340 nm. Retention times and peak areas were measured with an SP 4270 integrator (Spectra-Physics, Santa Clara, CA, U.S.A.).

Patients' samples

The patients' samples were those submitted for therapeutic drug monitoring to the clinical laboratory of the University Hospital of Utrecht. The total VPA concentration of the patients' plasma had been determined with the use of a fluorescence polarization immunoassay (FPIA) with a TDx analyser (Abbott, Irving, TX, U.S.A.). After the FPIA analysis the samples were immediately analysed using the HPLC procedure or were stored at −20°C prior to analysis.

RESULTS AND DISCUSSION

The principles of the procedure for the derivatization of carboxylic acids in the presence of the micellar system are described in more detail in the accompanying paper[6]. It was suggested that the mechanism is based on phase-transfer catalysis (PTC)[7]. In micellar PTC (MPTC), the carboxylic acid is extracted with a lipophilic quaternary ammonium cation from the aqueous bulk phase into the micellar pseudophase. In the micelle the reaction of the carboxylic acid with the label occurs[6].

In the other study[6] it was found that optimal derivatization rates at 70°C and pH 7.0 could be obtained with a micellar system that consisted of 50 m*M* Arkopal N-130 and 36 m*M* THxABr, and this composition was adopted throughout this study.

Derivatization in plasma

Effect of proteins. When plasma samples were incubated at 70°C in the micellar solutions, problems occurred with the derivatizations owing to the precipitation of the plasma proteins. The plasma proteins could in principle be removed prior to the derivatization, *e.g.*, by the addition of acids (*e.g.*, perchloric acid) or organic solvents (*e.g.*, acetonitrile or methanol)[9]. However, the addition of these deproteinizing reagents to the plasma sample strongly affects the micellar system and thereby the derivatization rates[6].

Another, more general, problem with deproteinizing procedures is that VPA, like many other drugs, is strongly protein bound (80–90%)[3] and could coprecipitate with the proteins. This makes the quantitative determination of VPA more difficult. We tried to circumvent the deproteinization step by simply diluting the plasma sample with buffer prior to the derivatization step in the micellar system. With a plasma content of 25% or less in the micellar solution only a slight or no protein clotting at 70°C was observed. Therefore, in further incubations a plasma content of 20% in the micellar solution was used.

Effect of BrMMC concentration. The derivatization of VPA in 20% plasma (method 1) using 1.5 m*M* BrMMC gave only a *ca.* 60% yield of the VPA derivative compared with derivatizations in buffer and acetone. In addition, it was found that in plasma, in contrast to buffer and acetone, the BrMMC was completely absent after the incubation. The obvious reason for this low recovery is that in plasma other compounds are present, such as free fatty acids (FFA)[10], which are derivatized with BrMMC. It was considered that with plasma the BrMMC concentration in the micellar solution had to be increased in order to ensure the presence of an excess of BrMMC during the introduction. However, it was recognized that with derivatization method 1 the reagent concentration could hardly be increased, owing to the limited solubility of the reagent in organic solvents and to the inhibiting effect of these solvents on the derivatization reaction in the micellar system[6]. Therefore, we evaluated other possibilities of adding the reagent to the micellar solution.

A major problem is caused by the low rate of dissolution of crystalline BrMMC in the micellar solution. However, if the BrMMC was precipitated in the presence of the surfactant and the ion-pair reagent from acetone (see Experimental), then the label readily dissolved after the addition of water to the incubation vial. An additional advantage of this new approach is that the BrMMC is stable in these prepared incubation vials for at least 1 month if stored at −20°C. Therefore, the incubation vials can easely be prepared in advance in large amounts and the actual sample preparation (method 2) is rapid and simple.

Fig. 1 shows the influence of the BrMMC concentration on the yield of the derivatization (method 2, $n=4$) of 350 μM VPA in plasma (100 μl) that was diluted with buffer (400 μl). The yield in plasma is expressed as a percentage of those obtained in pure aqueous micellar solutions and acetone. Fig. 1 shows that the yield of the VPA derivative reaches *ca.* 100%, which indicated that both the free and the protein-bound fractions of the VPA are determined. Apparently the bond between the VPA and the proteins is completely disrupted during the derivatization.

From Fig. 1 it can also be concluded that with a 20% plasma solution at least *ca.* 4 m*M* BrMMC is required for a 100% yield of the VPA derivative. In subsequent experiments we used a BrMMC concentration of *ca.* 5.3 m*M* in the incubation mix-

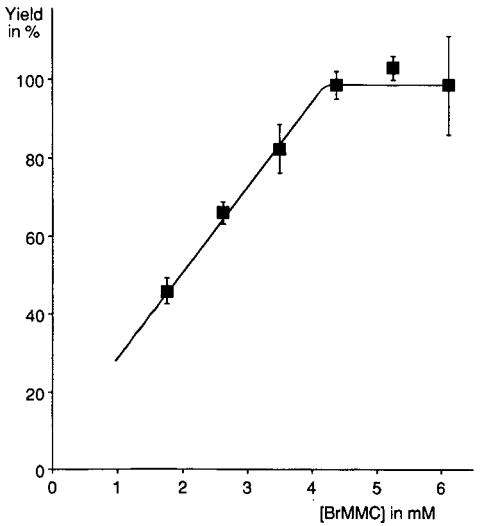

Fig. 1. Influence of BrMMC concentration on the yield of the derivatization of 350 μM valproic acid in plasma using incubation method 2.

ture to ensure the presence of an excess of the label during the incubation. At BrMMC concentrations higher than *ca.* 6 mM problems with the solubility of the label in the micellar system occurred, leading to a lower reproducibility of the derivatization.

Derivatization rate. Fig. 2 shows the kinetics of the derivatization of 350 μM VPA in plasma with 5.3 mM BrMMC using the conditions described in method 2. Fig. 2 illustrates that the derivatization of VPA is completed within *ca.* 35 min. In addition, it shows that the derivative is reasonably stable under the incubation conditions.

The VPA derivative is stable for at least 16 h in the acetonitrile-diluted micellar solution at room temperature. If stored at −20°C the same sample is stable for at least 1 month.

Linearity. The linearity of the determination in plasma for VPA concentrations from 7 to 1042 μM (n = 12) was investigated. The calibration graphs passed through the origin and had a correlation of r = 0.9476 without the use of an internal standard and r = 0.9997 if the peak-area ratio relative to 10-undecenoic acid was used.

Reproducibility. The coefficient of variation (C.V.) of the determination of VPA in plasma was examined. At a spiked concentration of 50 μM VPA the C.V. of the peak area was 7.4% (n = 6). A similar reproducibility was observed with sodium valproate (C.V. = 7.2%, n = 5) and with the internal standards nonanoic acid (C.V. = 7.6%, n = 6) and 10-undecenoic acid (C.V. = 7.5%, n = 6). The reproducibilities of the peak areas was not substantially improved at higher concentrations of the acids. In spiked plasma the C.V. of the peak-area ratio relative to 10-undecenoic acid as an internal standard was 2.5% (n = 6) at a concentration of 670 μM VPA and 3.2% (n = 6) with 38 μM VPA. At a concentration of 670 μM VPA similar reproducibilities of the peak-area ratios (C.V. = 2.9%, n = 6) were found with nonanoic acid as an internal standard.

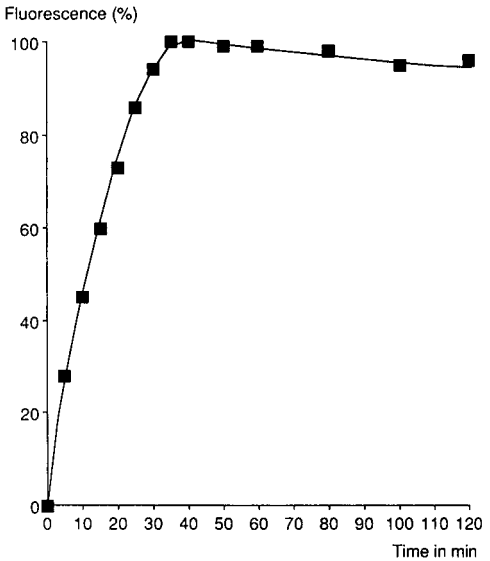

Fig. 2. Derivatization of 350 μM valproic acid in plasma with 5.3 mM BrMMC using incubation method 2.

From these results, it can be concluded that the use of an internal standard is advisable. However, the choice of the internal standard seems not to be critical.

Limit of detection. The limit of detection (LOD) of VPA in spiked plasma and buffer was investigated. In plasma the LOD was *ca.* 7 μM, owing to the presence of small interfering peaks in the chromatogram. For the monitoring of VPA at therapeutic levels (260–840 μM) this LOD value is sufficient. In buffer the LOD was *ca.* 25 nM. This shows that with this derivatization technique the carboxylic acids could in principle be detected sensitively without the need for tedious sample preparation procedures.

Patients' samples

The VPA concentrations in fifteen patients' samples were determined with the HPLC procedure using method 2. The chromatogram of such a derivatization is shown in Fig. 3a and that of a healthy subject in Fig. 3b. From these chromatograms it can be concluded that both VPA and undecenoic acid are well separated from the other plasma constituents.

The levels determined were compared with those obtained using a standard fluorescence polarization immunoassay (FPIA) in the clinical laboratory (Fig. 4). The correlation between the two methods is satisfactory ($r = 0.980, n = 15$). The relationship between the VPA concentration determined with the HPLC method, $[VPA]_h$, and that determined by FPIA, $[VPA]_f$, is $[VPA]_h = -7\ \mu M + 1.01[VPA]_f$. At a VPA concentration of 590 μM the C.V. of the peak-area ratio in the patients' samples was 2.9% ($n = 5$). At the same concentration the reproducibility of the FPIA method was 2.5%.

The main advantage of HPLC over the FPIA method is the possibility of

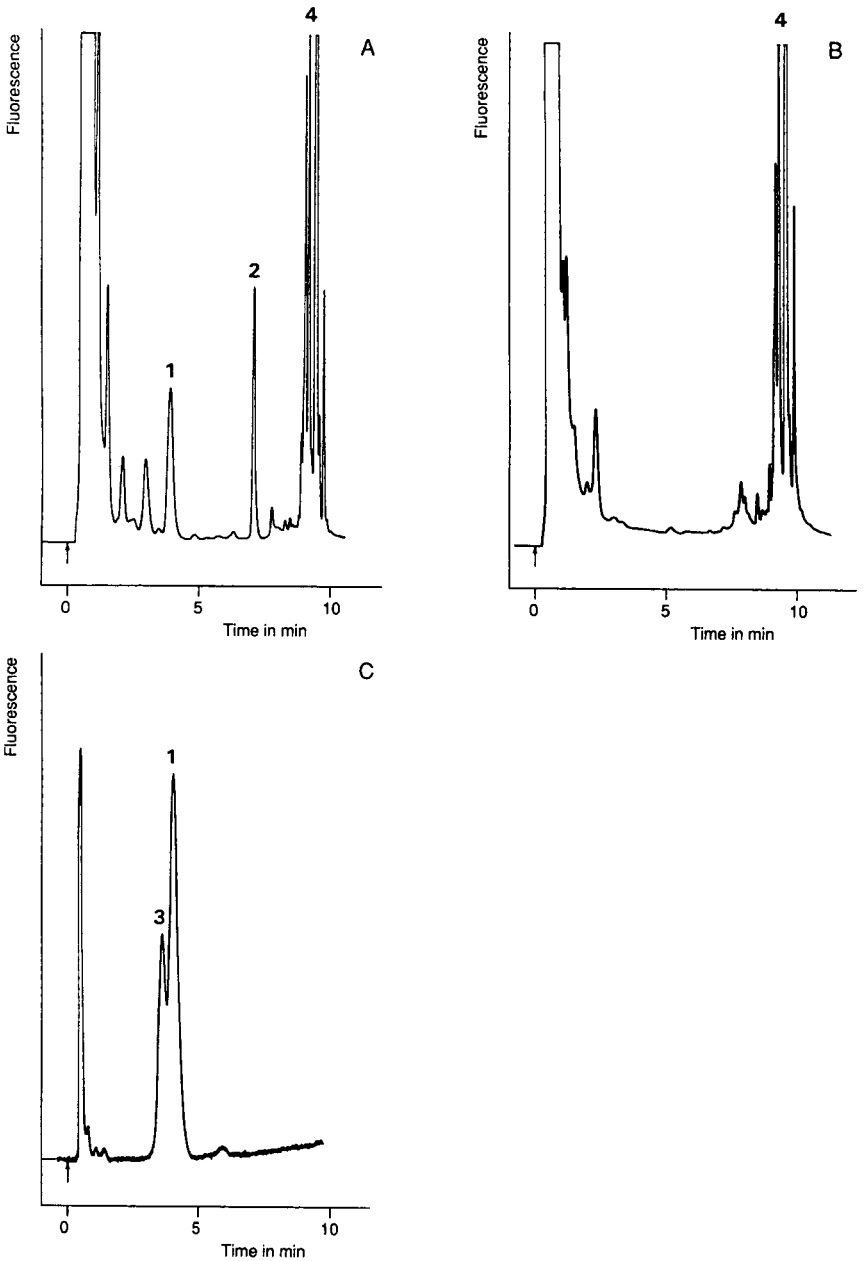

Fig. 3. Chromatograms obtained after derivatization (method 2) of: (A) sample from patient under treatment with VPA with UA (250 μM) as internal standard; (B) plasma from a healthy subject; (C) synthetic (*E*)-2-en-VPA and VPA. Peaks: 1 = VPA; 2 = 10-undecenoic acid; 3 = (*E*)-2-en-VPA; 4 = plasma-free fatty acids.

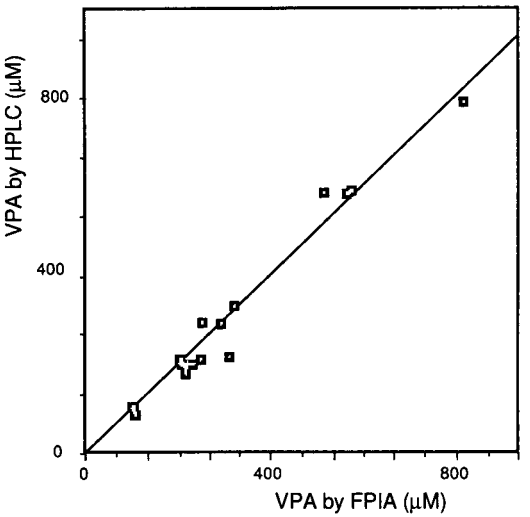

Fig. 4. Comparison of the FPIA and HPLC methods for the determination of VPA. VPA concentrations were determined (n = 15) in plasma samples from patients under VPA treatment. The solid line indicates the best least-squares fit (r = 0.980) between the results of the assays.

determining several bio-active compounds simultaneously. With VPA, especially its metabolites are of clinical importance. Normally only small amounts (< 8%) of metabolites of VPA are present in plasma [mainly (*E*)-2-propyl-2-pentenoic acid and 3-oxo-2-propylpentanoic acid][11,12]. However, elevated metabolite levels in plasma might be related to fatal liver injuries[12]. The deviations in the metabolite pattern could easely be monitored by the HPLC method. Although this field of research is outside the scope of this study, we performed some tentative experiments on the detection of one of the main metabolites of VPA in plasma, namely (*E*)-2-propyl-2-pentenoic acid [(*E*)-2-en-VPA][11,12]. Synthetic (*E*)-2-en-VPA was derivatized (method 2) and determined by HPLC (Fig. 3c). It can be seen that the (*E*)-2-en-VPA derivative and the VPA derivative eluted separately. Additional studies need to be performed, however, to investigate whether the HPLC method is suitable for monitoring the pathological VPA metabolism in patients' samples.

Some of the patients were also treated with other anti-epileptic drugs (*e.g.*, phenobarbital, phenytoin and carbamazepin). We therefore investigated whether the presence of these compounds interfered with the determination of VPA. In contrast to carbamazepin, both phenobarbital and phenytoin gave a fluorescent derivative after reaction with BrMMC (method 2). This observation is in agreement with other studies[13,14] in which barbiturate-like compounds were derivatized with BrMMC in aprotic solvents. However, the derivatives of phenobarbital and phenytoin eluted near the void volume of the column and therefore did not interfere in the determination of VPA.

CONCLUSIONS

This study of the derivatization of VPA in plasma has shown that MPTC is a useful derivatization procedure for biologically active carboxylic acids. The main

advantage of MPTC over conventional derivatization procedures for carboxylic acids is that the extraction of the drug into a suitable organic solvent prior to the derivatization step can be omitted. This makes the determination of carboxylic acids faster and easier to perform.

Another interesting advantage of MPTC is that in principle it is suitable as an on-line derivatization procedure for carboxylic acids. However, with BrMMC as a label too long derivatization times are required for convenient interfacing with liquid chromatography. In plasma the precipitation of proteins at elevated temperatures would cause additional problems. We are now investigating MPTC systems that perform well at ambient temperatures, by means of which a automated on-line derivatization procedure for carboxylic acids is possible.

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STUDY OF THE INFLUENCE OF AQUEOUS MICELLAR SYSTEMS ON THE DERIVATIZATION OF UNDECYLENIC ACID WITH 4-BROMOMETHYL-7-METHOXYCOUMARIN

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SUMMARY

The effects of aqueous micellar systems on the derivatization of a fatty acid, 10-undecenoic acid (UA), with a fluorophore, 4-bromomethyl-7-methoxycoumarin (BrMMC), were examined. High derivatization rates were obtained in solutions of the non-ionic surfactants Triton-X 100 and Arkopal N in the presence of cationic ion-pair reagents such as tetrahexylammonium bromide. The derivatization mechanism is probably based on phase-transfer catalysis. Especially high reaction rates are obtained in turbid non-ionic micellar solutions. This opaqueness is connected with an important optimizing parameter of the derivatization rate, the so-called cloud temperature of a micellar system. Under the optimal conditions the derivatizaton of UA with BrMMC is complete within 45 min at 70°C.

INTRODUCTION

High-performance liquid chromatography (HPLC) is a versatile technique for the analysis of drugs that are present in biological matrices. However, HPLC analysis can be hampered by the fact that the drug may be difficult or impossible to detect. This problem can be overcome by derivatizing the drug with, *e.g.*, a fluorescence label[1,2]. Unfortunately, with carboxylic acids the derivatization reactions are often incompatible with water[3]. This means that in bioanalysis the substrate has to be extracted from the aqueous matrix into a suitable organic solvent. Extraction procedures are often tedious and can cause problems concerning the drug recovery and the reproducibility of the analysis[4,5].

A possible alternative to the extraction of a substrate into an organic solvent could be to introduce an organic "pseudo"-phase into the aqueous matrix by means of micelles. Micelles are small, more or less spherical aggregates of amphiphilic molecules[6–8]. The core of the micelle may be more or less deprived of water[9,10] and the properties are similar to those of hydrocarbons[10]. If the substrate and the reagent are solubilized in the micellar core then the necessary derivatization conditions may be

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fulfilled. In this manner it could be possible to circumvent the tedious procedures for extraction of the drug.

Micelle-enhanced reactions have been studied extensively[11–13]. It is surprising, however, that very few studies have dealt with the labelling of bio-active compounds (*e.g.*, amines[14,15]) prior to HPLC analysis.

The need for an alternative, more convenient, derivatization procedure is most marked with carboxylic acids that are present in aqueous biological matrices. Therefore, we have extended our investigations to the derivatization of carboxylic acids with a fluorescence label in aqueous micellar systems. As a fluorescence label we selected 4-bromomethyl-7-methoxycoumarin (BrMMC)[16–20]. In general, the derivatization of carboxylic acids with BrMMC has to be carried out in water-free[19,20] aprotic dipolar solvents such as acetone to which 18-crown-6 ether and potassium carbonate have been added to increase the reaction rate[17].

This paper describes part of a study on the mechanism of the derivatization of aliphatic carboxylic acids with BrMMC in the presence of aqueous micellar systems.

We report here on the influence of the micellar system [type and concentration of the surfactant and presence of additives (such as ion-pair reagents)] on the derivatization of a model substrate, undecylenic acid (10-undecenoic acid, UA), with BrMMC. The influence of substrate-related factors on the derivatization reaction in the micellar system will be discussed elsewhere[21].

Micelle-mediated labelling with BrMMC has recently been applied to plasma samples of patients treated with an anti-epileptic drug, valproic acid[22]. The ultimate aim of our studies is to develop a fully automated on-line labelling procedure for carboxylic acids that is based on micelle-enhanced derivatizations and which can be applied to biological matrices.

EXPERIMENTAL

Chemicals and solutions

Millipore water (Milford, MA, U.S.A.) was used throughout. Except for the non-ionic surfactants the chemicals were of analytical-reagent grade.

The non-ionic Arkopal N surfactants (condensates of nonylphenol with polyoxyethylene) were kindly supplied by Hoechst Holland (Amsterdam, The Netherlands). These surfactants are mixtures of molecular species varying in their polyoxyethylene (POE) chain length. The Arkopal N surfactants contained a POE moiety with averages of 8, 9, 10, 11, 13, 15, 18 and 23 oxyethylene units, and are denoted N-80, N-90, N-100, N-110, N-130, N-150, N-180 and N-230, respectively. The mean POE chain lengths are used to calculate the molecular weights of the non-ionic surfactants.

The non-ionic surfactants of the Brij type (alkoxy-POE condensates) were purchased from Sigma (St. Louis, MO, U.S.A.) and Servo (Delden, The Netherlands).

Triton X-100, cetyltrimethylammonium bromide (CTAB) and sodium dodecylsulphate (SDS) were purchased from Merck (Darmstadt, F.R.G.). Tetrabutylammonium bromide (TBuABr), tetrapentylammonium bromide (TPeABr), tetrahexylammonium bromide (THxABr) and 4-bromomethyl-7-methoxycoumarin (BrMMC) were supplied by Fluka (Buchs, Switzerland). Acetonitrile, acetone, methanol and 18-crown-6 ether were obtained from Merck. Undecylenic acid (UA)

was supplied by OPG (Utrecht, The Netherlands). All chemicals were used as obtained.

In a sealed amber-coloured flask the BrMMC reagent was added to acetone at the level of 8 mg/ml. This saturated solution was stored at 4°C and prepared freshly every week. Prior to incubation the BrMMC was completely dissolved in acetone by heating to *ca.* 50°C. Undecylenic acid was dissolved in acetone at a concentration of 5 m*M* and stored at 4°C. The 18-crown-6 ether was dissolved in acetone at a concentration of 3 mg/ml and stored at room temperature.

General incubation procedure

The micellar solutions were prepared by dissolving known amounts of the surfactant and the ion-pair reagent in 10 m*M* phosphate buffer adjusted to pH 7.0 with sodium hydroxide. Other additives, such as organic solvents, salts and urea, were dissolved in the micellar solution prior to the incubation. Of the incubation solutions 965 μl were pipetted into type 3814 reaction vessels (Eppendorf, Hamburg, F.R.G.) and 10 μl of undecylenic acid solution (final concentration 50 μ*M*) were added. This mixture was pre-incubated for 5 min in a water-bath at the incubation temperature. The incubation was started by the addition of 50 μl of BrMMC stock solution. The incubations were carried out protected from light and were performed at 70 ± 1°C, if not stated otherwise. At given times (depending on the derivatization rate) 75-μl samples were taken from the incubation mixture and diluted with 75 μl of acetonitrile in a type 3810 Eppendorf vessel. These samples were stored at −20°C. The derivatization product of UA with BrMMC obtained from the micellar systems was tentatively identified by comparing the retention time and the excitation and emission spectra of the product with those obtained from incubations in acetone.

The incubations in acetone were carried out according to ref. 17. A 10-μl volume of the undecylenic acid solution was added to a type 3814 Eppendorf vessel that contained 935 μl of acetone, 30 μl of 18-crown-6 ether solution and *ca.* 10 mg of fine-grained potassium carbonate. After the addition of 20 μl of the BrMMC solution, the reaction was carried out at 60°C for 30 min. Next, samples were taken as described above.

Chromatographic system

Samples of 10 μl of the diluted incubation mixture were injected into a fully automated HPLC system, using a laboratory-filled 10-μm LiChrosorb RP-18 column (300 × 4.6 mm I.D.) (Merck). The HPLC system consisted of two M 6000 A pumps and an automated gradient controller (Waters Assoc., Milford, MA, U.S.A.), controlled by a Model 231 automatic sampler injector (Gilson, Villiers le Bel, France). A linear gradient was run in 10 min from methanol–water (60:40, v/v) to 100% methanol. All solvents were filtered through a 0.2-μm filter and deaerated ultrasonically prior to use. A Model 650 fluorescence detector (Perkin-Elmer/Hitachi, Tokyo, Japan) was used. The optimized excitation and emission wavelengths were 330 and 395 nm, respectively. Retention times and peak areas were measured with an SP 4270 integrator (Spectra-Physics, Santa Clara, CA, U.S.A.).

Data analysis

The apparent pseudo-first-order rate constant, k, was calculated from[23]

$$I(t) = I^* (1 - e^{-kt}) \quad (1)$$

where $I(t)$ is the peak area of the derivative at a given time, t, and I^* is the peak area after complete derivatization of the substrate. A non-linear Marquardt optimization[24] of eqn. 1 through at least eight data points was used to calculate the best approximation of the rate constant.

Micelle size determination

Dynamic light scattering was used to determine the apparent mean diameter of the micellar aggregates. The surfactants and ion-pair reagent were dissolved at various concentrations in 10 m*M* phosphate buffer. All solvents were filtered through a 0.2-μm polycarbonate filter (Nucleopore, Pleasanton, CA, U.S.A.) prior to use. Triplicate determinations were carried out at 50°C (instrumental limitation) by the use of Malvern 7027 particle analyser controller and additional equipment (Malvern, U.K.). A 100-mW He–Ne laser (λ = 632.8 nm) (NEC, Tokyo, Japan) was used as a light source.

Cloud point determination

To determine the cloud temperature (T_c) of the various micellar solutions, 3-ml aliquots of the micellar solutions were pipetted into stoppered glass-walled tubes. By means of a temperature-controlled water-bath the temperature dependent change from clear to opaque solutions was determined (in duplicate) by eye.

RESULTS AND DISCUSSION

Preliminary experiments

Preliminary derivatization experiments were carried out in pure surfactant systems without any additives except buffer. Irrespective of the type and concentration of the surfactant used, only insignificant amounts of MMC derivatives were formed. This can be explained as follows.

It is generally accepted that nucleophilic substitution (S_N2) reactions involving carboxylic acids are inhibited in aqueous solutions owing to solvation of the carboxylic acid[3,25].The aqueous bulk phase and the strongly hydrated interface of the micelles[26], therefore, can be ruled out as possible reaction sites.The micellar core may be more or less deprived of water[9,10]. This means, in principle, that the derivatization reaction can occur in the micellar core. In the micellar solution the acid will be present in a protonated (HA) and a deprotonated (A^-) form. It is likely that only the uncharged species, HA, can penetrate into the hydrocarbonaceous environment of the micellar core. However, this species lacks the nucleophilicity that is necessary for an S_N2 reaction[3,25]. Therefore, only the A^- species present in the core of the micelle can be derivatized in the micellar system. However, it is unlikely that the ionic A^- can penetrate into the hydrocarbonaceous core of the micelle. The species A^- can be extracted, however, into the micellar core using a cationic ion-pair reagent (*e.g.*, a quaternary ammonium salt). Following extraction, the reaction with the label (BrMMC) could take place in the micellar core. In non-micellar two-phase systems this principle is known as phase-transfer catalysis (PTC)[27–29].

Two equilibria mainly determine the derivatization rate in conventional PTC

systems. The first is the extraction of the analyte, $(A^-)_w$, with an ion-pair reagent, $(Q^+)_w$, from the aqueous phase into the organic phase. In the organic phase the analyte is present as the electro-neutral complex, $(QA)_0$. The extraction equilibrium is described by

$$[(QA)_0] = K_{ex}^{qa} [A^-_w] \cdot [Q^+_w] \quad (2)$$

where K_{ex}^{qa} is the extraction constant. The value of K_{ex}^{qa} depends strongly on the hydrophobicity of the ion-pair reagent and of the analyte[27–29].

The second equilibrium is the derivatization rate in the organic phase. If the label (BrMMC) is present in excess, then the derivatization rate, v_0, can be described as a pseudo-first-order equation[30]:

$$v_0 = k[(QA)_0] \quad (3)$$

where k is the pseudo-first-order reaction constant.

We have studied the possible use of ion-pair reagents in the derivatization of carboxylic acids in aqueous micellar systems. To our knowledge, no study has been reported on phase-transfer catalysis in micellar systems.

The addition of TBuABr to SDS solutions led to the precipitation of the surfactant with the ion-pair reagent. When the ion-pair reagent was added to CTAB only a slight improvement in the reaction rate was found. In contrast, the addition of TBuABr to non-ionic micellar systems, especially Triton X-100 and Arkopal N, led to a marked improvement in the reaction rate and was, therefore, studied in more detail.

Influence of the ion-pair reagent

Fig. 1 shows the influence that the concentration of TBuABr, TPeABr and THxABr has on the derivatization rate of UA with BrMMC at 70°C in the presence 50 m*M* Triton X-100 in 10 m*M* phosphate buffer (pH 7.0). The Triton X-100 concentration was chosen arbitrarily. However, a surfactant concentration range of 20–100 m*M* is often used in micellar catalytic systems[11–13].

Comparison of Fig. 1A–C illustrates that the derivatization rate is strongly affected by the type and concentration of the ion-pair reagents. At an equimolar concentration of the ion-pair reagents the derivatization rate increases sharply from TBuABr to THxABr. This phenomenon is generally seen in PTC, and is related to the fact that the extraction constant, K_{ex}^{qa} (eqn. 2), increases with increasing number of carbon atoms in the ion-pair reagent[29].

In PTC the derivatization rate gradually increases with increasing ion-pair reagent concentration[29]. At low analyte concentrations the derivatization rate initially increases linearly with increasing ion-pair reagent concentration. Finally, it reaches asymptotically a certain plateau value[21]. This can be explained as follows. Eqn. 3 shows that, when the label is present in excess, the derivatization rate is proportional to the concentration of the ion-pair complex, $(QA)_0$, in the organic phase[30]. With a fixed analyte concentration the amount of the analyte extracted into the organic phase initially increases linearly with increasing concentration of the ion-pair reagent (eqn. 2). When the concentration of the ion-pair reagent is increased still further it will become increasingly difficult to extract the last traces of analyte into the organic phase.

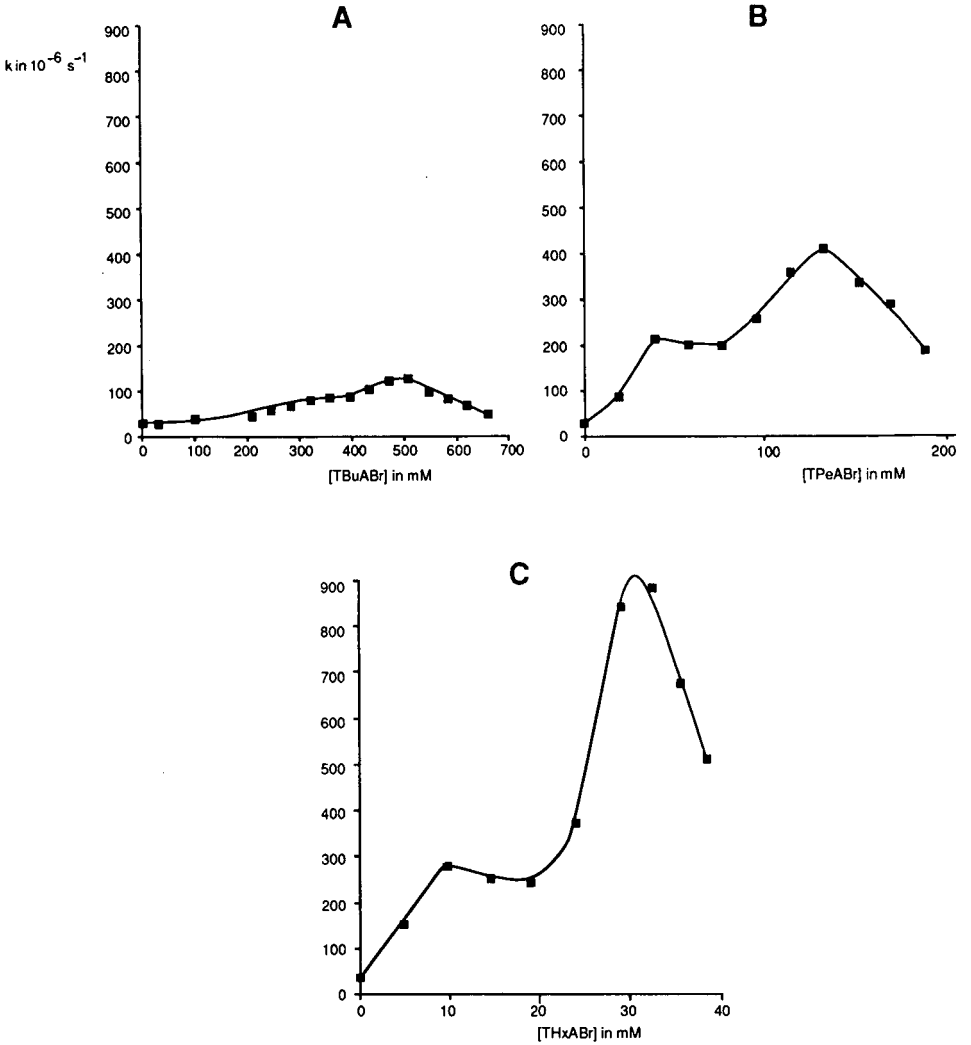

Fig. 1. Influence of the concentration of the ion-pair reagents (A) TBuABr, (B) TPeABr and (C) THxABr on the apparent rate constant (k) of the reaction between UA and BrMMC at 70°C in Triton X-100 (50 mM) in 10 mM phosphate buffer (pH 7.0).

Finally, the concentration of $(QA)_0$ and, therefore, the derivatization rate will reach a certain plateau value. A more quantitative description of the relationship between the ion-pair reagent concentration and the derivatization rate can be found elsewhere[21].

The relationship described can also be observed in Fig. 1 at lower ion-pair concentrations (*e.g.*, <80 mM TPeABr or <20 mM THxABr). Initially the derivatization rate increases with increasing concentration of the ion-pair reagent. With a further increase in the ion-pair reagent concentration the derivatization rate

levels off. However, in a particular concentration range of the ion-pair reagents (*e.g.*, 100–180 m*M* TPeABr or 25–40 m*M* THxABr) higher, deviant, derivatization rates were observed compared with the calculated values[21]. This aberrant behaviour is caused by changes in the properties of the micellar system and is discussed in the following section.

Effect of cloud temperature

Non-ionic micellar systems are often clear solutions. Most of the incubation mixtures that were used for the set-up shown in Fig. 1 also looked clear. However, at the incubation temperature some of these incubation solutions were more or less opaque. In some of these solutions (*e.g.*, 32–40 m*M* THxABr; Fig. 1C) the deviant, high derivatization rates were observed. Obviously there is a relationship between the high derivatization rates and the opaqueness of the solutions. This relationship was studied in more detail.

The opaqueness of non-ionic surfactant solutions is connected with the so-called cloud temperature (T_c), at which there is a transition from normal to very large micellar aggregates[31]. It is these large structures that are responsible for the turbid appearance of the micellar solutions. T_c can be affected by the presence of additives such as salts and organic solvents[31]. At higher temperatures than T_c the solution begins to flocculate and finally separates into two phases. One layer is surfactant-rich whereas the other is deprived of micelles[31,32].

The influence of the concentration of the ion-pair reagents on the T_c of Triton X-100 (50 m*M*) is shown in Fig. 2. At low ion-pair reagent concentrations the T_c of Triton X-100 gradually increases with increasing concentration of the ion-pair reagents. The steepness of the increase in T_c clearly depends on the number of carbon atoms in the ion-pair reagent. Similar results have been reported for other hydrophobic additives such as hydrocarbons[31]. Fig. 2. shows that the micellar systems become turbid again at low temperatures within a small concentration range of the ion-pair reagents, *e.g.*, 30–40 m*M* THxABr (Fig. 2C). This uncommonly steep change in T_c probably coincides with the saturation curve of the ion-pair reagents. This notion is based on the observation that, at room temperature, above *ca.* 40 m*M* THxABr the surplus ion-pair reagent remained present as small droplets in the more or less turbid micellar solutions.

A discrepancy is observed between the ion-pair reagent concentration at which a maximum derivatization rate is obtained in the more or less turbid incubation solutions (Fig. 1) and the concentration at which a T_c of 70°C was determined (Fig. 2). The discrepancy can be explained by the presence of BrMMC in the incubation mixtures. This causes a further decrease in the T_c of these mixtures. The decrease in the derivatization rate, *e.g.*, > 150 m*M* TPeABr (Fig. 1B), occurred in the region in which phase separation was observed (Fig. 2B). One should therefore try to prevent phase separation.

From the above experiments we concluded that the cloud temperature is a very important parameter in optimizing the derivatization rate in micellar systems.

It must be emphasized that the deviant, high derivatization rates are not restricted to a particular composition of the incubation solution, *e.g.*, 50 m*M* Triton X-100 and 33 m*M* THxABr (Fig. 1C). Additional experiments indicated that similar derivatization rates can also be obtained with different compositions of the micellar

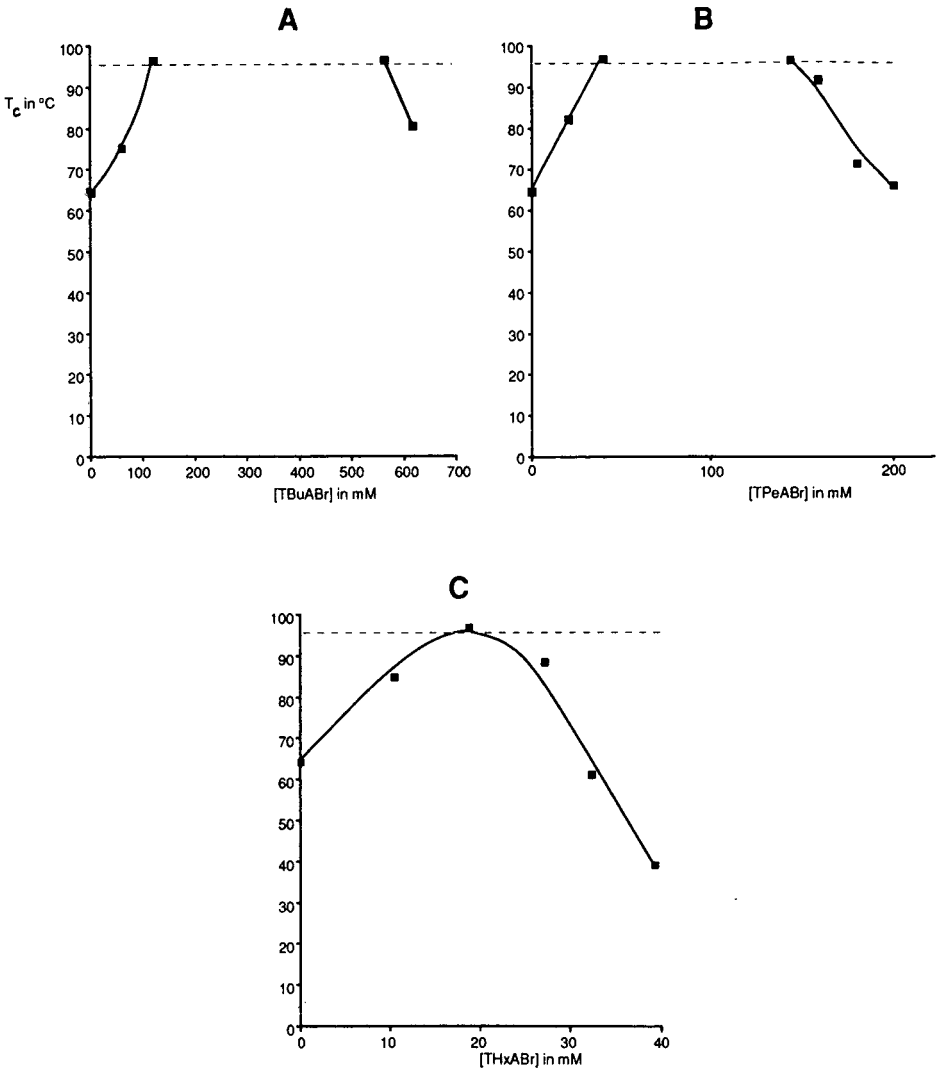

Fig. 2. Influence of the concentration of the ion-pair reagents (A) TBuABr, (B) TPeABr an (C) THxABr on the cloud temperature (T_c) of Triton X-100 (50 m*M*) in 10 m*M* phosphate buffer (pH 7.0). The broken line indicates the experimental limit.

system (*e.g.*, 70 m*M* Triton X-100 and 40 m*M* THxABr). The only important requirement for obtaining a high derivatization rate in a particular micellar system is, as far as we know, that the solution must be turbid at the incubation temperature.

T_c can easily be adjusted by changing the surfactant or the ion-pair reagent concentration. One should try, however, to prevent phase separation.

Effect of micellar size on reaction rate

In the previous section it was concluded that the cloud temperature plays an important role in the derivatization rate in micellar solutions. At the cloud temperature very large micellar aggregates form. Obviously, the size of the micellar aggregates might be an important parameter affecting the reaction rate. This was therefore investigated in more detail. These and subsequent experiments were performed with Arkopal N surfactants, because a better derivatization performance (*e.g.*, reaction rate) was obtained in the Arkopal N systems than in Triton X-100. It is conceivable that in general the results with the Arkopal N systems can be applied to other nonionic surfactant systems.

Fig. 3 shows the effect of the THxABr concentration on the apparent mean micelle size (Φ_{app}) of Arkopal N-130 at a fixed concentration of 50 m*M* in buffer at 50°C (experimental limitation). The determined value of the apparent mean micellar size of the pure micellar solution is in reasonable agreement with ref. 33. Fig. 3 shows that the micellar size is almost constant at lower ion-pair reagent concentrations (< 20 m*M* THxABr). After a small decrease the micellar size increases almost asymptotically beyond 30 m*M* THxABr. Above *ca.* 36 m*M* the micellar solutions were opaque, as a result of which the size of the very large micellar aggregates was indeterminable.

At a concentration of 50 m*M* Arkopal N-130 and 36 m*M* THxABr a cloud temperature is reached at *ca.* 50°C (Fig. 4). The difference between the THxABr concentrations that induce a T_c of 50°C and 70°C (incubation temperature), respectively is small (Fig. 4). Therefore, it is conceivable that a similar relationship to that depicted in Fig. 3 would be found if the micellar size could be determined at 70°C. Fig. 3 also shows the relationship between the apparent rate constant and the

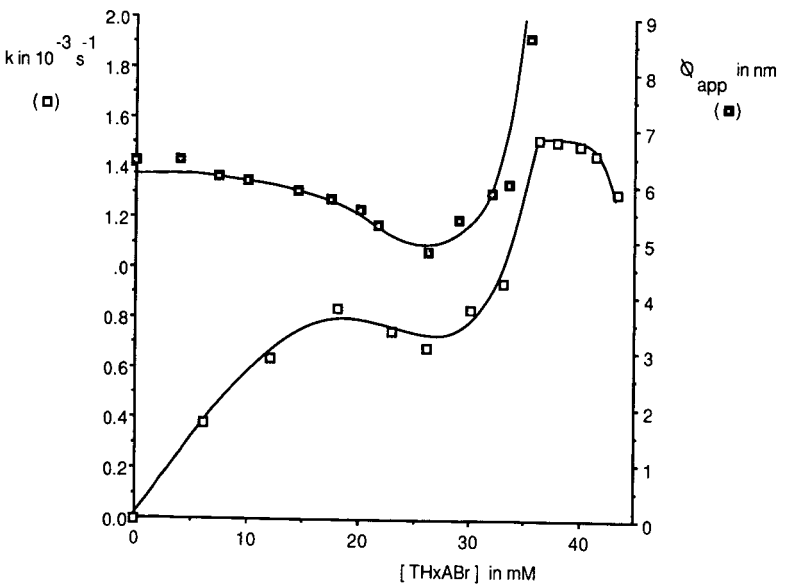

Fig. 3. Influence of THxABr concentration on the apparent mean micellar size, Φ_{app} (■), at 50°C and the apparent rate constant, k (□), of the reaction between UA and BrMMC at 70°C in a surfactant system of 50 m*M* Arkopal N-130 in 10 m*M* phosphate buffer (pH 7.0).

concentration of the ion-pair reagent in the same micellar system at 70°C. This rate profile is similar to that found in Triton X-100 (Fig. 1). However, higher derivatization rates are obtained. Fig. 3 shows that the derivatization rate increases up to 20 m*M* THxABr. Obviously, in this concentration range the increase in the derivatization rate is not related to changes in the micellar size. However, as argued previously, this increase can be related to the amount of the acid that is extracted into the micellar core[21]. Between 25 and 36 m*M* THxABr a clear relationship can be observed between the increase in the derivatization rate and the increase in the size of the micelles.

It is not known why the derivatization rate increases with increasing micelle size. One possible explanation is that the increase in the micelle size leads to a decrease in the water content within the micellar aggregates. It is reported that the reaction with BrMMC is strongly inhibited by the presence of water[19,20].

Also of interest is the observation that the optimum in the rate profile in the Arkopal N-130 system (Fig. 3) is less critically related to the THxABr concentration than in the Triton X-100 system. This means that it is more advantageous to use the Arkopal N-130 surfactant than Triton X-100.

We studied whether the presence of large micellar aggregates alone with the T_c of 70°C, hence without the presence of ion-pair reagents, could be responsible for the increase in the derivatization rate. However, insignificant reaction rates were found in ion-pair reagent-free micellar solutions in which the presence of large micelles was induced by the presence of 100 m*M* sodium chloride. This finding also indicates a PTC-like derivatization mechanism in the micellar systems.

Influence of polyoxyethylene chain length

It is well known that the POE chain length of non-ionic surfactants strongly affects their properties. If the POE content increases, then the aggregation number (N)[26,34] and the micellar size[26,34] decrease, whereas the critical micelle concentration (CMC)[34,35] and T_c[31] increase. Therefore the influence of the POE content on the catalytic properties of the micellar system was investigated.

Fig. 5 shows the influence that the concentration of several Arkopal N surfactants has on the reaction rate of UA with BrMMC in the presence of a fixed THxABr concentration of 36 m*M* at pH 7.0 and 70°C. It illustrates that on increasing the surfactant concentrations the rate constants in the various micellar systems approach an almost constant value.

On decreasing the surfactant concentration the derivatization rate increases more or less steeply to high values. With a further decrease in the surfactant concentration flocculation occurs and/or the solubility limit of the ion-pair reagent is exceeded. The surfactant concentration at which the optimal derivatization rate is obtained depends on the POE chain length of the surfactant. This might be related to a different solubility of the ion-pair reagent in the Arkopal N solutions. Fig. 4 shows that the solubility limit of the ion-pair reagent increases with increasing POE chain length. This notion is based on the previous arguments that the solubility limit is connected with the ion-pair concentration at which T_c decreases.

Fig. 5 demonstrates that the maximum derivatization rate that can be obtained in a micellar system is apparently independent of the POE chain length. Obviously the POE chain length does not have strong influence on the maximal derivatization rate in opaque solutions. Fig. 5 shows that in the non-opaque solutions the derivatization rate

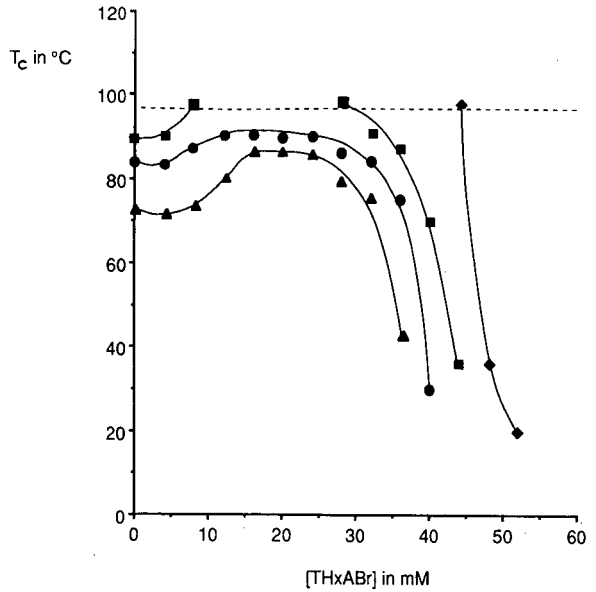

Fig. 4. Influene of THxABr concentration on the cloud temperature (T_c) of different Arkopal N surfactants (50 m*M*) in 10 m*M* phosphate buffer; ▲, N-110; ●, N-130; ■, N-150; ◆, N-230. The broken line indicates the experimenal limit.

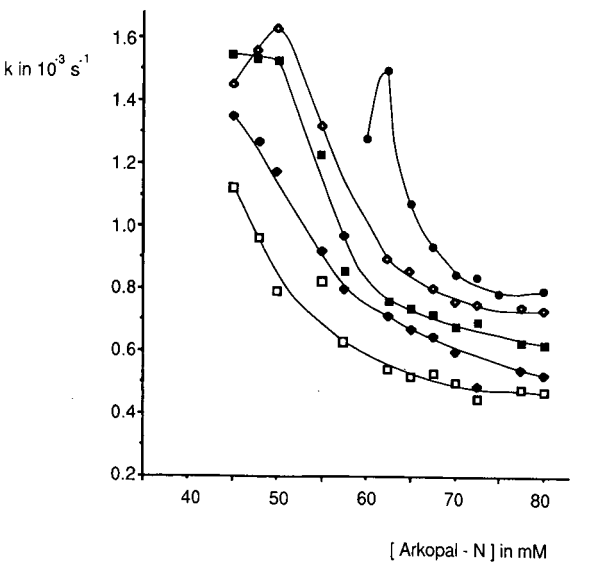

Fig. 5. Influence of the concentration of different Arkopal N surfactants on the apparent rate constant (k) of UA with BrMMC at 70°C in the presence of THxABr at a fixed concentration of 36 m*M*; ●, N-80; ◇, N-110; ■, N-130; ◆, N-150; □, N-230.

increases with a decrease in the POE chain length of the surfactant. The possible reason for this is that the micellar size increases with a decrease in the POE chain length[26]. It was concluded in a previous section that the derivatization rate increases with increasing size of the micelle. Further experimental evidence for this assumption is given by Fig. 6, which shows that in non-opaque Arkopal N systems the derivatization rates (70°C; $n=3$) increase with the apparent micellar size (Φ_{app}; 50°C) of the five surfactants. The concentration of the surfactants was 70 mM in all instances and that of THxABr was 32 mM.

Influence of organic solvents

To ensure high derivatization rates the BrMMC must be present in excess. We investigated how the label could be added to the incubation solutions. The BrMMC is almost insoluble in aqueous solution and slightly soluble (*ca.* 2.5 mM) in the micellar solutions. Thus aqueous stock solutions of BrMMC could not be used. Crystalline BrMMC dissolves only slowly in the micellar solution. Therefore, it was necessary to dissolve BrMMC at high concentration in an organic solvent before adding it to the micellar system. Originally acetone was used for the preparation of the stock solutions of BrMMC. The solubility of BrMMC in acetone is *ca.* 3 mg/ml at 25°C. To ensure the presence of an excess of BrMMC, relatively large amounts of acetone had to be added to the micellar solution. This was considered to be undesirable because organic solvents could strongly influence the properties of micellar systems[8,36]. Therefore, we tested several other solvents to dissolve BrMMC. The solubility of BrMMC in

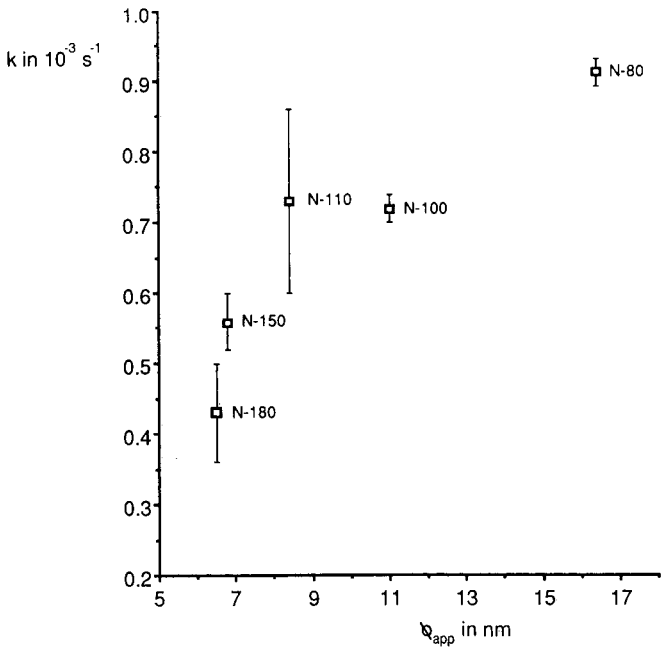

Fig. 6. Relationship between the apparent mean micellar size (Φ_{app}) at 50°C and the apparent rate constant (k) at 70°C of various Arkopal N surfactant systems. The micellar systems were composed of 70 mM Arkopal N surfactant and 32 mM THxABr in 10 mM phosphate buffer (pH 7.0).

acetonitrile and ethyl acetate was similar to that in acetone and no further consideration was given to their use. Although BrMMC could be solubilized more easily in dioxane (*ca.* 8 mg/ml at 25°C), this solvent could not be used because it strongly inhibited the reaction between UA and BrMMC in the micellar system even at low percentages. The reason for this behaviour is unknown. However, dioxane shows a similar behaviour to incubations performed in acetonitrile[20].

Because of these poor alternatives we investigated how much acetone could be added to the micellar systems without affecting the derivatization rate. Fig. 7 shows that with the addition of up to 6% acetone no significant changes in the reaction rate could be seen in a micellar system of 50 m*M* Arkopal N-130 and 36 m*M* THxABr. At higher acetone percentages the derivatization rate decreased sharply. Above *ca.* 35% acetone the derivatization reactions were almost completely inhibited because the micelles were no longer present under this condition[36]. Obviously, unimpaired micellar aggregates are a prerequisite for the catalytic properties. On the other hand, the rate-inhibiting effect of organic solvents was utilized to terminate the derivatization reaction by the addition of an equal volume of acetonitrile to the micellar solutions.

With regard to the addition of the reagent, it was concluded that the percentage of acetone should be kept low (<6%). However, the addition of 60 μl of BrMMC stock solution (3 mg/ml) would result in only 0.7 m*M* BrMMC in the incubation solution (1 ml). Therefore the solubility of BrMMC in acetone was increased to *ca.* 8 mg/ml by heating the BrMMC stock solution prior to the addition of the reagent. An

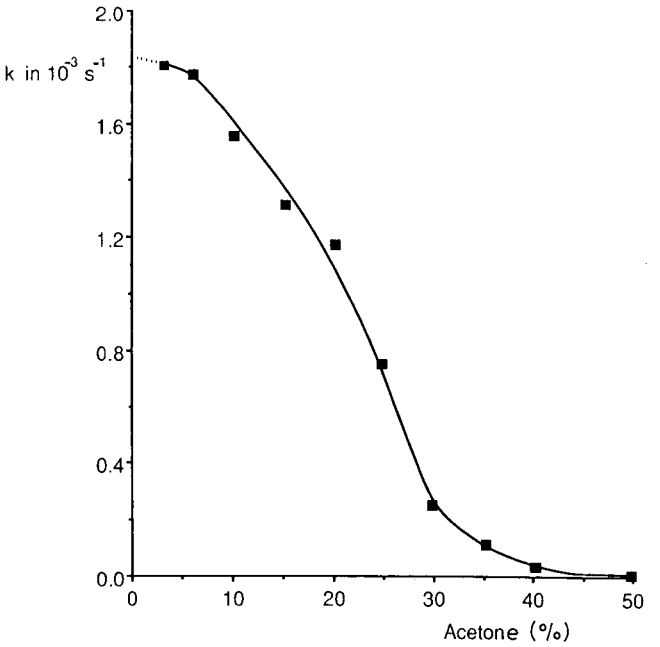

Fig. 7. Influence of the percentage of acetone present in the incubation mixture on the derivatization rate constant (k) of UA with BrMMC at 70°C. The micellar system consisted of 50 m*M* Arkopal N-130 and 36 m*M* THxABr in phosphate buffer (pH 7.0).

alternative and more convenient method of adding the BrMMC has been reported recently[22].

Influence of salts

The final aim of this study is to develop micelle-mediated derivatizations of carboxylic acids in biological matrices, *e.g.*, plasma and urine. These matrices contain salts and urea, which will affect the properties of the micellar system. Salts increase the aggregation number[8,37] and decrease the CMC[36,38] and T_c of non-ionic surfactants ($ClO_4^- \gg Br^- > Cl^- >$ urea)[8,39]. Anions can also compete with UA for the extraction into the micellar core [$ClO_4^- \gg Br^- > Cl^-$ (refs. 27–29)], which may result in lower derivatization rates.

Fig. 8 shows the influence of ClO_4^- (a well known protein precipitant), Cl^- and urea (plasma constituents) on the derivatization rate in 50 m*M* Arkopal N-130 and 36 m*M* THxABr at pH 7.0. Fig. 8 clearly illustrates that the perchlorate ion strongly inhibits the derivatization reaction, whereas chloride anion and urea do this to a much lesser extent. It is difficult to say for certain that the decrease in the derivatization rate is caused entirely by the competition of the anions with extraction of the acid[22]. Also, the induced flocculation of the micellar systems could have affected the derivatization rate. Owing to the the marked decrease in the derivatization rate it can be concluded, however, that perchloric acid cannot be used as a protein precipitant in combination with the micellar derivatization systems.

Derivatizations were carried out in a 50% Hanks balanced salt solution (BSS)[39] at pH 7.0 in order to study whether the micelle-enhanced derivatization reactions are

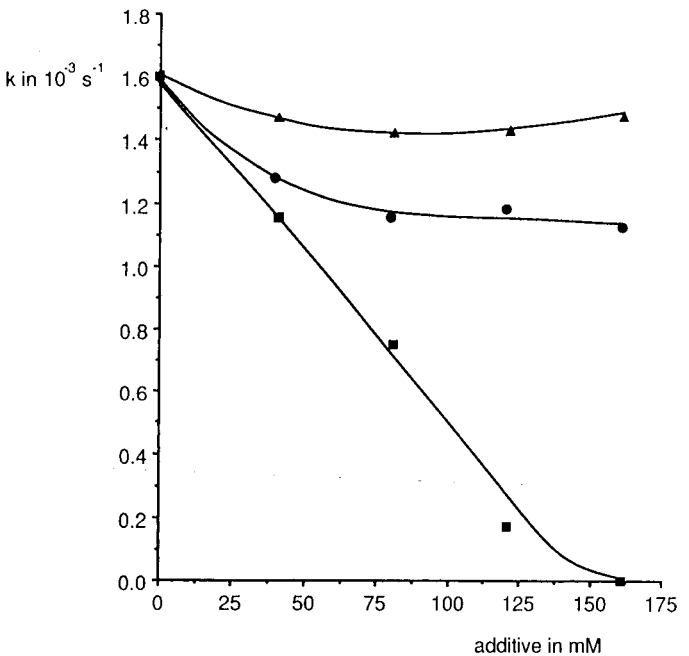

Fig. 8. Influence of the concentrations of urea (▲), NaCl (●) and $NaClO_4$ (■) on the apparent rate constant (k) at 70°C. The micellar solution consisted of 50 m*M* Arkopal N-130 and 36 m*M* THxABr in 10 m*M* phosphate buffer (pH 7.0).

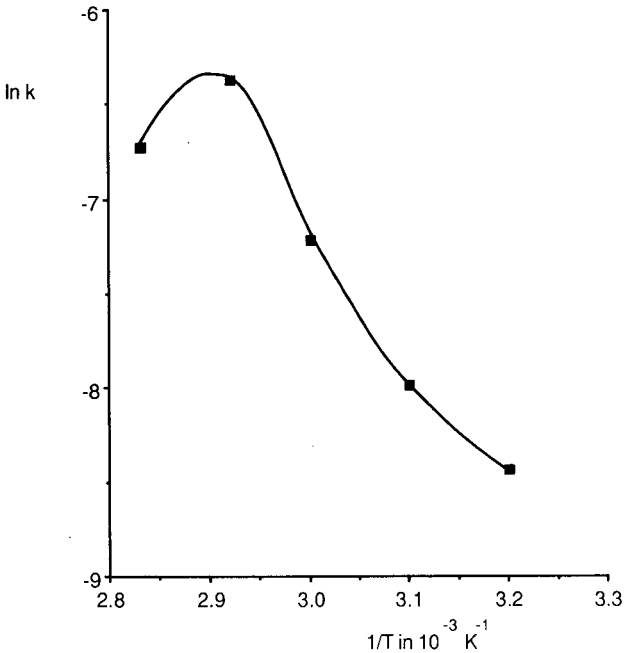

Fig. 9. Arrhenius plot of the influence of temperature on the derivatization rate in a micellar solution of 50 m*M* Arkopal N-130 and 36 m*M* THxABr in 1 m*M* phosphate buffer (pH 7.0).

compatible with the salt composition of biological fluids. With a 50% BSS solution it is assumed that the biological matrix is diluted with an equal volume of concentrated micellar solution. The apparent rate constant found for the reaction of UA with BrMMC was $1.4 \cdot 10^{-3}$ s^{-1}. In a micellar solution with 10 m*M* phosphate buffer (pH 7.0) a rate constant of $1.8 \cdot 10^{-3}$ s^{-1} was obtained. In other words, the salt composition of biological fluids will not seriously limit the use of micelle-enhanced derivatizations in biological matrices.

Influence of temperature

Fig. 9 shows the Arrhenius plot[23] of the influence of temperture on the derivatization rate in a micellar system that consisted of 50 m*M* Arkopal N-130 and 36 m*M* THxABr. This micellar system has a T_c of 60–70°C (Fig. 4). The relationship is not linear, which indicates that mechanisms other than purely kinetic ones affect the reaction rate in the micellar system[23]. An explanation of this behaviour is that micellar size increases with increase in temperature[8]. The micellar size increases particularly near T_c and, therefore, so does the derivatization rate. The decrease in the reaction rate at 80°C is caused by phase separation.

We investigated whether the derivatization rate at 40°C could be improved by lowering the T_c of the micellar system to *ca.* 40°C by the addition of extra THxABr. However, in these turbid solutions the derivatization rates found at 40°C were not significantly higher than those in clear solutions. Obviously, high derivatization temperatures are still required in order to overcome the enthalpy of reaction in the presence of large micelles.

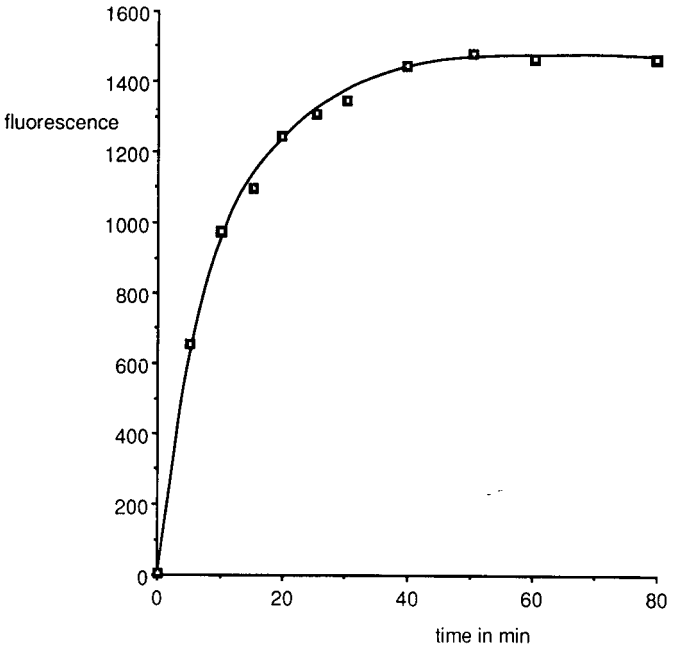

Fig. 10. Relationship between the derivatization time and the peak area of the 10-undecenoic derivative obtained in an incubation solution of 50 m*M* Arkopal N-130 and 36 m*M* THxABr in 10 m*M* phosphate buffer (pH 7.0) at 70°C.

Validation of the micelle-mediated derivatizations

Fig. 10 illustrates that the derivatization of 50 μM UA with BrMMC in a micellar system of 50 m*M* Arkopal N-130 and 36 m*M* THxABr is complete within 45 min at 70°C.

The chromatogram of the MMC derivative of UA after derivatization for 60 min in the same micellar solution is shown in Fig. 11. Although several hundred injections were performed on a single HPLC column, no deterioration in the performance of the column could be observed. This demonstrates that the injected surfactant and the ion-pair reagent do not affect the chromatographic process in the long term[40].

The linearity of the determination of 10-undecenoic acid in 10 m*M* phosphate buffer solution that contained 50 m*M* Arkopal N-130 and 36 m*M* THxABr was satisfactory from 2 n*M* to 2 m*M* UA ($r = 0.997$; $n = 17$). The reproducibility of the determination of 50 μM UA in the same solution was 3.7% ($n = 6$).

These tentative results indicate that the derivatization performed in the micellar system is satisfactory compared with other pre-column derivatization procedures for carboxylic acids that are present in aqueous solutions[20,41,42].

CONCLUSIONS

This study has demonstrated that carboxylic acids can be derivatized in aqueous solutions using micelles. The proposed derivatization procedure involves the use of a non-ionic surfactant (*e.g.*, Arkopal N or Triton X-100) and a cationic ion-pair

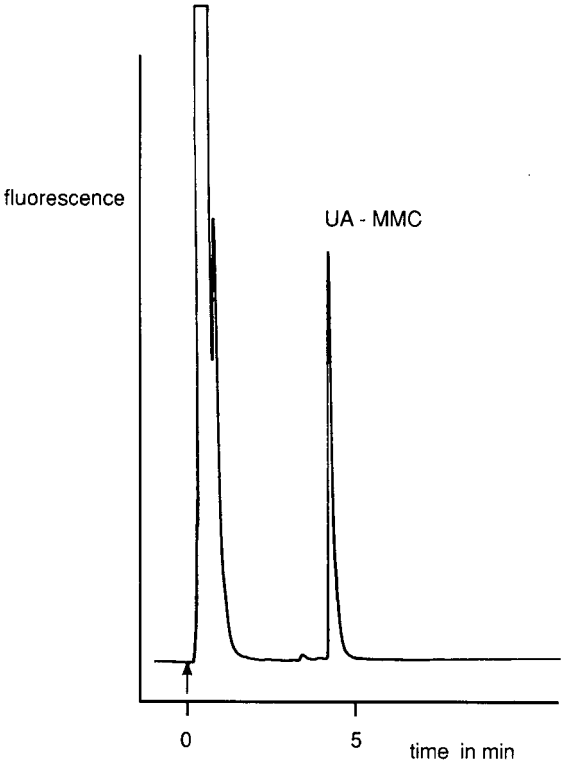

Fig. 11. Chromatogram of 20 μl of the 10-undecenoic derivative (UA-MMC, 50 μM) obtained from incubation in 50 mM Arkopal N-130 and 36 mM THxABr at pH 7.0 and 70°C.

reagent (*e.g.*, quaternary ammonium salt). The mechanism of the derivatization of the acid is likely to be related to phase-transfer catalysis. The micelles act here as an organic "pseudo"-phase, in which the actual derivatization reaction occurs.

The derivatization rates increase with increasing size of the micellar aggregates. Especially high reaction rates are observed if the cloud temperature of a particular micellar system, at which giant micellar aggregates are formed, approaches the incubation temperature.

In a subsequent paper we shall discuss the influence that the acid-related factors have on the derivatization rate of aliphatic carboxylic acids in the micellar systems[21]. In addition, a model will be presented of the mechanisms involved in the derivatization of the acids in the micellar systems.

It has been demonstrated recently that with the use of the micellar systems the tedious extraction steps that are involved in conventional derivatization procedures can be circumvented[22]. This could make the principle of micelle-mediated derivatization reactions very well suited for an on-line pre-column derivatization procedure for carboxylic acids that are present in aqueous biological matrices.

The interesting new approach of the use micellar systems in the automated on-line pre-column derivatization of bio-active acids that are present in plasma samples is currently under investigation.

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Note

Effect of chemically bonded alkyl chain length on the recovery of serotonin and its metabolite from urine by a solid extraction clean-up procedure

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Serotonin is a neurotransmitter in cerebral centres and its perturbations can produce humour and behavioural disorders. The investigation of tryptophan (TRP) and serotonin (5-HT) metabolism is important in neurochemistry also for the early detection and supervision of treatment of carcinoid tumours.

Different procedures have been devised for the determination of the tryptophan, serotonin and the major metabolite of serotonin, *i.e.*, 5-hydroxyindole-3-acetic acid (5-HIAA). Ultraviolet absoprtion, spectrophotometric and fluorimetric determination have been thoroughly reviewed[1]. Radioenzyme methods mass spectrometry and gas chromatography–mass spectrometry have been also used[2,3]. Because of its high separating power, the application of high-performance liquid chromatography (HPLC) is still increasing in importance. Many investigators employ fluorescence detection[4–6] but it generally requires a derivatization step for high sensitivity. LC methods using UV detection suffer from a lack of sensitivity[7,8]. Most simple and sensitive for neurochemical applications seems to be the use of HPLC with electrochemical detection (ED)[9–11]. ED and UV methods have been compared and the superiority of the former has been demonstrated[12]. The sensitivity of ED is higher for 5-hydroxytryptophan (5-HTP) and 5-HIAA whereas the opposite holds for the fluorimetric detection of serotonin and tryptophan[6,13–15].

Tryptophan metabolites have been determined in brain tissue[14–22], cerebrospinal fluid[23,24], urine[23], serum and plasma[17,23] and biological materials[25,26]. There are different methods for the isolation of material of biological origin[23], *e.g.*, TRP and 5-HTP using a strong cation-exchange resin, *e.g.*, Dowex AG-50, 5-HT on a weak cation exchange resin, *e.g.*, Amberlite GC-50, and 5-HIAA on Sephadex G-10 gel. "Off-line" isolation pre-columns are usually used. Attempts to use "on-line" preconcentration columns resulted in a *ca.* 100-fold increase in detection sensitivity[17,20].

Dense layered bonded stationary phases have been used successfully for the isolation of 5-HIAA from urine[27] and in HPLC separations[28]. Some attempts to isolate tryptophan and its metabolites have been made[29,20].

The properties of sorbents with alkyl bonded stationary phases are strongly dependent on many factors, such as coverage density[31], porous structure and chemical nature of the solid silica support[32,33], amount of remaining silanol groups (endcapping) and bonded layer structure (functionality of silane modifier). Monochloro-

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dimethylalkylsilanes seem to be the most promising modifiers because their use allows precisely defined and dense alkyl bonded layers to be obtained[31,34,35]. Inconvenient interaction of remaining silanol groups is usually eliminated by secondary silanization (end-capping) and its effectiveness may differ depending on how dense a layer is obtained in primary silanization[36,37].

EXPERIMENTAL

The chromatographic system consisted of an HPP-4001 pump (Laboratorní Přístroje, Prague, Czechoslovakia), a Model 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.) fitted with a 50-μl sample loop and 100 × 4.6 mm I.D. stainless-steel column (Chemical Reagents Factory, ZOCh, Lublin, Poland) packed with LiChrosorb RP-18 (5-μm) (Merck, Darmstadt, F.R.G.). An ELDEC 102 electrochemical detector (Chromatofield, Chateauneuf-Les Martiques, France) with a glass carbon electrode was employed at a potential of +0.6 V (5-HT, 5-HIAA) *vs.* a silver–silver chloride reference electrode.

The specific surface areas (S_{BET}) of the sorbents for the pre-columns were determined by the BET method from nitrogen adsorption data using a Sorptomatic Model 1800 instrument (Carlo Erba, Milan, Italy). The degree of surface coverage of the sorbents (α_{RP}) was calculated by means of the Berendsen equation[38] on the basis of the carbon loading (% C) determined by a Model 185 CHN analyser (Hewlett-Packard, Palo Alto, CA, U.S.A.).

LiChrosorb Si-60 was chemically modified by C_6, C_8 and C_{18} monochlorodimethylsilanes (Petrarch Systems, Levittown, PA, U.S.A.) in the absence and presence of an activator according to the method proposed by Buszewski *et al.*[35,39]. C_1 and secondary silanization (end-capping) were performed using hexamethyldisilazane (HMDS) (POCh, Gliwice, Poland).

Analytical-reagent grade chemicals used for off-line clean-up isolation and for chromatography were obtained from POCh. The mobile phase consisted of analytical-reagent grade chemicals dissolved in water [0.15 *M* sodium phosphate buffer (pH 4.2) containing 13% (v/v) of methanol]. Doubly distilled, deionized water was used.

The standards used were tryptophan (Reanal, Budapest, Hungary), serotonin (Merck), 5-hydroxyindole-3-acetic acid (Koch-Light Labs., Colnbrook, U.K.). Standard solution consisted of TRP (10 μl/ml, 5-HT (1 μg/ml) and 5-HIAA (2 μl/ml) dissolved in water, prepared freshly every month and kept frozen until used.

The off-line clean-up pre-columns consisted of 2-ml plastic extraction tubes packed with various types of synthesized stationary phases to yield a 1.8-cm high bed. The simultaneous isolation of 5-HT and 5-HIAA from the standard solutions and urine was carried out according to the procedure described in ref. 29.

RESULTS AND DISCUSSION

From the viewpoints of detectability, a constant potential of the working electrode (+0.6 V) and the most interesting information in neurochemistry, we investigated the recoveries of 5-HT and 5-HIAA using the pre-column clean-up procedure (Table I). The recovery of TRP was also tested at a detector potential of +0.9 V and

it was close to the values for 5-HIAA. The physico-chemical characteristics of off-line clean-up pre-columns with various sorbents are given in Table I.

The results may be discussed in terms of the chemically bonded surface hydrophobicity and availability of the remaining silanol groups for solute molecules. Surface hydrophobicity is directly proportional to the number of bonded main alkyl groups[31,34,35] or, more precisely, inversely proportional to the relaxation time (alkyl chain mobility) in CP-MAS NMR measerements[36,37]. Our results support the conclusions of Bayer *et al.*[36] that a high hydrophobicity (low relaxation times) may be obtained not only by dense coverage with main alkyl groups but also by exhaustive secondary end-capping of the partially covered silica surface. This is in agreement with our results for shorter bonded alkyl chains (C_6 and C_8) (sorbents 5, 6, 9 and 10 in Table I). A similar situation exists for the sorbents with a long C_{18} bonded alkyl chain (sorbents 13 and 14 in Table I), but there are evident hindrances to narrow pore penetration in bare silica gel[39].

Comparison of C_1 sorbents cannot be taken into account because primary and secondary silanization were effected with the use of the same modifier, *i.e.*, HMDS, which introduces the same trimethylsilyl radicals on the silica surface. The presence of an activator does not influence the surface reaction (lower surface coverage of sorbent 2 than 1; Table I) and, moreover, the recoveries of 5-HT and 5-HIAA are low and non-reproducible. Of course, the highest coverage density of the surface with main alkyl chains and hence the highest silica gel surface hydrophobicity may be obtained in the presence of morpholine activator[34,35].

Considering the influence of bonded alkyl chain length on most hydrophobic surfaces (EC and A + EC), it can be seen that the best 5-HT and 5-HIAA recoveries are given by C_8 pre-columns (sorbents 9 and 10, Table I). Similar results were obtained for C_{18} packings (sorbents 13 and 14, Table I). Bonding of shorter alkyl chain leads to lower recoveries of 5-HT for C_1 and C_6 (except sorbent 5, Table I) and a high 5-HIAA recovery (except sorbent 6, Table I). The coefficients of variation of the results obtained with the above sorbents, calculated for the average values from six independent measurements of recovery, are relatively low (Table I).

The above discussion of recoveries is valid for the results obtained with a standard two-component solute mixture. Test measurements with human urine showed that of the two available groups of alkyl-bonded sorbents of highest hydrophobicity (EC and A + EC), the latter gives a better performance (Fig. 1). Sorbents obtained without an activator gave a poor recovery of 5-HT from urine samples, in contrast to those obtained for standard solutions (Table I).

The 5-HT and 5-HIAA recoveries shown in Table I were obtained using a standard clean-up procedure[29] in which the investigated compounds were sorbed on "off-line" pre-columns from 2 ml of standard solution and the same volume of displacing agent [0.1 *M* ammonia solution containing 25% (v/v) of methanol] was used. Samples of 10 μl were taken from the eluate injected into the chromatograph.

This method guarantees the use of less than half of the clean-up pre-column capacity, because the breakthrough volumes correspond to *ca.* 5 ml of a standard solution or urine. Hence it is possible to obtain an almost 2-fold urine sample enrichment if 4 ml of urine and only 2 ml of displacing agent are used. Fig. 1 shows chromatograms that illustrate the 5-HT and 5-HIAA recoveries obtained for double urine samples on clean-up pre-columns with different sorbents. Because of the low

TABLE I

CHARACTERISTICS OF SORBENTS FOR OFF-LINE CLEAN-UP PRE-COLUMNS AND COMPARISON OF RECOVERIES OF 5-HT AND 5-HIAA FROM STANDARD SOLUTION (n = 6)

No.	*Type of sorbent for pre-column**	*Coverage density*		*Recovery of 5-HT ± S.D. (%)*	*Coefficient of variation*	*Recovery of 5-HIAA ± S.D. (%)*	*Coefficient of variation*
		C (%)	α_{RP} *(μmole/m²)*				
1	SG-Si-60 + C_1	6.45	5.81	4.2 ± 1.5	35.7	91.8 ± 3.0	3.3
2	+ C_1 + A	4.49	3.90	8.5 ± 2.7	31.8	Irreproducible.	
3	+ C_6	6.26	2.07	2.0 ± 0.7	35.0	84.6 ± 2.5	3.0
4	+ C_6 + A	10.59	3.89	5.6 ± 1.9	33.9	90.0 ± 1.3	1.4
5	+ C_6 + EC	10.43	5.87	48.4 ± 2.3	4.8	74.6 ± 1.9	2.5
6	+ C_6 + A + EC	13.95	5.60	23.4 ± 1.3	5.6	44.1 ± 2.3	5.2
7	+ C_8	7.89	2.12	2.6 ± 0.5	19.2	82.8 ± 3.1	3.7
8	+ C_8 + A	13.01	3.08	3.5 ± 1.0	28.6	85.7 ± 1.2	1.4
9	+ C_8 + EC	11.98	5.66	64.9 ± 2.2	3.4	86.3 ± 2.7	3.1
10	+ C_8 +A + EC	15.10	5.54	31.9 ± 2.4	7.5	60.0 ± 2.9	4.8
11	+ C_{18}	10.16	1.39	4.0 ± 0.9	22.5	79.0 ± 3.4	4.3
12	+ C_{18} + A	17.26	2.65	1.7 ± 0.4	23.5	79.5 ± 2.3	2.9
13	+ C_{18} + EC	13.49	4.22	46.5 ± 1.8	3.8	80.5 ± 3.2	3.9
14	+ C_{18} + A + EC	18.66	3.80	56.1 ± 1.9	3.4	62.7 ± 2.2	3.5

* SG-Si-60 = bare silica gel. S_{BET} = 348 m²/g, mean pore diameter (D) = 8.6 nm, pore volume (V_p) = 0.884 cm³/g, particle size (d_p) = 40–60 μm. A = activator. EC = end-capped (HMDS). C_1–C_{18} = length of bonded alkyl chain. Modifiers: C_6–C_{18} monochlorodimethylalkylsilanes.

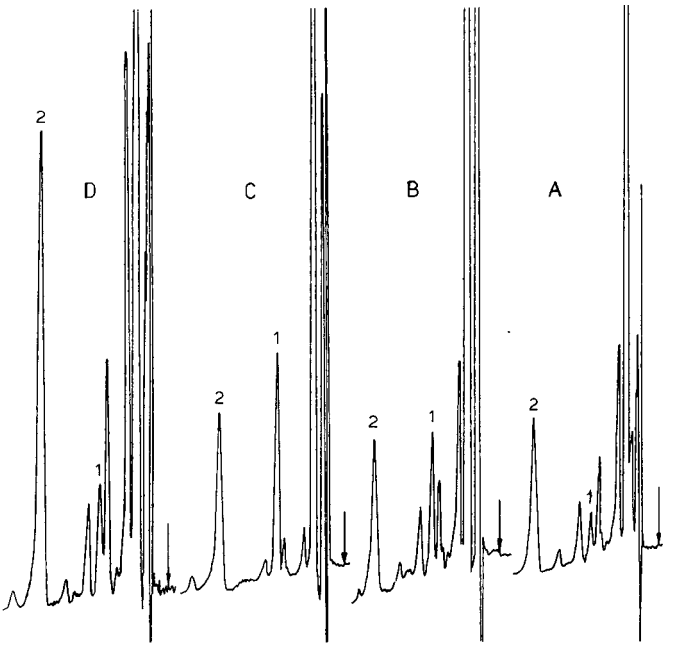

Fig. 1 Chromatograms of 5-HT and 5-HIAA obtained for double human urine samples using clean-up pre-columns with different sorbents. (A) Untreated urine; (B) urine with addition of 0.5 μl/g of 5-HT standard; after pre-column osilation and preconcentration, (C) C_{8+A+EC} and (D) C_{8+A+EC}. Chromatographic conditions: C_{18} column (250 × 4 mm I.D.) packed with 7-μm spherical particles (ZOCh); electrochemical detector (+0.6 V); mobile phase, 0.15 *M* sodium phosphate buffer (pH 4.2) containing 12.5% (v/v) of methanol; flow-rate, 0.9 cm^3/min; inlet pressure, 80 bar; sample volume, 10 μl. Peaks: 1 = 5-HT; 2 = 5-HIAA.

serotonin concentration (*ca.* 0.1 μg/ml), standard 5-HT solution (0.5 μl/ml) was added to common urine in order to show the real differences in the investigated pre-columns.

On a C_{8+A+EC} pre-column (C, Fig. 1) real enrichment of both solutes takes place. The $C_{18+A+EC}$ pre-column (D, Fig. 1) gives a better pre-concentration of 5-HIAA but poorer that might be expected for 5-HT, which may be the result either of a lower coverage density (sorbents 13 and 14, Table I) or of a slightly different sorption mechanism (poor pre-cleaning). This problem may be partially solved by the use of larger pore silicas[29,30], but even this procedure does not permit the same mechanism and the sample capacity in relation to that of C_{8+A+EC} localized on the surface of Si-60 silica gel.

A clear explanation of the phenomena discussed is difficult on the basis of the data in Table I. Taking into account literature data[29–31] and our latest results[40], we suggest that the most active compound towards silanol groups, 5-HT (amine), needs the best screening of the silica surface with a chemically bonded film to avoid undesirable interactions. This is probably achieved on $C_{18+A+EC}$ sorbent bonded on silica gel Si-60, where the narrow pores are completly closed with alkyl groups, in contrast to the effect of the shorter C_8 alkyl chain.

The disadvantage of the $C_{18+A+EC}$ material in practical pre-cleaning applications is its low selectivity, and from this point of view C_{8+A+EC} sorbent has a better performance (see the chromatograms in Fig. 1). It is noteworthy that the end-capping process is important, but it cannot solve the problem of elimination of surface activity because of steric hindrance (a trimethylsilyl group has *ca.* twice the cross-sectional diameter of a hydroxyl group) and allows the elimination of only half the silanol groups.

The different chemical natures of the investigated solutes influences their recoveries strongly and are similiar shapes of the molecules are less important. The low recovery of 5-HT is connected with irreversible sorption during the pre-cleaning procedure rather than its degradation and the latter effect is obviated through the standardization procedure during analysis.

We consider that the combination of clean-up samplers with different chemically bonded materials and a suitable choise of solvents for sorption–desorption processes will allow real practical applications to urine analysis. The limits of detection were established as 1 ng of 5-HIAA and 0.5 ng of 5-HT. If larger samples (up to 50 μl) are injected on to the HPLC column and a simple enrichment procedure is used, the limit of detection may be lowered by *ca.* one order of magnitude.

CONCLUSIONS

The hydrophobicity of the bonded alkyl layer and the availability of the remaining silanol groups on the silica gel surfaces to solute molecules are the main factors influencing the recoveries of 5-HT and 5-HIAA. Exhaustive silanization of the silica gel surface, including end-capping, gives usable sorbents for the isolation of 5-HT and 5-HIAA. C_8 sorbents synthesized on the basis of SI-60 silica gel in the presence of an activator show sufficient selectivity and recovery of 5-HT and 5-HIAA from urine samples and a sample capacity that allows simple enrichment before analysis.

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Note

Determination of benzo[*a*]pyrene in coke tars

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Polycyclic aromatic hydrocarbons (PAHs) are toxic substances found in increasing concentrations in the environment. Benzo[*a*]pyrene is carcinogenic and particularly hazardous, and methods for its determination in environmental and industrial samples have been investigated[1–5].

The aim of this work was to establish a method for the determination of benzo[*a*]pyrene in the tars originating from the coke plants. The method was intended to consist in the collection, extraction and isolation of benzo[*a*]pyrene from the coke tar, and its qualitative and quantitative determination by means of high-performance liquid chromatography (HPLC). For each stage we attempted to propose simpler approaches then those already established[6–8].

EXPERIMENTAL

The tar samples investigated originated from different black coals that were processed in different coke plants, and for this reason the results given here should not necessarily be considered as representative of products that may eventually be produced on a commercial scale.

A tar sample was homogenized with a mechanical stirrer until a uniform consistency was obtained. The sample was then dissolved in benzene using an ultrasonic stirrer. The insoluble parts gave a stable emulsion in the benzene solution obtained. To remove the insoluble part, a portion of this solution was placed on top of a non-pressurized chromatographic column filled with silica (Kieselgel H, 0.08–0.20 mm; E. Merck) that had been conditioned at 120°C and eluted with benzene. The first 50 cm^3 of eluate were collected, of which 1 cm^3 was applied to ready-made preparative chromatographic glass plates covered with silica (Kieselgel 60; E. Merck) and conditioned at 120°C for 0.5 h. On the same plates benzo[*a*]pyrene was additionally spotted as a chromatographic standard.

The chromatograms were developed with benzene–cyclohexene (3:2, v/v). The developed and dried chromatograms were examined under UV light and the band corresponding to benzo[*a*]pyrene was marked. It was then removed from the plate and eluted with benzene. From the solution obtained benzene was evaporated under the vacuum and the dry residue was dissolved in 1 cm^3 of cyclohexane.

To determine the mass loss of benzo[*a*]pyrene during the collection, extraction and isolation procedures we had to repeat all the above-described steps on a carefully

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prepared "dummy" sample. Therefore, we added an appropriate and accurately weighed aliquot of benzo[*a*]pyrene to a known amount of silicone grease and homogenized this "dummy" sample using an ultrasonic stirrer.

The cyclohexane solution obtained was further analysed by HPLC using a chromatograph (Laboratorní Přístroje, Prague, Czechoslovakia) consisting of an HPP 5001 pump, an LCD 2563 UV–VIS detector with a 290-nm filter, a TZ-4620 recorder, and a CI-100 integrator. Separation was achieved on a 150 × 3.6 mm I.D. column packed with 5-μm Separon SGX-C_{18} (octadecylsilica) (Laboratorní Přístroje). The mobile phase was methanol–water (85:15, v/v) at a constant flow-rate of 0.7

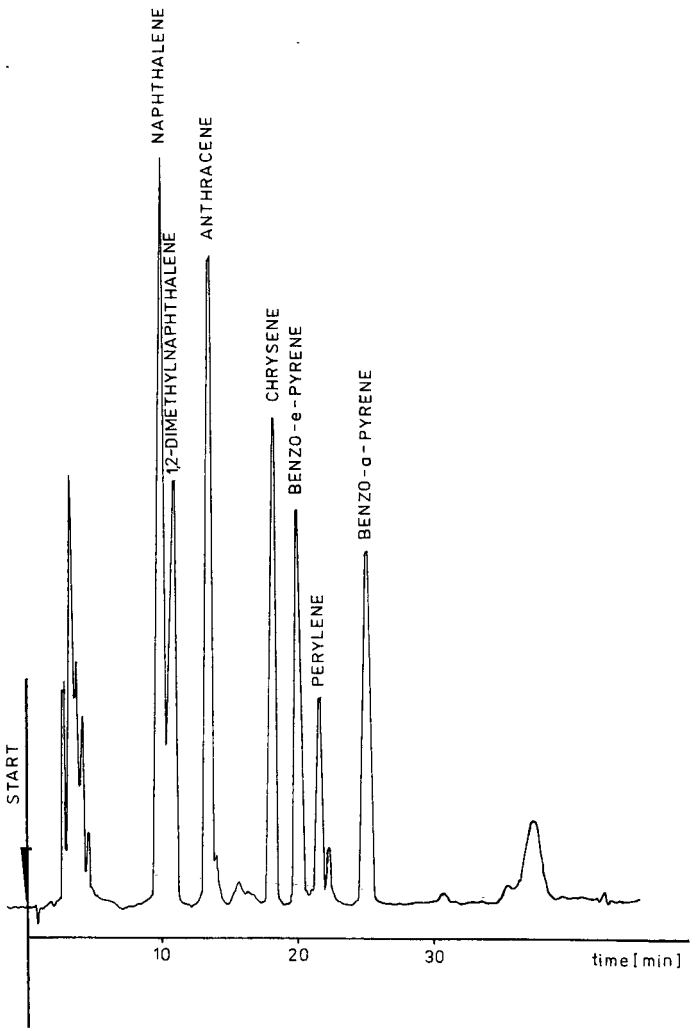

Fig. 1. Separation of a standard PAH mixture on a Separon SGX-C_{18} column (150 mm × 3.3 mm I.D.). Mobile phase, methanol–water (85:15, v/v); flow-rate, 0.7 cm^3/min; pressure, 80 atm; detection, UV–VIS at 289 nm.

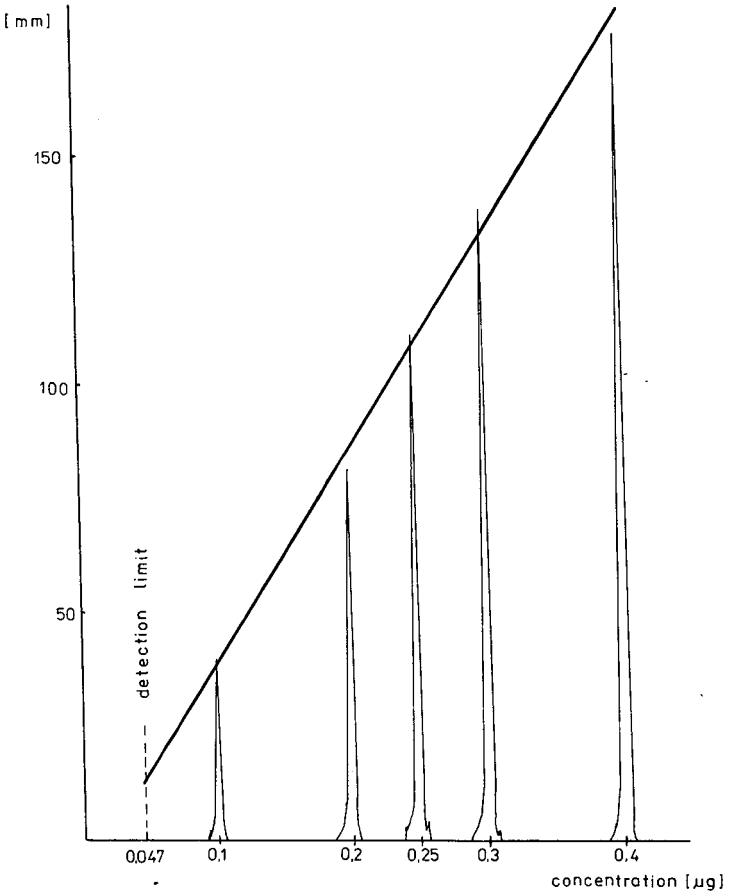

Fig. 2. Peak height *vs.* concentration of benzo[*a*]pyrene.

cm^3/min. These conditions provided a good separation of benzo[*a*]pyrene from benzo[*e*]pyrene and other PAHs (see Fig. 1). Quantification was based on a previously constructed calibration graph (Fig. 2).

TABLE I

DETERMINATION OF THE BENZO[*a*]PYRENE (BaP) LOSS DURING SAMPLE PURIFICATION AND DETERMINATION OF THE CORRECTION FACTOR *k*

k was determined from the equation: actual amount of BaP = k · (determined amount of BaP).

Initial amount of BaP (mg/g of sample)	*Determined amount of BaP (mg/g of sample)*	*Mean determined amount of BaP (mg/g of sample)*	*k*
10	8.08		
10	8.15	8.11	1.233
10	8.10		

TABLE II

AMOUNT OF BENZO[*a*]PYRENE (BaP) IN THE COKE TARS FROM DIFFERENT SOURCES

Sample No.	*Amount of BaP determined (mg/g of sample)*	*Mean*
1	1.27, 1.32, 1.30	1.30
2	2.73, 2.82, 2.75	2.77
3	3.18, 3.15, 3.20	3.18
4	3.88, 3.89, 3.85	3.87

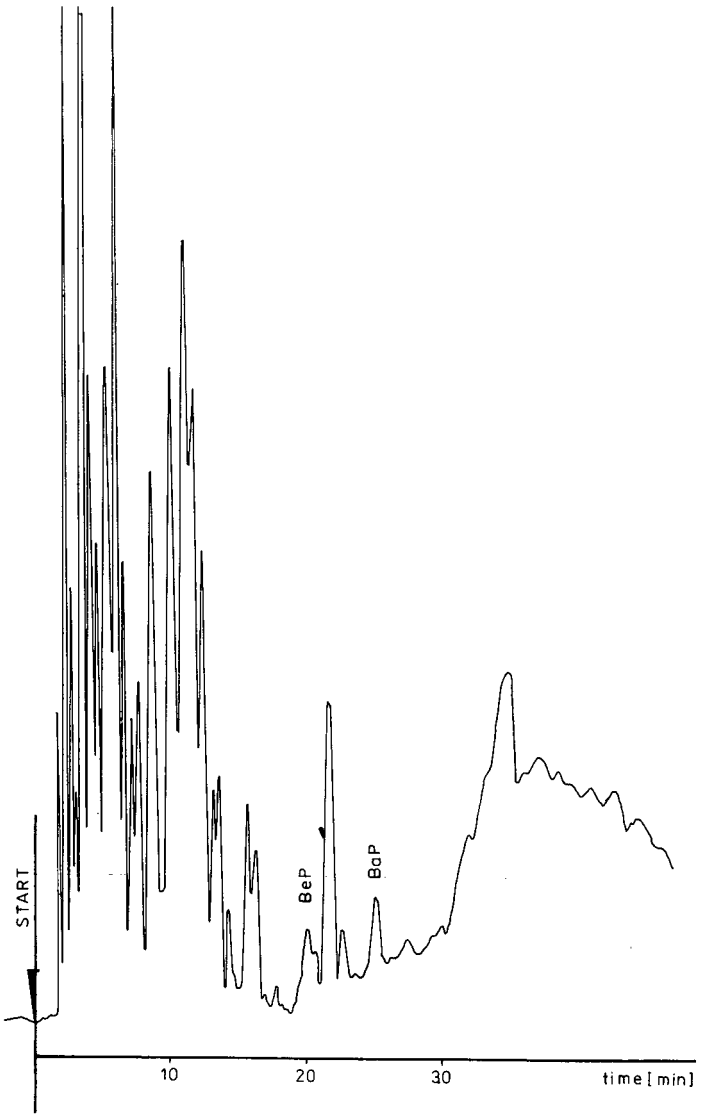

Fig. 3. Separation of a PAH mixture originating from the coke tar. Conditions as in Fig. 1. BaP = benzo[*a*]pyrene; BeP = benzo[*e*]pyrene.

RESULTS

On the basis of the results obtained for the reference benzo[*a*]pyrene samples, carried through the routine purification procedure, we calculated the losses of this compound during the analytical procedure. The results are given in Table I.

Examples of the determination of benzo[*a*]pyrene in coke tars originating from different plants are shown in Table II. A chromatogram of PAHs separated from coke tar is given in Fig. 3.

CONCLUSIONS

The proposed method permits the separation and determination of benzo[*a*]-pyrene in coke tars. The content of benzo[*a*]pyrene depends on the origin of the tar (see Table II). The method can be extended to the determination of other PAHs, provided that the necessary calibration graphs are prepared in advance.

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THERMAL DESORPTION–CAPILLARY GAS CHROMATOGRAPHY FOR THE QUANTITATIVE ANALYSIS OF DIMETHYL SULPHATE, DIETHYL SULPHATE AND ETHYLENE OXIDE IN THE WORKPLACE

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SUMMARY

Sampling on solid adsorbents followed by thermal desorption and gas chromatography was evaluated as a simple method for the quantitative analysis of dimethyl sulphate, diethyl sulphate and ethylene oxide in the workplace environment. Tenax TA and Carbosieve S-III (for ethylene oxide) were found to be suitable adsorbents. The charged tubes can be stored at 22°C for 4 days. The recovery is better than 98% (> 80% for ethylene oxide) in the mass range 1 ng–1 μg. The recovery is not dependent on air humidity. The limits of detection tested were at the lower parts per billion level.

INTRODUCTION

Industrial users of chemical substances have a duty to protect their workers from any adverse effects of the substances used in their processes. Thus the monitoring of toxic pollutants in the workplace environment is an essential task in industrial hygiene in order to control potential health hazards resulting from exposure to these substances. For many substances, threshold values have been defined. In the F.R.G. these are known as MAK or TRK values[1]. In Switzerland the SUVA sets the threshold values[2]. The monitoring of workers exposures requires analytical methods well suited for a reliable quantitative analysis at the required concentrations. For some substances, particularly for established carcinogens such as dimethyl sulphate (DMS), diethyl sulphate (DAS) or ethylene oxide (ETO), the TRK values are to low for a direct analysis. To lower the detection limit, sample preconcentration is necessary. The most common method involves trapping of the target compound on solid adsorbents followed by liquid desorption. Based on this technique, various procedures have been developed for DMS[3–7], including its derivatization[3,4]. A surveillance monitor based on solid adsorbent sampling/thermal desorption has been developed by Widmer *et al.*[8]. Diethyl sulphate can be analyzed analogously to dimethyl sulphate[4]. Sampling of ethylene oxide is usually done on activated charcoal followed by liquid[9–12] or headspace desorption[13]. Some procedures include a derivatization of the ethylene oxide in order to obtain a more stable analyte for the subsequent quantitation[9,10]. A comparison of commercially available solid adsorbent samplers has

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been published by Kring *et al.*[14]. In this paper, solid adsorbent sampling followed by thermal desorption and gas chromatography (GC) is described for the quantitative analysis of DMS, DAS and ETO.

EXPERIMENTAL

Materials and apparatus

DMS, DAS and ETO were obtained from Fluka (Buchs, Switzerland), cyclohexane and hexane from Merck (Darmstadt, F.R.G.). For the desorption, a thermal desorption unit TDAS 5000 (Carlo Erba, Italy) was used. The TDAS 5000 was coupled via an heated transfer capillary (1 m × 0.53 mm × 2.65 μm HP-1) to a Hewlett-Packard 5890 gas chromatograph. A10-cm length of the transfer capillary was cooled with liquid nitrogen to −150°C in order to focus the broad sample train desorbed from the adsorbent. This cold trap was controlled by a MFA 515 (Carlo Erba) control module. The cold trap was heated rapidly to allow a rapid injection of the substances onto the column. A split/splitless type injector (valve with vent to the atmosphere, controlled by the MFA 515, to permit higher desorption flows in the thermal desorption step) was used. For DMS and DAS a flame ionization detector or a flame photometric detector operated in the S-mode (393 nm) was used, for ETO a flame ionization detector. Helium served as the carrier gas. The chromatographic conditions are given in Table I.

Solid adsorbents

For the enrichment of organic atmospheric trace compounds, various solid adsorbents are known[15]. For thermal desorption the best compromise between the following properties has to be evaluated: chemical and physical stability of the ad-

TABLE I

CHROMATOGRAPHIC CONDITIONS

	DMS, DAS	*ETO*
Cold trap		
Splitless time (s)	30	Not in action;
Start temperature (°C)	−80	focusing of sample
Final temperature (°C)	150	at the column head
Column		
Type	DB-1 (J+W.Sci.) 15 m x 0.52 mm x 1.5 μm	GS-Q (J+W.Sci.) 30 m x 0.52 mm
Flow-rate (ml/min)	6.5	12
Start temperature (°C)	60	35
Start time (min)	2	0
Rate 1 (°C/min)	10	10
Temperature 1 (°C)	100	110
Time 1 (min)	0	0
Rate 2 (°C/min)	30	30
Final temperature (°C)	250	240
Final time (min)	0	5

sorbent under the experimental conditions; high retention volume for the substances of interest at ambient temperatures; very low retention volumes at the desorption temperature; high selectivity which means high affinity for the target compounds and a concomitant low affinity for water, CO_2, NO_x, SO_2, O_3) and high capacity to allow for sampling of larger amounts of substance. For DMS and DAS, Tenax TA[15,16] and for the low boiling ETO, Carbosieve S-III[17] were found to be ideal. Two different glass sample tubes (100 mm × 4 mm I.D.) were either filled with 150 mg Tenax TA, 20–35 mesh (Chrompack, The Netherlands), or with 600 mg of Carbosieve S-III, 60–80 mesh (Supelco, Switzerland), and fixed at both ends with silanized glass wool plugs. Heating in a helium carrier gas stream of 40 ml/min for 20 min at 300–350°C (Tenax TA) or for 20 min at 330–400°C (Carbosieve S-III) was sufficient for clean blanks.

Calibrations

For external standard calibration standard solutions of DMS or DAS in *n*-hexane or ETO in cyclohexane were injected directly (on-column). The standard solutions were prepared gravimetrically. The calibration of the whole analytical procedure was done by two methods.

(1) The tubes were charged directly through evaporation of the standard solution in a GC injector. The standard solution was then completely transferred to the adsorbent tube by a nitrogen flow of 40 ml/min. In the case of ETO most of the cyclohexane was adsorbed in a Tenax TA tube, mounted prior to the Carbosieve S-III tube. To ensure that all ethylene oxide breaks through the first (Tenax) tube, the tubes were flushed with carrier gas for 5 min.

(2) Alternatively, the tubes were charged dynamically from a gas stream containing the substance at the parts per billion level. For example, a standard solution containing 9.5 mg DMS per ml hexane was injected continuously into a GC injector by means of a syringe pump, evaporating the solution at a rate of 0.33 μl/min into a nitrogen flow of 2 l/min. This yields a DMS concentration of 0.3 ml/m^3 corresponding to 1.57 mg/m^3 (22°C). Other concentrations were generated by variation of the concentration of the standard solution or by variation of the injection or the flow-rates. A fraction of this gas mixture was then sucked through the adsorbent tube for a defined time with a defined flow-rate. A standard personal low flow sampling pump SKC 222 (SKC, Eighty Four, PA, U.S.A.) was used. The flow-rate must be checked with the adsorbent tube investigated prior to use! The tubes were capped immediately after charging with plastic caps.

For calibration of the whole procedure, at least five different samples (each triplicate) with the expected amounts of substances (1 ng to 1 μg) were analyzed. Due to the non-linear response of the flame photometric detector, more calibration points had to be used with this detector for reliable interpolations. The chromatographic conditions are listed in Table I, the parameters for thermal desorption in Table II. The experimental set up for thermal desorption is shown in Fig. 1.

Gas chromatography and thermal desorption

The temperatures of the valve, interface and transfer line (Table II) were adjusted to ensure a quantitative transfer of the respective substance. To remove air from the system, the tubes were purged with the carrier gas and then preheated

TABLE II

PARAMETERS USED FOR THERMAL DESORPTION (TDAS 5000)

	DMS	*DAS*	*ETO*
Valve temperature (°C)	130	130	120
Interface temperature (°C)	130	130	250
Transfer line temperature (°C)	130	130	250
Purge flow (ml/min)	26	26	28
Purge temperature (°C)	≈25	≈25	≈25
Purge time (s)	10	10	2
Preheating time (s)	60	60	0
Desorption flow (ml/min)	13.5	13.5	12
Desorption temperature (°C)	190	190	320
Desorption time (s)	120	180	180
Cleaning time (min)	10	10	0

(except for the highly volatile ETO for which there is a risk of losses.). The desorption parameters were adjusted to allow for a quantitative desorption within the temperature limits (400°C) of the apparatus and the adsorbents. The parameters chosen for cold trapping are listed in Table I. During the thermal desorption process DMS and DAS were trapped at −80°C. For the injection of the substance the trap was then heated very rapidly to 150°C and the split valve of the injector was closed for 30 s. For

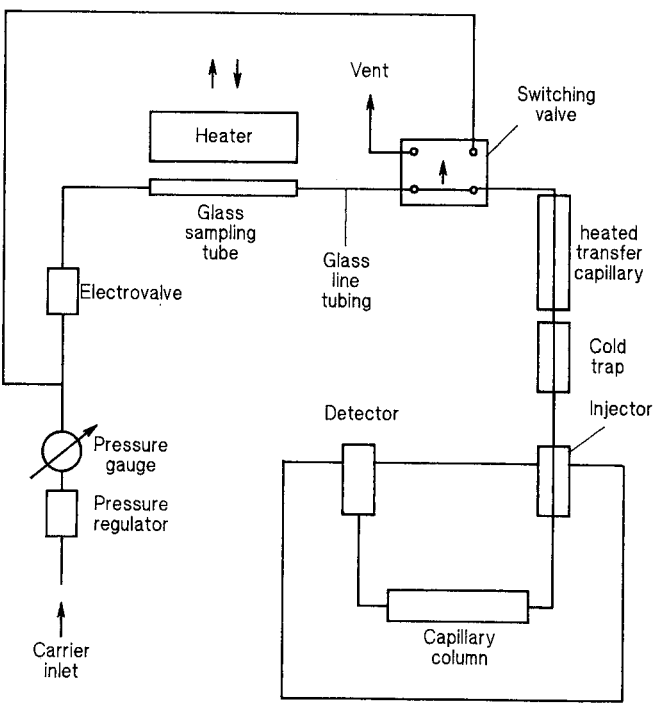

Fig. 1. Experimental set up (TDAS 5000).

TABLE III

RECOVERY OF SUBSTANCE, IN A STREAM OF HUMID NITROGEN

	DMS	*DAS*	*ETO*
Absorbed substance (μg)	5	1	1.7
Volume flow (l/h)	12	12	6
Purge time (h)	4	4	4
Total flow (l)	48	48	24
Relative humidity (%)	0, 40, 80	0, 40, 80	0, 40, 80
Temperature (°C)	≈27	≈25	≈25
Loss of substance (%/h)	<0.5	<0.5	<5
Recovery (%)	>98	>98	>80

ETO the cold trap could not be used. Compared with Tenax TA, Carbosieve S-III traps more water which regularly blocked the cold trap by ice formation. This problem could not be overcome by changing the transfer capillary (larger diameter or other coatings). Thus "focusing" of the desorbed ETO was done at the column head of the GS-Q column. The temperature programmes are given in Table I. The chromatographic conditions (Table I) were primarily optimized to separate the solvents and the purge gas impurities (see method tests). The field samples usually did not present chromatographic problems.

RESULTS AND DISCUSSION

Method tests

The comparison of the results from the calibration graphs with the on-column injection (external standard) shows good agreement. This means that the adsorption of the substance from the dry nitrogen calibration mixtures and the subsequent thermal desorption were complete under the conditions specified. In order to check for potential losses during field sampling, charged tubes were exposed to a stream of nitrogen with varying relative humidity. Water is known to be an efficient eluent,

TABLE IV

RESULTS OF METHOD TESTS

	DMS	*DAS*	*ETO*
Minimum detectable quantity*			
by FID (ng)	1	0.1	0.5
by FPD (ng)	≈1	≈1	–
Maximum quantity tested (μg)	5	1	15
Range tested (mg/m^3)	0.05–2.5	0.005-0.5	0.0005–7.5
Relative standard deviation (%)	1.0	0.8	5.3
n = 6 (95% confidence limit)	(5 μg)	(1 μg)	(1 μg)
	4.0	2.3	4.8
	(0.1 μg)	(0.01 μg)	(0.01 μg)

* FID = flame ionization detection; FPD = flame photometric detection.

which could change the time-dependent recoveries. First the tubes were charged with a defined amount of substance, see Table III. The tubes were then purged (in the same direction as used for the adsorption) with nitrogen at 12 l/h and 0, 20 or 80% relative humidity (DMS, DAS). A total flow of 48 l was applied in each experiment. The tubes were desorbed thermally. The recoveries found for the total purge time are given in Table III. The various relative humidities were generated by volumetric mixing of dry nitrogen with nitrogen which had been saturated with water by passing through a water-filled washing bottle. The effective relative humidity was measured by a hygrometer. For DMS and DAS the recoveries were almost complete within the relative standard deviation of the whole method. For ETO the losses were somewhat higher, only 80% of the amount dosed being recovered after purging with 24 l of nitrogen at 6 l/h, independent of the relative humidity. For practical purposes this is acceptable, as the test conditions are much more rigorous than in field sampling (e.g. sampling at 20 ml/min for 8 h yields a total flow of 9.6 l. The further results of the method tests are listed in Table IV. The range of analyses is limited by the substance's minimum detectable quantity and by the loading capacity of the sampling tube and the cold trap. For our practical needs the range tested was limited to concentrations close to the respective TRK values. There exists the potential to extend the range to extremely low concentrations, even lower than tested for ETO. The relative standard deviation of the whole method is relatively low (0.8–4.8%), dependent on the amount of substance injected.

CONCLUSIONS

Solid adsorbent sampling/thermal desorption has proved successful for industrial hygiene monitoring. The main advantages of the method are the extremely low limits of detection, the high selectivity for the target compounds, particularly when selective (flame photometric) detectors are used, the wide applicability with respect to the compounds to be analyzed, the simplicity of sample preparation and the relative ease of operation. The main disadvantages are the still limited choice of solid adsorbents and the fact that only one analysis per sample is possible.

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APPLICATION OF HEADSPACE GAS CHROMATOGRAPHY TO THE MEASUREMENT OF ORGANIC EMISSIONS

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SUMMARY

A method is presented for the determination of volatile organic compounds in industrial air emissions, based on sampling by means of either active or passive adsorption on active charcoal and silica gel, followed by headspace gas chromatographic analysis. An improvement in the identification and determination of priority pollutants is achieved by coupling the headspace gas chromatograph with a mass spectrometer (ion-trap detector). The detection limits conform to current legal requirements. The method can easily be used for similar applications in other industries.

INTRODUCTION

Most industrial processes generate some waste, both organic and inorganic, often in the form of atmospheric gas emissions. These can include dust and aerosols, gases and vapours. The principal origins of the organic compounds are internal combustion engines and industrial processes. In order to arrive at the ambient air quality defined by the Swiss Ordinance for the Maintenance of Clean Air, both the concentration and the total emission discharge must be within set limits[1]. Control of such limits implies efficient and easily automated analytical methods. Although continuously recorded on-line measurements[2] would bring advantages, the current state of the art is not yet developed and today's sensors, IR and mass spectrometric (MS) monitors do not reflect all the practical needs.

The application of headspace gas chromatography (GC) offers a cost-efficient off-line analysis[3] of volatile organic compounds (VOCs) such as those listed in Table I. A method for the sampling and determination of VOCs in air emissions is described. The method is based on taking samples by means of adsorption samplers, followed by headspace GC coupled with MS for identification.

EXPERIMENTAL

Instrumentation

A Perkin-Elmer Model HS-100 headspace sampler coupled with a Sigma 2000 gas chromatograph and equipped with instruments for flame ionization, electron-

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TABLE I

VOLATILE ORGANIC COMPOUNDS SUITABLE FOR HEADSPACE GC DETERMINATION

Compound	*Boiling point (°C)*	*Compound*	*Boiling point °C*
Ethylene oxide	11.5	Isopropanol	82.4
Diethyl ether	34.6	*n*-Propanol	97.4
Dichloromethane	40.0	Benzene	110.0
Acetone	56.2	Toluene	111.0
Chloroform	61.7	2-Pentanone	116.0
Methanol	64.9	*n*-Butanol	117.0
Tetrahydrofuran	67.0	Tetrachloroethylene	121.0
Hexanes	70.0	*n*-Butyl acetate	127.0
Ethyl acetate	77.0	Chlorobenzene	132.0
Acrylonitrile	77.0	Xylenes	144.4
Ethanol	78.5	*o*-Dichlorobenzene	180.5
2-Butanone	80.0	White spirit	190.0

capture and nitrogen–phosphorus detection was used. A duplicate system was coupled with a heated interface to a mass spectrometer (Finnigan ion-trap detector). The connection was made via a splitter at the end of the chromatographic column.

A J&W Scientific fused-silica capillary column (30 m × 0.32 mm I.D.) with a 1-μm DB-1701 chemically bonded phase was used. After a 6-min isothermal period at 45°C, the temperature was increased from 45 to 120°C at 20°C/min, followed by a holding time of 15 min at 120°C. The detector temperature was 250°C. The carrier gas was helium at a flow-rate of 1 ml/min.

Mass spectra were acquired in the full-scan mode, in the range 25–260 a.m.u., at a scan rate of 1 scan/s. The multiplier voltage was set to 1450 V and the transfer line temperature was 250°C.

PROCEDURES

Charcoal and silica gel samples were loaded into 20-ml headspace vials and, after addition of 1 ml of benzyl alcohol (pro analysi; Merck) and 1 ml of doubly distilled water, respectively, were sealed with caps having aluminium-coated silicone septa. After a thermostating time of 20 min at 80°C for charcoal samples and 70°C for silica gel samples, an aliquot of the gas phase was injected into the chromatographic column (injection time 0.08 min). Blanks of active charcoal and benzyl alcohol and also of silica gel and doubly distilled water were run together with each sample series. Quantitation was effected using an external standard method.

For the calibration, linearity and sensitivity experiments, two standard solutions of mixtures of up to ten components in benzyl alcohol prepared volumetrically in headspace vials, which were filled to the top in order to avoid the presence of any gas phase: in the first solution, each component was present at a concentration of about 80 μg/μl, depending on the density; in the second, the concentration was about 4 μg/μl. A series of dilutions were prepared taking samples of 0.5–10 μl of the standard solutions by means of a Hamilton syringe and injecting them into sealed vials containing the appropriate adsorbent and the corresponding elution solvent. To

avoid flashing in the gas phase, the standard solution was injected directly into the adsorbent–eluent phase. The adsorbent was 150 mg of an active charcoal (NIOSH approved; SKC) or 225 mg of a silica gel (SKC). A minimum of four vials were analysed for each concentration value.

RESULTS AND DISCUSSION

Sampling

Air monitoring involves air sampling and sample preparation followed by the measurement of pollutants. A basic characteristic of most air sampling methods is that a representative portion of the volumetric flow is taken from flue gas and the sample is concentrated outside the stack. Fundamental for the reliability and accuracy of any method are correct sampling and sample-preparation techniques. For our purposes both must be simple, reproducible and precisely defined. The most commonly chosen technique is pollutant enrichment[4,5], either actively or passively, on an adsorbent.

In active adsorption a representative sample is drawn from the chimney flue through an adsorbent tube at a constant rate by means of a pump (Fig. 1). The flow-rate is usually between 0.5 and 10 l/h; normally National Institute for Occupational Safety and Health (NIOSH, Cincinnati, OH, U.S.A.) approved tubes[6] filled with active charcoal, silica gel, Tenax or certain Chromosorbs are used. Using this technique, the individual breakthrough volume[4,7–9] is determined in our laboratory by varying the amount of adsorbent (100–1000 mg) and the exposure time (30 min to 24 h). Additionally for on-site sampling, the eventual breakthrough is checked by connecting two tubes in series. Active adsorption has proved to be especially suitable for short-term measurements.

Passive adsorption samples are taken by means of "diffusion samplers" developed at Sandoz (Fig. 2). They consist of adsorbent-filled glass tubes with a precisely defined diffusion path where gases and vapours are adsorbed solely by free diffusion[4,10]. They are mounted directly to the chimney flue as illustrated in Fig. 2. The sampler design and the type and amount of adsorbent are varied according to the particular needs of the measurements. Two diffusion sampler tubes were tested: 11 cm × 0.7 cm and 5 cm × 0.7 cm I.D. The sampling time was varied between 4 h and 1 week; the best results were obtained with the 11 cm × 0.7 cm I.D. tubes.

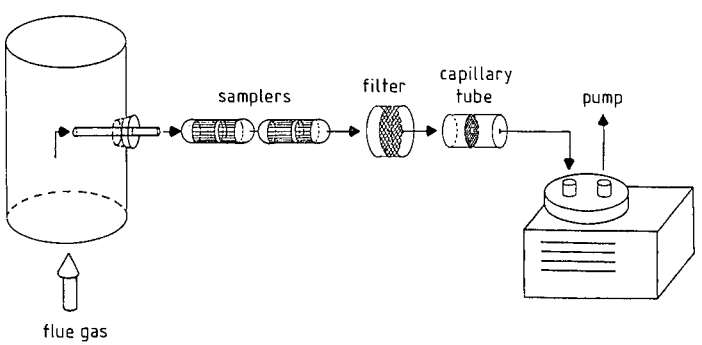

Fig. 1. Active sampling.

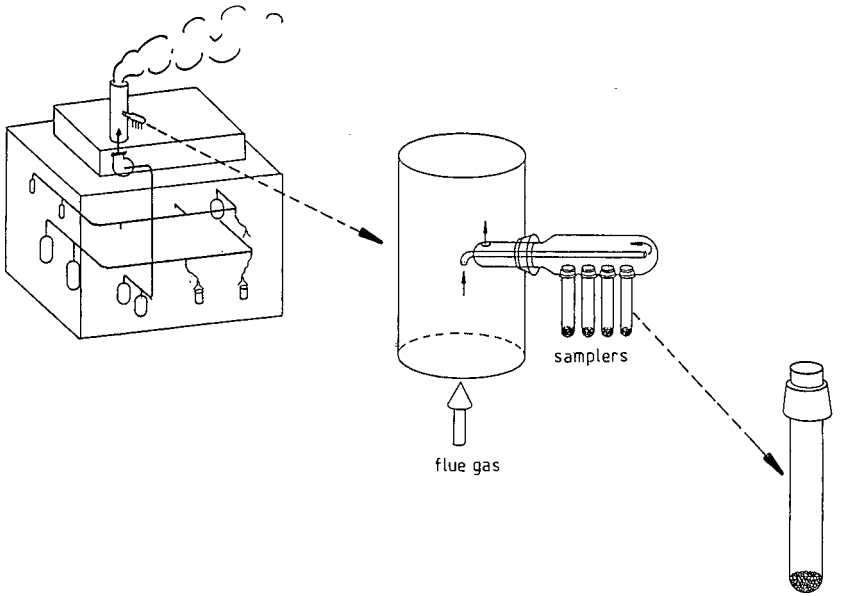

Fig. 2. Passive sampling.

Both active and passive adsorption tests were carried out with dynamic gas mixtures, or with dosing from solution via a syringe. For each compound, concentrations from 4 to 290 mg/m^3 were dosed at 25°C and a relative humidity of 80% and 40%, respectively.

It is virtually impossible to adsorb completely a series of compounds having widely differing physico-chemical properties[4,11] on a single adsorbent, and normally tubes containing active charcoal and tubes containing silica gel are used simultane-

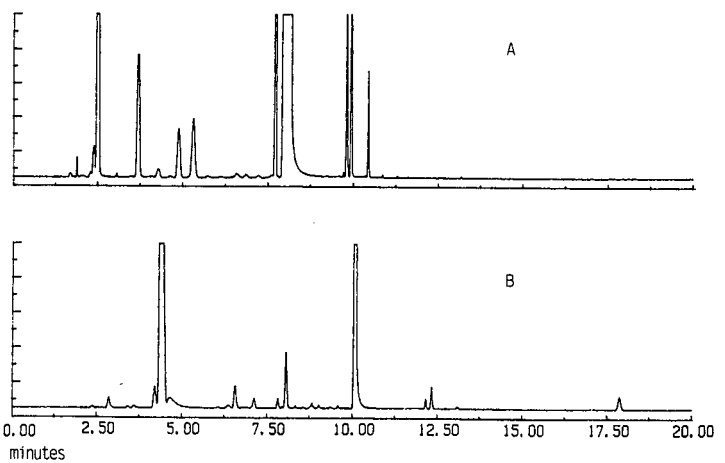

Fig. 3. Headspace gas chromatograms of the same sample showing the differences between (A) silica gel and (B) active charcoal.

ously. Active charcoal has a high affinity for non-polar and silica gel for polar compounds. Both adsorbents have a high adsorption capacity, which is an essential characteristic for sampling where concentrations are relatively high. The striking differences between the adsorption characteristics of charcoal and silica gel for a specific emission are shown in Fig. 3. For headspace elution benzyl alcohol is used for charcoal[3,12] and doubly distilled water for silica gel. Careful prior testing of adsorbents and eluents is necessary.

Headspace gas chromatography

The sample preparation is independent of the adsorption procedure and can be carried out in three ways: solvent elution, thermal desorption or by the headspace technique. The separation and determination of the individual components are performed by chromatography (Fig. 4). Most in the emissions in the chemical industry consist of volatile organic compounds. This is one of the reasons which makes the use of headspace GC attractive, as none or little of the less volatile components reaches the separation column.

In headspace GC the gas phase, ideally in equilibrium with the liquid phase, is analysed. This equilibrium is a function of the matrix, among other factors. If a multiple headspace technique is not applied[3], the influence of the adsorbent, as a major component of the matrix, must also be taken into account. For most substances active charcoal did not influence the GC "response factors"[12], with a few exceptions, *e.g.*, butyl acetate and *o*-dichlorobenzene (Fig. 5). In comparison, the influence of silica gel on the response factors is much more marked. The influence of the adsorbent must also be taken into account for the calibration. Two methods are commonly used: determination of the matrix effect by pre-testing or calibration in the presence of each individual adsorbent; we prefer the latter method.

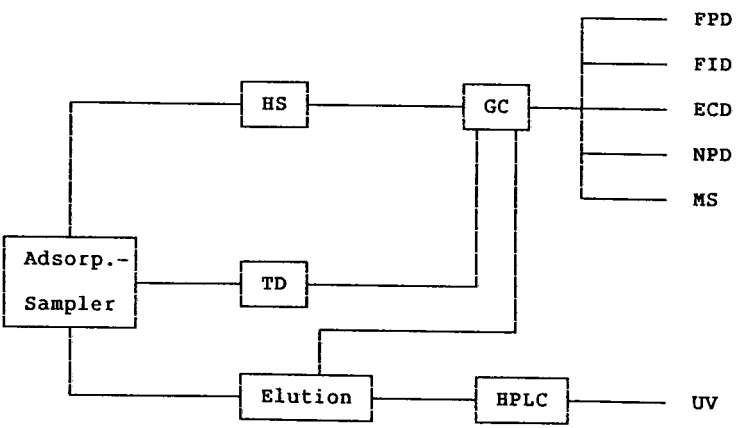

Fig. 4. Analytical methods for an enriched air emission sample. Abbreviations: Adsorp. = adsorption; ECD = electron-capture detection; FID = flame ionization detection; FPD = flame photometric detection; GC = gas chromatography; HPLC = high-performance liquid chromatography; HS = headspace; MS = mass spectrometry; NPD = nitrogen–phosphorus detection; TD = thermal desorption; UV = ultraviolet absorbance detection.

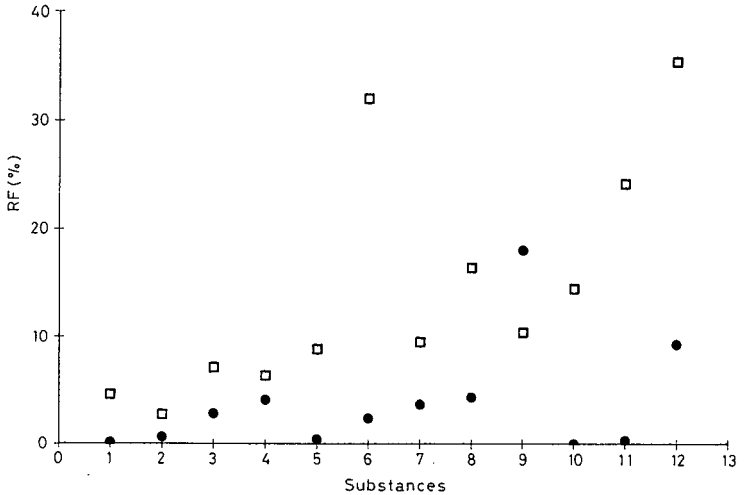

Fig. 5. Influence of the matrix on response factors. Relative difference, $RF(\%) = (RF'' - RF')\,100/RF'$, where RF'' = RF in an adsorbent–eluent matrix and RF' = RF in eluent. □, Silica gel–water; ○, active charcoal–benzyl alcohol. Substances: 1 = methanol; 2 = ethanol; 3 = isopropanol; 4 = *n*-butanol; 5 = acetone; 6 = tetrahydrofuran; 7 = dichloromethane; 8 = tetrachlorethylene; 9 = butyl acetate; 10 = toluene; 11 = xylene; 12 = *o*-dichlorbenzene.

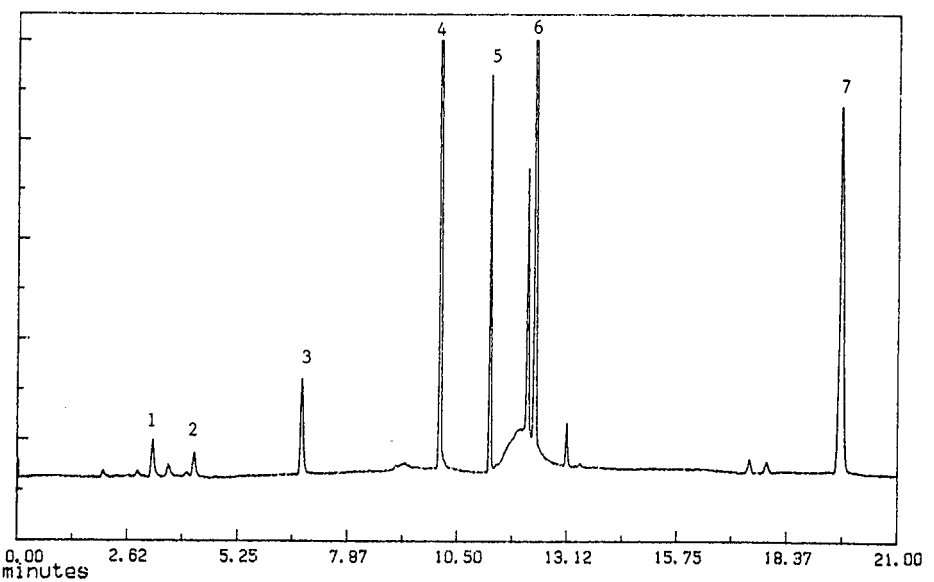

Fig. 6. Headspace gas chromatogram showing the detection of several substances at low concentration. Peaks: 1 = ethanol (1.97 μg/ml); 2 = isopropanol (1.94 μg/ml); 3 = tetrahydrofuran (2.19 μg/ml); 4 = toluene (2.16 μg/ml); 5 = *n*-butyl acetate (2.17 μg/ml); 6 = xylenes (2.14 μg/ml); 7 = *o*-dichlorobenzene (3.24 μg/ml).

TABLE II

LIMITS OF DETECTION OF THE HEADSPACE GC METHOD COMPARED WITH THE LIMITS SET BY THE SWISS CLEAN AIR ACT (1986)

Compound	*Legal limit* (mg/m^3)	*Passive** (mg/m^3)	*Active*** (mg/m^3)
Ethylene oxide	5	0.50	0.12
o-Dichlorobenzene	20	0.60	0.06
Toluene	100	0.45	0.06
Ethanol	150	1.30	0.25

* Passive adsorption: 104 h sampling.
** Active adsorption: 8 h sampling at 1 l/h.

In this comprehensive headspace GC method the linearity range is very large. In general, satisfactory linearity is achieved in the concentration range needed for our emission measurements, which currently lies between 5 and 700 μg/ml. The linearity plots for most of the substances studied show correlation coefficients higher than 0.995.

An example of the signals detected using this method at low concentrations is given in Fig. 6. The signal-to-noise ratio is well above 3:1. The sensitivities achieved are compared with the requirements of ref. 1 in Table II. The use of a universal flame ionization detector, with both active and passive sampling, permits detection limits at least ten times better than those legally prescribed in Switzerland. The passive sampling alone allows determinations, for most compounds, at levels up to 100 times lower than those required by Swiss law. By modifying the sampling conditions, *e.g.*, sampling time, sampling gas flow-rate or sampler design, the sensitivity can easily be increased. The application of specific detectors permits a further lowering of the detection limits for specific substance groups.

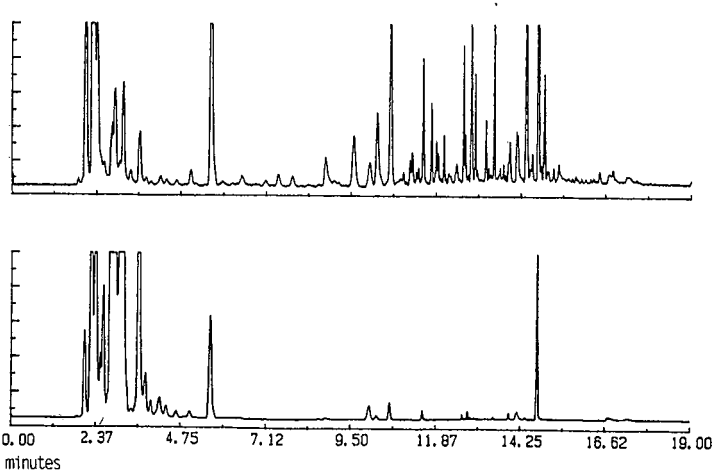

Fig. 7. Headspace gas chromatograms showing results of two consecutive 1 week samplings from a chimney stack.

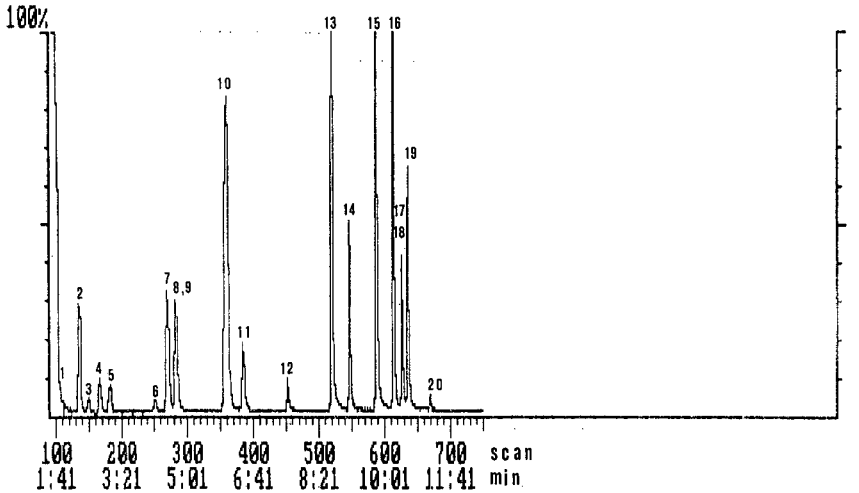

Fig. 8. Mass chromatogram of the gases in the extraction flue of a wastewater collector. Peaks: 1 = methanol; 2 = diethyl ether; 3 = ethanol; 4 = acetone; 5 = dichloromethane; 6 = *n*-propanol; 7 = ethyl acetate; 8 = tetrahydrofuran; 9 = 2-butanone; 10 = isopropyl acetate; 11 = *sec.*-butanol; 12 = *n*-butanol; 13 = toluene; 14 = tetrachloroethylene; 15 = *n*-butyl acetate; 16 = di-*n*-butyl ether; 17 = chlorobenzene; 18 = *p*-xylene; 19 = *o*-xylene; 20 = *m*-xylene.

Coupling of headspace GC with MS

Most of our products are manufactured in batches using multi-purpose plants and the emissions at any individual measuring point can vary considerably. Fig. 7 shows a typical example, both chromatograms being of samples taken from the same flue during two consecutive weeks with diffusive sampling for 1 week in each instance. It is not only the sample composition that can change but also the relative concentrations in a given sampling operation, and these factors complicate both qualitative and quantitative analysis.

Coupling of headspace GC with MS has proved to be of particular value for the identification of components. Fig. 8 shows the mass chromatogram of a sample drawn from the extraction flue of an industrial effluent system, where up to 20 components were identified. In addition to the identification possibilities offered by the ion-trap detector, this particular system coupling allows two-dimensional quantitation, which gives a useful increase in reliability for the evaluation of priority substances.

CONCLUSIONS

The application headspace GC to the determination of organic gases and vapours in emissions has the following advantages:

(1) Sampling can be performed with high-capacity adsorbents. This allows long-term sampling and is also suitable for short-term sampling in the presence of high concentrations.

(2) The substance range is simplified in that only the more volatile components are submitted to chromatographic analysis. The greater part of organic emissions in

chemical industry consists of such compounds which, consequently, have a preferential position in our examination.

(3) The eluents used in headspace GC interfere minimally in the chromatographic analysis.

(4) The sensitivity of the method is well suited to the control requirements of current Swiss law and a sensitivity increase can be achieved without complex modifications.

(5) Headspace GC can be highly automated and, consequently, it can cope with a high sample throughput.

(6) The method presented here, is a commercially viable off-line technique of the measurement of organic pollutants in industrial emissions.

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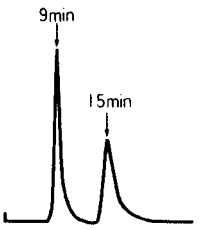

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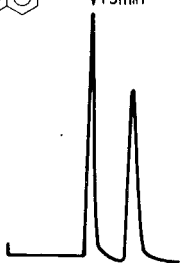

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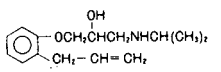

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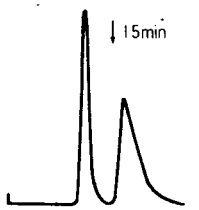

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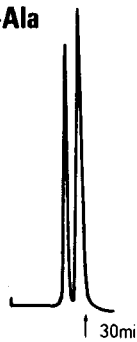

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