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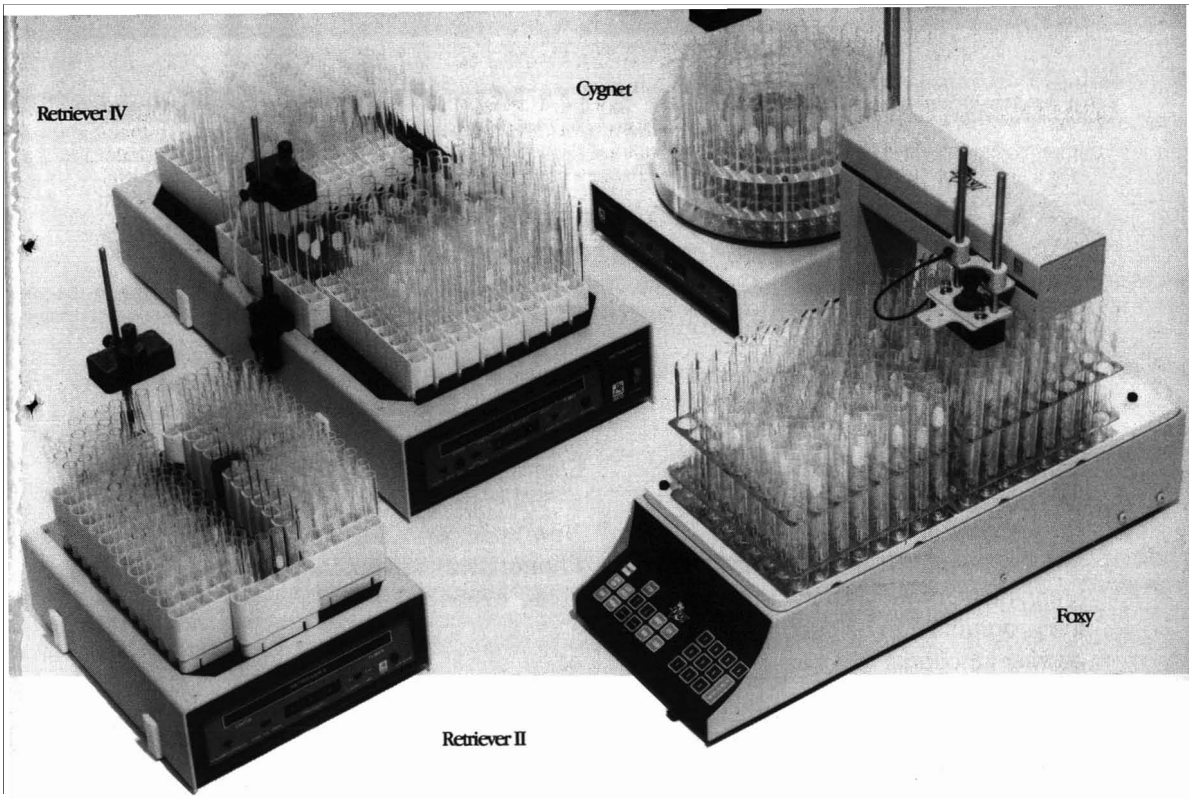

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INTERNATIONAL SYMPOSIUM ON COUPLED COLUMN SEPARATIONS

TECHNIQUES FOR INTEGRATED MULTISTAGE SEPARATION IN LIQUID, GAS AND SUPERCRITICAL FLUID CHROMATOGRAPHY AND ELECTROPHORESIS

Uppsala (Sweden), October 26–27, 1988

Guest Editor

B. D. WESTERLUND

(Uppsala)

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PREFACE

The symposium on Coupled Column Separations in Uppsala, Sweden, October 26 and 27, 1988 attracted approximately 120 participants. The programme consisted of 17 lectures, 13 posters and a concluding discussion session, which was skilfully convened by professor Udo Brinkman from the Free University in Amsterdam. The social events of the symposium included a reception in the main university building and a banquet.

One of the major challenges in modern analytical chemistry is the determination of trace amounts of analytes in complex matrices. In traditional analytical techniques multistep sample work-up procedures are necessary in such applications. However, the accuracy and precision of analytical methods are normally improved by decreasing the number of steps in the procedure. Coupled column separations provide powerful possibilities for facilitating the analyses of complex materials: biological fluids, petroleum products and environmental samples can in many cases be injected directly into coupled column chromatographic systems.

The purpose of the symposium was to highlight advances in the field and recently developed techniques. There was a special emphasis on the possibilities of on-line coupling of different separation principles, such as liquid chromatography–gas chromatography, electrophoresis–liquid chromatography, etc. The first coupled column technique introduced was of the gas chromatography–gas chromatography type. However, most research and application activities today involve liquid chromatography–liquid chromatography techniques. This was reflected at the symposium where 50% of the papers dealt with this methodology. As Dr. Peter Hupe remarked, there are still many combinations to explore: *e.g.*, gas chromatography–supercritical fluid chromatography, gas chromatography–electrophoresis, supercritical fluid chromatography–liquid chromatography, supercritical fluid chromatography–electrophoresis and electrophoresis–electrophoresis.

Evidently, there are many reasons for starting a series of symposia on "Coupled Column Separations", and it is indisputable that such techniques will continue to grow in importance and sophistication in the years to come, and they will certainly be a significant and growing part of the larger general symposia on chromatography in the future. It was my impression that the symposium fulfilled its goals, which was mainly due to the high quality of all the contributions.

Uppsala (Sweden) DOUGLAS WESTERLUND

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APPLICATIONS OF ON-LINE COUPLED LIQUID CHROMATOGRAPHY–GAS CHROMATOGRAPHY

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SUMMARY

On-line coupling of two efficient separation methods, liquid and gas chromatography (LC–GC), is proving to be a very powerful two-dimensional technique. The main reasons for adoption of this coupled method are simplification of sample preparation by minimizing the pre-separation and clean-up steps, efficient elimination of interfering components, better repeatability, improvement of quantitation, reduction of analysis times, more information about the sample components and automation. A survey of LC–GC applications is given.

SAMPLE TRANSFER THROUGH VAPORIZING INJECTION

The first on-line liquid chromatography–gas chromatography (LC–GC) interface based on a GC autosampler injector that was modified with a flow-through side-arm syringe was presented at the 1979 Pittsburgh Conference[1,2] and a paper describing the coupling and some applications was published 1 year later[3]. However, according to subsequent publications (Fig. 1), the method was not accepted even though the interface was commercially available and the technique was automated. Because conventional-size LC columns (4.6 mm I.D.) were used, only a small fraction of the LC peak could be transferred into the vaporizing injector of the GC instrument. The system was mainly suitable for the qualitative analysis of concentrated

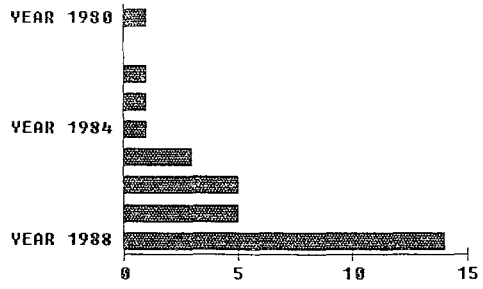

Fig. 1. LC–GC applications according to publication year.

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samples. The technique was applied to the analysis of atrazine in sorghum[3], *n*-alkanes and terpenoids in desert shrubs[4] and hydrocarbon group type in gasoline and diesel fuels[5].

The coupling was further automated in 1987 with pneumatic valves and applied to the analysis of folpet in hop samples. The transferred fractions were still small (2 μl)[6].

In order to reduce the volume of sample liquid introduced into the GC system, a bundled multi-capillary stream splitter placed between the LC and GC instruments has also been used[7,8]. An eluent splitter allows the interfacing of a conventional LC column with a GC system without the need for a retention gap. Multi-capillary splitters permit the simultaneous monitoring of the eluent for accurate zone sampling and transfer. However, the splitters decrease the sensitivity, because only a small volume of the sample is introduced into the GC system. The system was applied to the LC–GC analysis of a coal tar sample and LC–GC–mass spectrometry (MS) of a solvent-refined coal sample, with the three- and four-ring fractions being separated and identified[7–9].

TRANSFER OF THE WHOLE LC FRACTION INTO THE GC SYSTEM

The major problem connected with the direct transfer of an LC fraction into a GC system was the large volume of liquid, until the basis for a technique that allows the introduction of several hundred microlitres of liquid was developed[10,11]. The use of a retention gap pre-column was an important aspect[12].

The whole LC fraction (270 μl, 3 mm I.D. LC column) was directly transferred into a GC system for the first time in 1984[13]. The effluent was pumped through an on-column injector into a long retention gap (50 m) that was coupled to a separation column (30 m). The LC eluent spreads into an uncoated pre-column, which must be at least as long as the zone flooded with the solvent. This conventional retention gap technique was applied to the analysis of azulene in toothpaste. The transfer of the whole LC fraction guarantees more reliable qualitative and quantitative results. After 1984 the number of publications on LC–GC applications started to increase (Fig. 1).

It is possible to keep the volume of the LC effluent below 100 μl by using microbore LC columns and below 50 μl if efficient packed capillary columns are used. The sample capacity might then cause problems, especially with samples that contain large amounts of by-products. Chlorinated benzenes in fuel oil have been determined by on-line LC–GC using a 250-μm I.D. packed fused-silica capillary LC column[14].

Most of the applications of coupled LC–GC have involved normal-phase LC. With reversed-phase LC, problems occur with very polar solvents (methanol and water) that do not wet the surface of the GC columns and that destroy very rapidly the deactivation of the pre-column. In 1985, a packed reversed-phase capillary LC column was used for the analysis of polychlorinated biphenyls by LC–GC[15]. The solvent was pure acetonitrile. Diazepam in urine has also been analysed by reversed-phase micro-LC–GC[16]. The mobile phase was methanol–water (80:20) containing 0.1% H_3PO_4, but the fraction volume was only 2 μl.

In addition to the conventional retention gap technique, partially concurrent solvent evaporation and fully concurrent solvent evaporation techniques have been reported for handling even larger sample volumes transferred from the LC into the

GC system with decreasing solvent evaporation times by using shorter retention gaps and an on-column interface[17]. Partially concurrent solvent evaporation has been applied to the group-type analysis of gasoline[18].

Fully concurrent solvent evaporation involves complete eluent evaporation during the transfer into the GC system. It eliminates restrictions on the volume of LC fractions transferred, but it is suitable only for the analysis of components eluted at relatively high column temperatures.

In 1986, a loop-type interface for concurrent solvent evaporation was introduced. It was applied to the analysis of raspberry ketone in a raspberry sauce[19]. LC–GC coupling via a loop-type interface is very convenient, as the only parameter to be selected is the column temperature during the introduction of the LC fraction. The conventional retention gap technique and also partially concurrent solvent evaporation techniques with an on-column interface need three parameters to be adjusted with respect to each other: carrier gas flow-rate, column temperature and the rate of eluent introduction.

Manual loop-type interfaces have been used in many applications, *e.g.*, in the analysis of wax esters in olive oil[20], polychlorinated biphenyls in fish[21], broxaterol in plasma and urine[22], diisooctyl phthalate in salad oil[23] and dicamba in tobacco[24]. The LC fraction volumes varied between 250 and 1250 μl.

We have used a loop-type interface with a ten-port rotating switching valve that makes it possible to cut two different fractions and analyse them independently. The interface has been modified slightly from our earlier system[25] (Fig. 2). Our coupling method has been applied to the determination of metals as diethyldithiocarbamate (DEDTC) chelates. A mixture of DEDTC chelates of Pd^{II}, Hg^{II}, Cu^{II}, Sn^{II}, Co^{III} and Fe^{III} was injected into the LC system with a silica column (2.1 mm I.D.). The 250-μl fraction containing Cu^{II}, Hg^{II} and Pd^{II} chelates was transferred into the GC system, where all three were successfully separated (Fig. 3). In Fig. 4, Se^{II}, Cu^{II} and Hg^{II} chelates were collected after high-performance liquid chromatographic (HPLC) sep-

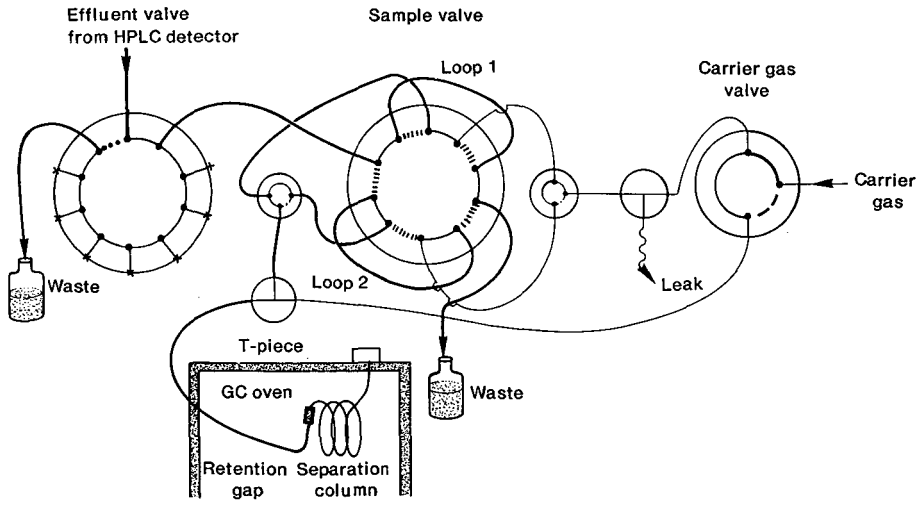

Fig. 2. Schematic diagram of a loop-type ten-port valve LC–GC interface.

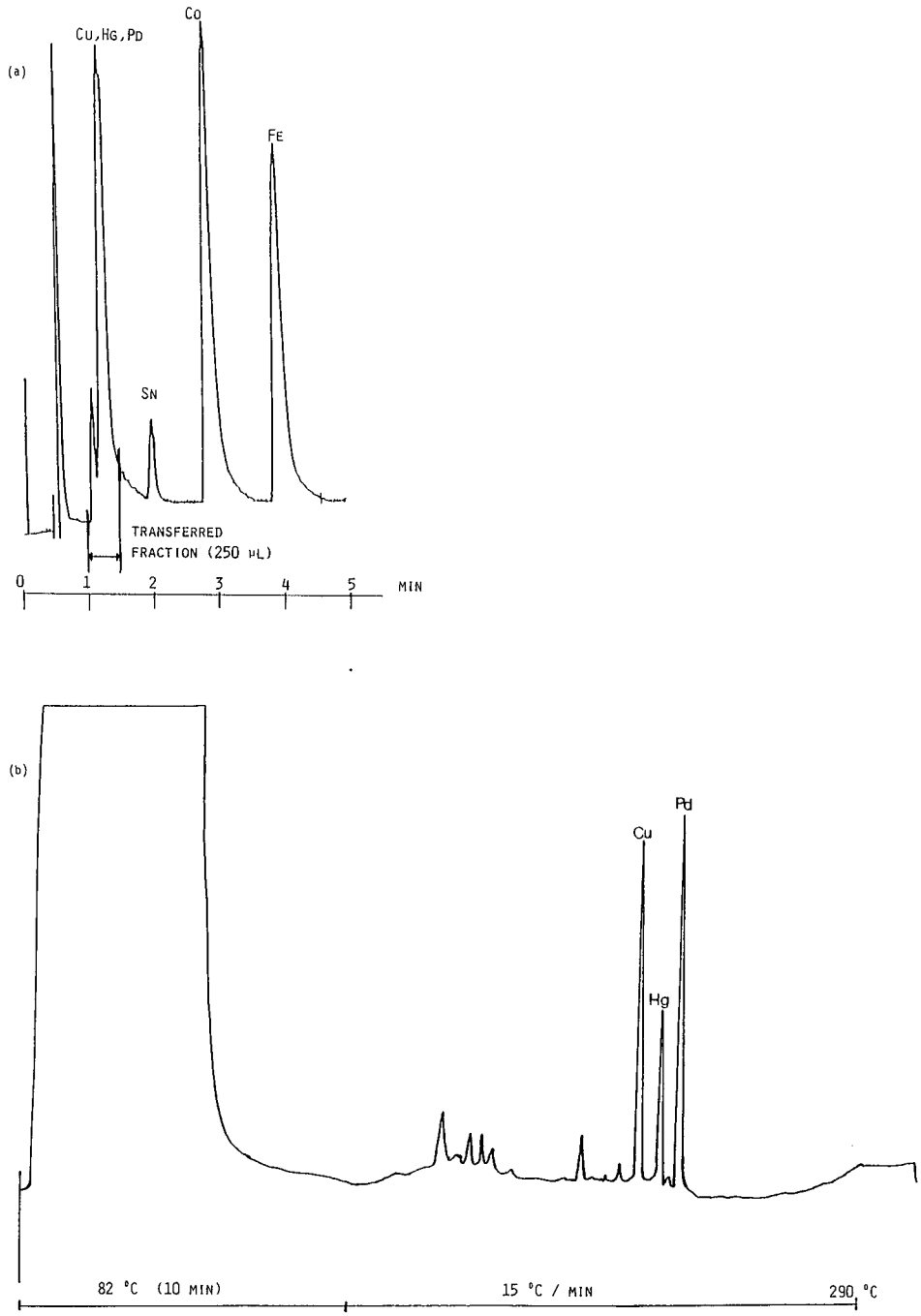

Fig. 3. (a) LC of some metal diethyldithiocarbamate chelates. Hypersil silica (5 μm) column (100 × 2.1 mm I.D.); eluent, hexane–dichloromethane (1:1, v/v). (b) GC of metal chelate fraction [$Cu(DEDTC)_2$, $Hg(DEDTC)_2$ and $Pd(DEDTC)_2$] after on-line LC separation. SE-54 (0.25 μm) column (5 m × 0.32 mm I.D.). Retention gap, 2 m × 0.53 mm I.D.

aration (NH_2-bonded silica, 2.1 mm I.D. column) in the sample loop 1 (400 μl) and Pd^{II} and Co^{III} chelates in loop 2 (250 μl). The metal chelates were separated with good resolution by GC. LC serves as an excellent pre-separation method for metal chelates. The best results were obtained when the sample was flushed into the GC system with additional solvent. The use of coupled LC–GC for the determination of metals has been applied to water samples[26].

Most of the automated on-line LC–GC systems use a loop-type interface. Automated LC–GC has been used for, *e.g.*, the identification of two- to four-ring polycyclic aromatic compounds in diesel fuel. A ten-port valve interface and conventional retention gap technique were employed[27]. Rapid analysis for free sterols, esterified sterols and wax esters in oils and fats has been described[28]. This work was done on a prototype of the automated Carlo Erba LC–GC instrument. The volume of LC fraction transferred was 750 μl (2 mm I.D. silica column). A fully automated LC–GC system was also applied to the determination of free erythrodiol in olive oil. The LC fraction was as large as 850 μl, but the total analysis time was only 25 min[29]. In the last application, a solvent vapour exit was used to decrease the volume of solvent introduced into the GC system.

Recently, an LC–GC interface was described that allows the removal of the LC eluent by an early solvent vapour exit and simultaneous cold trapping of the solutes followed by splitless transfer of the solutes into the GC oven[30]. A pre-column was not used in the GC system. The method was applied to the determination of chlorinated pesticides in water.

From Fig. 1, it is seen that the number of publications on LC–GC applications increased considerably in 1988. Fig. 1 is based on the data in Table I. It can be seen that largest LC fractions are transferred by the loop-type interface. The applications can be grouped roughly into four areas: fuels, foodstuffs, environmental and medical samples. Fuel analyses have mainly been qualitative. Most of the samples analysed using on-line LC–GC were either fuels or foodstuffs. The division of applications according to the interface is not unambigious.

CONCLUSIONS

There are at least three approaches for coupling LC with GC: (1) modification of LC to meet the requirements of GC, that is, the use of microcolumns in LC; (2) modification of GC to "accept" large sample volumes; or (3) reduction of the sample liquid introduced into the GC system by using effluent splitters.

Micro-LC columns, especially packed capillary columns, permit high separation efficiencies. Because of the low flow-rate of the eluent, the LC fraction of interest can be introduced directly into the GC system. The saving in eluent costs is also an important aspect, in addition to short analysis times. The only drawback is the low sample capacity, especially with samples that contain large amounts of by-products. An important role of LC is to minimize clean-up and pre-separation steps before the final GC analysis. For trace analysis, conventional LC columns seem to be the best alternative.

It is possible to introduce large volumes of liquid into the GC system by using either on-column or loop-type interfaces. Long retention gaps used in conjunction with an on-column interface lengthen the analysis times and many parameters must

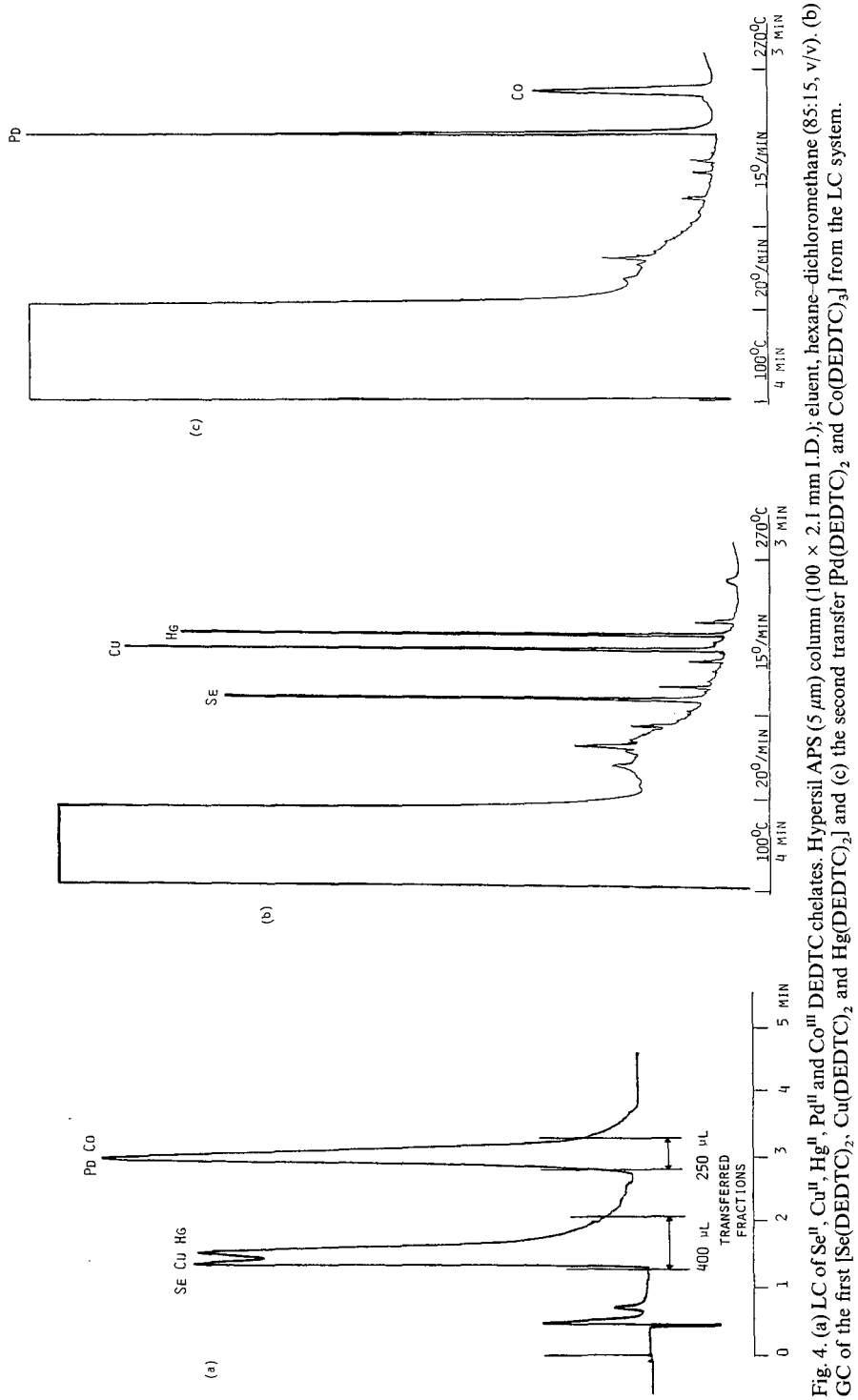

Fig. 4. (a) LC of Se^{II}, Cu^{II}, Hg^{II}, Pd^{II} and Co^{III} DEDTC chelates. Hypersil APS (5 µm) column (100 × 2.1 mm I.D.); eluent, hexane–dichloromethane (85:15, v/v). (b) GC of the first [$Se(DEDTC)_2$, $Cu(DEDTC)_2$ and $Hg(DEDTC)_2$] and (c) the second transfer [$Pd(DEDTC)_2$ and $Co(DEDTC)_3$] from the LC system.

TABLE I

LC–GC APPLICATIONS, VOLUME OF LC ELUENT TRANSFERRED TO THE GC SYSTEM AND THE INNER DIAMETER OF THE LC COLUMN INVOLVED, GROUPED ACCORDING TO THE INTERFACE

System	*Sample analysed*	*Analytes*[a]	*Fraction volume (µl)*	*LC column I.D. (mm)*	*Ref.*
Automated LC–GC	Sorghum	Atrazine	8	4.0	3
	Desert shrub	*n*-Alkanes and terpenoids	9	4.6	4
	Gasoline and diesel fuel	Chemical classes	2	4.6	5
	Hop	Folpet	2	4.6	6
	Coal liquids	Chemical classes	0.1	–	7, 8
	Diesel exhaust particulates	PACs	150	1.0	31
	Turkish lignite	Chemical classes	150	1.0	32
	Kerosine and diesel fuel	Chemical classes	150	1.0	33
	Diesel fuel	PACs	150	1.0	27
	Urine	Heroin metabolites	500	2.0	34
	Oil, fat	Sterols	750	2.0	28
	Olive oil	Erythrodiol	850	3.0	29
On-column interface	Toothpaste	Azulene	270	3.0	13
	Petroleum	Chemical classes	100	2.0	18
	Bovine urine	Diethylstilbestrol	–	3.0	35
	Sediment	PCBs	180	0.7, 1.1	36
	Gasoline	PACs	2–3	0.32	37
	Urine	Diazepam	1–3	0.32	16
	Aqueous samples	Chlorinated pesticides and PCBs	100	1.1	38
Loop-type interface	Raspberry sauce	Raspberry ketone	450	2.0	19
	Fuel oil	Chlorinated benzenes	22	0.25	14
	Coal tar	PCBs	40	0.1, 0.3	15
	Olive oil	Wax esters	450	2.0	20
	Fish	PCBs	400	3.0	21
	Plasma and urine	Broxaterol	500	3.9	22
	Triglyceride	Di(2-ethylhexyl)phthalate	1200	4.6	23
	Dicamba	Tobacco	250	2.0	24
	Plasma	I-Moprolol β-blocker	500	4.0	39
Others	Coal	Aromatics	10	1.0	7–9
	Aqueous samples	Chlorinated pesticides	–	–	30

[a] PACs = polycyclic aromatic compounds; PCBs = polychlorinated biphenyls.

be adjusted with conventional retention gap and partially concurrent solvent evaporation techniques. A loop-type interface is fairly simple and easily automated for both conventional retention gap and concurrent solvent evaporation techniques. For the latter, only one parameter, the temperature of the oven during the introduction of the sample, needs to be adjusted. For eliminating contamination, very pure solvents are necessary and flushing of the sample loop with solvent may be needed. Loop-type interfaces are applied in the analysis of compounds eluted more than about 50°C

above the column temperature during sample introduction, but by using co-solvent trapping early peaks can also be analysed[40].

The introduction of the whole LC fraction into the GC system gives the most reliable results. Removal of LC solvent vapour by a vapour exit accelerates transfer and also minimizes problems that large sample volumes might cause for GC detectors.

The applications can be grouped into four areas; fuels, foodstuffs, environmental and medical samples. Polymers seem to be a new area for LC–(pyrolysis) GC[41]. Most of the applications have been qualitative and judgements regarding the significance of the results (precision, accuracy, repeatability, reproducibility, detection limit, determination limit, etc.) have not been common.

The coupling of reversed-phase LC with GC needs further development, although many useful attempts have been carried out.

The use of diode-array detectors in LC would improve the reliability of cutting LC fractions and LC–GC–MS, LC–GC–Fourier transform IR spectrometry and LC–multi-dimensional GC would provide more information about sample components.

It is evident that full automation of on-line LC–GC improves the repeatability of the analyses. An automated system is fast and easy to use. The availability of fully automated commercial instruments is probably the best way to increase the potential of this two-dimensional chromatographic system and to widen the areas of application.

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AUTOMATED LIQUID CHROMATOGRAPHIC ANALYSIS OF DRUGS IN URINE BY ON-LINE SAMPLE CLEANUP AND ISOCRATIC MULTI-COLUMN SEPARATION

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SUMMARY

A multi-column system has been developed for automated analysis of basic drugs in urine. Two polymeric pre-columns, containing PRP-1 and Aminex A-28, were used to isolate the drugs. A short reversed-phase column, coupled to a 150 × 4.6 mm I.D. silica column, produced the analytical separation. Sample preparation consisted of dilution and centrifugation. The entire procedure required less than 30 min. Careful optimization of mobile phase conditions led to retention of benzoylecgonine and barbiturates. For most drugs, levels of 0.3 mg/l were sufficient to produce peaks that could be matched against stored spectra with a computerized library search program.

INTRODUCTION

Analysis of drugs in biological fluids by liquid chromatography (LC) has been automated by a variety of techniques for on-line sample processing (OSP)[1,2]. The use of a short pre-column to separate the analytes from proteins and salts has been explored for over ten years[3,4]. Many current approaches and applications were recently reviewed[5,6]. Other useful approaches include zone electrophoretic sample treatment[7] and dialysis[8]. Furthermore, direct analysis of biological fluids is possible by the use of columns with hydrophilic surfaces[9,10] or of a micellar mobile phase that solubilizes proteins[11]. A device for the complete automation of sample handling for LC was recently described[12].

Many published applications of OSP have been restricted to a single drug and its metabolites. This undoubtedly reflects the widespread use of the technique for pharmacokinetic and bioavailability studies of new drugs. In some cases, drugs with similar chemical structures have been analyzed simultaneously. Examples include the analysis of benzodiazepines[13], amphetamines[14], barbiturates[15], and tricyclic antidepressants[16,17].

A number of difficulties have prevented the use of OSP for screening multiple classes of drugs in a single procedure. First, conditions for adsorption and desorption of drugs from a pre-column require careful optimization. Different strategies are appropriate for hydrophilic, moderately hydrophobic, and very hydrophobic drugs[18]. Second, techniques for broad-spectrum LC analysis of drugs have been reported in the

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literature[19,20] but have not been widely used. This reflects the success of gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS), which have gained more prominence for drug screening. Also, there is little qualitative information available from LC with detection at a single wavelength, as it is currently performed in most toxicological laboratories.

Recently, there have been several reports of LC screening techniques for toxicology where off-line sample preparation was used. Search programs were developed to analyze spectra collected with a diode-array detector. In order to determine acidic, neutral, and basic drugs in a single analysis, Demorest *et al.*[21] used an acetonitrile gradient at pH 2.1. Hill and Lagner[22] used two different gradients and two different columns for separate analyses of acids and bases. Isocratic methods for screening a limited group of hydrophobic drugs were reported by Minder *et al.*[23] and Jinno *et al.*[24].

In general, isocratic analysis would be preferable for full-scan UV detection[25]. The background contributed by the mobile phase would then not change during chromatography, and spectral libraries could be collected without reference to background spectra at a given retention time. This is particularly important at trace levels. However, isocratic reversed-phase chromatographic retention of multiple drug classes is difficult to achieve. In particular, amphetamines and opiates are very hydrophilic and are not readily analyzed under the conditions appropriate for benzodiazepines and tricyclic antidepressants.

Cation-exchange chromatography permits isocratic analysis of drugs under conditions where hydrophobic interactions are minimized. Jane[26] demonstated the use of underivatized silica, a weak cation exchanger, for drug analysis in 1975. Three different types of eluents have been reported: methanol containing ammonia[26–28], a completely aqueous mobile phase at a low pH[29], or mixtures of aqueous buffers with organic solvents[30–33]. In the latter two cases, a basic amine modifier was added to improve peak symmetry. The published methods are best suited for the analysis of strongly basic drugs; weak bases, like the benzodiazepines, show little retention on silica, even at basic pH[26]. Comparisons of the performance of underivatized silica to hydrophobic bonded silica have been published[34,35], and the optimization of mobile phase components has been investigated[16,35–37]. The interlaboratory reproducibility of retention times on underivatized silica has been reported[38].

Coupled-column chromatography is a useful isocratic alternative to solvent gradients, especially when repetitive analysis is required[39]. The versatility of underivatized silica may be extended by combination with a column displaying a different selectivity. Complex pharmaceutical mixtures have been analyzed by using column switching techniques and combinations of silica and reversed-phase packings[40]. Other investigators have coupled cation-exchange and reversed-phase columns. The combination of a 250 × 4.6 mm I.D. strong-cation-exchange column, coupled with a 50 × 4.6 mm I.D. reversed-phase column for drug analysis has been reported[41]. Illicit heroin samples were analyzed with a 45 × 2.1 mm I.D. C_{18} column, coupled with a 250 × 4.6 mm I.D. alumina column[42].

We have developed a multi-column system for the analysis of drugs in urine. Primary sample clean-up is performed with a 10 × 2.1 mm I.D. PRP-1 cartridge. A 10 × 3.2 mm I.D. anion-exchange column is used to selectively retain hydrophobic neutral and acidic drugs. Under carefully optimized conditions, barbiturates are

slightly retained by the anion-exchange column. Finally, coupled reversed-phase (25 × 3.2 mm I.D.) and silica (150 × 4.6 mm I.D.) columns permit the separation of barbiturates, benzodiazepines, amphetamines, tricyclic antidepressants, and opiates. Column switching is employed so that only a small eluate volume from the first two columns reaches the final two columns. A diode-array detector is utilized for monitoring and identification of the eluted peaks.

EXPERIMENTAL

Apparatus

The apparatus was constructed from two high-pressure pumps, three high-pressure switching valves, one solvent selection valve, two pre-columns, two analytical columns, a heater, an automatic sampler, a UV detector and a system controller, as shown in Fig. 1.

A Bio-Rad (Richmond, CA, U.S.A.) Model 402 gradient controller with an ACER 710 personal computer controlled the functions of pump A (Model 1330 isocratic pump, Bio-Rad), the 8-port high-pressure valve (Valco, Houston, TX, U.S.A.) and two 4-port high-pressure valves (Valco). The controller initiated sampling via a signal to the Model AS-48 automatic sampler (Bio-Rad), equipped with a 500-μl sample loop. When the filled injection loop was rotated into the flow path, the

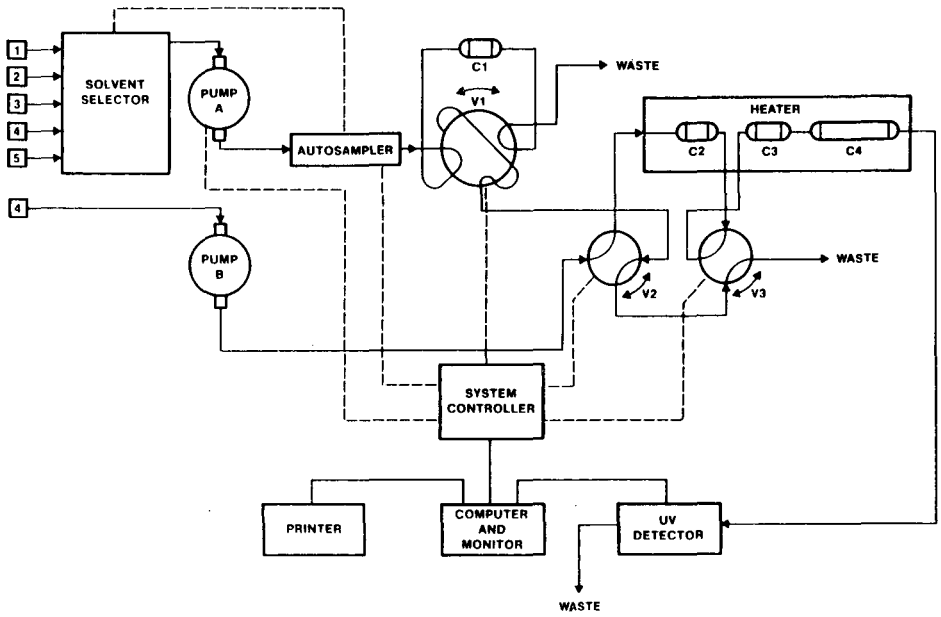

Fig. 1. Experimental set-up for on-line analysis of basic drugs in urine according to the final procedure described in the text. V1 = 8-port switching valve; V2, V3 = 4-port switching valves; C1 = 10 × 2.1 mm I.D. PRP-1 column, 16 μm; C2 = 10 × 3.2 mm I.D. Aminex A-28 column, 11 μm; C3 = 25 × 3.2 mm I.D. C_8 column, 5 μm; C4 = 150 × 4.6 mm I.D. silica column, 5 μm; 1 = 0.1% potassium borate buffer (pH 8.0) (buffer A); 2 = 6 m*M* KH_2PO_4, 5 m*M* tetramethylammonium hydroxide, 2 m*M* dimethyloctylamine, adjusted to pH 6.50 with phosphoric acid (buffer B); 3 = 60% buffer B, 40% acetonitrile; 4 = 67% buffer B, 33% acetonitrile; 5 = 30% buffer B, 70% acetonitrile.

automatic sampler signalled back to the controller and at the same time sent a signal to the solvent selector (FIATRON, Oconomonoc, WI, U.S.A.). These signals initiated the valve sequence and solvent selection sequence. A second Model 1330 isocratic pump ran at a constant flow-rate, independent of the controller. The first column was operated at ambient temperature, the other three were maintained at 40°C in a column heater (Bio-Rad). The chromatograms were routinely monitored at 210 nm and 235 nm with a Model 1040A diode-array detector (Hewlett-Packard, Avondale, PA, U.S.A.), which included a Model 85B microprocessor. Chromatograms generated at 210 nm were stored by the system controller. A Model 3392A Integrator (Hewlett-Packard) monitored the signal at 235 nm and performed quantitation at either wavelength, as required. For identification of drug spectra, a commercially available toxicology program (Library Search/HP 1040A, Central Pathology Laboratory, Santa Rosa, CA, U.S.A.) was used. A Model E Microfuge (Beckman, Fullerton, CA, U.S.A.) was employed in sample preparation.

Stationary phases and columns

Initial urine purification and drug concentration was performed with a 10 × 2.1 mm I.D. stainless-steel pre-column, packed with PRP-1, a spherical 12–20 μm poly(styrene–divinylbenzene) co-polymer (Hamilton, Reno, NV, U.S.A.). Further purification was performed with a 10 × 3.2 mm I.D. stainless-steel pre-column, packed with Aminex A-28 11-μm resin (Bio-Rad). Both pre-column cartridges were slurry-packed at 5000 p.s.i. and were held in Brownlee cartridge holders (Rainin, Emerville, CA, U.S.A.). The 25 × 3.2 mm I.D. reversed-phase cartridge contained 5-μm octylsilica (Phenomenex, Rancho Palos Verde, CA, U.S.A.) and was slurry-packed at 5000 p.s.i. The 150 × 4.6 mm I.D. column contained 5-μm spherical silica, 50-Å pore size (Machery-Nagel, Duren, F.R.G.) and was slurry-packed at 7500 p.s.i. A small cartridge containing ACT-1, a C_{18}-derivatized PRP column (Interaction Chemicals, Mountain View, CA, U.S.A.) and a small reversed-phase cartridge (Bio-Rad) were used in comparison studies.

Chemicals

HPLC-grade acetonitrile was obtained from Alltech Assoc. (Los Altos, CA, U.S.A.); HPLC-grade potassium dihydrogenphosphate was from Fisher (Santa Clara, CA, U.S.A.); tetramethylammonium (TMA) chloride and hydroxide were obtained from Fluka (Ronkonkoma, NY, U.S.A.), and N,N-dimethyloctylamine was from Aldrich (Milwaukee, WI, U.S.A.). All other laboratory chemicals were of analytical grade. Water was purified using an in-house ion-exchange system and was equivalent to HPLC grade. Drugs and organic acids were obtained from Alltech (State College, PA, U.S.A.) or Sigma (St. Louis, MO, U.S.A.). Other drugs were gifts from their respective manufacturers.

N-Ethylnordiazepam (7-chloro-1,3-dihydro-1-ethyl-5-phenyl-2H-1, 4-benzodiazepin-2-one) was synthesized in-house by N-ethylation of nordiazepam with ethyl iodide. It was recrystallized from methanol and showed a single peak when analyzed by reversed-phase chromatography under conditions where it is fully resolved from nordiazepam[43].

RESULTS AND DISCUSSION

Initial urine purification

The simultaneous extraction of drugs with varying charges and polarities from urine requires a very hydrophobic packing. XAD-2 resin has been employed for this purpose in an open-column mode for many years[44]. PRP-1 is a highly cross-linked polymer of identical chemical composition. The utility of this packing for extraction of methaqualone in serum was reported by Hux *et al.*[45]. The versatility of PRP-1 is illustrated by published procedures which demonstrate the direct extraction of both barbiturates and amphetamines from urine[14,15].

Our investigations verified the retention on PRP-1 of basic, neutral, and weakly acidic drugs from urine, buffered to the pH range 7.5–9.5. In particular, hydrophilic drugs, such as benzoylecgonine and ethchlorvynol, were retained under these conditions. When reversed-phase cartridges were evaluated under identical conditions, these hydrophilic drugs were not retained, and no single pH could be determined where both barbiturates and amphetamines were extracted from urine. The breakthrough volumes of all drugs in pH 8.0 buffer was sufficiently large, so that the flow through the PRP-1 column could be reversed without any losses. This permitted rinsing of the front end of the column, where particulates accumulate. The final wash volume was determined by the length of time required to rinse weakly retained urine components from the column, as determined by direct observation of the baseline at 210 nm.

The PRP-1 cartridge was very tolerant of biological specimens under the conditions employed. Fouling of the column by urine samples (which had been previously centrifuged at 11 000 *g*) was never observed. Experiments performed with pre-buffered and centrifuged serum indicated that at least 50 ml of serum could be injected before a substantial increase in backpressure occurred.

Elution from the first pre-column

The elution of drugs from the PRP-1 pre-column was attempted, using three different approaches: pH changes, addition of competing ions, or addition of organic solvents. Combinations of these methods were also evaluated.

A change in pH alone was sufficient to elute amphetamines and benzoylecgonine. Benzodiazepines and tricyclic antidepressants were not eluted, even at a very low pH, unless an organic modifier was added. However, low pH was not compatible with the use of a silica column as a cation-exchanger. Small amounts of the acid would inevitably reach that column, causing a reduction in the number of ionized silanol sites and a dramatic loss of retention. Elution at pH 6.5 required a higher concentration of acetonitrile than elution under acidic conditions.

Although amphetamines and other hydrophilic drugs were eluted from the PRP-1 cartridge in volumes below 100 μl, the hydrophobic drugs were eluted much more slowly; elution volumes for amitriptyline and diazepam were closer to 500 μl. Furthermore, elution of these drugs typically began after the hydrophilic drugs had been released. Since it was unlikely that the compounds had penetrated the column in the forward-flow mode, it would appear that the interaction with the packing was particularly strong. Experiments with reversed-phase cartridges of similar dimensions yielded peak widths of the eluted drugs that were only half as large. Cartridges packed

with ACT-1, a poly(styrene–divinylbenzene) co-polymer which contains covalently bonded octadecyl groups, yielded 2- to 3-fold increases in the elution volumes for diazepam and amitriptyline. PRP-1 cartridges of different length and inner diameter yielded nearly identical elution volumes.

Addition of basic competing agents to increase the efficiency of elution for hydrophobic drugs was also evaluated. Dimethyloctylamine concentrations (0.005 *M*) and tetramethylammonium chloride (0.4 *M*) led to no significant changes in the width of eluted peaks.

Because the two analytical columns contain silica and would be adversely affected by basic buffers, the preliminary replacement of borate buffer with pH 6.5 phosphate buffer was studied. The dilute borate buffer was displaced with 250 μl of 6 m*M* buffer (containing the competing bases). Larger volumes led to the elution of amphetamines. This buffer replacement step had a significant effect on the lifetime of the silica columns. In the absence of any wash step, a large void would sometimes form at the front end of the silica column after less than 50 injections. With the displacement step, this column could be used for over 200 injections. The buffer replacement step also had a favorable effect on the peak shapes of the hydrophobic drugs. These compounds were concentrated at the head of the subsequent column. A stepwise-gradient was produced by solvent switching; drugs were transferred from the rear of the PRP-1 column to the front of the anion-exchange column at the reduced acetonitrile concentration and were briefly retained there.

Further purification of the urine extract on an anion-exchange column

Although most carboxylic acids are completely ionized at pH 8.0, many organic acids were retained on the first precolumn after the initial clean-up. We previously studied the hydrophobicity of many endogeneous phenolic and indolic acids[46]. Under the conditions employed here for clean-up, hippuric acid was not retained at all; indolepropionic acid was retained in the initial (forward) wash but was slowly washed off the column in the reverse wash. Acidic drugs, such as ibuprofen and indomethacin, were fully retained by PRP-1.

The use of a second pre-column, containing anion-exchange resin was therefore investigated. A polymeric material was most appropriate because of its high capacity and pH stability. Initial experiments with pre-packaged 30 × 4.6 mm I.D. Aminex A-27 cartridges (Bio-Rad) demonstrated extensive retention of endogenous and exogenous carboxylic acids. (Benzoylecgonine contains a carboxyl group, but is amphoteric; at pH 6.5 it displays weakly basic behavior.) However, barbiturates were also retained, along with oxazepam and several other benzodiazepines. To minimize this retention, smaller columns (10 × 3.2 mm I.D.) were prepared; also, a smaller particle size was employed (Aminex A-28; 11 ± 2 μm) to improve the peak shape of the weakly retained drugs. Attempts to pack narrower columns were not successful due to swelling of the resin in the presence of organic solvents.

When barbiturates were eluted from the A-28 column alone under isocratic conditions, the retention times were shorter than those observed following transfer from the PRP-1 column. This is due to the gradient which is generated on the A-28 column (Table I). In the absence of solvent, none of the acids are eluted when buffers with low ionic strength are employed. The addition of organic solvents helps to elute the barbiturates, but at intermediate solvent concentrations (*e.g.* 20%) phenobarbital

TABLE I

RETENTION DATA (k') FOR SELECTED COMPOUNDS ON A 10 × 3.2 mm I.D. AMINEX A-28 COLUMN

Eluent A = 6 m*M* KH_2PO_4, 5 m*M* tetramethylammonium chloride, 2 m*M* dimethyloctylamine (pH 6.50); eluent B = acetonitrile. Flow-rate, 1.0 ml/min; 40°C. The total void volume was 0.18 ml, as measured by injection of 50% aqueous methanol. φ is the phase ratio (B/A + B).

Compound	φ						
	0.1	*0.2*	*0.3*	*0.4*	*0.5*	*0.6*	*0.7*
Indolepropionic acid	>50	>50	>50	>50	39.1	26.6	17.9
Indomethacin	>50	>50	>50	41.5	31.6	20.7	11.0
Ibuprofen	>50	>50	>50	35.0	29.4	13.1	6.8
Phenobarbital	22.4	10.0	5.0	2.7	0.9	1.0	1.0
Secobarbital	10.5	4.6	1.8	0.8	0.5	0.4	0.3
Oxazepam	25.5	6.8	2.3	1.0	0.8	0.5	0.2
Nordiazepam	35.7	7.7	2.0	0.9	0.7	0.4	0.3
Diazepam	20.2	4.2	1.2	0.5	0.6	0.2	0.3
Imipramine	0.9	0.6	0.1	0.1	0.1	0.1	0.1
Amphetamine	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Caffeine	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Morphine	0.1	0.1	0.1	0.1	0.1	0.1	0.1

(pK_a 7.2) is released more slowly than secobarbital (pK_a 7.9). Furthermore, TMA in the mobile phase competed with the resin under conditions where partition occurred; an increase in the TMA concentration speeded elution of barbiturates. It was important to optimize anion-exchange conditions in order to minimize the contribution of endogenous acids to the background absorbance at 210 nm in the final chromatograms.

Coupled analytical columns

Silica columns have been used for drug screening in both basic and neutral solutions. Extended column stability at high pH was probably due to the very low concentration (less than 10%) of water in the mobile phase. Under conditions where the mobile phase would be useful for coupled-column chromatography with reversed-phase columns, the water content would be greater than 50%. Therefore, neutral eluents are more suitable for coupled-column analysis.

Mobile phase conditions appropriate for the separation of benzodiazepines on reversed-phase columns typically lead to lengthy retention of the more hydrophobic tricyclic antidepressants and phenothiazines. The addition of a competing base to the mobile phase[47] minimizes the difference in retention. This approach is successful because a significant proportion of retention is due to ionic interaction with the negatively charged silanol groups remaining on the bonded surface.

We recently demonstrated the use of N,N-dimethyloctylamine to reduce effectively the retention of tricyclic antidepressants on a reversed-phase column[43]. Therapeutically important benzodiazepines and tricyclic antidepressants were analyzed in 30% acetonitrile (pH 6.4) on a 100 × 2.1 mm I.D. 3-μm octylsilica column. For the screening procedure described here, reduction of column dimensions to give

minimum acceptable resolution of benzodiazepines was desirable. Resolution of chlordiazepoxide and its metabolite, norchlordiazepoxide, and of diazepam and its metabolites, nordiazepam and oxazepam, was evaluated. Analysis of the second group was complicated by the slight retention of oxazepam and nordiazepam on the A-28 column. A 25 × 3.2 mm I.D. reversed-phase column and 33% acetonitrile yielded acceptable resolution ($0.5 < R < 1.0$). With longer columns amitriptyline and morphine or other opiates were unresolved.

Quaternary amines were reported to improve the peak shapes of basic drugs, chromatographed on silica[30,31]. Furthermore, the total analysis time is responsive to both the concentration of quaternary amines and the cations in the buffer. Alkylamines also lead to improved peak shapes on bare silica. In agreement with earlier observations[37], we found that tetramethyl- and tetraethylammonium salts were not appreciably adsorbed on silica, and both improved peak shapes when present in small amounts. Ammonium buffers also improved peak shapes and appeared to speed preferentially the elution of primary amines.

In Table II the elution order of the coupled column system is compared to results obtained for the silica column alone. Some results are also shown from the extensive toxicological survey reported by Jane *et al.*[28], who employed a 125 × 4.9 mm I.D. Spherisorb 5-SW column; the mobile phase was methanolic ammonium perchlorate (10 m*M*, pH 6.7). It may be seen that the retention time and the elution order for silica with 33% acetonitrile is quite similar to that in the earlier study in which 90% methanol was used. Apparently, cation exchange is the primary retention mechanism under both conditions. Addition of the short reversed-phase cartridge resulted in slightly increased retention of benzodiazepines and permitted increased differentiation within this class. The acetonitrile concentration chosen for the analytical separation (33%) was lower than the optimal concentration for elution from PRP-1 at pH 6.5 (40%). However, a small amount of the higher concentration could be tolerated by the reversed-phase column without measurable effect on the reproducibility of retention times; this volume was experimentally determined to be 0.2 ml. The combination of this step with the borate buffer replacement described above led to compression of hydrophobic drugs on the anion-exchange column and improved peak shapes in the final separation.

The silica column must offer sufficient retention to assure elution of amphetamines after benzodiazepines (which are substantially retained on the reversed-phase column). Retention on underivatized silica is proportional to the surface area of the packing[48,49]. A 50-Å 5-μm packing with a surface area of 450 m^2/g produced sufficient retention in a 150 × 4.6 mm I.D. column to achieve the desired selectivity.

The coupled analytical columns and the anion-exchange column were maintained at 40°C to increase the reproducibility of retention times. This also improved peak shapes and lowered the backpressure.

Regeneration of the pre-columns

The elution of the PRP-1 column in the back-flush mode led to the release of most hydrophobic drugs, *e.g.*, buclizine, butaclamol, and emetine. Cyclosporine was the only one of the compounds evaluated that was retained. Experiments with drug-free serum revealed a significant peak not seen in urine. It was eluted at

TABLE II

RETENTION DATA (k') FOR DRUGS IN SINGLE- AND MULTI-COLUMN SEPARATIONS

Compound	*Complete system*[a]	*Coupled analytical columns*[b]	*Silica alone*[c]	*Literature*[d]
Caffeine	0.7	0.5	0.6	0.2
Cotinine	0.9	0.8	0.7	0.2
Benzoylecgonine	1.0	0.6	0.9	0.9
Secobarbital	1.0	0.6	0.4	N.A.
Oxazepam	1.1	0.6	0.5	N.A.
Phenobarbital	1.1	0.5	0.4	N.A.
Nordiazepam	1.3	0.8	0.5	0.2
Diazepam	1.6	1.0	0.5	0.1
N-Ethylnordiazepam	2.1	1.3	0.5	N.A.
Phenylpropanolamine	2.2	2.2	2.3	0.9
Phentermine	2.4	2.8	2.9	0.6
Amphetamine	2.5	2.5	2.5	0.9
Phenmetrazine	2.7	2.7	3.0	1.7
Lidocaine	2.7	2.4	2.5	0.6
Ephedrine	2.7	2.8	2.4	1.0
Pentazocine	2.8	3.4	3.5	1.8
Methamphetamine	3.1	3.1	3.1	2.1
Desipramine	3.1	3.1	3.1	2.1
Nortriptyline	3.3	3.0	3.0	2.0
Diphenhydramine	3.4	3.2	3.5	3.3
Methadone	3.8	3.4	3.4	2.2
Methadone metabolite[e]	4.1	4.1	4.2	2.8
Imipramine	4.2	3.7	3.7	4.2
Flurazepam	4.2	3.8	4.0	1.3
Amitriptyline	4.3	3.6	3.4	3.3
Morphine	5.0	5.1	5.7	3.8
Codeine	5.7	5.6	6.4	4.8
Chlorpheniramine	5.9	5.7	6.4	3.9
Hydromorphone	7.0	6.9	7.6	7.9
Hydrocodone	8.0	7.8	9.1	7.1

[a] Analysis in fully automated four-column system. Void volume, 1.9 ml; "injection point", time when fastest drugs are eluted from column 1.

[b] Isocratic analysis on reversed-phase and silica columns only. Void volume, 1.7 ml.

[c] Isocratic analysis on silica column only. Void volume, 1.5 ml.

[d] Reported retention data[28]. Mobile phase, 10 mM ammonium perchlorate (pH 6.7) in 90% aqueous methanol; column, 125 × 4.9 mm I.D. Spherisorb 5 SW. N.A. = data not available.

[e] 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine.

acetonitrile concentrations above 26% and was attributed to lipids. The A-28 column, which has the same polymeric structure as PRP-1, is eluted in the forward direction. Hydrophobic anions are strongly retained on the anion-exchange column, as are hydrophobic neutral compounds, including the lipids. A 70% acetonitrile wash facilitated the elution of these compounds. Furosemide was not eluted, even with 70% acetonitrile, due to the low ionic strength of the eluent.

Sample application

Experiments were conducted to determine the amount of buffer required to

neutralize an acidic urine (pH 5.0). A 1:1 mixture of urine with 2% potassium borate (pH 8.0) was required. Higher borate concentrations could not be employed due to limited solubility. Ammonium acetate was then examined because of its high solubility in water and acetonitrile. A 1:1 mixture of urine with 2 *M* ammonium acetate (pH 8.0) offered adequate buffering and also better peak shapes than the borate buffer. A 5:1 mixture of urine with 6 *M* ammonium acetate (pH 8.0) gave equivalent buffering capacity and permitted the injection of a more concentrated sample.

Final procedure

A total of five different eluents are employed in the complete system. Based on their function, these may be described in the following manner: (1) application buffer, (2) pH exchange buffer, (3) strong eluting buffer, (4) mobile phase, (5) pre-column wash solvent. The final experiments were performed using the instrumentation and formulations shown in Fig. 1.

Urine from healthy laboratory employees was spiked with eight drugs from a variety of chemical classes and with two internal standards, N-ethylnordiazepam and chlorpheniramine. Caffeine was present in almost all urine and was eluted near the solvent front. Spiked sample (0.5 ml) was mixed with 0.1 ml 6 *M* ammonium acetate (adjusted to pH 8.0 with 2 *M* potassium hydroxide) and centrifuged 30 s at 11 000 *g*. The 1.5-ml microcentrifuge tube was placed in the autosampler; the sampling needle was raised a few mm above the bottom of the cup to prevent contact with precipitate. Following injection, the pump A flow-rate was raised to 5 ml/min for 0.6 min. The flow-rate was briefly lowered while valve 1 (V1) was reversed, then the PRP-1 column was backflushed with 8 ml of the application buffer. The flow-rate was lowered to 1.0 ml/min. The solvent selector was switched to buffer 2 (0.25 ml) so that buffer 2 reached the PRP-1 column after the new flow-rate was achieved. The time of elution for amphetamine and morphine was determined by direct detection in the eluate of the first column; V2 was switched 3 s before this time to permit transfer of drugs to the A-28 column. V3 was switched 6 s later to permit transfer of rapidly eluted drugs to the coupled analytical columns. The time of elution of the slowest-eluted drugs from the A-28 column (secobarbital and oxazepam) was determined by direct detection in the eluate from that column. At this time, V3 was switched to disconnect the clean-up columns from the analytical columns. During this time, the solvent selector was switched to buffer 3 (0.2 ml), speeding elution of drugs from the PRP-1 column, and then to buffer 4. The total volume transferred from the PRP-1 column was 1.6 ml.

Following transfer of all drugs to the coupled analytical columns, the flow was maintained by pump B at 1.0 ml/min until analysis was complete. This pump delivered mobile phase continuously at a constant flow-rate; its output was diverted to waste during the transfer steps. While the analytical separation was completed, the pre-columns were rinsed with 7 ml of wash reagent (70% acetonitrile in buffer). The PRP-1 column was rinsed first with 2 ml; then the PRP-1 column and A-28 column were rinsed together. Next, both clean-up columns were equilibrated with mobile phase. When analysis was completed, V2 was switched so that pump B delivered mobile phase to columns 2, 3, and 4; pump A was used to equilibrate the PRP-1 column in borate buffer, while the next sample was loaded.

Fig. 2 shows the performance of the final three columns, eluted isocratically, after injection of 50 µl of an aqueous mixture containing 2–5 mg/l of each drug. Figs.

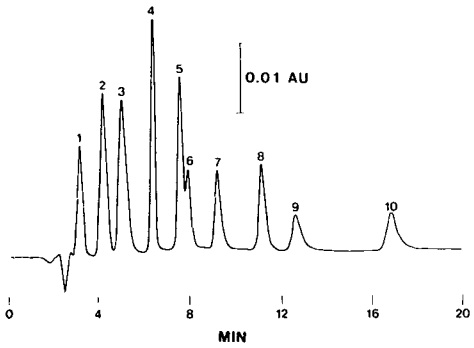

Fig. 2. Chromatogram produced by injection of 50 μl of an aqueous mixture into columns 2, 3, and 4. (Column 1 was replaced by a short length of 0.25 mm I.D. tubing.) Drugs shown are: (1) secobarbital (5 mg/l), (2) diazepam (2 mg/l), (3) N-ethylnordiazepam (4 mg/l), (4) amphetamine (5 mg/l), (5) methamphetamine (5 mg/l), (6) diphenhydramine (2 mg/l), (7) imipramine (2 mg/l), (8) morphine (2 mg/l), (9) chlorpheniramine (3 mg/l), (10) hydrocodone (2 mg/l). Mobile phase, 6 m*M* KH_2PO_4, 5 m*M* tetramethylammonium hydroxide, 2 m*M* dimethyloctylamine, pH 6.50 containing 33% acetonitrile; detection, 210 nm.

3 and 4 show the performance of the complete system for 500-μl injections of drug-free and drug-supplemented urine. It may be seen that preconcentration on PRP-1 resulted in noticeable broadening of the benzodiazepine and imipramine peaks; for other drugs the 500-μl injection caused very little broadening.

The system repeatability for ten consecutive injections is shown in Table III. Peak height and retention time precision were excellent for concentrations of 2–5 mg/l. Although little retention variability may be observed on a daily basis, changes may occur as the reversed-phase and silica columns age and retention decreases. The two internal standards may be useful to monitor these changes; N-ethylnordiazepam retention reflects the performance of the reversed-phase column, while chlorpheniramine retention reflects the performance of the silica column.

Linearity data for concentrations of 0.3, 1, 2, 5, 10, and 25 mg/l are shown in Table IV. Concentrations as high as 100 mg/l did not lead to carry-over in subsequent specimens.

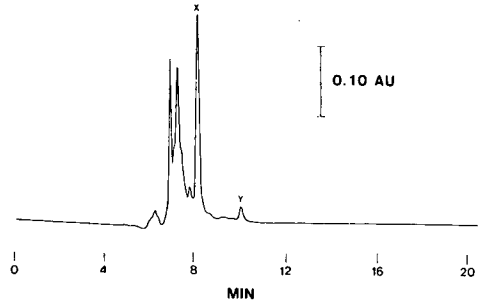

Fig. 3. Chromatogram of 500 μl of buffered drug-free urine, analyzed with the complete system. X and Y are endogenous peaks. Caffeine is eluted with the solvent front. Detection, 210 nm. Other conditions as in Fig. 1.

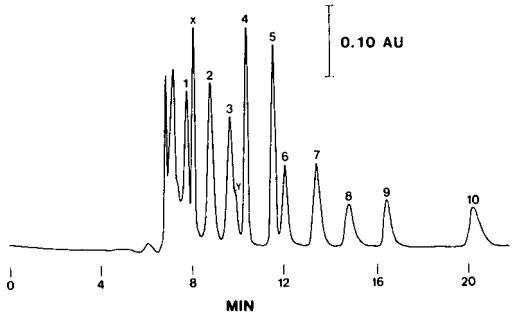

Fig. 4. Chromatogram of urine to which drugs have been added. Identity and concentration of drugs is given for Fig. 2. Sample, 500 μl of buffered urine; detection, 210 nm. For other conditions, see Fig. 1.

The detection limit of the system may be defined by the lowest concentration that yields a spectrum which can be matched against the stored spectral library. The library requires 10 mAU at λ_{max}, which will be different for different compounds. Using a 10-mAU cutoff at 210 nm, the minimal detectable amounts shown in Table IV were obtained. Obviously much smaller peaks can be observed. In practice, the detection limit for compounds eluted between caffeine and diazepam may be higher, owing to background contributions by caffeine and endogenous compounds. Benzoylecgonine (λ_{max} = 233 nm) is more readily detected at 235 nm, the secondary detection wavelength, where background contributions are reduced, as compared to 210 nm.

Suitability for toxicology screening

The College of American Pathologists distributes proficiency samples on a quarterly basis to toxicology laboratories for qualitative analysis. The 1988 program included a total of 77 compounds. To determine the utility of our method for toxicological screening, stock solutions of 71 drugs were prepared and added to urine to obtain concentrations of 2 mg/l. (Six volatile compounds were not evaluated.) Each sample was analyzed; the retention times and the absorbance at 210 nm and 235 nm

TABLE III

REPEATABILITY DATA FOR AUTOMATED MULTI-COLUMN SCREENING OF URINE

Compound	*Amount added (μg) to 500 μl urine*	*Retention time ± S.D. (min)*	*Peak height R.S.D. (%)*
Secobarbital	2.5	7.85 ± 0.003	2.7
Diazepam	1.0	8.89 ± 0.006	1.0
N-Ethylnordiazepam	2.0	9.73 ± 0.007	1.8
Amphetamine	2.5	10.43 ± 0.012	0.7
Methamphetamine	2.5	11.58 ± 0.009	0.6
Diphenhydramine	1.0	12.06 ± 0.012	0.7
Imipramine	1.0	13.29 ± 0.011	1.2
Morphine	1.0	14.61 ± 0.024	1.2
Chlorpheniramine	1.5	16.33 ± 0.023	1.2
Hydrocodone	1.0	19.90 ± 0.045	1.0

TABLE IV

LINEARITY DATA FOR AUTOMATED MULTI-COLUMN SCREENING OF URINE

Number of injections at each concentration = 3.

Compound	*Concentration range (mg/l)*	*Regression line*			*Detection limit*[a] *(mg/l)*
		Slope	*r*	*Intercept (mg/l)*	
Diazepam	0.3–25	$8.3 \cdot 10^{-5}$	0.9999	−0.04	0.3
Amphetamine	0.3–10	$4.5 \cdot 10^{-5}$	0.9999	−0.02	0.2
Methamphetamine	0.3–10	$4.8 \cdot 10^{-5}$	0.9999	−0.02	0.2
Diphenhydramine	0.3–25	$3.9 \cdot 10^{-5}$	0.9999	0.01	0.2
Imipramine	0.3–10	$4.4 \cdot 10^{-5}$	0.9999	0.03	0.3
Morphine	0.3–10	$9.4 \cdot 10^{-5}$	0.9991	−0.26	0.3
Hydrocodone	0.3–10	$1.2 \cdot 10^{-4}$	0.9997	−0.15	0.3

[a] Concentration producing a peak height of 10 mAU at 210 nm.

were recorded. The full spectra (195–375 nm) were also stored on hard disc. Of the 71 compounds injected 61 were retained on the sample preparation cartridges and produced symmetrical peaks which were separated by at least 0.5 min from the caffeine peak. (The proficiency program does not include caffeine.) Of the compounds that could not be analyzed, four were acids, four were neutral, and two were weak bases with no appreciable UV absorbance. Even weakly absorbing drugs such as phencyclidine produced observable peaks at concentrations of 50–100 μg/l, but their spectra could not always be matched against the stored spectra. The analytical system,

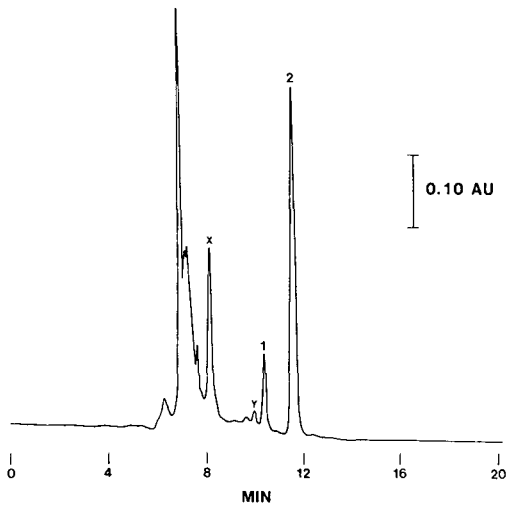

Fig. 5. Chromatogram of a urine specimen which was positive for amphetamines by thin-layer chromatography. Peak identities were confirmed by comparison with stored library spectra. Concentrations were determined by comparison with urines supplemented with known concentrations of drugs: 1 = Amphetamine (1.8 mg/l), 2 = methamphetamine (8.2 mg/l); detection, 210 nm. For other conditions, see Fig. 1.

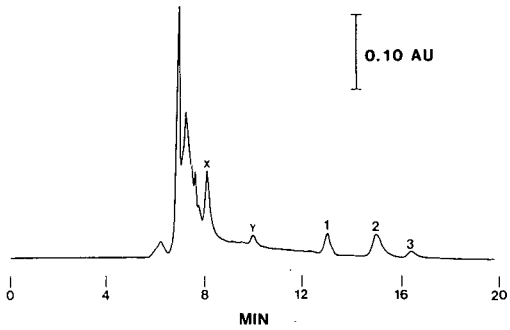

Fig. 6. Chromatogram of a urine sample which was positive for opiates by thin-layer chromatography. Peaks: 1 = monoacetyl morphine (0.6 mg/l), 2 = morphine (1.0 mg/l), 3 = codeine (0.2 mg/l). Detection, 210 nm. For other conditions, see Fig. 1.

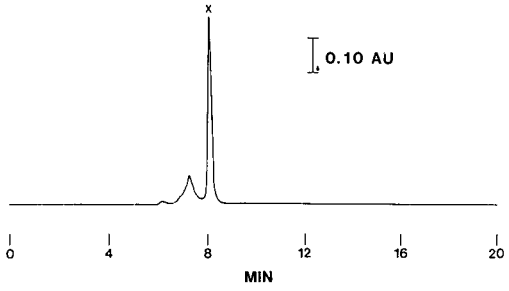

Fig. 7. Chromatogram of a drug-free urine sample. Detection, 235 nm. Note the absence of the endogenous peak Y. For other conditions, see Fig. 1.

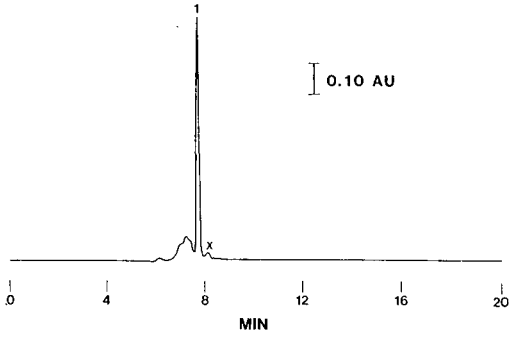

Fig. 8. Chromatogram of a urine specimen which was positive for benzoylecgonine by thin-layer chromatography. Peak: 1 = benzoylecgonine (22.4 mg/l). Note the small size of the endogenous peak X in this urine. Detection at 235 nm. For other conditions, see Fig. 1.

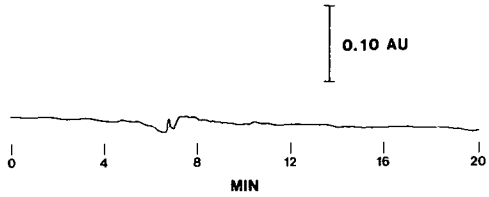

Fig. 9. Chromatogram of 500 μl of buffered drug-free serum. Detection, 210 nm. For other conditions see Fig. 1.

therefore, has broad applicability as a qualitative screening technique, and could be part of a confirmatory scheme for many drugs when concentrations exceed 300 μg/l.

Physiological samples may contain more than one drug, as well as their metabolites; these samples can be successfully analyzed only if the drug combination does not produce overlapping peaks. Because the elution order produces a separation by class, samples containing two different drug classes (*e.g.* amphetamines and opiates) are more readily analyzed than samples containing two drugs of the same class (*e.g.* imipramine and amitriptyline).

Analysis of biological specimens

Chromatograms were obtained for specimens previously analyzed by a thin-layer chromatographic technique (Figs. 5–8). Positive identification of benzoylecgonine, amphetamine, methamphetamine, 6-monoacetyl morphine, morphine, and codeine was possible by computerized evaluation of the chromatograms, using the library search routine.

Several techniques could extend the applicability of the system in toxicological analysis. Larger volumes of urine could be preconcentrated off-line by solid-phase or solvent extraction. Extraction of 5 or 10 ml urine might lead to a 10- to 20-fold reduction in detection limits.

Many drugs are difficult to analyze in urine because of extensive metabolism and conjugation. Serum samples would be more appropriate for such drugs, and blood is usually available for emergency toxicological screening. Fig. 9 shows the chromatogram obtained from a drug-free serum which was processed in the manner described above for urine. The nearly complete absence of background, including the endogenous peak observed (at 8 min) in almost all urines, suggests that serum samples may be especially useful for detection of benzodiazepines and barbiturates, which are eluted in the first few minutes of chromatography.

CONCLUSIONS

The use of two pre-columns for sample clean-up and two coupled analytical columns permitted the analysis of a broad range of drugs of toxicological interest. Elution under isocratic conditions eliminated the need for re-equilibration of the analytical columns and produced a constant spectral background, which simplified the storage and processing of UV spectra. Resolution was generally sufficient to permit separation of drugs from their hydroxylated and demethylated metabolites. Levels of 300 μg/l yielded peaks that were large enough for processing by a computerized library

search routine. Complete analysis required less than 30 min. This analytical system may be useful for toxicological screening in cases where very rapid identification (or exclusion) of a broad range of drugs is required.

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CHROMSYMP. 1613

DETERMINATION BY COUPLED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–GAS CHROMATOGRAPHY OF THE β-BLOCKER LEVOMOPROLOL IN PLASMA FOLLOWING OPHTHALMIC ADMINISTRATION

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SUMMARY

Levomoprolol is a β-blocking agent used in the topical treatment of glaucoma. The necessity for comparing the plasma levels of a drug administered by the ophthalmic route with those obtained following systemic treatment requires increasingly sensitive methods in order to determine the low plasma concentrations produced by the administration of eye drops. On-line high-performance liquid chromatography–gas chromatography and concurrent solvent evaporation proved to be advantageous in the determination of levomoprolol in human plasma. Levomoprolol was determined by capillary gas chromatography (GC) with electron-capture detection (ECD) after solid-phase extraction from plasma and derivatization. Quantitation was based on the internal standard method. The detection limit of 0.2 ng/ml is 50 times lower than that obtained with previous GC methods involving on-column injection and ECD.

INTRODUCTION

In trace analysis, detection limits are commonly restricted by the level of simultaneously eluted interfering material and/or non-volatile materials in the sample matrix. Hence, detection limits depend primarily on the efficiency of the sample clean-up. Optimum sample preparation is based on few, but highly efficient steps. High-performance liquid chromatography (HPLC) is probably the most efficient method for isolating trace components of interest from a complex material. Direct coupling of HPLC to gas chromatography (GC) simplifies the procedure and renders it rapidly. Levomoprolol or 1-(2-methoxyphenoxy)-3-isopropylamino-2-propanol is a β-blocking agent[1] in use for a long time by the oral route in antihypertensive therapy[2]. Recently, it proved to be successful in the topical treatment of glaucoma[3–5]. A previous method[6] used for the determination of levomoprolol in plasma and urine of healthy volunteers after oral treatment provided a detection limit of 10 ng/ml.

Determination of the drug in human plasma after topical administration, in particular by the ocular route, requires analytical methods with very low detection limits. Its purpose is to assess the drug absorption and to carry out bioavailability

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studies in comparison with a systemic reference dosage form to evaluate toxicological aspects.

This paper reports the results obtained by an on-line HPLC–GC method and a concurrent solvent evaporation technique[7–13]. This method permits the detection limit to be lowered to 0.2 ng/ml, allowing the determination of levomoprolol in the plasma of subjects treated by the ocular route. This approach also enables the extent of absorption to be compared with that of the systemic reference dosage form (oral route).

EXPERIMENTAL

Materials and reagents

Levomoprolol hydrochloride and 1-(3-chloroisoxazol-5-yl)-2-(*tert.*-butylamino)ethanol (used as an internal standard) were supplied by Zambon Group (Milan, Italy). All solvents and reagents were purchased from Merck (Darmstadt, F.R.G.) and were of analytical-reagent grade, except the derivatizing agent, trifluoroacetic anhydride, obtained from Supelco (Bellefonte, PA, U.S.A.).

Bond-Elut CN columns (1-ml capacity) were purchased from Analytichem International (Harbor City, CA, U.S.A.) and a solid-phase extraction (SPE) vacuum manifold from Supelco. The centrifuge was a Sorvall RT 6000 B supplied by DuPont Instrument Systems (Wilmington, DE, U.S.A.). *n*-Pentane and diethyl ether were distilled before use.

The HPLC system consisted of a Jasco (Tokyo, Japan) Model BIP-1 pump, a Jasco Uvidec 100-V UV detector operating at 223 nm on-line with the pump and the HPLC–GC interface and a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 561 recorder.

The gas chromatograph was a 5300 Mega series from Carlo Erba (Milan, Italy), equipped with a Model ECD 400 ^{63}Ni electron-capture detector. The output of the detector was recorded on a Spectra-Physics (San Jose, CA, U.S.A.) Model 4270 integrator.

The interface between the HPLC and GC instruments, not commercially available, was assembled in our laboratories, using a Porter Instrument (Hatfield, PA, U.S.A.) Model VCD 1000 A-10 flow regulator and a Valco (Houston, TX, U.S.A.) Model C-6-W six-port switching valve, equipped with a Supelco 500-μl sample loop. When the switching valve was in the load position, the HPLC eluent from the UV detector passed through the sample loop. The switching valve was then turned to transfer position when the HPLC fraction was fully eluted in the sample loop, *i.e.*, at the end of the fraction as observed on the liquid chromatogram recorder. At this time the carrier gas was introduced into the sample loop behind the plug of liquid and pushed it into the capillary precolumn. The vapour pressure in front of the liquid plug counterbalanced the pressure of the carrier gas behind the plug, allowing the liquid plug to evaporate within the section of the precolumn inlet. Evaporated liquid was automatically replaced, as the plug was moved forward, by the carrier gas, up to completion of solvent evaporation.

Sample preparation

Sample preparation was performed with a Bond-Elut CN column containing silica modified with bound cyanopropyl groups. It was attached to the Luer fittings

over the vacuum manifold cover, having the capacity for twelve columns. The column was conditioned by rinsing with one volume of dichloromethane, and successively with one volume of methanol and one volume of carbonate buffer (pH 10), taking care to avoid drying of the column. To 1 ml of plasma were added 1 ng of internal standard (10 μl of a 0.1 μg/ml aqueous solution) and 0.5 ml of the carbonate buffer. The sample was applied to the activated Bond-Elut CN column, which was then washed with two volumes of carbonate buffer. The Bond-Elut CN column was centrifuged at 4000 rpm for about 15 s, then transferred to a test-tube and eluted with two volumes of dichloromethane by centrifugation at 1000 rpm for 3 min. The organic phase was evaporated to dryness under a gentle stream of nitrogen at room temperature, then 1 ml of diethyl ether and 0.25 ml of trifluoroacetic anhydride were added and allowed to react at room temperature for 45 min. Final evaporation under a stream of nitrogen afforded the residue for the chromatographic analysis. The residue was dissolved in 0.5 ml of HPLC eluent [*n*-pentane–diethyl ether (55:45)] and 100 μl were injected into the HPLC system.

Chromatography

The HPLC column was a Hibar LiChrosorb CN (5-μm) column (250 mm × 4 mm I.D.) (Merck). The HPLC pre-separation was carried out with *n*-pentane–diethyl ether (55:45) as eluent at a flow-rate of 1 ml/min. The column was kept at room temperature.

The column performance and eluent flow-rate were carefully checked by periodic injections of an amount of sample and internal standard that could be revealed by the UV detector (see also Fig. 1, right). Further, the HPLC eluent was kept under a slight pressure of nitrogen to avoid the formation of bubbles, which could change the HPLC retention time. Under the conditions described, levomoprolol and the internal standard were eluted at approximately the same retention time. The HPLC fraction corresponding to the levomoprolol was then transferred to the gas chromatograph by means of the HPLC–GC interface.

For GC analysis a Permabond SE-54 fused-silica column (25 m × 0.32 mm I.D.) supplied by Macherey-Nagel & Co. (Düren, F.R.G.) was used. A fused-silica capillary precolumn (2.5 m × 0.32 mm I.D.) silylated with diphenyltetramethyldisilazane was coupled to the separation column by means of a press-fit connection. Hydrogen was used as the carrier gas at a flow-rate of 3 ml/min and an inlet pressure of 300 kPa behind the flow regulator. Nitrogen was used as make-up gas for electron-capture detection (ECD) at a flow-rate of 30 ml/min. Eluent introduction occurred at 79°C. The column temperature was programmed linearly at 10°C/min to 200°C and subsequently at 40°C/min to 250°C. It was then kept constant at 250°C for 3 min. Under the conditions described, 300 analyses can be done before loss of efficiency.

Calibration

A calibration graph covering the range from 0.2 to 5 ng/ml was obtained as follows. Into each of the seven human plasma samples (1 ml) a constant amount of internal standard (1 ng) was added by means of a microsyringe, together with increasing amounts of levomoprolol (0.2, 0.5, 0.8, 1, 2, 3 and 5 ng).

Extraction was performed as described under *Sample preparation*. Blank plasma was also extracted to ensure that no interfering peaks were present. The cali-

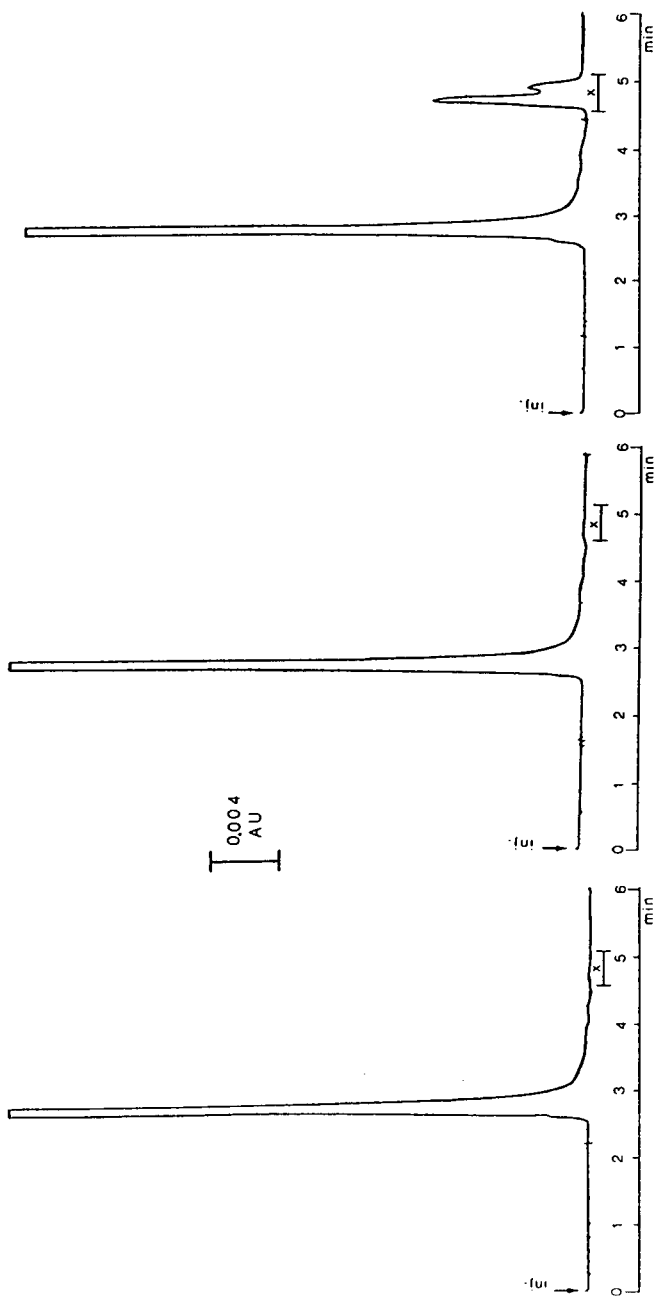

Fig. 1. Typical liquid chromatograms of (left) blank human plasma, (middle) blank human plasma spiked with 1 ng/ml of levomoprolol and 1 ng/ml of internal standard and (right) blank human plasma, containing 0.1 μg/ml of levomoprolol and 0.1 μg/ml of internal standard, to provide evidence for the transfer of the cut to the GC system (marked ×).

bration graph was plotted with levomoprolol concentration on the abscissa and the ratio of the levomoprolol and internal standard peak heights on the ordinate.

RESULTS

In Fig. 1, liquid chromatograms are presented for blank human plasma and for blank human plasma to which 1 ng/ml of levomoprolol and 1 ng/ml of internal standard had been added. Under these conditions, 1 ng/ml of levomoprolol cannot be detected on the chromatogram. Fig. 1 also shows the liquid chromatogram of a blank human plasma to which 0.1 μg/ml of levomoprolol and 0.1 μg/ml of internal standard had been added. This chromatogram shows that only a very large amount of sample (in this instance 100 times higher than 0.1 ng/ml) can be detected by UV spectrophotometry, indicating the liquid fraction that must be transferred to the GC system.

The gas chromatograms of three samples transferred from the HPLC system to the gas chromatograph are shown in Figs. 2, 3 and 4. They show that levomoprolol and the internal standard are eluted from the GC column without significant in-

Fig. 2. Typical gas chromatogram of blank human plasma.

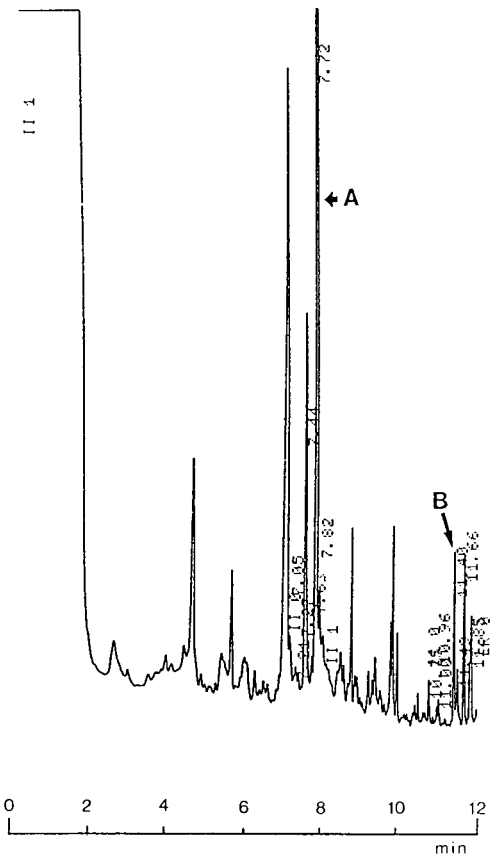

Fig. 3. Typical gas chromatogram of blank human plasma spiked with (B) 1 ng/ml of levomoprolol and (A) 1 ng/ml of internal standard.

terference (Fig. 2); the gas chromatogram of a blank plasma to which 1 ng/ml of levomoprolol and 1 ng/ml of internal standard had been added (Fig. 3) and that of a blank plasma spiked with 0.2 ng/ml of levomoprolol and 1 ng/ml of internal standard (Fig. 4) indicate a detection limit below 0.2 ng/ml.

The calibration graph was obtained as described under Experimental. Each concentration was determined at least in triplicate. The calibration graph was linear in the range 0.2–5.0 ng/ml. Linear regression gave the equation $y = 0.145x - 0.003$ and a correlation factor of $r^2 = 0.995$.

The results for precision and accuracy evaluation are given in Table I. They were obtained by determining seven different concentrations of levomoprolol in 1 ml of human plasma, ranging from 0.2 to 5 ng/ml. This analysis was repeated three times on 0.8, 1 and 2 ng/ml samples and six times on 0.2, 0.5, 3 and 5 ng/ml samples. The results indicate a recovery ranging from 92.7 to 113.8% and a relative standard deviation of 15.7% for a 0.2 ng/ml concentration. These values were obtained by comparing the amount of levomoprolol recovered with the known amount added to blank human plasma (see Table I).

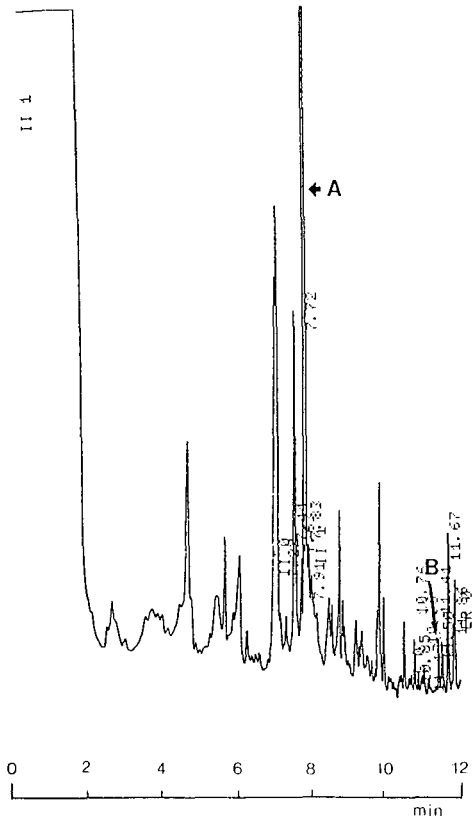

Fig. 4. Typical gas chromatogram of blank human plasma containing (B) 0.2 ng/ml of levomoprolol and (A) 1 ng/ml of internal standard.

TABLE I

ACCURACY AND PRECISION OF LEVOMOPROLOL DETERMINATION

Levomoprolol added to plasma (ng/ml)	*Recovery*		*C.V. (%)*
	ng/ml	*%*	
0.2	0.20 (n = 6)	100.0	15.7
0.5	0.53 (n = 6)	106.0	17.5
0.8	0.91 (n = 3)	113.8	16.1
1	0.98 (n = 3)	98.3	20.8
2	1.95 (n = 3)	97.5	18.5
3	2.78 (n = 6)	92.7	19.4
5	5.12 (n = 6)	102.4	13.8

TABLE II

INDIVIDUAL AND MEAN (± S.E.M.) LEVOMOPROLOL PLASMA CONCENTRATIONS FOLLOWING OCULAR AND ORAL TREATMENT

Administered dose: ocular, 540 μg; oral, 75 mg.

Subject	*Levomoprolol (ng/ml)*													
	Oral administration							*Ophthalmic administration*						
	Time (min)							*Time (min)*						
	30	*60*	*120*	*180*	*240*	*360*	*480*	*30*	*60*	*120*	*180*	*240*	*360*	*480*
1	362.3	350.0	252.7	125.4	107.8	72.1	36.8	0.50	0.70	0.80	0.57	0.52	0.31	0.21
2	314.4	382.7	441.8	281.5	263.0	141.8	92.3	2.3	3.1	1.5	1.4	0.97	0.53	0.32
3	115.8	130.8	102.1	57.9	49.6	30.3	18.5	1.2	1.7	1.1	0.85	0.50	0.36	0.20
4	299.2	360.9	400.3	265.5	201.3	105.1	39.9	2.9	3.5	1.6	1.1	0.93	0.58	0.37
5	260.5	355.5	341.5	211.9	210.1	97.8	41.2	2.0	2.9	1.8	1.5	1.1	0.63	0.35
6	160.0	192.4	180.2	100.0	75.6	46.5	23.5	1.5	1.9	1.6	0.93	0.62	0.87	n.d.
Mean ± S.E.M.	252.0 ± 38.9	295.4 ± 43.3	286.4 ± 53.7	173.7 ± 37.7	151.2 ± 34.8	82.3 ± 16.7	42.0 ± 10.7	1.7 ± 0.35	2.3 ± 0.43	1.4 ± 0.15	1.1 ± 0.14	0.78 ± 0.1	0.45 ± 0.06	0.24 ± 0.05

Pharmacokinetics of levomoprolol

The method was used for the evaluation of the plasma concentrations of levomoprolol after ocular administration. It allowed a comparison of the results with those obtained for the same subjects after oral administration. The study was carried out on six healthy volunteers (four males and two females) aged 28–51 (37 ± 8) years and weighing 48–90 (72 ± 15.6) kg. Each subject was apparently healthy, in particular as far as the renal, hepatic and gastrointestinal functions are concerned. A "cross-over" experimental design was adopted. Each subject received the two preparations (0.9% levomoprolol eye drops, 1 drop into each eye = 540 μg of levomoprolol, and Levotensin tablet = 75 mg of levomoprolol, 7.5 μg/kg body weight and 1.04 mg/kg body weight, respectively), in two treatment sessions, carried out 2 weeks apart, randomizing the order of administration. During each of the two sessions, basal samples of venous blood were drawn from each fasting subject prior to administration of the eye drops or the tablet. Further blood samples were also collected 30, 60, 120, 180, 240, 360 and 480 min after treatment. The plasma samples obtained after centrifugation were analysed as described under Experimental. The individual and mean (± S.E.M.) values of levomoprolol plasma concentrations obtained at the different sampling times for the two treatments are given in Table II.

The following pharmacokinetic parameters were evaluated: the plasma concentration peak (C_{max}), the time at which the plasma concentration peak was reached (T_{max}), the half-life of the elimination phase ($T_{1/2}$) and the extrapolated area under the concentration–time curve from time zero to infinity (AUC_{tot}). C_{max} reached 2.3 ng/ml at 70 min after ocular administration and 313.9 ng/ml at 75 min following oral administration. $T_{1/2}$ was 150.8 min after ocular administration and 145.1 min following oral treatment. The AUC_{tot} values were 545.8 and 83 750.8 ng · min/ml after ocular and oral administration, respectively. The extent of absorption after ocular treatment was thus 90.5% of the absorption after oral administration, calculated by comparison of the total AUC values and taking into account the ratio of the administered doses.

DISCUSSION

The results confirm that coupled HPLC–GC is a versatile method, overcoming problems due to inadequate detection limits of methods involving GC alone. The method proved to be very useful for preliminary clean-up of samples and for trace enrichment, allowing large volumes to be injected into the capillary GC column. When analysing tissues and biological fluids, detection limits are primarily constrained by interfering sample components. The possibility of reducing the detection limits renders coupled HPLC–GC very suitable for pharmacokinetic and metabolic studies. The on-line HPLC–GC technique proved to be essential for the pharmacokinetic study of levomoprolol after ocular administration. In fact, it allowed the determination of plasma concentrations in humans following ocular administration and comparison with those obtained after oral administration of the tablets used as a reference dosage form.

The results obtained from the pharmacokinetic study indicate that levomoprolol is very well absorbed through the cornea after ocular administration, as demonstrated by the ratio of the total AUC values compared with the administered dose.

However, the plasma concentration peak after ocular administration is more than 100 times lower than that obtained following oral treatment. In spite of the large extent of absorption, the plasma levels are not so high as to compel us to perform systemic toxicity studies following ocular administration. Generally, accurate and ultrasensitive methods such as on-line HPLC–GC for the determination of low drug concentrations after ocular treatment permit the establishment of pharmacokinetic *vs.* pharmacodynamic relationships and pharmacokinetic studies comparing the ocular (or generally topical) administration and a reference dosage form for bioavailability, activity and tolerance evaluation purposes. Further, it allows the detection of the drug in the eye and monitoring of pharmacokinetic studies designed to define long-acting formulations or formulations in which drug absorption is not desired.

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CHROM. 1621

ANALYSIS OF DICAMBA IN TOBACCO BY ON-LINE COUPLED LIQUID CHROMATOGRAPHY–GAS CHROMATOGRAPHY

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SUMMARY

A new analysis procedure for determining dicamba in dried tobacco is presented. The method consists of a simple sample extraction followed by esterification and a final analysis using on-line coupled liquid chromatography–gas chromatography (LC–GC). Clean-up and preseparation of a sample is by LC and the fraction of interest is analysed using GC with electron-capture detection. The conditions during sample transfer produce concurrent solvent evaporation and vapours are vented out through a solvent vapour exit placed after the separation column. The detection limit is low and the method is simple and sensitive.

INTRODUCTION

Dicamba (3,6-dichloro-2-methoxybenzoic acid) is a herbicide, widely used for selective post-emergence purposes. It acts on broad-leaved weeds but not on grasses. The reported oral LD_{50} in rats is 1 g per kg. The compound is sold under a wide range of commercial names and is used in the culture of maize, cereals, sorghum, sugar cane, pastures and also in the early stages of the culture of flax, rape cabbage oil palm and rice.

This compound should not be found in tobacco. The latter is susceptible to it, and the only reason for its presence on tobacco is careless application to other crops or, as some say, for accelerated yellowing of the leaves. No maximum allowable limit for tobacco is foreseen in, *e.g.*, the legislation of the Federal Republic of Germany. However, in foods of plant origin the limit is 0.05 mg per kg (1982). The rules on tobacco inspection issued by the US Department of Agriculture state a maximum allowable residue level for dicamba of 0.5 mg per kg (1986).

The commonly applied methods for determining dicamba in tobacco involve repeated liquid–liquid extractions to isolate the acids from the rest of the sample, followed by esterification with diazomethane and clean-up by column liquid chromatography. The final analysis is generally by gas chromatography (GC) with electron-

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capture detection (ECD)[1]. These methods are lengthy. Furthermore, the recoveries of dicamba from tobacco tended to be low and poorly reproducible.

The method proposed here reduces the procedure to a simple extraction, followed by esterification. The sample clean-up is carried out by normal-phase liquid chromatography (LC), coupled on-line to a gas chromatograph for the final analysis. This greatly reduces the work involved in the analysis of a sample, but also improves the recovery of dicamba and reduces detection limits through more efficient removal of interfering by-products.

Application of coupled LC–GC to pesticide analysis was first described by Majors[2] for the determination of atrazine in sorghum. More recently, Ramsteiner[3] applied a similar technique for the analysis of the plant protection agent Folpet in hop samples. In both cases, transfer was achieved through a conventional GC autosampler, injecting up to a few microlitres into a vaporizing injector. This transfer technique, providing a heart-cut analysis of the LC peak, sensitively relies on accurate LC retention times, as the transfer should occur at the maximum of the LC peak. Furthermore, only a small proportion of the LC fraction is transferred (at most a few percent), either resulting in modest sensitivity or necessitating loading a large amount of sample material onto the LC column.

The transfer technique applied here involves introduction of the complete LC fraction into the gas chromatograph by the concurrent solvent evaporation technique[4].

Concurrent eluent evaporation means evaporation during transfer. This avoids a flow of liquid into a GC column, which occurs when the retention gap transfer technique is applied[5]. Concurrent eluent evaporation has the important advantage that large fraction volumes can be transferred to the gas chromatograph by employing short (2–3 m) precolumns before the analytical column. An inherent problem is peak broadening of early eluted peaks, *i.e.*, peaks eluted up to 50–80°C above the GC column temperature used during eluent transfer[6]. Using a moderately volatile eluent, such as *n*-pentane and diethyl ether, the first sharp peaks can be expected at elution temperatures of 100–140°C. The methyl ester of dicamba is eluted within the critical temperature range, considering peak broadening, when standard thin-film GC columns are used.

To achieve sharp dicamba peaks with the concurrent eluent evaporation technique the following factors must be considered. Broadening of the dicamba peak depends on the column oven temperature used during eluent transfer. The minimum transfer temperature is determined by the requirement that the column temperature must exceed the eluent point to produce a vapour pressure exceeding the pressure of the carrier gas pushing the liquid into the column. As the eluent boiling point increases with pressure (10–20°C/bar), the lowest transfer temperature is possible at low inlet pressure. However, high inlet pressure is of interest for efficient discharge of the eluent vapours through the column. For the analysis of dicamba, this problem was solved, first, by applying a minimum inlet pressure during transfer, and secondly, by use of a GC column of intermediate polarity and somewhat elevated film thickness, which increased the elution temperature of dicamba.

EXPERIMENTAL

Apparatus

The LC system consisted of a Orlita membrane-type pump, a Waters U6K injector and a Perkin-Elmer LC-75 UV detector. Valco W-type valves were applied for interfacing the liquid and gas chromatographs. The gas chromatograph was a Carlo Erba Fractovap GI, equipped with an electron-capture detector (Model 40) and a loop-type interface as described in ref. 7.

Sample preparation

A 6-g amount of tobacco taken from different brands of cigarettes was extracted by mixing the sample for 15 min with 40 ml of *n*-hexane (E. Merck, Darmstadt, F.R.G.; p.a. grade)–diethyl ether (May & Baker, Dagenham, U.K.; HPLC grade) (1:1), containing 2 ml of 10% H_2SO_4 (Merck) in ethanol, in an ultrasonic bath. After removal of the extract, the tobacco was rinsed with *n*-hexane–diethyl ether (1:1) to give a final extract volume of 100 ml. A 15-ml volume of water was added to the extract to allow separation of the liquid phases. Half of the organic phase (equivalent to 3.0 g of tobacco) was evaporated to dryness, using a rotary evaporator. The residue was dissolved in 5 ml of methyl *tert.*-butyl ether (MTBE, Merck, HPLC grade). Methylation with diazomethane was carried out as described in ref. 8. After addition of 2 ml of *n*-heptane (Merck, p.a.), the MTBE was evaporated in a stream of nitrogen. The final solution was made up to 3 ml with *n*-heptane and centrifuged at 670 *g* for 5 min.

LC preseparation

The LC preseparation was carried out on a 100 mm × 2 mm I.D. column (Knauer, Bad Homburg, F.R.G.) packed with 5-μm Spherisorb S-5-W silica. *n*-Pentane (technical grade, distilled over sodium) containing 1.5% diethyl ether (May & Baker, HPLC grade) was the eluent at a flow-rate of 300 μl/min. A 10-μl volume of the centrifuged crude sample was injected. After the elution of dicamba methyl ester, the LC column was backflushed with 1 ml of MTBE to remove remaining polar material. The LC fraction of interest was cut out by a 250-μl stainless-steel sample loop placed in the ten-port sample valve (Fig. 1).

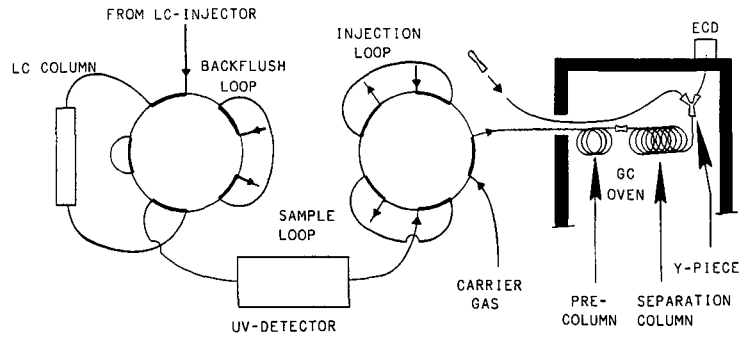
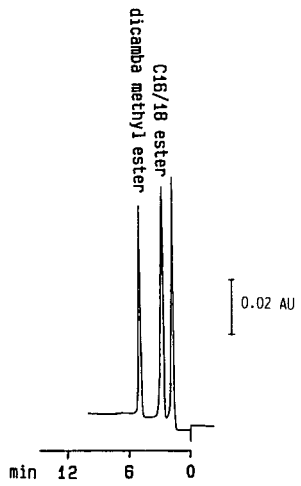


Fig. 1. Schematic diagram of the LC–GC set-up: ——, LC separation and GC analysis; ———, LC backflush and sample introduction into the gas chromatograph.



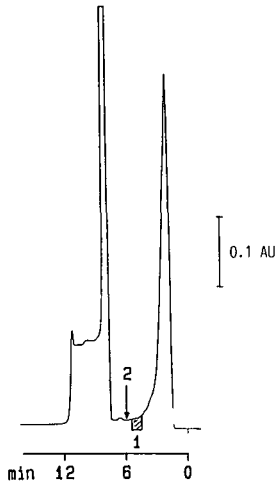


Fig. 2. LC separation of dicamba methyl ester and the C_{16-18} wax ester.

Fig. 3. Liquid chromatogram of a tobacco extract (tobacco spiked with 10 μg/kg dicamba), detected at 220 nm. Backflushing with 1 ml of MTBE occurred at the point indicated. The dicamba methyl ester fraction (250 μl), determined by previous coinjection, is marked. 1 = fraction; 2 = start of backflush.

Removal of wax esters by LC

With the simple extraction method applied, the most important by-products are the wax esters from the tobacco leaves. They must be removed as they would overload the GC capillary column and cause severe broadening of the early-eluted peaks (such as dicamba) and necessitate baking out of the column at high temperatures after each experiment. The proposed LC preseparation of dicamba and the wax esters was checked by using a mixture of dicamba and hexadecyl stearate. Fig. 2 shows an LC chromatogram, obtained with 1.5% diethyl ether in *n*-pentane as the eluent (10 μg of dicamba methyl ester and 1 mg of hexadecyl stearate in 1 μl of *n*-hexane, 10 μl injected). Fig. 3 shows an LC chromatogram of a tobacco extract with the dicamba fraction cut out, using the apparatus described in Fig. 1.

Memory effects

Special care must be taken to avoid memory effects, since large amounts of dicamba methyl ester (up to 10 μl of 10 μg/ml solution) must be injected into the LC system to determine the LC retention time ($<$ 10 ng of dicamba methyl ester cannot be reliably detected by an UV detector). Injections of LC standard solutions and real samples should be carried out by using different syringes. It should be regularly checked for possible memory effects originating, *e.g.*, from the LC injector, by injecting eluent into the LC system and analyzing the fraction with the retention time of dicamba.

GC separation

The GC separation was carried out on a 15 m × 0.32 mm I.D. glass capillary column, coated with OV-61-OH of film thickness 0.3 μm. The exit of this column was

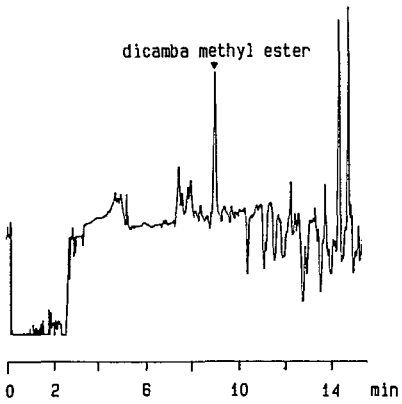


Fig. 4. LC–GC–ECD chromatogram of a tobacco sample containing 10 μg/kg of dicamba (the 250-μl fraction from Fig. 3).

connected to a press-fit Y-piece made of glass, which served as a solvent vapour exit during solvent evaporation. This Y-piece was connected to the detector by a 30 cm × 0.1 mm I.D. fused-silica capillary. It was closed by a stopper (made of a press-fit connector) after completion of the solvent evaporation step. The column inlet was equipped with a 3 m × 0.32 mm I.D. fused-silica precolumn (retention gap), deactivated by phenyldimethyl silylation. The precolumn was connected to the separation column using a press-fit connector. Transfer of the LC fraction was carried out at an inlet pressure of an 1.5 bar (rather low pressure to keep the transfer temperature low) with an oven temperature of 66°C. The flow regulator was set to a flow-rate of 3 ml/min (hydrogen). After completion of solvent evaporation, the oven temperature was programmed at 8°C/min to 280 °C where it was kept for 10 min. The elution temperature of dicamba methyl ester was 134°C.

RESULTS AND DISCUSSION

Fig. 4 shows a LC–GC–ECD chromatogram of a tobacco sample corresponding to 100 pg of dicamba injected. It shows that the achievable detection limits are very low, despite the short analysis procedure. Recoveries of dicamba added to tobacco samples before extraction ranged between 50 and 70%. Considering the recovery and sensitivity obtained, our analyses showed no traces of dicamba in commercial cigarettes. The proposed method for analysing dicamba in tobacco is simpler and more convenient compared to commonly applied procedures. The sensitivity is rather good, although the recovery might possibly be improved by optimizing the sample extraction step, which would improve both the sensitivity and reliability of the analysis.

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CHROMSYMP. 1620

AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF 5-S-CYSTEINYL-3,4-DIHYDROXYPHENYLALANINE IN URINE

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SUMMARY

An automated high-performance liquid chromatographic (HPLC) method has been developed for measurement of 5-S-cysteinyl-DOPA in urine (DOPA = 3,4-dihydroxyphenylalanine). The urinary sample was injected into an HPLC boronate column. With a mobile phase of 0.1 *M* phosphate buffer containing 0.2 m*M* disodium ethylenediaminetetraacetate (Na_2EDTA) (pH 6.0) mixed with methanol (9:1), 5-S-cysteinyl-DOPA was adsorbed while most other compounds were washed away. By column switching, the column flow was reversed and 5-S-cysteinyl-DOPA was desorbed by a mobile phase of 0.1 *M* formic acid and 0.2 m*M* Na_2EDTA at pH 3.0 and chromatographed on a reversed-phase column. The precision, as estimated from repeated analysis of an urinary sample and from duplicate analysis of a number of samples, ranged from 1.4 to 5.2% (coefficient of variation), and the analytical recovery was 93 ± 4.1%. The method is suitable for use in the clinical laboratory.

INTRODUCTION

Pigment formation in melanocytes proceeds by a number of reactions and the first part of the metabolic pathway is fairly well known[1,2]. The enzyme tyrosinase first hydroxylates tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and then oxidizes the latter compound to dopaquinone[3]. This compound is an highly reactive electrophilic intermediate which can undergo an internal ring closure to give indole derivatives[4]. In the presence of thiols the SH group reacts by a nucleophilic attack on dopaquinone to give DOPA-thioether compounds. Several such compounds are known, and among them 5-S-cysteinyl-DOPA is quantitatively dominant[5]. The urinary excretion of this compound increases during UV stimulation[6,7], and its measurement is of great value for the study of melanoma metastases in patients with melanoma[8].

A number of high-performance liquid chromatographic (HPLC) methods for the determination of 5-S-cysteinyl-DOPA has already been developed[9–14]. Recently, we described the determination of 5-S-cysteinyl-DOPA in human urine by direct

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injection in a coupled-column HPLC system with electrochemical detection (ED)[15]. In this method, the crude urinary sample was manually injected directly into a purification column of silica to which 3-aminophenylboronic acid is covalently bonded. After on-column washing with a mobile phase of 0.1 *M* phosphate buffer and 0.2 m*M* Na_2EDTA (pH 6.0) mixed with methanol (9:1), the eluent was changed to a mobile phase of 0.1 *M* formic acid and Na_2EDTA (pH 3.0) which desorbed 5-S-cysteinyl-DOPA and transferred it to an analytical reversed-phase column. We have now investigated the prerequisites for automated analysis of 5-S-cysteinyl-DOPA in urine and report the validation of that method.

EXPERIMENTAL

Materials

The catecholamines, dopamine (3-hydroxytyramine hydrochloride), norepinephrine (L-norepinephrine bitartrate), epinephrine (L-epinephrine bitartrate) and 3,4-dihydroxybenzylamine (DHBA) hydrobromide, as well as 3,4-dihydroxyphenylacetic acid (DOPAC) were obtained from Sigma (St. Louis, MO, U.S.A.). 3,4-Dihydroxy-L-phenylalanine (L-DOPA) was from Merck (Darmstadt, F.R.G.). The DOPA-thioether compounds, 5-S-, and 2-S-L-cysteinyl-L-DOPA and 2,5-S,S-di-L-cysteinyl-L-DOPA[16], 5-S-D-cysteinyl-L-DOPA[17], 5-S-L-cysteinylglycine-L-DOPA[18] and 5-S-glutathionyl-L-DOPA[18] were synthesized as described earlier.

Sodium dihydrogenphosphate, disodium hydrogenphosphate, disodium ethylenediaminetetraacetate (Na_2EDTA), sodium metabisulphite and hydrochloric acid were from Merck, methanol from Rathburn Chemicals (Walkerburn, U.K.) and formic acid from Fluka (Buchs, Switzerland).

Stock solutions (1 m*M*) of L-DOPA, epinephrine, norepinephrine, dopamine and dihydroxybenzylamine were prepared in a solution of 10 m*M* hydrochloric acid and 0.2 m*M* Na_2EDTA. Stock solutions of DOPA-thioethers were prepared in 1 *M* hydrochloric acid solutions. Working solutions were prepared by dilutions in water, except for the 5-S-L-cysteinyl-L-DOPA standard, which was prepared in 0.1 *M* acetate buffer, containing 0.2 m*M* Na_2EDTA (pH 4.0).

Mobile phases

I, pH 6.0. A 0.1 *M* phosphate buffer, containing 0.2 m*M* Na_2EDTA, was prepared from sodium dihydrogenphosphate and disodium hydrogenphosphate and adjusted to pH 6.0. This buffer was mixed with methanol (9:1) and suction filtered through a 0.45-μm cellulose acetate filter (Sartorius, Göttingen, F.R.G.). Similar mobile phases with other pH values were prepared for experimental purposes.

II, pH 3.0. A solution of 0.1 *M* formic acid and 0.2 m*M* Na_2EDTA was adjusted to pH 3.0 with sodium hydroxide. The solution was filtered as for mobile phase I.

The components of the chromatographic system were all from Waters Assoc. (Milford, MA, U.S.A.). The boronate affinity column (35 mm × 3.1 mm I.D.) was packed with acetylated boronic acid-silica (ABA-silica) from Perstorp Biolytica (Lund, Sweden). This material consists of LiChrosorb (10 μm) to which 3-aminophenylboronic acid has been bound covalently[19]. The analytical column (250 mm × 4.6 mm I.D.) was a Supelcosil C_{18} (5 μm) column from Supelco (Bellefonte, PA, U.S.A.). The system configuration is depicted in Fig. 1. It consisted of a Model 590

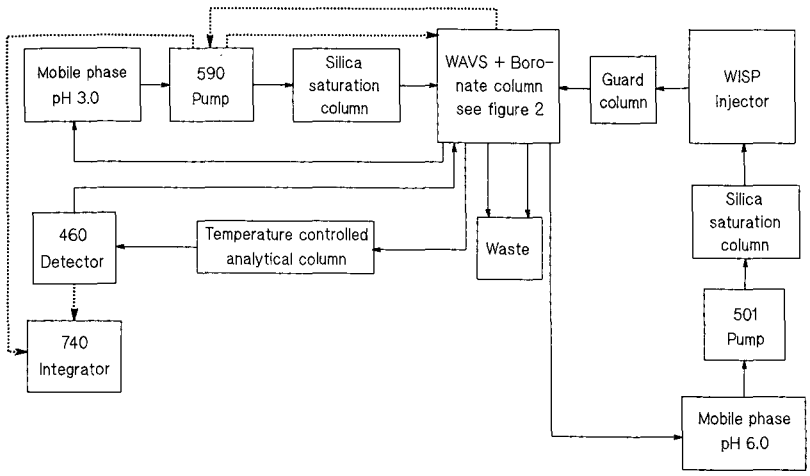

Fig. 1. System configuration for automated determination of 5-S-cysteinyl-DOPA. Dotted lines indicate electrical connections, and solid lines indicate fluid connections. All components were from Waters Assoc.

solvent delivery system, pumping the mobile phase II to the analytical column. This pump was also programmed to control the operation of the WAVS valve station. The samples were transported from the WISP 712 autoinjector to the boronate column with a Model 501 HPLC pump. The silica saturation columns (150 mm × 4.6 mm) before the WAVS and WISP were dry-packed with Polygosil C_{18}, 40–63 μm (Macherey-Nagel, Düren, F.R.G.), and the guard column before the boronate column was a 15 mm × 3.2 mm New Guard RP-18 Column from Brownlee Labs. (Santa Clara, CA, U.S.A.). The analytical column was maintained at 28°C by a Temperature Controle Module thermostat and column heater.

Detection was performed with a glassy carbon working electrode at +0.60 V *versus* Ag/AgCl/3 *M* KCl using a Model 460 electrochemical detector (Waters Assoc.). Assuming a two-electron oxidation of cysteinyl-DOPA, we obtained *ca.* 6% conversion in the electrochemical cell, calculated as A/nFN, where A = area of the chromatographic peak expressed in coulombs, n = number of electrons involved in the electrochemical process (here $n = 2$), F = Faraday's constant, 96 500 C/mol, and N = amount of cysteinyl-DOPA injected in moles.

Integration and calculations were performed by a Model 740 data module from Waters Assoc.

The connections of the WAVS valve station are shown in Fig. 2, upper panel, and an automated sample cycle is described in the same figure, lower panel.

Manual injection was also performed by using the high-pressure valve 1 as an injection valve. This configuration was used to study the recovery from the boronate column.

Methods

Urine was collected for 24 h in bottles containing 5 ml of 0.7 *M* thymol in 2-propanol[20] to inhibit bacterial growth. Aliquots of the urines were stored at −20°C until analyzed.

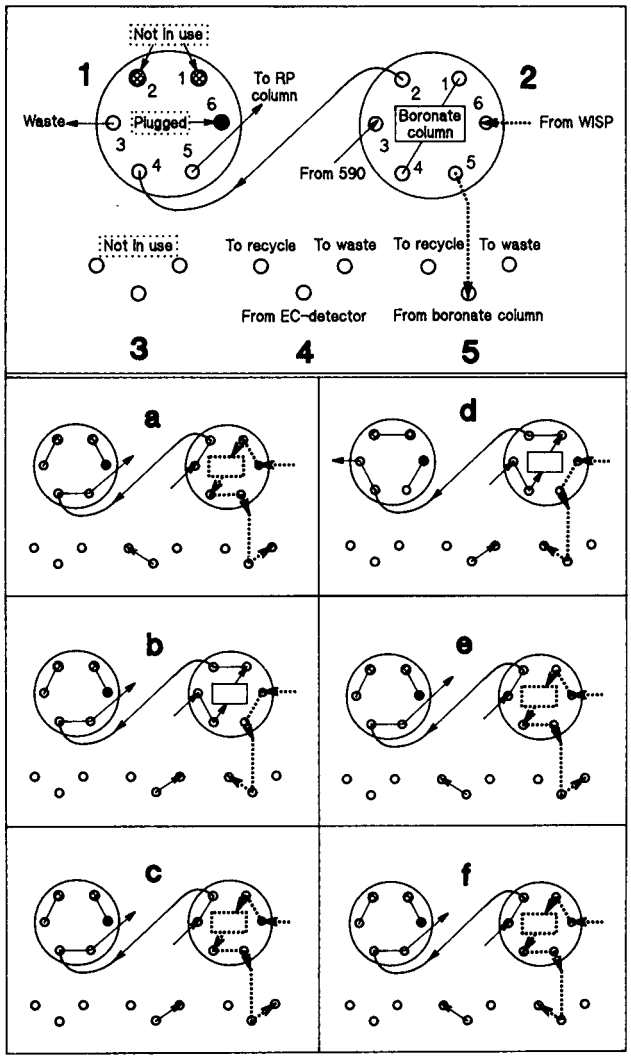

Fig. 2. Usage of the Waters automated valve station. We used both the high-pressure valves (1 and 2) and two of the three low-pressure valves (4 and 5). Upper panel: plumbing for automated analysis of 5-S-cysteinyl-DOPA. This is a key to the lower panel. Lower panel: operation cycle of the automated determination of 5-S-cysteinyl-DOPA. The two pumps are pumping at a flow-rate of 1.0 ml/min unless stated otherwise. Solid lines indicate mobile phase II and dotted lines indicate mobile phase I. (a) Sample is introduced into and adsorbed onto the boronate column. Interfering compounds are washed away for 8 min. (b) The flow direction of the boronate column is reversed, and mobile phase II desorbs cysteinyl-DOPA for 0.7 min. (c) Desorption is completed, and cysteinyl-DOPA is chromatographed on the analytical column. The 501 pump is not turned off. (d) Cysteinyl-DOPA has now been detected. The 590 pump is programmed to decrease the flow-rate slowly to 0. The high-pressure valve 1 is switched so that the boronate column can be washed for *ca.* 3 min at 2 ml/min with mobile phase II. The total wash volume is 6.9 ml. (e) Reequilibration for 10 min of the boronate column is started. (f) The system is now ready for a new injection. Both mobile phases are recycled until the sample is injected.

Urine samples were thawed in cold water and mixed thoroughly, and a 10-ml aliquot was clarified by centrifugation at 1300 *g* for 5 min. A measured volume of the urine was then diluted in two volumes of a 100 m*M* acetate buffer, containing 0.2 m*M* Na_2EDTA (pH 4.0). The diluted urine was transferred to ampoules and placed in the autoinjector.

In routine analysis we usually started with a standard sample, followed by urines, and a new standard sample for each ten samples. If the results were above 3 μM and quantitative analysis was needed, the sample was further diluted in the acetate buffer (see above) and reanalyzed.

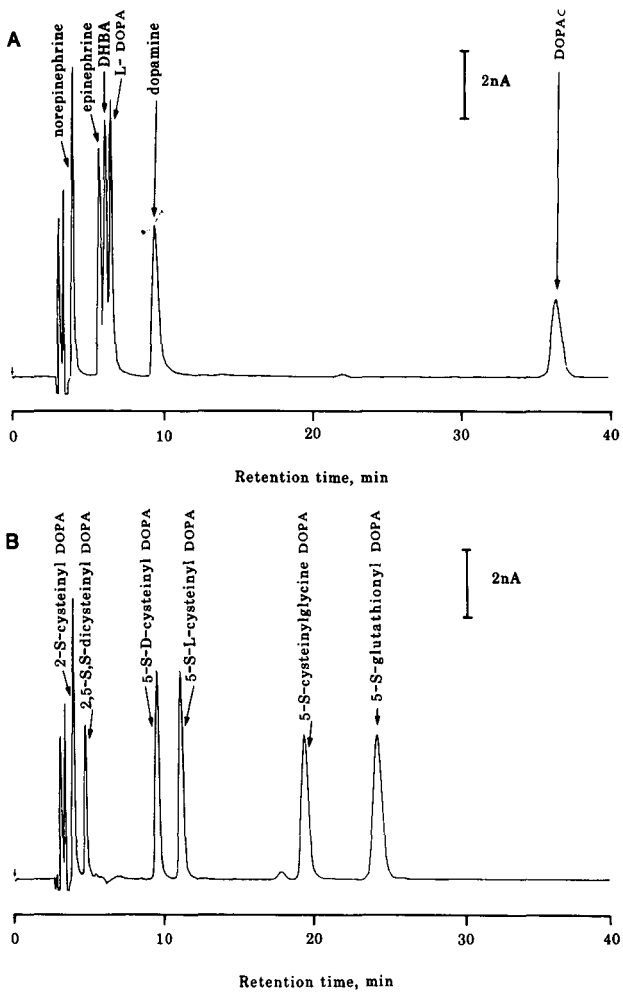

Fig. 3. Separation of synthetic catecholamines, L-DOPA and DOPA-thioethers. Injection was performed directly into the analytical column. (A) Norepinephrine, epinephrine, DHBA, L-DOPA, dopamine and DOPAC. (B) 2-S-Cysteinyl-DOPA, 2,5-S,S-dicysteinyl-DOPA, 5-S-D-cysteinyl-L-DOPA, 5-S-L-cysteinyl-L-DOPA, 5-S-cysteinylglycine-DOPA and 5-S-glutathionyl-DOPA.

RESULTS

Fig. 3 depicts the separation of catecholamines, L-DOPA and DOPA-thioethers on the analytical reversed-phase column only. There was satisfactory separation of the compounds of interest, except that dopamine and 5-S-D-cysteinyl-L-DOPA were not separated, both having a retention time of *ca.* 9.6 min.

Fig. 4 depicts the variation of the peak area for 5-S-L-cysteinyl-L-DOPA as a function of the desorption time. Complete elution was achieved after 0.7 min. Since a ghost peak was also found which increased with time, we chose 0.7 min as our standard desorption time.

When injection of the compounds into the boronate column was followed by column switching, satisfactory separation was also obtained (Fig. 5). In general, the retention times changed very little, and no broadening of peaks occurred. Thus for 5-S-L-cysteinyl-L-DOPA, injected directly into the analytical column or by column switching, the efficiency, $N = 16\ (t_R/w_b)^2$, was about 5500. The resolution, $R_S = 2\Delta t/(w_{b1} + w_{b2})$ (w_b is the peak width at its base, Δt is the difference in retention time of the two peaks), for the separation of 5-S-L-cysteinyl-L-DOPA and 5-S-D-cysteinyl-L-DOPA, as tested for the two types of injection, was about 2.9, and the asymmetry factor, B/A at 10% peak height[21], varied between 1.1 and 1.2 with both types of injection, even after 355 injections. No deterioration of the column was observed after 1000 injections.

From a comparison of Fig. 5 with Fig. 3 it is seen that L-DOPA and DOPAC were not detected after adsorption on the boronate gel at pH 6.0, and a ghost peak was obtained with a retention time of 7.4 min when the boronate column was eluted into the reversed-phase column.

Prolonged washing of the boronate column at pH 6.0 resulted in almost complete elution of the catecholamines, while 5-S-cysteinyl-DOPA remained on the column (Fig. 6).

We compared the peak area of 5-S-L-cysteinyl-L-DOPA after injection with a calibrated loop directly into the analytical column and via the boronate column. The recovery from the boronate column was 92%.

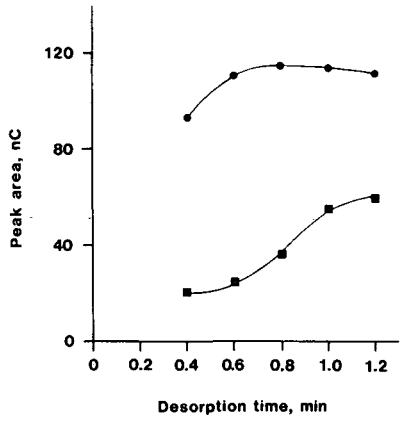

Fig. 4. Desorption of 5-S-cysteinyl-DOPA from the boronate column as a function of the elution with mobile phase II. (●) 5-S-L-cysteinyl-L-DOPA; (■) ghost peak.

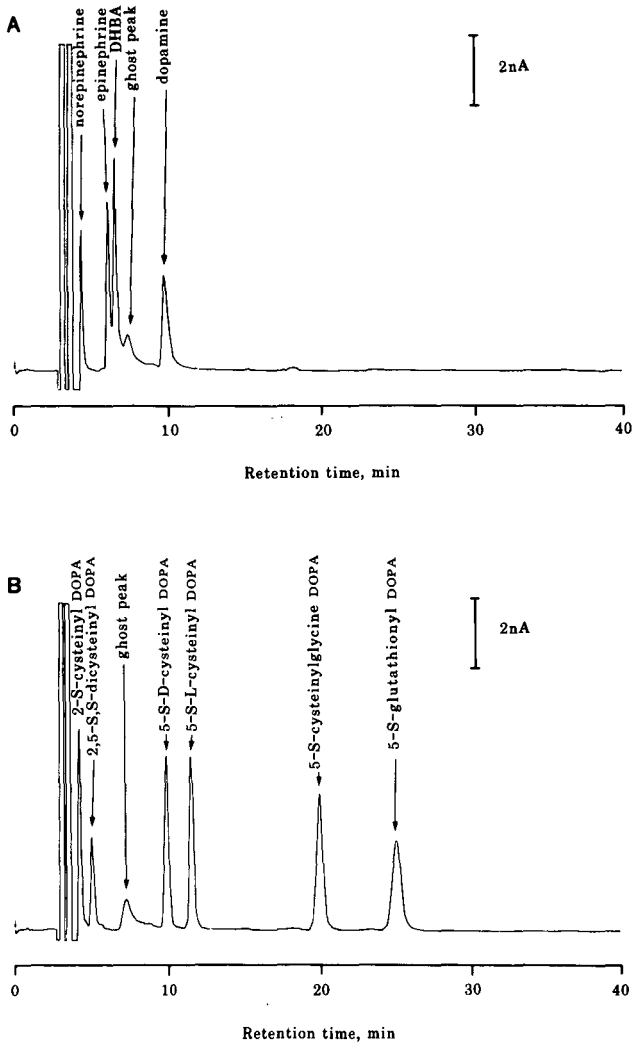

Fig. 5. Separation of compounds of interest after injection into the boronate column and chromatographed after column switching on the reversed-phase column. (A) and (B) as in Fig. 3.

The carry-over was estimated by measurement of the 5-S-L-cysteinyl-L-DOPA peak, obtained in a chromatogram after injection of pure water, following chromatography of a standard of 5-S-cysteinyl-DOPA at a concentration of 50 μM. The carry-over was 0.87% in the first water sample, and 0.22% in the second water injection. Trace amounts of 5-S-cysteinyl-DOPA were also detected in the following two chromatograms corresponding to pure water injection. Because the 5-S-cysteinyl-DOPA concentration can vary considerably in melanoma urines, we did not accept this carry-over, but washed the boronate column with mobile phase II after each injection. By this procedure the carry-over was reduced to 0.03%.

In our first application of a boronate HPLC column to the determination of

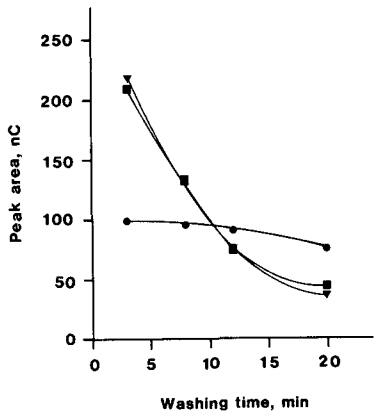

Fig. 6. Stability of 5-S-L-cysteinyl-L-DOPA (●) on the boronate column during prolonged washing, as compared with that of norepinephrine (■) and dopamine (▼). Washing was with mobile phase I, pH 6.0.

5-S-cysteinyl-DOPA we prepared the standard in mobile phase I. The short-term (< 4 h) stability seemed satisfactory, but in extended series 5-S-cysteinyl-DOPA deteriorated, and this solution could not be used for preparing a standard solution because of the risk of oxidation. 5-S-Cysteinyl-DOPA was stable for at least 30 h in 0.1 *M* acetate buffer containing 0.2 m*M* Na_2EDTA (pH 4.0). This was also the case when EDTA was not added. Addition of 5 m*M* sodium metabisulphite to the acetate buffer with EDTA changed the stability of 5-S-cysteinyl-DOPA for the worse. Thus, in subsequent experiments the standard was prepared in 0.1 *M* acetate buffer (pH 4), containing 0.2 m*M* Na_2EDTA.

The choice of optimum washing time depended on the results from the chromatography of urines. We found that a washing time of 8 min was adequate for minimizing the appearance of extraneous peaks. Figs. 7 and 8 depict chromatograms of

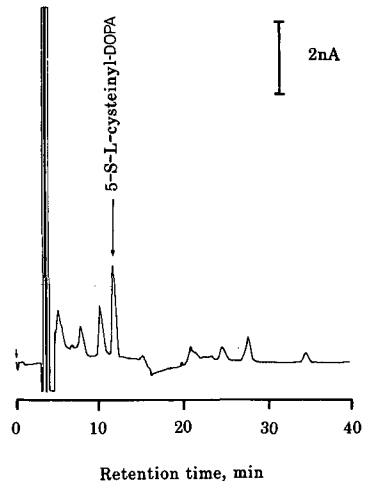

Fig. 7. Urinary chromatogram from an healthy subject. The 5-S-cysteinyl-DOPA concentration was 0.89 μM.

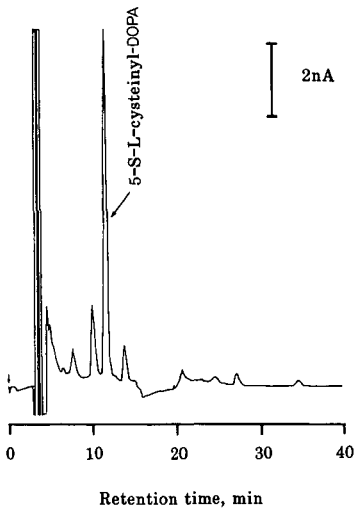

Fig. 8. Urinary chromatogram from a patient with melanoma metastases. The 5-S-cysteinyl-DOPA concentration was 3.42 μM.

urines from an healthy subject and a patient with known melanoma metastases.

The identity of the peak from urine was first confirmed by adding synthetic 5-S-cysteinyl-DOPA to urine and recording of its identical retention. Furthermore, we incubated the urine with the enzyme tyrosinase which oxidizes 5-S-cysteinyl-DOPA to its quinone. This procedure completely abolished the peak and confirmed its authenticity.

We studied the stability of 5-S-cysteinyl-DOPA in urinary samples when applied to the automated sampler standing by for injection. For these studies, we used freshly collected urines, and to obtain fresh alkaline samples, volunteers were given 4–8 g of sodium bicarbonate by mouth 4–8 h before providing urine. It appeared that 5-S-cysteinyl-DOPA in urines at pH > 6.5 was unstable. For routine analysis, we therefore always diluted the urines in two volumes of acetate buffer, which gave a pH value between 4 and 5. This ensured stability of the samples for more than 24 h.

The precision was estimated from repeated (n = 20) analysis of an urinary sample, with standardization before and after ten injections. The mean concentration obtained was 0.767 μM and the standard deviation was 0.011 μM, which gives a coefficient of variation of 1.4%. Furthermore, duplicate analysis of urinary samples with different 5-S-cysteinyl-DOPA concentrations gave similar precision values (Table I). From these data the detection limit for 5-S-cysteinyl-DOPA in urine was calculated as 0.03 μM.

For estimation of the analytical recovery, 5-S-cysteinyl-DOPA in six normal human urines was determined in duplicates (initial concentration 0.18–0.63 μM) and 5-S-cysteinyl-DOPA was added to increase the concentration by 0.59 μM. The increase was estimated to be 0.51-0.57 μM corresponding to a mean (± S.D.) recovery of 93.5 (± 4.1)%.

Comparison of our results with those obtained by the earlier method[12] showed that the present method gave similar values. Regression analysis gave a straight line

TABLE I

PRECISION CALCULATED FROM DUPLICATE ANALYSIS OF 5-S-CYSTEINYL-L-DOPA IN URINE

Number of samples	*Concentration (μM)*		*Standard deviation (μM)*	*Coefficient of variation (%)*
	Mean	*Range*		
32	0.30	0.08–0.52	0.016	5.2
22	0.97	0.55–3.1	0.013	1.4
5	27	5.4–98	0.39	1.4

according to the equation:

$$\text{present method} = 0.96\ (\text{comparison method}) - 0.05\ \mu M\ (n = 58,\ r = 0.97)$$

About 25 determinations can be performed per day with only a few hours of technicians' time. This is a major advantage compared to earlier non-automated methods.

DISCUSSION

Chromatographic separation of catecholamines and catecholic amino acids by use of boric derivatives bound to a support matrix was described more than 10 years ago[22,23]. A boronic affinity HPLC column was later developed[19], and we recently reported its use in a manual column-switching method for determination of 5-S-L-cysteinyl-L-DOPA. Thus, without prior purification, the urine sample is injected into the system at pH 6.0, and 5-S-L-cysteinyl-L-DOPA is adsorbed on the boronate column. By switching to another mobile phase (pH 3.0), the 5-S-L-cysteinyl-L-DOPA is desorbed and chromatographed on the reversed-phase column. Such methods have also been published for the determination of, *e.g.*, dopamine, norepinephrine, epinephrine and DOPAC[24–27].

De Jong *et al.*[27] have described the prerequisites for on-line sample pretreatment on small alumina and dihydroxyboryl-silica for the analysis of catecholamines. The retention of catecholamines and derivatives was determined by injection of standards, applying eluents of pH 2–8. At low pH (< 5), neither the catecholamines nor their derivatives were retained. At pH 8, only derivatives possessing the catechol function (DOPA, α-methyl-DOPA, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylethylene glycol, 3,4-dihydroxyphenylethanol), and compounds containing the amine function (normetanephrine, metanephrine and 3-methoxytyramine) and the classical catecholamines (dopamine, norepinephrine and epinephrine) were retained. Only catecholamines were retained very strongly. The authors concluded that for preconcentration a pH of *ca.* 8 is the best choice.

Cysteinyl-DOPA contains, in addition to the catecholic function, two amino acid groups. This may be the reason why its adsorption on the boronate affinity column is even stronger than that of norepinephrine[15]. We therefore chose a lower pH value (pH 6.0) for adsorption. At this pH value 5-S-cysteinyl-DOPA is retained, while norepinephrine and dopamine are partly eluted (Fig. 6).

We have also tried to use a smaller boronate affinity column (36 mm × 2.0 mm

I.D.). Also with this column satisfactory results were obtained. The precision is similar, but the analysis time cannot be shortened, because the flow-rate has to be reduced during elution at increased pressure.

As is seen from Fig. 3, the retention of dopamine on the analytical column was nearly identical to that of 5-S-D-cysteinyl-L-DOPA. In earlier methods[12,14] we employed this 5-S-cysteinyl-DOPA diastereomer as an internal standard, but due to interference from dopamine it would be unsuitable in the present method. Although use of an internal standard is very convenient in some situations, its use in the present method is unnecessary.

Melanin production has classically been considered to follow two pathways, the DOPA-thioether pathway, which gives pheomelanin, and the indolic pathway, which gives eumelanines. The analysis of melanocyte metabolites as markers of malignant melanoma has been performed since the days of Thormählen, *ca.* 100 years ago[28]. The Thormählen test is positive for indole compounds with a special substitution pattern[29]. Thus this test is not specific for a single compound but for a group of substances, and in the last decade it has been replaced by analysis of 5-S-cysteinyl-DOPA for monitoring melanoma patients[2].

In recent years, modern methods for the analysis of individual indole compounds have been published[4,30]. An increase in their concentrations was found for several of the eumelanin metabolites in the urines of patients with known melanoma metastases[30], but low values have also been reported for a patient with extensive metastases and an high urinary 5-S-cysteinyl-DOPA concentration[31]. Thus, at this time, 5-S-cysteinyl-DOPA seems to be the best marker of malignant melanoma metastasis.

The rapid increase in the incidence of malignant melanoma reported by several countries will greatly increase the demand for a clinical routine method in the near future. The automated method presented in this paper is well suited for the follow-up of patients with suspected melanoma metastases.

ACKNOWLEDGEMENTS

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CHROMSYMP. 1589

COUPLED-COLUMN CHROMATOGRAPHY ON IMMOBILIZED PROTEIN PHASES FOR DIRECT SEPARATION AND DETERMINATION OF DRUG ENANTIOMERS IN PLASMA

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SUMMARY

Columns packed with immobilized α_1-acid glycoprotein and albumin were used in coupled-column chromatography to increase their utility for determining low concentrations of enantiomers in biological samples. The two enantiomers eluted from the protein columns were trapped and compressed on two separate columns, packed with hydrophobic stationary phase, and subsequently transferred to a fourth column for final separation. The overall effect was an increase in efficiency and selectivity. Examples are given of separations of the enantiomers of terbutaline, metoprolol, oxazepam and bupivacaine in plasma. For quantitative determination a single calibration can be used for both enantiomers.

INTRODUCTION

Liquid chromatography (LC) on columns is currently the most useful technique for the separation and determination of enantiomers in biological samples. LC allows great latitude in the chromatographic conditions for special applications. Separation can be achieved either indirectly after derivatization to diastereomers or directly[1]. In the latter instance, a chiral stationary or chiral mobile phase can be used.

Progress in direct separation by LC has been summarized in several books and papers[1–4]. Some guidance on how to choose suitable chromatographic systems for special applications can be obtained from the current literature[5]. However, most of the applications described are for samples in an uncomplicated matrix. As was pointed out in a recent paper[6], application of direct separation to biochemical analysis is not straightforward, especially when very low concentrations of drugs and their metabolites are to be determined. Some of the more severe problems were addressed, *e.g.*, restricted chromatographic conditions, low efficiency, internal standardization and loadability. It was shown that LC with coupled columns and the combination of LC with mass spectrometry can be used to overcome some of these problems. Recently,

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Chu and Wainer[7] utilized LC with coupled columns for the measurement of warfarin enantiomers in serum.

In this work, coupled-column chromatography (CCC) has been further explored with columns packed with immobilized proteins for use in biochemical analysis. Two stationary phases of this type are commercially available, α_1-acid glycoprotein and albumin. Although it has been shown that this type of phase can be used for the separation of a number of compounds, their applications to biochemical analysis are limited. Most important, broad asymmetric peaks are often obtained, which will hamper their use in quantitative work at low concentrations. A means of circumventing this problem is to concentrate the samples eluted as broad peaks by utilizing the effect of peak compression. There are several ways to achieve this, but in principle, peak compression can best be obtained if an analyte is introduced into a column in a "non-eluting" solvent. In reversed-phase chromatography, water is a "non-eluting" solvent, and large volumes can be introduced into columns packed with a hydrophobic chemically bonded phase, *e.g.*, C_{18}. In biochemical analysis, peak compression was used to allow the injection of large sample volumes[8,9], and in CCC to avoid band broadening when analytes were transferred from one column to another[10]. In most applications of protein columns high concentrations of water are used in the mobile phase[11,12].

Thus, in principle, peaks eluted from a protein column can be trapped on a column with a hydrophobic stationary phase for compression and subsequently transferred to another hydrophobic stationary phase for final separation. In this way, the efficiency and symmetry of peaks can be improved, and this will also increase the overall sensitivity and selectivity. This principle has been used here and is exemplified for some selected chiral drugs in plasma samples. The possibility of determining plasma concentrations by CCC is also discussed.

EXPERIMENTAL

Materials

The structures of the chiral drugs used in this study are shown in Fig. 1. Racemic bupivacaine hydrochloride, (*R*)-bupivacaine and (*S*)-bupivacaine, used as the hydrochlorides, were obtained from Astra (Södertälje, Sweden). Racemic metoprolol was used as the succinate and *S*-(−)-metoprolol as hydrochloride. Both were obtained from Hässle (Mölndal, Sweden). The racemate of oxazepam was obtained from KabiVitrum (Stockholm, Sweden). Racemic terbutaline was used as the sulphate and (−)-terbutaline and (+)-terbutaline were used as the hydrobromides. All three were obtained from Draco (Lund, Sweden). Albumin (A7030) and glutathione were obtained from Sigma (St. Louis, MO, U.S.A.), ethanol (99.5%, spectroscopic grade) from Kemetylprodukter (Bromma, Sweden) and methanol and acetonitrile from Rathburn Chemicals (Walkerburn, U.K.). Water was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Bond-Elut C_{18} cartridges were obtained from Analytichem International (Harbor City, CA, U.S.A.). All other chemicals were of analytical-reagent grade and were obtained from E. Merck (Darmstadt, F.R.G.).

The configuration of the CCC system is shown in Fig. 2. The pretreated sample was injected into the protein column (column 1) for separation of enantiomers. For collection and quantitative transfer of each enantiomer eluted from column 1 into

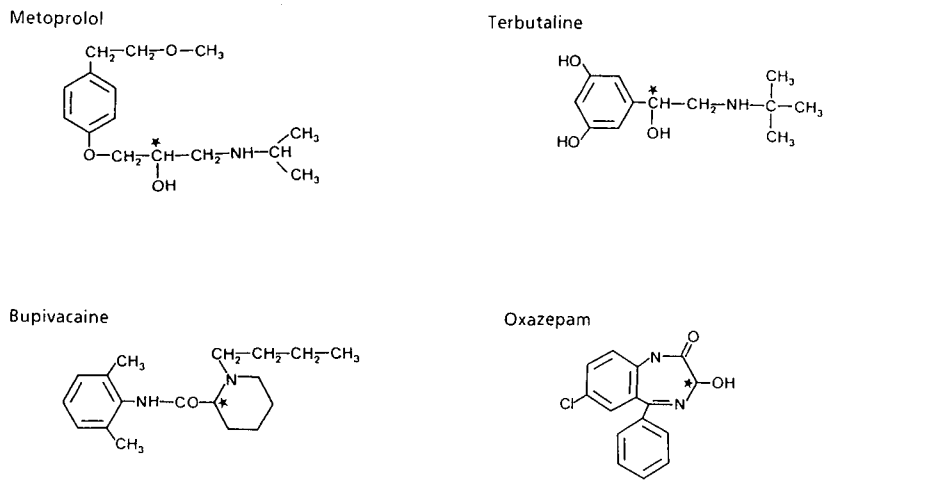

Fig. 1. Structure of chiral drugs used.

columns 2 and 3, a suitable retention time "window", defined by exact times for switching the six- and ten-port valves, was chosen. The "window" was determined by coupling column 1 directly to the detector, and injecting representative amounts (calculated from estimated plasma concentrations) of the racemates. When the first enantiomer started to emerge from column 1, the six-port valve was switched so that the eluate was entering column 2 for trapping of that enantiomer. When the second enantiomer started to emerge from column 1, the ten-port valve was switched so that it could be trapped on column 3. At the same time mobile phase 2 was directed to column 2 for elution of the trapped enantiomer into the achiral column (column 4). After elution of the first enantiomer from that column, the second enantiomer was eluted from column 3 into column 4. All of the eluate from column 1, except for the enantiomers, was directed to waste.

The CCC system was composed of two LKB 2150 pumps (LKB, Bromma, Sweden), a Valco Model CV-6-UHPa-N60 valve (Valco, Houston, TX, U.S.A.) for

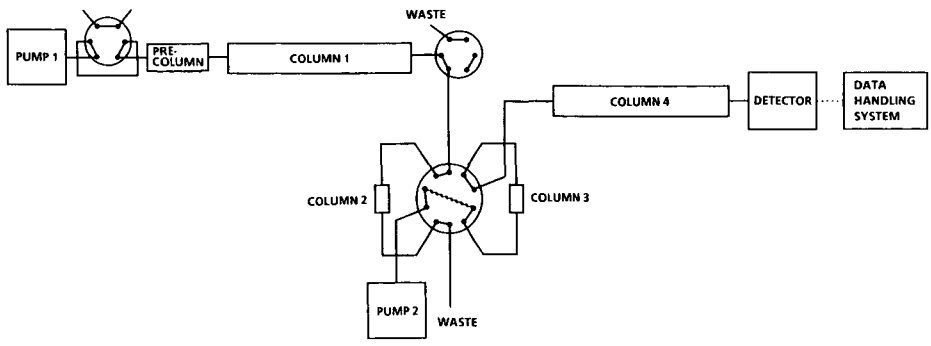

Fig. 2. Schematic diagram of the coupled-column chromatography system used for the separation of some selected chiraBl drugs in plasma: column 1 = chiral (protein); columns 2 and 3 = achiral for trapping; column 4 = achiral.

injection of samples (loop volumes 20–100 μl), and a Valco A-C6U and AC10W (ten-port) valve for column switching. A Model 490 UV detector (Waters Assoc., Milford, MA, U.S.A.) set at 210 nm was used for bupivacaine and a Spectromonitor 111 UV detector (LDC/Milton Roy, Riviera Beach, FL, U.S.A.) set at 230 nm for oxazepam. A Model Rf 535 fluorescence detector (Shimadzu, Tokyo, Japan) set at λ_{ex} = 272 nm and λ_{em} = 306 nm was used for detection of metoprolol. For detection of terbutaline an LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) with a TL-5 glassy carbon electrode and an Ag/AgCl (NaCl, 3 *M*) reference electrode was used. The working potential was 0.9 V. A Chromguard precolumn (Chrompack, Middelburg, The Netherlands) (10 × 3 mm I.D.) packed with a 20-μm pellicular, chemically bonded CN phase was used to protect the protein column.

For separation of the enantiomers (column 1) of bupivacaine, metoprolol and terbutaline, columns (100 × 4 mm I.D.) packed with immobilized α_1-acid glycoprotein of a newly manufactured type from ChromTech (Stockholm, Sweden) were used. Enantiopac columns (100 × 4 mm I.D.) obtained from LKB were used in some additional experiments. Columns packed with bovine serum albumin are commercially available, but in this study bovine serum albumin was immobilized *in situ* on to Nucleosil 300-5 silica (Macherey, Nagel & Co., Düren, F.R.G.) by a procedure described elsewhere[13]. This column was used for separation of the enantiomers of oxazepam.

The mobile phases used for separation of the enantiomers (mobile phase 1) consisted of 20 m*M* phosphate buffer (pH 7.5) for terbutaline, 10 m*M* phosphate buffer (pH 7)–2-propanol (91:9, v/v) for bupivacaine, 20 m*M* phosphate buffer (pH 7)–2-propanol (99.75:0.25, v/v) for metoprolol and 50 m*M* phosphate buffer (pH 6.8)–1-propanol (98:2, v/v) for oxazepam. The flow-rates were 0.8 ml/min and 1.0 ml/min (oxazepam).

Waters Guard-Pak columns (4 × 5 mm I.D.) packed with μBondapak C_{18} was used as trapping columns (columns 2 and 3). For achiral chromatography (column 4), the chemically bonded phases 5-μm Nucleosil C_{18} and Nucleosil C_8 (bupivacaine) were used packed in 100 × 4 mm I.D. (bupivacaine), 100 × 4.6 mm I.D. (metoprolol, terbutaline) and 200 × 4.6 mm I.D. (oxazepam) stainless-steel columns. The mobile phases (mobile phase 2) consisted of 50 m*M* ammonium acetate buffer pH 6.0–methanol (90:10, v/v) for terbutaline, 50 m*M* phosphate buffer (pH 3.0)–acetonitrile (72:28, v/v) for bupivacaine, 500 m*M* phosphate buffer (pH 3.0)–water–acetonitrile (10:70:20. v/v/v) for metoprolol and 2 m*M* potassium dihydrogenphosphate–acetonitrile–methanol (42:1:57, v/v/v) for oxazepam. The flow-rates were 1.0 ml/min and 1.5 ml/min (oxazepam).

Methods

Bond-Elut C_{18} solid-phase extraction columns were used for sample work-up by published methods[6,14,15] for terbutaline, oxazepam and metoprolol. Bupivacaine was extracted from plasma with hexane from alkaline medium by a previously described method[16]. The sample work-up procedures were followed in detail, *e.g.*, the same volumes of plasma as given therein were used.

Standards were prepared by adding racemate to plasma at relevant concentrations. For metoprolol and oxazepam calibration studies were also undertaken and the

concentration ranges were 7–3500 nmol/l and 0.8–17 μmol/l, respectively. For calibration the area under the chromatographic peak was measured and correlated with the actual concentrations.

The maximum overall efficiency (number of theoretical plates) that could be obtained was determined by injection of standard solutions of the respective drugs directly into the achiral column (column 4) with a loop of negligible volume (20 μl). These values were compared with those obtained by using the whole CCC system.

Known injected amounts of the respective racemates were used to calculate the recovery in the transfer from column 1 to column 4. Peak areas after passage through the whole CCC system were determined at relevant concentrations and compared with those obtained after direct injection into column 4. For calculation of peak areas the Nelson Analytical (Cupertino, CA, U.S.A.) software program Access Chrom was used. The total absolute recovery, including sample work-up, was only determined for metoprolol by adding known amounts of the racemate to blank plasma and taking it through the whole analytical procedure. For bupivacaine, metoprolol and terbutaline pure enantiomers were used to determine their order of elution on the protein columns.

RESULTS AND DISCUSSION

The strategy for the separation of the enantiomers on protein columns (column 1) was taken from the literature[11,12]. In each instance, complete separation could be achieved by using high concentrations of water, and thus the heart-cut sample could be compressed by using a hydrophobic packing material for trapping the enantiomers eluted from the chiral column (column 1). The eluate from column 1 could either be directly transferred to column 4 or trapped in a loop or on a small column. Sampling via a loop or direct transfer of the relatively large volumes used here (1.4–4.2 ml) was tried, but gave rise to pressure surges and a high background, making quantitative work at low concentrations more difficult or impossible. Instead, small columns packed with a hydrophobic packing material and relatively large particles (> 20 μm) were chosen. Small columns reduced the trapped volumes to less than the void volume of the column (< 100 μl). Pressure surges during valve switching were also smoothed out.

The amount of retardation and thus peak compression in general are determined by the capacity factor for the analyte and can be judged from the following equation[10]:

$$V_i = V_0 \cdot \frac{1}{1 + k_i^{ci}}$$

where V_i = the contracted volume containing compound i, V_0 = trapped sample volume transferred to column i and k_i^{ci} = capacity ratio of compound i when using the injection medium of trapped volume as the mobile phase on column i.

In the two step transfer (Fig. 2) of the enantiomer from the protein column (column 1) via the trapping column (column 2 or 3) to the final achiral column (column 4), it is important not to introduce extra band broadening by the transfer process. For that reason, mobile phase 1 must be a stronger eluting solvent on column 1 than on the trapping column (column 2 or 3). Further, mobile phase 2 must be a stronger eluting solvent on the trapping column than on column 4.

TABLE I

EFFICIENCY OBTAINED FOR SOME SELECTED DRUG ENANTIOMERS ON PROTEIN PHASES ALONE AND AFTER COUPLED-COLUMN CHROMATOGRAPHY

Protein phase	*Drug*	*Number of theoretical plates*				*Amount injected (nmol)*
		Chiral column (column 1)		*CCC (column 4)*		
		First-eluted enantiomer	*Second-eluted enantiomer*	*First-eluted enantiomer*	*Second-eluted enantiomer*	
α_1-Acid glycoprotein	Metoprolol	800	920	3800	3800	0.04
	Terbutaline	1300	1400	3500	3500	0.05
	Bupivacaine	2200	1600	3500	3500	8
Albumin	Oxazepam	700	430	6400	6400	13

The maximum efficiency that could be obtained for the whole CCC system was estimated by injection of standards in small (negligible) volumes directly into column 4. In all instances, the chromatographic parameters and the packing material used in column 4 were based on earlier published methods[6,15,17,18]. It was shown that maximum efficiency of the CCC system was obtained in all instances, and this indicates an optimal design of the CCC system.

In Table I and Figs. 3 and 4, examples are given to show how the efficiency was improved when CCC was used. It should be noted that no attempt was made to choose the most efficient achiral column. Thus, an even greater increase in efficiency could possibly have been obtained.

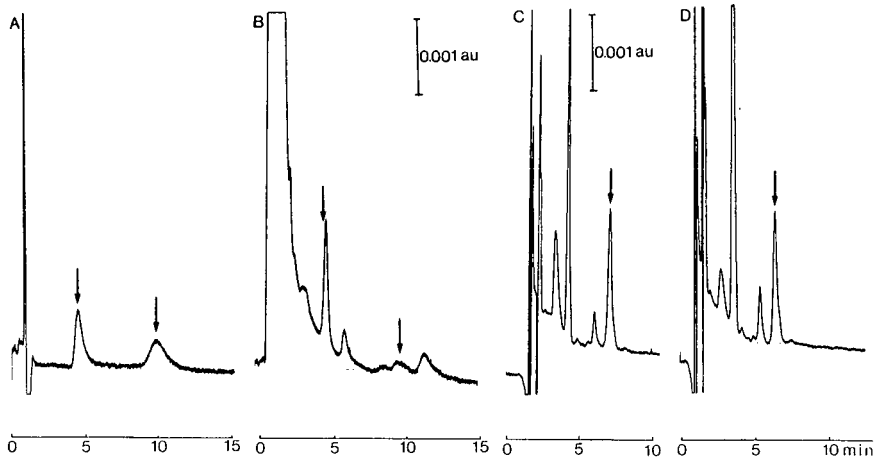

Fig. 3. Chromatograms obtained after separation of racemic oxazepam. (A) 70 pmol (racemate) of standard solution injected directly into the albumin column (column 1). (B) Blank plasma after separation on the albumin column alone. Arrows indicate positions of the enantiomers of oxazepam. (Only the racemate was used in the experiment and therefore no identification of the respective enantiomers is given.) (C) Spiked plasma, containing 3.3 μmol/l of racemic oxazepam after separation by CCC. Amount injected: 33 pmol (racemate). Arrow indicates oxazepam enantiomer (first-eluted enantiomer). (D) As in (C) (second-eluted enantiomer).

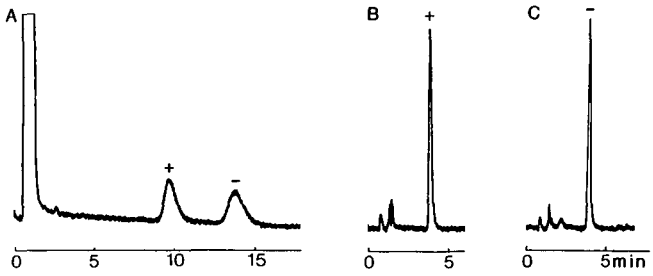

Fig. 4. Chromatogram obtained after separation of racemic metoprolol. (A) 35 pmol (racemate) of standard solution injected directly into the α_1-acid glycoprotein column (column 1). (B) Spiked plasma, containing 70 nmol/l of racemic metoprolol after separation by CCC. Amount injected: 58 pmol (racemate). (+)-Metoprolol, first-eluted enantiomer. (C) As in (B). (−)-Metoprolol, second-eluted enantiomer.

In Fig. 3 the selectivity achieved by using CCC is obvious. A more pronounced effect is exemplified in Fig. 5. In this instance, the gain in efficiency is only two- to three-fold (see Table I), but the selectivity is increased dramatically by CCC.

The recovery in the transfer from column 1 to column 4 was 100% in all instances. The absolute recovery, including sample work-up, was estimated to be 85% for metoprolol at 0.18–18 μmol/l.

In general on the achiral column, enantiomers at equal concentration will give the same area under the chromatographic peak. This is useful in practice because a single calibration graph can be used for their determination. For example, when peak area *versus* concentration was plotted for each enantiomer of metoprolol and

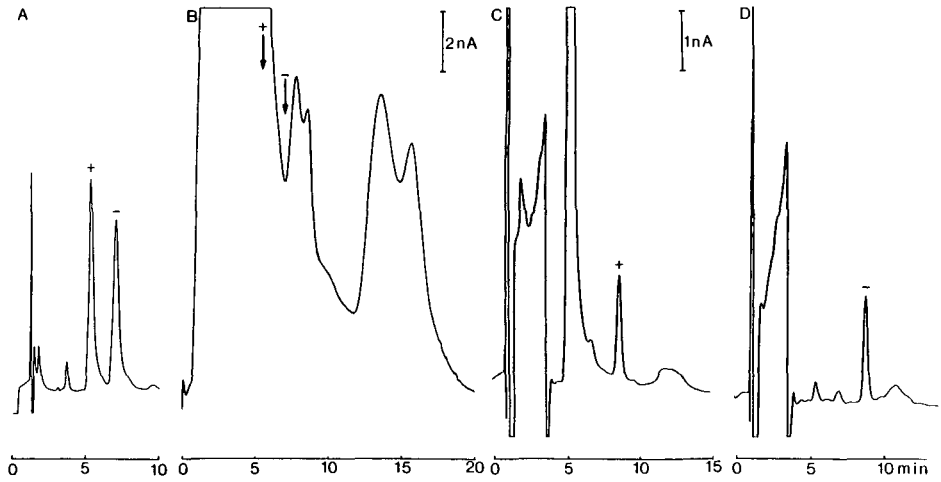

Fig. 5. Chromatograms obtained after separation of racemic terbutaline. (A) 50 pmol (racemate) of standard solution injected directly into the α_1-acid glycoprotein column (column 1). (B) Blank plasma after separation on the α_1-acid glycoprotein column alone. Arrows indicate positions of the enantiomers of terbutaline. (C) Spiked plasma, containing 50 nmol/l of racemic terbutaline after separation by CCC. Amount injected: 21 pmol (racemate). (+)-Terbutaline, first-eluted enantiomer. (D) As in (C). (−)-Terbutaline, second-eluted enantiomer.

oxazepam, overlapping calibration graphs were obtained for each pair of enantiomers ($r = 0.999$).

In the examples given here, simple mobile phases (phosphate buffers and propanol) were used on the protein columns, because chiral separation can often be achieved under such conditions. However, it has been shown that the addition of different kinds of modifiers to the mobile phase can be used to increase the chiral selectivity[11] and extends the applicability of protein columns to more compounds. It is believed that the proposed approach for application of CCC to chiral separation on immobilized protein columns can also be used in such instances.

The load capacity of protein columns is often low, and this might sometimes preclude the use of a protein column as the first column in the CCC system. In our experience, protein columns are not rugged and can lose their usefulness rapidly if handled incorrectly. Therefore, crude samples should not be injected directly into these columns. Instead, use of sample work-up and the use of a precolumn, as used in this work, is recommended.

For the proposed technique to work, resolution on the chiral column must be complete at all relevant concentrations. In Fig. 6, an example is given of the separation of bupivacaine on a first-generation α_1-acid glycoprotein column. Although the resolution could be judged as good (Fig. 6A), severe peak tailing can be seen. It was impossible to recover the respective enantiomers completely without contamination with the other enantiomer and it is not easy to observe the fronting on the second peak. Injection of equal amounts of the pure enantiomers revealed that severe fronting on the second enantiomer was hidden under the first-eluted enantiomer (Fig. 6B). In Fig. 6C, a separation is shown of the same amount of enantiomers of bupivacaine on a recently available α_1-acid glycoprotein column from ChromTech. In this instance, the resolution was nearly complete. As was mentioned above, the efficiency and symmetry of peaks on protein columns are often poor. Although the resolution seems to be complete, caution is recommended when utilizing the approach described in this work.

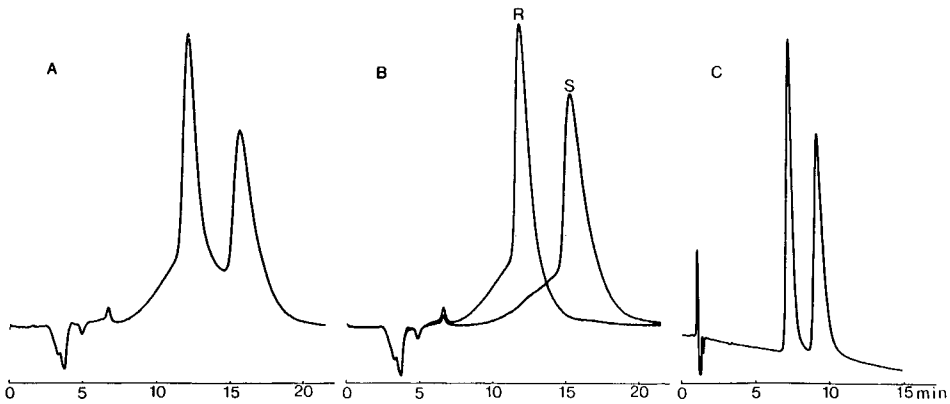

Fig. 6. Chromatograms of bupivacaine. (A) Racemic mixture separated on a first-generation α_1-acid glycoprotein column (Enantiopac). Amount injected: 3 nmol (racemate). (B) Enantiomers injected separately. Amount injected: 1.5 nmol (each enantiomer). (C) Racemic mixture separated on a newly manufactured α_1-acid glycoprotein column (ChromTech). Amount injected: 1.3 nmol (racemate).

CONCLUSION

The use of columns packed with immobilized proteins for the separation and determination of enantiomers in biological samples can be further extended if they are combined with achiral columns in a CCC system. It is possible to obtain an increase in efficiency (sensitivity), peak symmetry and overall selectivity. To achieve this it is necessary to run the whole CCC system in the reversed-phase mode, and to place the protein column first. For many of the enantiomers which have been separated on protein columns methods based on achiral reversed-phase LC have been described in the literature for the determination of the sum of the enantiomers in biological samples. In principle, it is therefore possible to combine such an achiral LC system directly with the chiral LC system to obtain a complete method for the determination of the enantiomers. This is exemplified here for four compounds of pharmaceutical interest.

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CHROMSYMP. 1608

INTRODUCTION OF WATER AND WATER-CONTAINING SOLVENT MIXTURES IN CAPILLARY GAS CHROMATOGRAPHY

I. FAILURE TO PRODUCE WATER-WETTABLE PRECOLUMNS (RETENTION GAPS)

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SUMMARY

The introduction of large volumes of water or water-containing solvent mixtures in capillary gas chromatography (GC) is of wide interest for the direct analysis of aqueous samples, and also for coupling reversed-phase liquid chromatography to GC. Water is a very difficult solvent for GC owing to its high surface tension (poor wetting properties), the very large volume of vapour produced per unit volume of liquid, the high boiling point and the poor properties concerning solvent effects. This paper deals with failures to transfer aqueous samples by retention gap techniques. Precolumns that could serve as retention gaps were either water-wettable or sufficiently inert for running GC, but never both at the same time.

INTRODUCTION

Water —an important sample matrix and solvent

A large proportion of the samples to be analysed by gas chromatography (GC) have an aqueous matrix, including most biological samples and many environmental samples. In some instances (*e.g.*, drinking water), more or less direct injection of the sample would be attractive for accelerating the analysis or to allow fully automatic on-line analyses. However, to achieve the sensitivity required, the volumes of water to be introduced must substantially exceed those introduced today.

For most samples (waste waters, biological fluids), an enrichment and a clean-up step are a prerequisite. Convenient on-line systems involve solid-phase extractions or (which is very similar) a preconcentration and preseparation by liquid chromatography (LC) on reversed-phase packing materials. Straight desorption or elution from these systems almost necessarily occurs with reversed-phase eluents, often containing substantial proportions of water. Switching to normal-phase LC systems with solvents of low polarity is possible, but presupposes complete removal of the water by

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evaporation as an intermediate step[1,2]. On-line extraction into solvents of low polarity is an alternative that can profit from work carried out for LC coupled with mass spectrometry[3] or flow-injection analysis (FIA)[4], and was used by Roeraade[5] and Fogelqvist *et al.*[6] for on-line water analysis. However, switching to normal-phase systems is attractive only if a more direct transfer within the reversed-phase system is impossible.

A further application, involving the transfer of large amounts of water to the capillary gas chromatograph, concerns reversed-phase LC coupled to GC for samples where preseparation by reversed-phase LC is superior to that by normal-phase LC.

Previous work

Direct injection of water is not new to GC. However, in capillary GC, injection of aqueous samples has been successful in only three instances. First, if very small volumes were introduced, *e.g.*, by the split method, where the presence of condensed water in the column inlet is unlikely or the amount is too small to form a droplet that can move further into the column (see Schomburg and co-workers[7,8], who also found that capillaries coated with Carbowax produced better results than apolar columns, tracing this back to wettability problems). Second, if the column temperature was high enough to rule out the flow of condensed water in the column; this was achieved by the splitless method or by on-column injection under special conditions[9].

Third, introduction of water by normal on-column injection (1–2 μl) was successful in a special case[10,11], the determination of volatile C_1–C_2 halocarbons in waters. A 1–2 μl volume of water is injected on-column into a capillary column coated with an extremely thick film of an apolar stationary phase. A substantial proportion of the water passes as a liquid through the whole column, as noted by the deposition of the dissolved salts in the column outlet, heated in the detector block. Solutes retained by the water are spread throughout the column (extreme band broadening space) and cannot be analysed. However, volatile, apolar components are not trapped and instantly evaporate from the water matrix in the column inlet. The use of uncoated precolumns is not only unnecessary, but also pointless as long as flooding into the separation column cannot be prevented.

An indirect injection method through multi-dimensional GC was proposed by Schomburg and co-workers[12,13]. It involves removal of water by GC on a column packed with Tenax. A different approach was used by Zlatkis *et al.*[14], who extracted the organics from the aqueous solution into the stationary phase of a GC capillary column before reconcentrating the sample and running the analysis. Experimenting with a similar approach, we were not very successful[15].

Water — a difficult solvent

Water certainly is one of the most difficult solvents for sample introduction in GC. It does not wet commonly used capillary surfaces (see below), has a high boiling point, requiring high solvent evaporation temperatures, and produces a very large volume of vapour per unit volume of liquid (about six times that of hexane). Further, it retains solutes poorly, giving rise to partial solvent trapping up to components eluted at high temperatures, and is chemically aggressive, destroying the deactivation of precolumns and possibly certain solutes.

Nevertheless, some transfer techniques seem to be promising, allowing the

introduction of water in fairly large amounts when some special precautions are taken. Results will be presented in a series of papers, describing different aspects and techniques. This paper reports experiments and observations made when we were trying to find water-wettable precolumns suitable for GC. Further papers will deal with the wettability of silylated surfaces by mixtures of organic solvents with water (*e.g.*, reversed-phase eluents) and their application to retention gap techniques, and the stability of precolumn deactivations when used for the introduction of water or mixtures containing water. Finally, promising results were obtained by concurrent evaporation of solvents (LC eluents) containing water when co-solvent effects were applied.

Retention gap technique

Retention gap techniques[16] are used for injecting large volumes of liquid, *e.g.*, by the on-column injection technique[17], or for coupling LC to GC (by the use of the conventional retention gap technique or partially concurrent eluent evaporation). In coupled LC–GC, retention gap techniques are preferred to the alternative transfer techniques, involving concurrent eluent evaporation, when it is important to obtain solvent trapping. Solvent trapping is the main solvent effect allowing elution of sharp peaks of accurate size, starting at the column temperature during eluent evaporation. Uncoated precolumns are also used as a column guard, retaining involatile sample by-products[18,19], but this is not of interest in this paper.

The sample liquid, driven by the carrier gas, is allowed to spread into a precolumn of low retention power (retention gap). As the carrier gas continues to flow during the sample transfer process, the solvent evaporates from the rear to the front of the sample film. This direction of the solvent evaporation, the prerequisite for creating solvent trapping effects[20], presupposes a column temperature below the solvent boiling point corrected for the current carrier gas inlet pressure.

To obtain solvent trapping, a price must be paid. The flow of the sample liquid into the column, caused by the flow of the carrier gas, spreads the sample material over a relatively long capillary section, the so-called flooded zone. The resulting "band broadening in space" must be reconcentrated at the entrance of the separation column by using the retention gap effect[21]. The latter is based on a large difference in retention powers between the "flooded" column inlet and the separation column. This is usually achieved by using a precolumn without a stationary phase film. This inlet must be of sufficient length to retain the liquid sample; no liquid must run into the separation column.

Wettability of the precolumn

Retention gap techniques rely on the formation of a sample film on the wall of the precolumn, which in turn presupposes wettability. For instance, a 1 m × 0.32 mm I.D. precolumn retains 3–6 μl of liquid if the surface is wetted (flooded zones of 17–30 cm/μl, depending primarily on temperature)[22,23]. However, a single microlitre of liquid runs through several metres of a capillary column, when not wetting the surface, leaving droplets here and there (Fig. 1). Under such conditions, unreasonably long precolumns would be required. Further, the volume of liquid retained per unit length of the precolumn becomes unreliable, as there is no control over how often a droplet of liquid remains on the capillary wall. Hence, retention gap techniques become awkward as soon as the sample does not wet the precolumn surface.

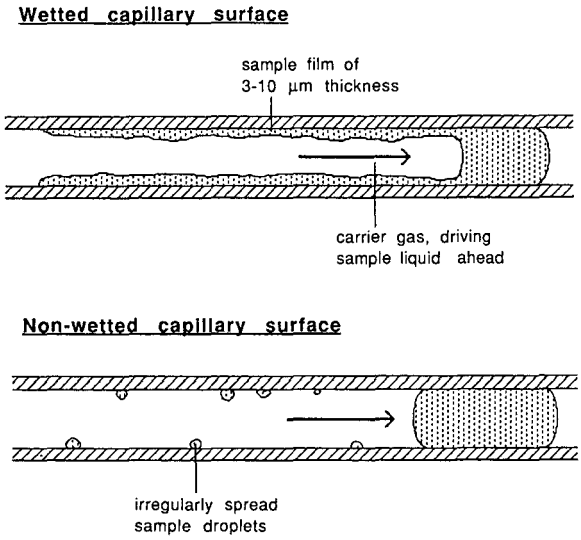

Fig. 1. Influence of wettability of the capillary precolumn wall on retention of liquid. Film formation causes 3–6 µl of liquid to be retained per metre of a 0.32 mm I.D. capillary, whereas this volume is far smaller and poorly reliable for a non-wetting liquid.

Wettability of the precolumn surface must be achieved by a compromise with other properties: the surface must be well deactivated to rule out adsorptivity towards the solutes to be analysed, *i.e.*, the precolumn must not deteriorate the performance of the GC system. Further, the deactivation must be thermostable up to high temperatures (350°C) and chemically stable when in contact with the solvents during solvent evaporation. As will be shown in a subsequent paper, the latter is a serious problem, particularly when dealing with water.

Surface tensions of solvents

Wettabilities of differently treated precolumns by the most important organic solvents have been tested previously[22]. It was pointed out that the critical parameter was the surface tension and not the polarity of the solvent. For instance, wettability with ethers, 1-propanol and 2-propanol was found to be more easily achieved than with benzene or dichloromethane.

As a first approximation, wettability is expected to be achieved if the surface tension of a liquid is below the critical energy of the surface to be wetted or, more pragmatically, if adhesion forces exceed the cohesion forces. Surface tensions for some important solvents are given in Table I (from refs. 24 and 25). Some of the values given are extrapolated from others at temperatures near that listed. Surface tensions decrease linearly with increasing temperature (Eötvös rule).

Acetonitrile, benzene, dichloromethane, dioxane and toluene are recognized as difficult solvents, but the surface tensions at 25°C only go up to 34 dyne/cm. Water, however, has a surface tension far exceeding that of all other solvents, namely 72 dyne/cm. This explains the experimental finding that it is very difficult to achieve a surface that is water-wettable.

TABLE I

SURFACE TENSIONS OF VARIOUS SOLVENTS

Solvent	*Surface tension (dyne/cm)*			
	25°C	*50°C*	*70°C*	*100°C*
Acetone	22.5	19.5		
Acetonitrile	28	24	22	
Benzene	27.7	23.8	22	18
Chloroform	26.2	22.5	20	
Cyclohexane	25.7	22.8	20.4	
Dichloromethane	27.5			
Diethyl ether	16.7	12.9	10.7	
Dioxane	34	30.9	28.4	
Ethanol	21.5	19	17	14.7
+20% water	24	22.5		
+50% water	29.5	28		
+75% water		34		
Ethyl acetate	22.2	19	18	14
n-Hexane	17		13.5	
Methanol	22.5		17.5	14.8
+10% water	25	22.5		
+20% water	27	25		
+50% water	35	33		
+75% water	46	43		
+90% water	58	55		
n-Octane	20	18	16	
2-Propanol	20.5	19	17	
1-Propanol	23	21	19	17
Toluene	27	23.5	22	19
Water	72	68	64	59

Critical surface energies

To achieve wettability by samples with a high surface tension, the capillary surfaces should have properties that create strong interactions with the sample liquid. These properties are usually described in terms of critical surface energies, the measurement of which was recently reviewed by Ogden and McNair[26]. Table II lists critical energies (tensions) of some glass or fused-silica surfaces, taken from refs. 27–31. Analogously deactivated glass and fused silica result in almost identical critical surface energies.

Many surface energies appear to be variable, as is known, *e.g.*, from problems concerning the coating of fused silica with Carbowax. Critical surface energies given for raw glasses appear to be very low, while the value for cyanopropylhydrosilioxane-treated fused silica is higher than expected from our experience concerning the wettabilities of similar surfaces.

From the critical surface energies given in Table II, the choice of a deactivation providing wettability for solvents with high surface tensions seems obvious: silylation with cyanopropylhydrosiloxane or deposition of a thin layer of immobilized Carbowax. However, both critical surface energies are still too low to allow water to spread on these surfaces.

TABLE II

CRITICAL SURFACE ENERGIES OF GLASS AND FUSED-SILICA SURFACES TREATED WITH DIFFERENT REAGENTS

Treatment	*Critical surface energy (dyne/cm)*
Untreated Pyrex glass	31.5
Untreated soft glass	30
Soft glass, HCl etched	>52
Raw fused silica	28–50
Treated fused silica:	
20% nitric acid, 200°C, 10 h	46
Hexamethyldisilazane (HMDS) or D_4	20.2
Diphenyltetramethyldisilazane (DPTMDS)	33.1
Tetraphenyldimethyldisilazane (TPDMDS)	35.3
Triphenylsilylamine	36.6
Cyanopropylhydrosiloxane	≥49
Carbowax	≈44

Data on critical surface energies and tensions must be considered with care. For instance, raw fused silica was poorly wetted by benzene or dichloromethane, and wettability of DPTMDS-treated glass or fused silica with methanol is critical, despite the fact that the surface tension of methanol is far below the critical energy of the phenylsilylated surface. Such discrepancies are probably due to the dependence of wettability on the nature of the interactions, which in turn depend on the chemical functionalities of the solvent and the surface.

EXPERIMENTAL

Etching of soft glass

Soft glass of 0.52 mm I.D. was etched by filling the capillary with 2–3% potassium hydroxide solution and heating at 110°C for 4 h. The capillary was thoroughly rinsed with water and heated at 350°C for dehydration. The resulting capillary is whitish owing to a strongly roughened internal surface.

Carbowax deactivation

Carbowax "baked" to the capillary surface. Carbowax deactivation was achieved in two ways. First, the classical method, introduced by Aue *et al.*[32] and Cronin[33] was applied, designed for deactivating capillary surfaces before coating them with a stationary phase. The capillary was rinsed with a 0.1% solution of Carbowax 20M in dichloromethane, followed by baking at 280°C for 2 h. The excess of Carbowax was rinsed out with dichloromethane, leaving behind a layer of non-extractable (bonded?) Carbowax.

Carbowax "bonded" to the surface. The capillary was statically coated with a very thin layer of immobilized Carbowax, using a method analogous to that originally introduced by Traitler *et al.*[34]. Glass capillaries were leached and dried in a stream of carrier gas at 350°C for 4 h. They were then coated with a 50 ppm solution

of Carbowax 20M in dichloromethane, containing glycidoxypropyltrimethoxysilane or vinyltriethoxysilane and dicumyl peroxide (10% of each, relative to the Carbowax). After pumping off the solvent, the capillary was flushed with nitrogen and sealed. The reagents were allowed to react at an oven temperature increasing at 3°C/min from 160 to 200°C; the final temperature was maintained for 8 h.

In situ polymerization of polyethylene glycol

A series of experiments were directed towards polymerizing ethylene oxide to short polyethylene glycol chains, bonded to the silanol groups of the leached glass surface. The leached and dried glass capillary was flushed with hydrogen chloride gas, then with ethylene oxide. The capillary ends were sealed in the flame and the capillary was heated at 200°C for 2 h. From determinations of the resulting retention power (comparing the retention times with those of columns of known film thickness), we deduced that nearly complete polymerization had occurred. However, about 60–80% of the polyglycol polymer was extractable with dichloromethane, alcohols or water, indicating poor attachment to the surface silanols.

Leaching and silylation

Soft glass was leached according to Grob[35] by filling 93% of the capillary with 18% hydrochloric acid and heating at 150°C overnight. The capillary was rinsed with 1% hydrochloric acid and dried statically at 280°C for 2 h, applying a vacuum at both ends. Silylation occurred with 1,3-bis(3-cyanopropyl)tetramethyldisiloxane (Petrarch, Bristol, PA, U.S.A.). A plug of the reagent was pushed through the column, the air was displaced with nitrogen and the capillary was sealed and heated at 360°C overnight. The capillary was then rinsed with toluene, methanol and diethyl ether. Duran glass was leached at 160°C, the other conditions being the same as above.

Barium carbonate treatment

The barium carbonate treatment corresponded to that applied for the preparation of polar columns (B1)[36] and resulted in a roughened surface.

Test of wettability

Wettability was tested on 5 m × 0.52 mm I.D. sections of the capillary of interest, with the exit connected to a 10 m × 0.32 mm I.D. whitish glass capillary that allowed us to observe water leaving the capillary. The volume of water injected was increased until some water passed from the capillary to be tested into the whitish capillary. The carrier gas flow-rate was adjusted to 10 ml/min (1 bar inlet pressure) and the oven temperature was 70°C. In some instances, the front of the flooded zone could be directly observed. Lengths of flooded zones below 30 cm/μl were interpreted as indications of wettability; longer flooded zones (and usually they rapidly exceed 1–2 m) indicate that no stable film of liquid was formed.

Adsorptivity of precolumns

Precolumns were connected to an apolar separation column and tested by injection of the standard test mixture[37] or a mixture of alkanes.

RESULTS AND DISCUSSION

Our intention was to find a glass or fused-silica surface that is well deactivated and wettable by water. We did not succeed, as we obtained either deactivation or wettability, but not both at the same time. Apparently, interaction forces (essentially the same as adsorption) get out of control as soon as the surface is of sufficient activity to be water-wettable. The forces between the liquid and the capillary surface, required to achieve wettability, must be so strong that the adsorptivity of solutes becomes unacceptable.

The results are summarized in Table III. Where wettability was achieved, the length of the zone flooded by water is indicated. Capillaries had an I.D. of 0.52 mm (causing flooded zones to become shorter than for 0.32 mm I.D. capillaries).

Raw fused silica was not wetted by water, in contrast to the raw glasses tested. However, raw glasses are too adsorptive to allow their use as GC precolumns. The same is true of leached glass (very high concentration of silanols on the surface).

Silylated surfaces (introducing cyanopropyldimethylsilyl or phenyldimethyl groups) were well deactivated but not wetted.

Carbowax deactivation

The most commonly applied technique for reducing the critical surface tension of water involves the use of detergents. Addition of detergents to aqueous samples is impossible as the detergents would accumulate at the front end of the flooded zone after evaporation of the water. Therefore, we wanted to bond them to the capillary surface. Carbowax was the non-ionogenic detergent chosen.

It was repeatedly found that Carbowax-deactivated precolumns were not wettable during the first test but that good wettability was observed later, independent

TABLE III

FUSED-SILICA AND GLASS SURFACES, TESTED FOR WATER WETTABILITY AT 70°C

Capillary surface	*Wettability*	*Length of flooded zone (cm/μl)*
Raw fused silica	No	
Raw soft glass	Yes	20
Deactivated with Carbowax	Yes	15[a]
Soft glass, etched (roughened) with KOH	Yes	17
+ Deactivated with Carbowax	Yes	14[a]
+ Leached + deactivated with dicyanopropylmethyldisiloxane (DCPTMDS)	No	
+ leached + deactivated with diphenyltetramethyldisilazane	No	
Raw Duran glass	Yes	13
Leached	Yes	13
Leached + deactivated with Carbowax	No	
Leached + ethylene oxide	No	
Leached + silylated with DCPMTDS	No	
Leached + $BaCO_3$ treatment	Yes	15
Leached + $BaCO_3$ treatment + Carbowax	Yes	16[a]

[a] Probably only after removal of the Carbowax by water.

of whether Carbowax was "baked" to the capillary surface or "bonded" to it, using silane reagents and cross-linking with a peroxide. We interpret these findings by assuming that the Carbowax layer was removed by the water. With a long glass capillary precolumn, some droplets of accumulated material (Carbowax), located near the front end of the flooded zone, could even be observed visually.

While freshly Carbowax-deactivated surfaces were inert, they became highly adsorptive after a few transfers of water. Finally, even alkanes were eluted as strongly tailing peaks. This confirms our assumption that the deactivation of water-wettable surfaces was completely destroyed. From these experiments, we conclude that smooth, Carbowax-coated surfaces are not wetted by water and that Carbowax, bonded to the surface either by "baking" or by bonding through silanes, is readily stripped away by water.

Roughened surfaces

As is known from earlier procedures used for capillary columns with polar stationary phases, roughening the surface of the internal capillary wall improves wettability. We hoped that this would compensate for the reduced surface energy resulting of better deactivation. However, these hopes were dashed; roughened, leached, and silylated surfaces were not wetted by water.

High resistance of water plugs

Using 0.32 mm I.D. precolumns and injecting 20–100 μl of water, we experienced very high resistance of the water plugs in water-wetted column inlets. At normal inlet pressures, the water plugs rapidly came to a stop and blocked the carrier gas flow. Inlet pressures exceeding 1 bar had to be applied to keep the water plugs moving at all. This prompted us to use wide-bore (0.52 mm I.D.) capillaries as precolumns for the introduction of water or solvent mixtures containing a high proportion of water.

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INTRODUCTION OF WATER AND WATER-CONTAINING SOLVENT MIXTURES IN CAPILLARY GAS CHROMATOGRAPHY

II. WETTABILITY OF PRECOLUMNS BY MIXTURES OF ORGANIC SOLVENTS AND WATER; RETENTION GAP TECHNIQUES

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SUMMARY

The wettability of phenyl- and cyanosilylated precolumn surfaces by mixtures of organic solvents and water (*e.g.*, typical reversed-phase eluents) was tested. Depending on the organic solvent, mixtures with high water concentrations, such as 1-propanol containing up to 70% water, still wet such standard precolumn surfaces. However, when retention gap techniques were applied, another requirement proved to be more limiting: water must evaporate at least as rapidly as the organic solvent, otherwise water is left behind the evaporating solvent mixture and, as water does not wet these surfaces, it floods the capillary. Hence high-boiling solvents are required; azeotropically boiling mixtures facilitate the work. It is shown that 1-propanol with up to 28% water allows the introduction of large volumes by the retention gap technique.

INTRODUCTION

In Part I[1] we described experiments that failed to find capillary precolumns of glass or fused silica that are both water-wettable and sufficiently well deactivated to suit gas chromatography (GC). Interactions of water with the support surface required to spread the water on such a surface are so great that interactions with the solutes (adsorptivity) get out of control. This is not surprising when one considers the large gap between the extremely high surface tension of water and the substantially lower critical surface energies obtainable through deactivation of glass or fused-silica surfaces.

Reversed-phase eluents for liquid chromatography

In reversed-phase liquid chromatography (LC) coupled on-line to GC, the eluent seldom consists of water. However, the surface tensions of mixtures of organic solvents and water tend to be far below those of water, rendering wettability easier.

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In fact, one of the earliest applications of LC–GC involving complete transfer of the LC fraction, published in 1985 by Cortes *et al.*[2], involved reversed-phase LC with acetonitrile as eluent (although probably carried out under conditions causing concurrent eluent evaporation, where wettability is less important). Recently, Duquet *et al.*[3] injected 1-μl volumes of methanol–water mixtures containing up to 50% water into 2 m × 0.25 mm I.D. GC precolumns. These precolumns were deactivated with hexamethyldisilazane (HMDS), aminopropylsilane and cyanopropylsilane. Unfortunately, no wettabilities were tested, and the tests on peak symmetry shown hardly serve as an indication of wettability.

This paper reports the wettabilities of silylated precolumn surfaces with various mixtures of organic solvents and water, of interest for reversed-phase LC–GC. Carbowax-deactivated precolumns were not considered because we were unable to bond Carbowax to the surface in such a way that it was not removed after a few injections[1]. Among the silylated surfaces, trimethylsilyl deactivation was not considered because of the known poor wettability[4]. Similarly, diphenylmethylsilylation was disregarded, because the resulting surfaces were found to be clearly less efficiently deactivated than phenyldimethylsilylated surfaces without offering noticeably better wettability.

EXPERIMENTAL

Deactivation of capillaries

Glass capillaries of 0.32 mm I.D. were leached and statically dried at 280°C according to Grob[5]. Silylation occurred with three reagents: (i) with a 1:1 mixture of hexamethyldisilazane (HMDS) and diphenyltetramethyldisilazane (DPTMDS) ("mesil/phesil"), (ii) with DPTMDS diluted with an equal volume of pentane ("phesil"), both heated overnight at 400°C, and (iii) with bis(cyanopropyl)tetramethyldisiloxane at 380°C ("cyanosil"), according to Blum[6].

Capillary rise experiments

For comparability purposes, the heights of rise of eluent mixtures of interest were measured. Approximately a third of a coil of a glass capillary was dipped into the liquid of interest, and the height of the meniscus above the surface of the liquid was measured.

Wettability tests

In 10 m × 0.32 mm I.D. sections of the three types of capillaries tested, lengths of flooded zones were determined as described[7]. A whitish glass capillary was attached to them, and increasing volumes of liquid were introduced by on-column injection until some liquid penetrated into the whitish capillary. The capillary length (10 m) was divided by the volume of liquid retained to yield flooded zones in terms of cm/μl. For determinations at elevated temperatures, the GC oven door was replaced by a pane of Pyrex glass.

GC experiments

The chromatographic experiments reported below were carried out with a 19 m × 0.32 mm I.D. glass capillary column coated with PS-255 (a methylsilicone) of 0.6

μm film thickness. It was coupled to a 15 m × 0.32 mm I.D. fused-silica precolumn, deactivated by dynamic coating with a 0.3% solution of OV-17 in dichloromethane, followed by heating at 320°C for 2 h and thorough rinsing with dichloromethane (see Part III[8]).

On-column injections of 50-μl volumes were carried out at an oven temperature of 100°C and an inlet pressure of 1.5 bar (hydrogen). After 3 min, the inlet pressure was reduced to 0.8 bar. This increase of the inlet pressure during injection helped to carry the sample liquid from the syringe needle further into the precolumn and to spread the sample plug at a suitable speed. High inlet pressures are preferable because of the high friction of water-containing solvents on wetted surfaces. In addition, the increased inlet pressure reduced the solvent evaporation time to about 3.5 min. When the pen of the recorder returned from the solvent peak, the oven temperature was programmed at 6°C/min to 270°C.

RESULTS AND DISCUSSION

Capillary rise experiments

Interaction forces between differently deactivated 0.32 mm I.D. (glass) capillaries and aqueous solvent mixtures were compared by the capillary rise method. Heights of rise determined at ambient temperature are listed in Table I. The listed heights of rise indicate that the critical energy of the mesil/phesil surface is noticeably below that of the phesil surface but that there is no significant difference between the phesil and cyanosil surfaces. In this situation, we prefer the phesil surface because of the chemical inertness of a phenyl group compared with the cyano group, which is rapidly hydrolysed.

Lengths of flooded zones/wettabilities

Lengths of flooded zones per microlitre of liquid in 0.32 mm I.D. capillaries are listed in Table II. Pragmatically, lengths up to *ca.* 30 cm/μl are interpreted as good wettability. Longer flooded zones indicate critical wettability, where the lengths of flooded zones may depend on small details of the capillary deactivation procedure and other unknown factors. Finally, lengths exceeding 1 m indicate a lack of wettability.

As expected, admixture of different organic solvents to water improves the wettability to different extents. Mixtures with acetonitrile are poorly suited to obtaining good wettability, as 10% of water in acetonitrile already causes the flooded zone to be elongated. Among the alcohols, the propanols produce the shortest flooded zones with the highest water concentrations. 1-Propanol containing up to 70% water wets phesil and cyanosil surfaces. Dimethylformamide and dioxane, of interest because of their high boiling points (see below), have poor wettability characteristics.

As observed previously[9], flooded zones are shortened when the oven temperature is increased. For 1-propanol containing up to 50% water, flooded zones of 13–15 cm/μl were determined at 100°C. We still do not fully understand this extreme shortening of flooded zones. The surface tension of liquids decreases with increasing temperature[1]. However, the height of rise of liquids did not increase with elevated temperature (in some instances it even slightly decreased), indicating more or less temperature-independent wettability (*i.e.*, there must be a decrease in critical energy of the surface to be wetted, corresponding to the decrease in surface tension).

TABLE I

HEIGHTS OF RISE OF SOME SELECTED REVERSED-PHASE ELUENTS IN GLASS CAPILLARIES DEACTIVATED WITH HEXAMETHYLDISILAZANE–DIPHENYLTETRAMETHYLDISILAZANE (1:1) (MESIL/PHESIL), DIPHENYLTETRAMETHYLDISILAZANE (PHESIL) AND DICYANOPROPYLTETRAMETHYLDISILOXANE (CYANOSIL)

Organic solvent	*Water (%)*	*Height of rise (mm)*		
		Mesil/phesil	*Phesil*	*Cyanosil*
Methanol	0	33	39	38
	10	32	38	
	20	23	37	
	30	19	30	
	50	8	25	
Ethanol	0	33	37	36
	10	31	36	35
	30	28	35	35
	50	22	32	32
1-Propanol	0	35	38	36
	10	34	36	35
	20	32	35	35
	50	24	34	34
	70	20	33	32
	80	16	29	29
2-Propanol	0	35	37	35
	10	32	36	35
	20	29	34	34
	50	27	33	33
	70	23	32	33
Dioxane	0	27	36	38
	10	26	36	38
	20	25	34	34
	30	20	32	33
Dimethylformamide	0	29	37	38
	10	17	37	35
	20		35	34

Sequence of solvent evaporation

The lengths of flooded zones listed above were determined before substantial proportions of the solvents evaporated. However, when uncoated precolumns are applied in the retention gap technique (on-column injection of large volumes, LC–GC involving the retention gap technique), formation of a film on the capillary wall by the solvent mixture is not the only condition to be fulfilled.

Most of the commonly used organic solvents evaporate more rapidly than water, which is due to the extraordinarily large volume of vapour created per unit volume of condensed water and also its relatively high boiling point. This causes a serious problem. The solvents do not evaporate evenly throughout the flooded zone, but from the rear towards the front of the sample film. The more rapidly evaporating organic solvent, responsible for the wettability of the surface, withdraws more rapidly from the rear towards the front of the flooded zone than water, leaving pure water behind, which no longer wets the capillary surface (Fig. 1).

TABLE II

LENGTHS OF FLOODED ZONES IN 0.32 mm I.D. CAPILLARIES FOR SOLVENTS AND SOLVENT MIXTURES USEFUL IN REVERSED-PHASE LC

Organic solvent	*Water (%)*	*Length of flooded zone (cm/μl)*			
		Phesil		*Cyanosil*	
		25°C	*60°C*	*25°C*	*60°C*
Acetonitrile	0	29		32	
	10	38			
	20	48			
	30	68			
	40	>100			
Methanol	0	29		28	
	10	36			
	20	39			
	30	47			
	50	>100			
Ethanol	0	26			
	10	27			
	20	29			
	30	35			
	50	48			
1-Propanol	0	28		25	
	10	33	23		17
	30	31	24		22
	50	28	23		18
	70	28	25		20
	80	>100			
2-Propanol	0	25		23	
	10	29	18		16
	30	26	19		18
	50	23	18		25
	70	>100	>100		>100
Tetrahydrofuran	0	27			
	20	28			
	30	33			
	50	>100			
Dioxane	0	28			
	10	28			
	20	28			
	30	>100			
Dimethylformamide	0		33		
	10		>100		
Diethylene glycol monomethyl ether	0		30		
	10		28		
	30		26		
	50		>100		

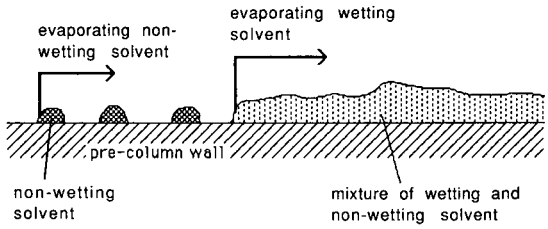

Fig. 1. Evaporation of a solvent mixture composed of a wetting organic solvent and a non-wetting solvent (such as water) on the surface of the capillary precolumn under conditions typical of retention gap techniques. Most organic solvents evaporate more rapidly than water, leaving the water behind, which no longer wets the capillary wall. Eventually, the resulting water droplets accumulate and penetrate further into the capillary column.

Accumulated water floods the column

First, this water forms small droplets, and the internal wall of the glass capillary turns whitish, like the fogging of a window. The stability of such droplets is delicate. In some instances, the droplets remain stationary until the water has evaporated. More often, however, one or a few water droplets start to move, driven by the carrier gas. They take other droplets with them, starting a breakthrough of water akin to an avalanche. Finally, the droplets become a plug, closing the capillary bore. This plug moves at the speed of the carrier gas, collecting all the water droplets encountered ahead. In fact, such a plug penetrates into the capillary column, like the droplets gliding down a window when it rains. If the organic solvent has been fully evaporated previously, the plug of water may not stop before having passed through the whole separation column. The whitish aspect of the capillary disappears, indicating almost complete removal of the water beyond the point where the "avalanche" started.

There is hardly any way to control whether or not the water droplets remain stable on the capillary wall until the water has evaporated. Large droplets clearly rupture more often than small droplets. As the size of the water droplets depends primarily on the concentration of the water in the solvent mixture, the "disaster" happens more regularly when high water concentrations are used. From visual observation, we have the impression that a 30% concentration of water in a much more rapidly evaporating organic solvent almost always causes a breakthrough. Temperature and gas flow-rates are other factors that influence the stability of the water droplets.

However, even if the water does not flood the separation column, there remains a problem concerning solvent trapping. After complete evaporation of the well retaining (strongly trapping) organic solvent, volatile solute material is partially released, as water is a poor solvent for trapping solutes of up to intermediate polarity. Resulting peaks are deformed, often starting with shoulders.

Effect of flooding water

The effect of the moving water has not been fully investigated yet. It was observed visually in glass capillaries (using a glass pane as an oven door) that it depends on the moment when the rupture occurs whether or not the water will run into the separation column. A droplet starting to move at an early stage is stopped by the solvent mixture forming a film ahead, the wetting liquid acting as a barrier. On the

other hand, a water droplet starting to move after all organic solvent has evaporated is hardly stopped anymore. However, even in this case, the "disaster" is not obvious. Water running into the separation column spreads all dissolved solute material throughout the flooded zone, possibly including the whole separation column. However, most components chromatographed by GC are hardly soluble in water at all, and one should expect that such substances rather tend to remain on the precolumn surface or dissolved in the stationary phase of the first part of the separation column. As long as there is no excessive spreading far into the separation column, there should be no peak distortion. Such uncertainties can only be clarified experimentally, although, as explained above, no simple, generally valid answer can be expected.

Water–1-propanol

1-Propanol and water form an azeotropic mixture containing 28% water. When 1-propanol–water mixtures are distilled at up to 1 bar, this composition was found to remain unchanged. This means that we can inject 1-propanol containing up to 28% of water without the risk that water will flood the column after evaporation of the wetting solvent. In fact, chromatographic experiments confirm this.

The chromatogram in Fig. 2 was obtained by injecting 50 μl of a solution of methyl esters in 1-propanol containing 25% water. All the peaks are well shaped, indicating successful reconcentration at the head of the separation column. A problem that is not shown here concerns the high polarity of the solvent mixture. Highly volatile components are only partially trapped and are eluted as peaks, deformed into "stools" or "chairs" (partial solvent trapping[10]).

On injecting the same sample in a solvent mixture containing more than 28% water, two kinds of deficiencies were observed, the reproducibility of which was limited. A solvent mixture containing 30% water caused the early peaks to be deformed (Fig. 3), while the later peaks were of perfect size and shape. The perfect shape of the peaks at elevated elution temperatures indicates that the very small

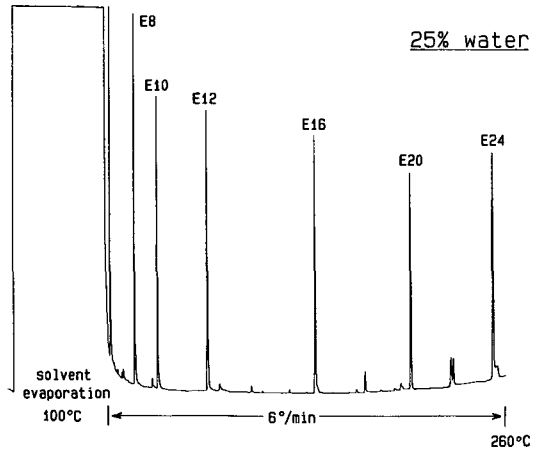

Fig. 2. Results of a 50-μl on-column injection of C_8–C_{24} methyl esters (E8–E24) in 1-propanol containing 25% of deionized water. Wettability of a standard "phesil" precolumn is no problem, and solvent trapping causes the early eluted ester peaks to be of almost perfect shape. For experimental details, see Experimental.

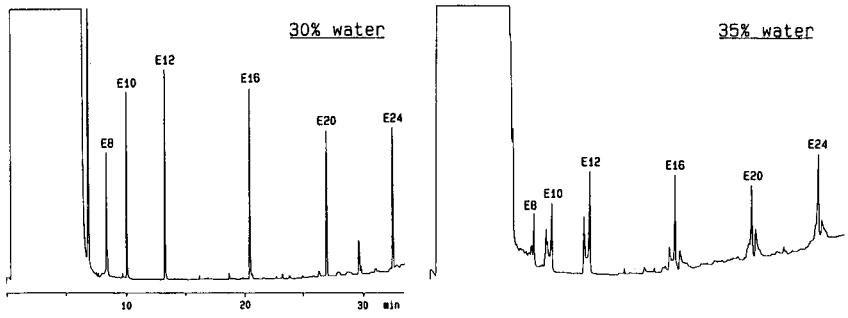

Fig. 3. Result of 50-μl on-column injections, as in Fig. 2, but with 30 and 35% water in 1-propanol. Now 1-propanol evaporates more rapidly than water, leaving behind water, which does not wet the precolumn surface.

amount of water left behind by the evaporating 1-propanol remained stationary without spreading the solute material. The broadening of the early eluted peaks, with the characteristics typical of "band broadening in time"[11], is due to water being a poor solvent for solvent trapping. As shown in Fig. 2, the methyl esters were fully trapped (completely retained) by the 1-propanol–water mixture. However, at the end of solvent evaporation, when all 1-propanol had left the inlet, it took some time to evaporate the remaining water. During this time, some of the methyl esters escaped.

When the test sample containing 35% water was injected, all methyl ester peaks were greatly distorted (not reproducibly in detail). This typical phenomenon of "band broadening in space" shows spreading of the solute material far into the separation column by the flooding effect. It indicates not only that the water droplets ruptured, but also that the water was able to carry along the sparingly soluble methyl esters. Peak distortion of solutes eluted at high temperatures was reduced or even disappeared when the column temperature was increased nearly to the boiling point of the solvent at the inlet pressure (experiments with 1-propanol containing 40% water). Such high oven temperatures produce concurrent eluent evaporation at the front end of the flooded zone, preventing the water from penetrating into the separation column. However, such techniques are difficult to carry out by conventional on-column injection (danger of back-flow), and peaks of volatile components are nevertheless severely distorted. Rather than using an on-column injector for sample introduction, such techniques should be carried out with a system corresponding to the loop-type LC–GC interface.

High-boiling organic solvents

Application of retention gap techniques to samples or LC eluents containing water appears to be restricted to mixtures with organic solvents that allow the water to evaporate first. This severely restricts the choice of organic solvents, almost ruling out all the more volatile, most commonly used solvents, such as methanol, ethanol and tetrahydrofuran. Some interesting azeotropic mixtures of higher boiling organic solvents with water are listed in Table III.

To evaporate water-rich organic solvents, organic solvents with high boiling

TABLE III
SOME AZEOTROPIC MIXTURES WITH WATER

Organic solvent	*B.p. (°C)*	*Water in mixture (%)*	*Azeotropic boiling point (°C)*
Ethanol	78	4	78
Acetonitrile	80	16	76
2-Propanol	82	13	80
1-Propanol	97	28	87
Pyridine	115	41	94
Butyloxyethanol	171	72	98
Ethylene glycol diacetate	191	85	94

points are required. Moreover, these solvents must be water-miscible and the aqueous mixture must wet the precolumn surfaces, which rules out solvents such as dimethylformamide (Table II).

CONCLUSIONS

Wettability tests have demonstrated that deactivation with DPTMDS, commonly used for capillary precolumns, produces surfaces of surprisingly good wettability. Not only do almost all organic solvents wet such phenylsilylated surfaces, but many of them still wet them when they contain considerable concentrations of water. The most commonly applied LC eluents, methanol and acetonitrile, are not those which wet at the highest water concentrations. Of the organic solvents tested, the best solvent in this respect is 1-propanol, still fully wetting the "phesil" surface when containing 70% water.

Wettability of precolumn surfaces is required for several techniques, including concurrent solvent evaporation with co-solvent trapping[12], and even for concurrent solvent evaporation it is of advantage (although not a prerequisite). Here, we have considered only retention gap techniques, for which wettability by the solvent mixture is just one requirement. The more severe restriction is due to the requirement that the water must evaporate more rapidly than the organic solvent. This limits the maximum water concentration in 1-propanol to 28% and in acetonitrile to 16%. For introduction of higher proportions of water by the retention gap technique, higher boiling solvents (or co-solvents) must be used.

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INTRODUCTION OF WATER AND WATER-CONTAINING SOLVENT MIXTURES IN CAPILLARY GAS CHROMATOGRAPHY

III. WATER-RESISTANT DEACTIVATION OF UNCOATED PRE-COLUMNS?

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SUMMARY

Uncoated capillaries, deactivated by silylation or by layers of non-extractable Carbowax or phenylsilicones, rapidly become active when water or (a far weaker effect) alcohols such as methanol are introduced. Apparently, the effect is far more pronounced for condensed water than for water vapour, as analogous separation columns hardly suffer under the action of water vapour. The resulting high adsorptivity of the precolumns is hardly noticed as long as the sample matrix contains water by itself. A thin layer of OV-17, "baked" on to raw fused silica, produced the best, but still unsatisfactorily, water-resistant deactivation.

INTRODUCTION

In Parts 1 and 2[1,2] we described the possibilities and limitations of introducing large volumes of water or water-containing solvent mixtures, concentrating on retention gap techniques. We did not find any precolumn surface suitable for gas chromatography (GC) that was water-wettable. On the other hand, admixture of organic solvents greatly improves the wetting properties. For instance, the addition of 30% of 1-propanol to water renders a phenyldimethylsilylated surface wettable. However, for retention gap techniques (with or without partially concurrent solvent evaporation), the problem still remains that organic solvents tend to evaporate before the water, leaving behind the non-wetting water.

Introduction of water or water-containing solvent mixtures presupposes the availability of uncoated precolumns (retention gaps) that are deactivated in such a way that deactivation resists water and alcohols. Such precolumns are required, regardless of whether retention gap techniques or concurrent solvent evaporation (with or without co-solvent trapping) are applied.

Experimentally, it was found that after a limited number of large transfers of

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water or water-containing mixtures, the adsorptivity of the GC system was strongly enhanced; the peaks started to tail, were strongly broadened or even disappeared. Typically, peak deformation became more pronounced towards the end of the analysis (*e.g.*, at increased temperature during long temperature programmes). Presumably, this is due to temporary deactivation of an adsorptive surface by adsorbed water, which loses its efficiency as more adsorbed water evaporates. Such activity was primarily observed when glass capillary precolumns were used. Fused-silica precolumns also became active, but not to the same extent.

This paper reports the alcohol and water resistance obtained with the deactivation procedures available. The result was disappointing: water destroyed all deactivation.

EXPERIMENTAL

Deactivation of capillaries

The following procedures were used to deactivate 10 m × 0.32 mm I.D. glass and fused-silica capillary tubes.

Silylation. After leaching (glass) or hydrothermal treatment (fused silica)[3], the capillaries were silylated with diphenyltetramethyldisilazane (DPTMDS) at 400°C (glass) or 370°C (fused silica). After silylation, the capillaries were rinsed with toluene, methanol and diethyl ether.

Carbowax deactivation by "baking". Raw fused silica was rinsed with 1% of Carbowax 20M in dichloromethane, flushed with carrier gas and heated at 280°C for 2 h. The capillaries were rinsed with *ca.* 2 ml of dichloromethane before testing.

Carbowax deactivation by "bonding". Capillaries were dynamically coated with a 0.3% solution of Superox 20M in dichloromethane, containing 10% (referred to the Superox) of dicumyl peroxide and 20% of vinyltriethoxysilane. They were flushed with nitrogen (removing dichloromethane and air) and sealed with a flame, then heated at 210°C for 2 h. The capillaries were again rinsed with dichloromethane before testing.

Coating with OV-17. Raw fused silica was dynamically coated with a 0.3% solution of OV-17 in dichloromethane at a speed of 2 cm/s, using a damping column at the exit. It was heated at 360°C for 2 h, then thoroughly rinsed with dichloromethane.

Coating with OV-61-OH. Leached glass or raw fused silica was dynamically coated with a 1% solution of OV-61-OH (33% phenyl) in dichloromethane (see above) and heated at 320°C for 30 min. Then the capillary was rinsed with azo-*tert.*-butane vapour in nitrogen, sealed and heated at 200°C for 2 h. Before testing, the capillary was rinsed with dichloromethane.

Testing of the deactivated capillaries

Testing of the deactivated capillaries was carried out by the retention gap test, described previously[4]. Capillaries were installed in a conventional split/splitless injector and a flame ionization detector was used. Before testing, the capillaries were heated at 250°C for 10 min. Tests were carried out isothermally at 70°C; the carrier gas inlet pressure was 0.15 bar (hydrogen). Gas hold-up times were determined by injecting 1 μl of fuel gas (methane). Then, 5–20 μl of the headspace gas from the test components (taken from the bottle) were injected by splitting (about 10:1), analysing at low attenuation to avoid excessive overloading. Retention times of reasonably shaped

peaks were measured with a stop-watch in order to calculate the adjusted capacity ratios (k'), which were then converted to "apparent film thicknesses", calibrated for an apolar stationary phase.

Test results were interpreted in terms of retention power and adsorptivity. Adsorptivity was deduced from peak shapes, but also from retention powers for polar compounds.

Treatment with methanol and water

To simulate the conditions during solvent evaporation in a reproducible way, capillaries to be tested for stability of the deactivation were filled to 90% with methanol, closed with a press-fit cap and heated at 90°C for 30 min, then rinsed and tested. Subsequently, the capillaries were filled to 90% with water, heated at 100°C for 30 min, rinsed and tested again. Finally, they were refilled with water and heated at 140°C for 30 min, followed by testing.

Resilylation

Silylated and silicone-coated capillaries were resilylated, after turning active due to methanol and water, using a technique described by Grob and Grob[5] for coated columns. An amount of 20–30 μl of a 1:1 mixture of DPTMDS and *n*-pentane was passed through the capillary at *ca.* 3 cm/s with nitrogen. Then, the capillary was installed in an injector, leaving the column exit hanging freely. The capillary was flushed with carrier gas for 5 min, the inlet pressure was reduced to 0.05 bar and the oven temperature was programmed at 3°C/min from 150 to 330°C, keeping it at the final temperature for 2 h. Finally, the capillary was rinsed with dichloromethane before being tested.

RESULTS AND DISCUSSION

Glass versus fused silica

Glass capillaries, deactivated by Carbowax or silylation, exhibited extremely high adsorptivity after water treatment, substantially exceeding that observed with fused-silica capillaries. This can easily be explained by the far higher concentration of silanol groups on the glass than on fused-silica surfaces. Deactivation by thin layers of OV-17 was less efficient on glass than on fused silica to begin with. For this reason, the results reported are restricted to fused-silica capillaries.

Carbowax-deactivated fused silica

The test results for a fused-silica capillary, raw as obtained (Polymicro Technologies, Phoenix, AZ, U.S.A.) and after deactivation with Carbowax by the "baking" method, are shown in Fig. 1. In the top row, test results for the raw fused silica show not only that the *n*-tetradecane (C-14) peak (upper left corner) is well shaped, but also that the capillary has a surprisingly high retention power (see Table I). The same is true for ethylnaphthalene. Methyl esters and alcohols were not eluted as detectable peaks, even when large amounts were injected, thus confirming the strong adsorptivity of the raw fused silica.

The second row of chromatograms was obtained with the freshly Carbowax-deactivated capillary. All test components were eluted as well shaped peaks. The

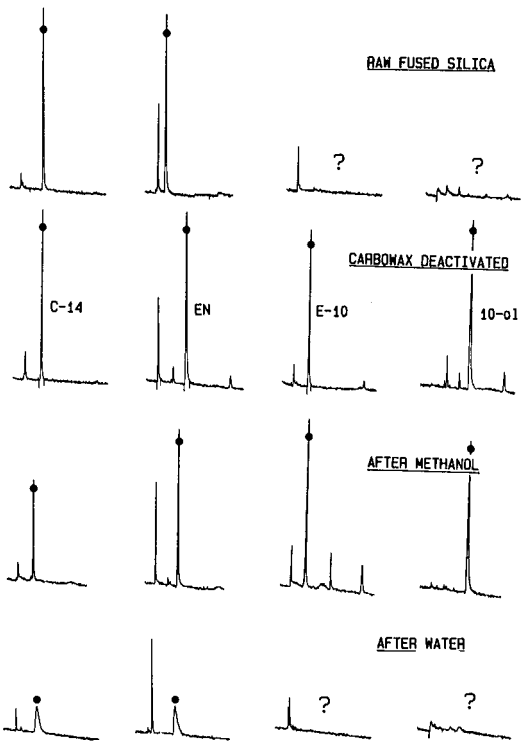

Fig. 1. Test results for raw fused silica, for the same capillary after Carbowax deactivation and after treatment of the latter with methanol at 90°C for 30 min and with water at 140°C for 30 min. Split injections of headspace gas of *n*-tetradecane (C-14), ethylnaphthalene (EN), methyl decanoate (E-10) and 1-decanol (10-ol). Corresponding peaks are marked by a spot; a question mark indicates that no peak is observed. Methanol hardly affects the deactivation, whereas water completely ruins it.

retention powers were moderate (except for the most polar test compound, showing the high polarity of the deactivation), but are acceptable for precolumns of up to intermediate length (requiring a moderate reconcentration efficiency). It is interesting that the retention power for the alkane was below that of the raw fused silica.

Methanol (after heating at 90°C) removed some of the Carbowax (as concluded from the slightly decreased retention powers) but did not seriously affect the deactivation (peak shapes are hardly affected). Water, however, left behind a fused-silica surface that was more active than the raw fused silica. Apparently, water even attacks the silica, opening some strained siloxane bonds and forming (adsorptive) silanol groups.

The results obtained with the "bonded" Carbowax were even slightly worse. The Carbowax film was relatively thick (see retention powers). Methanol removed more than half of it (see retention powers in Table I), and the remainder was completely ruined by the water. Thus "bonded" Carbowax, non-extractable by solvents including methanol and water at ambient temperature, becomes completely extractable after some hydrolysis.

On the other hand, we noted that separation columns coated with Carbowax-

TABLE I

RETENTION POWERS IN TERMS OF APPARENT FILM THICKNESSES OF AN APOLAR STATIONARY PHASE FOR DIFFERENTLY DEACTIVATED PRECOLUMNS WITH AND WITHOUT TREATMENT WITH METHANOL AND WATER

Deactivation	*Further treatment*	*Retention power (nm)*[a]					
		C-14	*EN*	*E-8*	*E-10*	*8-ol*	*10-ol*
Raw FS	–	5.2	3.1		–		–
"Phesil"	Fresh	1.4	0.5		1.4		3.6
	Methanol, 90°C	1.4	0.6		1.9		3.3
	Water, 140°C	1.8	0.9		–		–
CW "baked"	Fresh	3.4	7.5		7.4		22.7
	Methanol, 90°C	3.6	6.6		5.4		20.5
	Water, 140°C	5.4	8.3		–		–
CW "bonded"	Fresh	20.1	9.4				
	Methanol, 90°C	7.9	4.6				
	Water, 140°C	–	–				
1% OV-17	Fresh	6.1	13.7	35.3		30.5	
	Methanol, 90°C	5.9	16.3	33.8		41.8	
	Water, 100°C	6.5	17.9	46.9		68.2	
	Water, 140°C	5.4	16.5	66.6		83.2	
	Resilylated	5.2	13.4	21.3		21.8	
0.3% OV-17	Fresh	4.2	9.1	20.9		19.7	
	Methanol, 90°C	4.2	8.6	21.6		36.6	
	Water, 140°C	3.6	6.5	16.3		20.9	
1% OV-61-OH	Fresh	5.4	13.2	24.8		34.1	
	Methanol, 90°C	5.4	13.9	28.2		51.4	
	Water, 100°C	4.7	12.6	36.9		53.6	
	Water, 140°C	4.7	13.4	51.6		–	
	Resilylated	6.1	13.8	22.5		36.4	

[a] C-14 = *n*-tetradecane; EN = ethylnaphthalene; E-8 = methyl octanoate; E-10 = methyl decanoate; 8-ol = 1-octanol; 10-ol = 1-decanol.

type stationary phases resisted water well; column bleeding did not significantly increase even after the passage of very large amounts of water. This suggests that condensed water (within the flooded precolumn) does far more harm to Carbowax than does water vapour (in the separation column).

Silylated fused silica

Fig. 2 shows test results obtained with a fused-silica capillary, deactivated by phenyldimethylsilylation. All test solutes form well shaped peaks. The retention power is low (Table I). After heating the column with methanol at 90°C for 30 min, the capillary still had nearly the same inertness; the retention power also did not change. However, water strongly increased the adsorptivity. The retention powers measured for polar solutes increased owing to increased interaction with adsorptive sites of the surface. Alcohols hardly formed peaks any more.

From the test results, and from the fact that resilylation of the capillary restored inertness, we concluded that water hydrolyses the silyl ethers, in other words, the capillary surface silylates water!

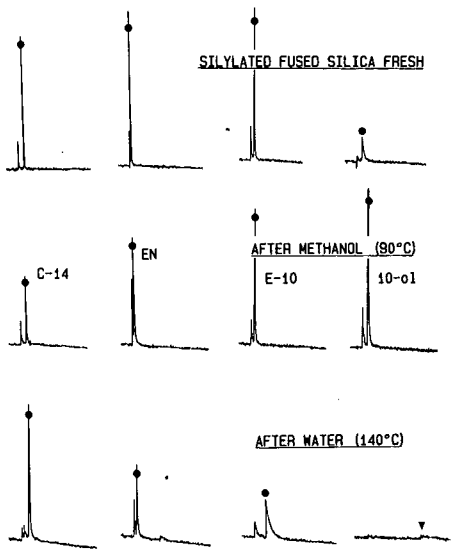

Fig. 2. Test results for a phenyldimethylsilylated fused-silica capillary (DPTMDS treatment), analogous to Fig. 1. Methanol hardly affects the inertness of the surface, whereas water ruins the deactivation.

Thin silicone films

Repeated testing revealed that silicone-coated glass capillary separation columns are hardly affected by water vapour; after passage of the vapour from several millilitres of water at up to 160°C, the 1-octanol peak of the standard test mixture still produced 40–60% of the possible peak height, and the methyl ester peaks had not deteriorated at all. This suggested coating of the precolumn with a thin layer of a stationary phase to obtain the same performance. Provided that short pieces (up to about 10 m × 0.32 mm I.D.) of precolumn are used, *e.g.*, for concurrent eluent evaporation, a thin layer of stationary phase produces a retention power sufficiently low to provide the reconcentration power required for shortening the bands broadened in space[6].

Simple coating with conventional silicone stationary phases results in films that are virtually completely extractable with organic solvents. However, as shown by Schomburg *et al.*[7], after heating at high temperatures (350–400°C) part of the silicone material becomes non-extractable. The method was used for deactivating capillaries before coating them with silicone stationary phases. We expected two benefits from coating with a silicone polymer. First, the capillary surface is shielded by a layer through which the water is unable to penetrate. This should prevent leaching of the silica and the formation of new silanol groups on the support surface. Second, deactivation should no longer be as easy to destroy. "Baking" silicones on active silica surfaces causes some degradation of the silicone, producing fragments that react with the available silanol groups of the support. Hence, silanols are derivatized by rather large molecules, and even if water hydrolyses such bonds again, these macromolecules, insoluble in water, are no longer removed by water. In contrast, simple heating should restore the reaction and cause the free silanol group to disappear again.

The choice of the silicone stationary phase was determined by three arguments: first, the stationary phase surface should be optimally wettable, calling for a high critical surface energy; second, water and alcohols should not penetrate into the stationary phase in order to obtain the desired protection of the support surface; and third, the stationary phase should be chemically stable. Phenyl substituents efficiently increase the surface tension but keep the solubility of polar solvents low. Cyano functions are unstable, as they tend to become hydrolysed.

The experimental results were conflicting. The deactivation achieved by a well bonded layer of OV-61-OH was hardly more water-resistant than silylation. In contrast to the silylated and the Carbowax-deactivated capillaries, methanol also caused some damage. Resilylation restored the original retention power, indicating that no silicone polymer was lost. Thus, methanol and water must have introduced highly adsorptive functional groups into the silicone (silanols?) rather than destroying the deactivation.

On the other hand, capillaries deactivated by a layer of conventional OV-17, "baked" to the surface, produced better results than all other deactivations (Fig. 3). The capillary became slightly more active on treatment with methanol but, in the case shown, water restored the original inertness rather than further degrading the deactivation.

As shown in Table I, deactivations by 1% and 0.3% solutions of OV-17 resulted in retention powers that were less different than might be expected (owing to the reduced viscosity of the more dilute solution, the amount of stationary phase deposited on the capillary wall by dynamic coating differed by a factor exceeding 3). This effect corresponds to what was previously found for Carbowax deactivations. A certain amount of stationary phase is bonded to the surface, while any extra amount remains extractable.

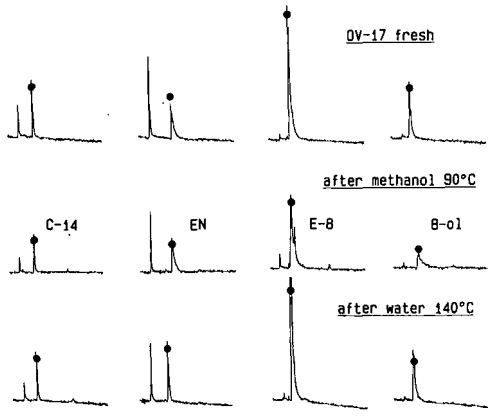

Fig. 3. Test results for a fused-silica capillary, deactivated by a thin layer of OV-17 (50% phenyl). As the retention power is relatively high, methyl octanoate (E-8) and 1-octanol (8-ol) were used as test components instead of E-10 and 10-ol. Methanol slightly affected the deactivation, whereas water almost restored the test result.

Resilylation

The silylated and silicone-coated capillaries were resilylated after becoming active with water. Resilylation is rapid and simple, and the re-deactivation is nearly complete. It confirms that water generates activity by introducing hydroxyl groups into the silica backbone or the silicone polymer of the stationary phase layer.

Consequences for practical GC

The above tests with the uncoated capillaries allow a rapid evaluation of precolumns, but there is no direct relationship between such test results and real GC analysis. This gap was filled by testing the precolumns as part of a separation system.

Precolumns (10–15 m × 0.32 mm I.D.) were joined with an excellent 15 m × 0.32 mm I.D. glass capillary, coated with PS-255 (a methylsilicone) of 0.3 μm film thickness (film thickness is an important parameter, as thicker films better reconcentrate bands broadened in the precolumn). The column quality test II[8] was injected by the on-column technique, using conditions standardized for the 15-m separation column.

The top test chromatogram in Fig. 4 was obtained with the Carbowax-deactivated precolumn shown in Fig. 1, after treatment of the latter with water (precolumn test, see bottom row in Fig. 1). Only alkanes produce reasonable peaks, all

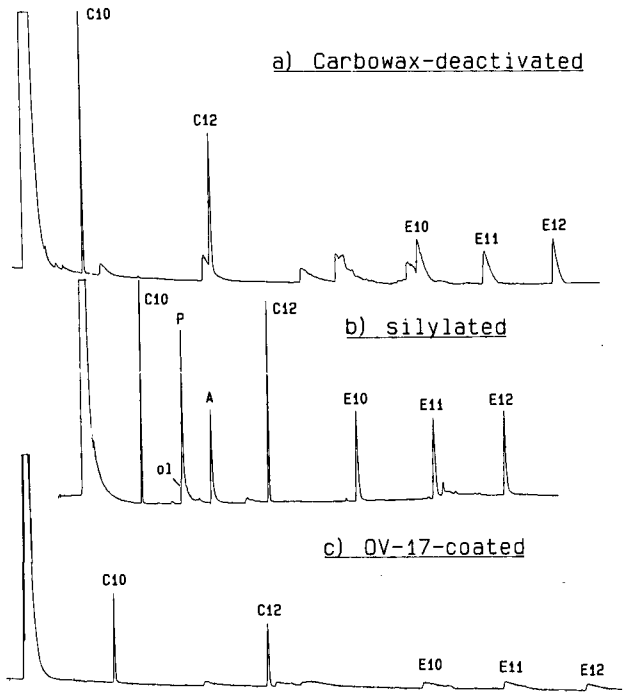

Fig. 4. Test chromatograms involving three precolumns, treated with water at elevated temperatures: (a) Carbowax deactivated; (b) silylated; (c) OV-17-coated. The 10–15 m × 0.32 mm I.D. precolumns were connected to an excellent apolar separation column; standard column quality test II. Peaks: C10, C12 = *n*-alkanes; E10–E12 = methyl esters; ol = 1-octanol; P = 2,6-dimethylphenol; A = 2,6-dimethylaniline. For details, see text.

other components being badly adsorbed. Not only are their peaks distorted, but also is their elution strongly retarded, and the elution pattern can no longer be recognized. The test chromatogram in the centre shows the effect of an active, initially silylated fused-silica capillary that was treated with water. The test on the uncoated capillary alone was similar to that shown in the bottom row of Fig. 2. The alcohol peak (ol) nearly disappeared, reflecting the strong adsorptivity also found by the test in Fig. 2. The peaks of the methyl esters are broadened and tail; their height should be similar to that for *n*-dodecane (C-12).

The last chromatogram in Fig. 4 gives a picture of a completely ruined precolumn, and one would not expect to obtain any reasonable peak, except for alkanes, when such a precolumn is used. However, this impression is wrong. This same fused-silica precolumn of 15 m length was previously used for injecting 10–60-μl volumes of 1-propanol containing water at various concentrations. The last chromatograms for these experiments were shown in Part II[2], where the methyl ester peaks (C_8–C_{24}) did not show any tailing. This confirms what we have observed in many other instances: the high activity of precolumns observed after the introduction of water affects GC analyses very differently, depending on whether water is still part of the sample matrix or not. Water appears to be very efficient in temporarily deactivating capillary surfaces.

It must be added that this last precolumn was deactivated by a thin layer of OV-17, which indicates that even the deactivation procedure showing the best test results does not yield an inertness that resists water over extended periods in practical work.

CONCLUSIONS

We were not previously aware of the detrimental effect of condensed water on uncoated precolumns. It must be assumed that the low water content of many every-day samples also rapidly destroys the deactivation of uncoated precolumns.

At present, uncoated precolumns with water-resistant deactivation are not available. This certainly is a need that should be filled in the future, but we have no idea how it could be achieved. Silicones and polyglycols appear to be hydrolysed and silica surfaces are leached. What other inert, thermostable and reasonably wettable surface coatings are available in GC? Nevertheless, the lack of water-resistant deactivation of capillary precolumns does not prevent work involving the introduction of water, such as in on-line coupled reversed-phase LC–GC. During all of the experiments carried out, the adsorptivity of the precolumn did not become a limiting factor, provided that we continued to introduce water-containing solvent mixtures. It did, however, preclude the use of glass capillary precolumns.

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INTRODUCTION OF WATER AND WATER-CONTAINING SOLVENT MIXTURES IN CAPILLARY GAS CHROMATOGRAPHY

IV. PRINCIPLES OF CONCURRENT SOLVENT EVAPORATION WITH CO-SOLVENT TRAPPING

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SUMMARY

Concurrent solvent evaporation of aqueous solutions causes loss and broadening of peaks up to high elution temperatures. This deficiency is largely eliminated by using co-solvent trapping. A 5–20% concentration of a high-boiling co-solvent (butoxyethanol) is added to the water or mixture containing water (reversed-phase eluent). This co-solvent does not completely evaporate concurrently, retaining the solute material in the uncoated precolumn until evaporated some time after the end of the sample transfer. The concept and the required properties of the co-solvent are discussed and demonstrated for a 1-ml sample of methyl esters in aqueous solution, using 20% butoxyethanol as co-solvent.

INTRODUCTION

Injection or liquid chromatographic–gas chromatographic (LC–GC) transfer of samples containing high proportions of water is a serious problem owing to the poor wetting characteristics of water, the large volumes of vapour produced per unit volume of liquid, poor solvent effects, and also because of the high boiling point of water. Application of the retention gap techniques (with or without partially concurrent eluent evaporation) is restricted because of poor wetting of the uncoated precolumn[1] by water-containing solvent mixtures. Up to 28% of water can be introduced if 1-propanol is the main solvent or 16% with acetonitrile.

The tolerable proportion of water in the solvent mixture increases when high-boiling organic solvents with good wetting characteristics are applied. However, in that event, concurrent solvent evaporation with co-solvent trapping, to be described here, is a more attractive method. Despite the large volumes of liquid that can be introduced in this way, only a short, uncoated precolumn is required. Short precolumns are of particular interest when working with water, as no precolumn with water-resistant deactivation is yet available[2]. Precolumns used for the evaporation of aqueous solvent mixtures rapidly turn adsorptive, and their further use relies on the continuing introduction of water, as the water temporarily deactivates the surface

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again. This makeshift arrangement is the more critical the longer the precolumns are.

Concurrent solvent evaporation does not rely on wettability of the precolumn surface, but is of limited usefulness because of strong peak broadening or losses of solute material with high elution temperatures. The method was successfully applied to on-line sample enrichment by reversed-phase LC–GC of atrazine in water[3]. However, the minimum elution temperature for atrazine was 240°C.

For reasons discussed further below, concurrent eluent evaporation with co-solvent trapping appears to be the method of choice for coupled reversed-phase LC–GC involving water-containing eluents. This paper describes the concept of this technique. Directions on its experimental optimization for water, and also for water–methanol and water–acetonitrile mixtures, are published separately[4]. Optimized sets of co-solvent concentrations and transfer temperatures are fairly generally applicable, as the solvent evaporation system is more or less independent of the separation column used and the components to be analysed.

CONCURRENT ELUENT EVAPORATION

Concurrent solvent evaporation means evaporation of the solvent during its introduction into the GC system[5]. This permits the introduction of very large volumes of liquid (up to many millilitres) by the use of uncoated precolumns only 2–5 m long. In our hands, this has become the most important technique for on-line transfer of LC fractions to GC.

However, concurrent solvent evaporation has an inherent drawback: evaporation occurs under conditions ruling out solvent trapping[6]. As solvent evaporation takes place at the front of the flooded zone, no condensed solvent remains ahead of the evaporation site that would retain solute material. As a result, the first solute material starts to migrate into the separation column long before the last material follows. If there is a solvent vapour exit in an early part of the separation column, a substantial part of these solute materials is lost. The resulting band broadening or losses reach components with high boiling points.

Band broadening cannot be estimated by considering only the transfer time. The lead of the most advanced material must be calculated from the retention volumes, *i.e.*, from the volume of vapour that flushes the most advanced material forward until the last solute material enters the GC system.

Below, it will be shown that 1 ml of water containing 20% butoxyethanol could be transferred within 5.5 min. However, band broadening far exceeds these 5.5 min. The vapour volume produced by the mixture is about 2500 ml. Assuming a carrier gas flow-rate of 2.5 ml/min during analysis, this would correspond to a difference in retention times between the first and the last solute material, *i.e.*, to an initial band width, of 1000 min, which is 16.7 h!

In conventional concurrent eluent evaporation (without co-solvent trapping), broad initial bands are reconcentrated by cold trapping and phase soaking[7]. In practice, this means that the first sharp peaks can be expected to be eluted 60–100°C above the column temperature during sample introduction (assuming organic solvents and fraction volumes of 500–2000 μl).

Concurrent solvent evaporation with water

Water is a very difficult solvent for concurrent solvent evaporation. First, the volume of vapour produced per volume of liquid is extremely large (about six times that of hexane). These vapours act as carrier gas, intensively flush the solutes forward and cause correspondingly broad initial bands (see above). In addition, vapours must be discharged through an early vapour exit in order to avoid excessively broad solvent peaks. Second, the boiling point of water is relatively high, compelling us to use high GC oven temperatures during introduction (110–140°C, depending on the inlet pressure). The combination of these two factors, together with the fact that water does not produce any phase soaking (or rather a reversed-phase phase soaking[8]), causes the first sharp peaks to be eluted between 230 and 260°C.

Co-solvent trapping

Two years ago we started experimentation with concurrent solvent (LC eluent) evaporation with co-solvent trapping[9], hoping to overcome the drawback of the conventional concurrent solvent evaporation technique described above. Co-solvent trapping, obtained from a high-boiling co-solvent, added in modest concentrations to the main solvent, serves to retain volatile components during evaporation of the main solvent (Fig. 1). This prevents these components from starting to migrate prematurely into the separation column, or loss through a solvent vapour exit, if such an exit is located in the early part of the column. Fully trapped components are retained in the co-solvent until the last portion of the co-solvent evaporates. Solutes start to be chromatographed with a delay, but as sharp bands released within a short time and from a short section of the precolumn. In fact, sharp peaks of correct size could be obtained with elution temperatures near the column temperature during transfer.

The feasibility of this technique was demonstrated for the solvent system *n*-pentane–*n*-heptane[10]. A 500-μl volume of a highly dilute gasoline solution was introduced into a 4-m uncoated precolumn. The co-solvent (*n*-heptane) concentration

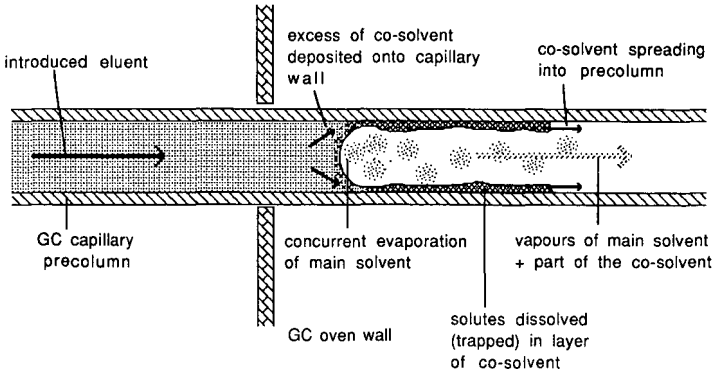

Fig. 1. Concurrent solvent evaporation with co-solvent trapping. The carrier gas pushes the sample into the oven-thermostated capillary precolumn. The oven temperature must be high enough to produce a vapour pressure, primarily of the main solvent, that stops the flow into the column. The main solvent evaporates, and just a small proportion of the liquid, consisting of a higher boiling mixture, primarily containing co-solvent, spreads along the walls into the uncoated precolumn. This layer retains the solutes and prevents volatile components from escaping prematurely through the solvent vapour exit (solvent trapping).

was 5%. The first perfect solute peaks were eluted before the xylenes. With the conventional concurrent solvent evaporation, almost all components of gasoline would have been lost through the solvent vapour exit. Previously, such a result could have been achieved only by the retention gap technique. Owing to the large volume of liquid introduced, partially concurrent solvent evaporation would have been presupposed in order to obviate the need for an excessively long uncoated precolumn.

Column temperature during transfer

Selection of a suitable column temperature during transfer is the most delicate point of the co-solvent trapping technique. The temperature must be within a range limited by the following aspects.

The lower end of the temperature range is determined by the fact that the concept involves concurrent solvent evaporation by the use of the loop-type interface[11]. Hence, the carrier gas pushes the sample liquid into the oven-thermostated precolumn (Fig. 1) against the vapour pressure of the sample (solvent). This means that the GC oven temperature must be high enough to produce a solvent vapour pressure exceeding the carrier gas inlet pressure. Hence, the minimum required oven temperature corresponds to the boiling point of the mixture of main solvent and co-solvent at the inlet pressure applied. At this temperature, primarily the main solvent evaporates, leaving behind a higher boiling mixture of main and co-solvent that is driven further into the precolumn by the vapours. This condensed main solvent–co-solvent mixture ahead of the main evaporation site is responsible for solvent trapping of the volatile solutes. The upper limit of the suitable temperature range is determined by the vapour pressure of the co-solvent. The vapour pressure of the co-solvent increases with temperature and thus increases the concentration of the co-solvent vapour in the gas phase. The concentration of the co-solvent vapour in the vapour mixture, discharged through the column, rapidly reaches that in the solvent mixture introduced. Hence, when the sample (LC fraction) is transferred at excessively high temperature, the co-solvent also completely evaporates, and the co-solvent trapping effect is lost again. In fact, the range of suitable oven temperatures during sample introduction is rather narrow (typically 5–10°C).

Terminology: partially concurrent solvent evaporation?

Partially concurrent solvent evaporation means that part of the solvent (LC eluent) evaporates during introduction while the non-evaporated part floods the GC precolumn[12]. Recently, Maris *et al.*[13] showed a nicely elaborated application of this technique for the LC–GC analysis of polychlorinated biphenyls (PCBs) in sediments.

It may be argued that concurrent solvent evaporation with co-solvent trapping should be classified as a partially concurrent solvent evaporation technique. Indeed, only part of the solvent evaporates during introduction, while another part, primarily co-solvent, pours into the GC precolumn. Nevertheless, classification as a concurrent solvent evaporation technique is preferred, because the co-solvent trapping technique is regarded as a sophisticated version of concurrent solvent evaporation. In fact, as long as the main solvent is considered, it still deserves the name (fully) concurrent solvent evaporation. In addition, instrumentation and working rules still very much resemble concurrent solvent evaporation, as a loop-type interface is applied, and the oven temperatures must be above the boiling point of the eluent.

REQUIREMENTS ON THE CO-SOLVENT

A co-solvent suitable for introducing water or water-containing solvent mixtures must fulfil a considerable number of requirements. Its selection should be made with care, also because a single co-solvent should be applicable to all kinds of samples, with the advantage that the co-solvent concentrations and GC conditions need to be optimized only once.

High boiling point

The optimum boiling point of the co-solvent is related to the required concentration of the co-solvent in the solvent. On the one hand, the required concentration of co-solvent should be small, as the co-solvent should be an additive, influencing the properties of the solvent mixture (*e.g.*, the eluent strength) as little as possible. Furthermore, work with short precolumns is easier when only small amounts of co-solvent are used (keeping the maximum volume of liquid flooding the precolumn small).

On the other hand, there is a minimum concentration of co-solvent required for a rapid build-up of a co-solvent film in front of the main evaporation site. Solvent evaporation tends to be a violent process. The front of the liquid often oscillates, *i.e.*, the liquid enters the oven-thermostated pre-column, *e.g.*, say 60 cm, evaporates and re-enters. Too small a co-solvent concentration would build up a layer of liquid that is periodically overrun by the liquid introduced for a long time, namely until a considerable part of the sample (LC fraction) has been introduced and the co-solvent layer has reached a sufficient length. Basically, this problem could be solved by a volume of pure co-solvent introduced ahead of the sample. However, this would complicate the system, particularly for automation, and experimentally we did not find any need for it as long as the co-solvent concentrations were not too low.

Work with low co-solvent concentrations presupposes that a high proportion of main solvent evaporates together with a small proportion of co-solvent, such that some condensed co-solvent remains in the precolumn, forming the layer required for solvent trapping. The boiling points of the two solvents must also be far apart to avoid the selection of the transfer conditions, primarily of the GC oven temperature, from becoming impractically critical (wide gap between the boiling and the condensation curves on the phase diagram).

The upper limit of the boiling point is determined by practical aspects. The evaporation rate must not be too low, as evaporation of the co-solvent at the end of the introduction process would otherwise become excessively time consuming (causing the solvent peak to become very broad). Even when added in small concentrations, the total amount of co-solvent to be evaporated easily reaches 50 μl. Further, the co-solvent should be minimally retained by the GC separation column, again to avoid an excessively broad solvent peak but also to prevent too many solute peaks from becoming obscured by the solvent peak.

Azeotropic mixture with water

Formation of an azeotropic mixture of the main solvent and the co-solvent, *i.e.*, with water in our case, has several advantages. First, evaporation occurs at a lower column temperature, allowing GC analyses to start at a relatively low temperature.

Second, the main solvent and the co-solvent co-evaporate in a well defined ratio, fairly independent of conditions. This is an important advantage over ideally evaporating solvent mixtures, such as the *n*-pentane–*n*-heptane mixture tested, where a small change in GC oven temperature or pressure has strong effects on co-solvent evaporation. With ideally evaporating mixtures, a change in the GC oven temperature by a few degrees causes a significant change in the volume of co-solvent–main solvent mixture flooding the precolumn, either in the direction of a full evaporation of the co-solvent or, *e.g.*, an extra 50 μl of liquid swamping the pre-column.

The stability of the main solvent–co-solvent ratio in the vapour phase is of particular importance when considering the effect of the pressure drop through the uncoated pre-column on the vapour composition, as discussed recently[10]. The pressure at the inlet of the precolumn corresponds to the carrier gas inlet pressure, whereas that at the outlet is only slightly above ambient pressure (depending on the pressure drop over the solvent vapour exit). If the solvents evaporate ideally, the concentration of the co-solvent in the vapour phase increases towards the outlet of the precolumn, because the vapour pressure of the co-solvent is constant (determined only by the temperature), whereas the total pressure decreases. If the pressure drop over the precolumn is large, the co-solvent is likely to evaporate completely at the front end of the flooded zone, releasing the volatile solutes (and causing their loss when an early solvent vapour exit is used). When the solvents evaporate as an azeotropic mixture (and as long as the composition of this mixture is fairly independent of pressure), such problems are eliminated. This allows us to apply increased inlet pressures (larger pressure drops through the precolumn), to accelerate the discharge of the eluent vapours.

Good wettability

The co-solvent spreading into the uncoated precolumn must wet the precolumn wall in order to form the film responsible for solvent trapping. A lack of wettability would cause an uncontrolled flow into the separation column or through the solvent vapour exit. Film formation is rendered more difficult by the fact that the co-solvent layer may contain a considerable concentration of water. Hence, to achieve wettability, the co-solvent must efficiently reduce the surface tension of water, an effect similar to that of detergents.

Chemical stability

The co-solvent must be chemically stable at the fairly high transfer temperatures usually required (100–130°C). Owing to the large amount of co-solvent introduced, a small concentration of a reaction (hydrolysis) product could seriously disturb the system.

LC compatibility

As the main application of the technique concerns coupled LC–GC, the co-solvent should be compatible with LC. Of course, the co-solvent could be added to the LC eluent only after the LC detector. However, this presupposes an additional LC pump, delivering the co-solvent into the LC effluent stream at a low flow-rate. Technically, it is simpler to add the co-solvent to the eluent, but this presupposes that the co-solvent does not excessively increase the viscosity of the eluent and that it does not interfere with UV detection.

Efficient solvent trapping

To obtain full solvent trapping, the co-solvent must be suitable for strongly retaining the solutes of interest. As the solutes amenable to GC analysis are of low to intermediate polarity, the maximum retention power is obtained with co-solvents of relatively low polarity. However, the co-solvent must be miscible with water, which requires some polar or polarizable functional groups.

BUTOXYETHANOL AS CO-SOLVENT

Among the high-boiling solvents tested, we found 2-butoxyethanol (ethyleneglycol monobutyl ether, butylcellosolve) to be best suited for our purpose. Its boiling point at ambient pressure is 171°C. At ambient temperature, butoxyethanol is miscible with water. However, the two solvents separate into two phases when heated to *ca.* 100°C (the GC temperatures during transfer).

Butoxyethanol of purum quality was obtained from Fluka (Buchs, Switzerland). This purity is insufficient for work with a flame ionization detector at higher sensitivity owing to interfering peaks (see the chromatogram in Fig. 4). In the near future, Fluka will offer a further purified butoxyethanol (Nr. 20398).

Phase diagram for butoxyethanol–water

For the mixture of butoxyethanol–water, data found in the literature[14] allowed us to draw the phase diagram shown in Fig. 2. This diagram shows an extremely flat boiling curve, indicating that a very large proportion of water evaporates within a narrow range of temperatures. This is important, as it causes efficient evaporation of the water within a few degrees above the boiling point of the sample mixture, leaving almost only butoxyethanol to flood the precolumn.

Ref. 14 does not give any indication about an azeotropic mixture, except that a mixture containing 20.8% butoxyethanol is mentioned to boil at 98.8°C. According to Horsley[15], the azeotropic mixture boils at 98.8°C and contains 27.1% (by weight)

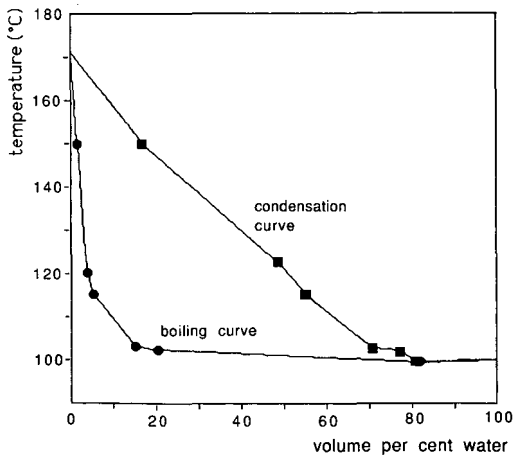

Fig. 2. Phase diagram of water–butoxyethanol after conversion of the commonly used mole fractions to volume percent.

of butoxyethanol. We distilled a water–butoxyethanol mixture, determining the composition of the distillate at various compositions of the boiling liquid. The compositions were analysed by measuring the refractive index of the liquid. With liquids containing 2.5–80% butoxyethanol (ten samples), the distillate always contained between 21.6 and 23.1% (by weight) butoxyethanol. Boiling points were at a minimum for mixtures containing 15–29% butoxyethanol (98.7°C).

Up to a pressure of at least 1 bar, the azeotropic mixture was found to be of constant composition, which is important regarding the pressure drop within the flooded zone. Neglecting possible problems with "shooting" liquid due to delayed evaporation, this allows us to work with both high and variable inlet pressures, but of course with the necessary corrections to the GC oven temperature.

Wettability characteristics

Wettability of a 0.32 mm I.D. phenyldimethylsilylated ("phesil") fused-silica capillary was tested, as described previously[16], by injecting increasing volumes of the solvent mixture to be tested until some liquid left the fused-silica capillary of interest and penetrated into the whitish glass capillary attached to it. Butoxyethanol was found to wet the "phesil" surface when it contained up to 30–35% of water.

INSTRUMENTAL

Concurrent solvent evaporation with co-solvent trapping was carried out with a device basically corresponding to the loop-type interface[11], although used for direct introduction by syringe. The design of the system is shown in Fig. 3. The test samples were drawn into the sample loop by a 10-ml plastic syringe. Steel capillary sample loops of 250 and 1000 μl were used.

Restriction instead of flow regulator

The pneumatic system for the carrier gas supply (located upstream of the carrier gas valve) differed from that of the standard loop-type interface. Instead of a flow regulator, as normally installed downstream of the pressure regulator, we mounted a restriction capillary (2 m × 0.25 mm I.D.), with a manometer ahead of and after the restriction. This arrangement served in the determination of the carrier gas flow-rate. During transfer, when almost no carrier gas flows, the two manometers show the same pressure. However, at the end of the transfer, the pressure measured on the second manometer drops to a level determined by the flow-rate of the gas passing the restriction. A more detailed description of the phenomena observed, and of their interpretation, will be given in a later paper[4].

T-Piece inside GC oven

In the standard loop-type interface, the T-piece, combining the sample supply line from the sample valve and the carrier gas line from the carrier gas valve, is located outside the GC oven. In this way, sample evaporation in the sample supply line before the T-piece can be ruled out (sample material deposited on the wall of the sample line is back-flushed as soon as the carrier gas valve is switched at the end of the transfer).

When working with co-solvents, two aspects differ from conventional concurrent eluent evaporation. First, there is no danger that sample material is lost in the

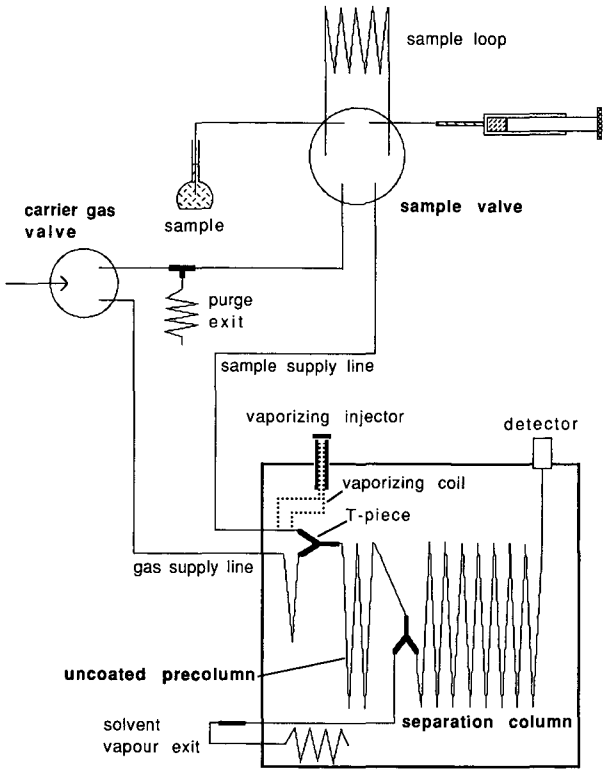

Fig. 3. Instrumental set-up used for the experiments described, basically involving a loop-type interface. However, the T-piece at the entrance of the uncoated precolumn and part of the carrier gas supply line are placed inside the GC oven. Further, there is no retaining precolumn ahead of the solvent vapour exit. The vaporizing coil entering the vaporizing injector was used for testing vaporization of the sample prior to introduction; an experiment to be described in a later paper.

sample supply line, even when the main evaporation takes place there (which is unlikely anyway with aqueous solvent mixtures, because the required heat of evaporation is large, and therefore the liquid penetrates relatively far into the oven-thermostated system). As there is a co-solvent–main solvent mixture pouring into the precolumn, solute material is carried safely through the T-piece under all conditions.

Second, the slow evaporation of the high-boiling co-solvent causes strongly broadened solvent peaks when the T-piece is kept outside the GC oven. At the end of the transfer, there is solvent within the T-piece and pushed backwards into the carrier gas line from the carrier gas valve. Switching the carrier gas valve causes the bulk of the liquid to be moved into the GC precolumn. However, there remains a film of liquid on the capillary wall that only can be transferred through evaporation. Although hardly 1 μl of liquid is involved, evaporation is disturbingly slow for high-boiling co-solvents.

For the loop-type interface used here, the T-piece and part of the carrier gas supply line were placed inside the GC oven. Both the carrier gas and the sample supply line consisted of 0.32 mm I.D. fused-silica capillaries, connected to a press-fit

T-piece. The oven-thermostated carrier gas supply line had a length of 50 cm; the remainder of the line outside the oven was *ca.* 40 cm long. The ratio of these two lengths must be chosen according to the pressure increase during transfer, as the extra pressure compresses the internal gas volume, pushing sample liquid into the line.

No retaining precolumn

Normally, concurrent solvent evaporation is carried out with two precolumns placed before the solvent vapour exit: an uncoated precolumn (2–4 m long) and a retaining precolumn about 3 m long, consisting of a piece of the separation column[17]. The retaining column is needed for retaining solute material of intermediate volatility, preventing their loss through the solvent vapour exit together with the solvent vapour. When working with co-solvent trapping, the retaining precolumn is not needed, as the co-solvent layer in the uncoated precolumn serves the same purpose (and retains volatile components far more efficiently than the stationary phase film in the retaining precolumn).

The solvent vapour exit was constructed of a press-fit T-piece and a 30 cm × 0.32 mm I.D. fused-silica capillary leaving the GC oven. To close this exit, a 1 m × 75 μm I.D. fused-silica capillary was attached to the outlet of the 0.32 mm I.D. capillary by a press-fit connector. For three reasons, this resistance capillary was re-introduced into the GC oven. First, recondensed, viscous solvent may completely block such a resistance for a long time, stopping the purge flow required to keep residual solvent vapour from the solvent vapour exit line away from the chromatographic path. Second, recondensation of solvent greatly reduces the volume, causing more vapour to be drawn into the solvent vapour exit (analogous to the recondensation effect in split and splitless injection[18]). This may have a strong impact on the (normally very small) proportion of the carrier gas–vapour mixture leaving through the solvent vapour exit, causing a loss of volatile solute material, co-evaporating with the co-solvent at the end of co-solvent evaporation. Finally, the resistance through the separation column increases with the oven temperature. To keep the proportion of the purge flow-rate with respect to the total carrier gas flow-rate constant, the resistance of the solvent vapour exit line must change with that of the separation column.

Wide-bore precolumn

In the interest of rapidly discharging the very large volume of vapour created by aqueous solvent mixtures, a wide-bore precolumn was used. In addition to the enhanced permeability, such precolumns offer an increased capacity for retaining liquid as a film on the precolumn wall. As this capacity increases proportionally with the inner diameter, a correspondingly shorter precolumn can be used, further reducing the resistance to the vapour flow.

There is probably an upper limit to the diameter of the precolumn; excessively wide precolumns used at excessively high flow-rates cause concurrent eluent evaporation to get out of control, as the liquid pushed into the precolumn by the carrier gas "shoots" too far. However, this limit has not been identified yet. The uncoated precolumn used here consisted of a 5 m × 0.53 mm I.D. fused-silica capillary deactivated with diphenyltetramethyldisilazane (DPTMDS), resulting in phenyldimethylsilylation.

RESULTS

Fig. 4 shows a chromatogram obtained from a 1-ml injection of water containing 20% of butoxyethanol. The sample components were C_{14}–C_{24} methyl esters; earlier peaks are obscured by the impurities in the butoxyethanol. Separation was carried out on a 12 m × 0.32 mm I.D. glass capillary column coated with PS-255 (a methylsilicone) of 0.3 μm film thickness. Transfer occurred at 114°C and 1 bar inlet pressure; during analysis, the inlet pressure increased with the oven temperature from 0.75 to 0.85 bar.

The solvent peak shown in the chromatogram has a width of 7.5 min. However, a closer analysis of the solvent peak observed at high attenuation and of the change of the inlet pressure during transfer (to be discussed in a later paper) makes it possible to explain this solvent peak width in more detail. Concurrent eluent evaporation took 5.5 min (starting about 1 min before the solvent peak began to be eluted). This means that the vapours passed through the solvent vapour exit at a remarkable rate of 440 ml/min. At the end of concurrent solvent evaporation, the solvent vapour exit was closed (connected to the high resistance). The additional 2 min of the solvent peak were due to evaporation of the small amount of co-solvent left in the precolumn, the vapour of which had to be discharged through the whole separation column and the GC detector. The last 1-min solvent peak width is due to impurities in the butoxyethanol.

Despite the fact that more than 100 ml of water passed through the system before the chromatogram shown was recorded, no tailing of the ester peaks was observed. This is worth noting after having experienced how rapidly precolumns become active when water is introduced.

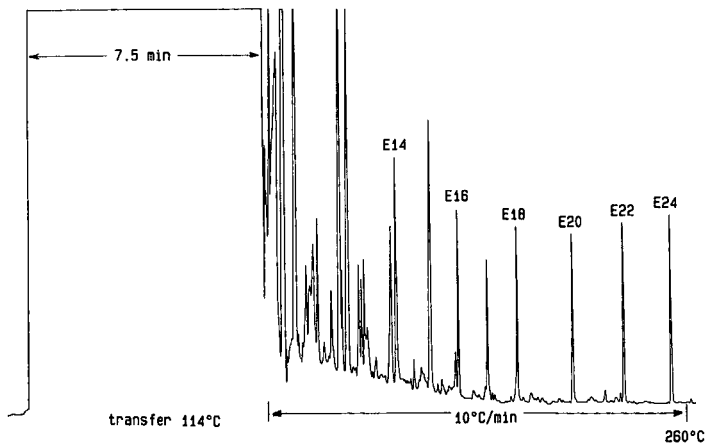

Fig. 4. Chromatogram resulting from the introduction of 1 ml of water containing 20% of co-solvent (butoxyethanol). Sample components, C_{14}–C_{24} methyl esters (E14–E24). With conventional concurrent eluent evaporation, all solute material was lost up to the last two peaks. More intensively purified butoxyethanol will be needed.

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CHROMSYMP. 1571

COUPLED REVERSED-PHASE LIQUID CHROMATOGRAPHY–CAPILLARY GAS CHROMATOGRAPHY FOR THE DETERMINATION OF ATRAZINE IN WATER

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SUMMARY

A fully automatable method for determining atrazine in water is described, based on enrichment of the atrazine from 10 ml of water on a small ODS liquid chromatographic column, desorption with methanol–water (60:40) + 5% 1-propanol and transfer to a gas chromatograph by concurrent eluent evaporation using a loop-type interface. The total analysis time is 35 min with a detection limit of *ca.* 3–5 ppt. Concurrent eluent evaporation of aqueous eluent mixtures suffers from a large temperature difference between transfer and elution of the first sharp peaks. On transfer at 112°C, the atrazine peaks were perfectly shaped only when eluted at about 250°C.

INTRODUCTION

Atrazine is one of the most widely used herbicides and it is frequently found in drinking water. Legal limits are low (0.1 ppb in Switzerland and some other European countries), calling for very sensitive analytical methods. Atrazine is determined in large numbers of samples, rendering a fully automated method desirable. Currently used methods involve sample enrichment by liquid–liquid[1] or solid-phase extraction[2], followed by reconcentration and analysis by liquid chromatography (LC) with UV detection or gas chromatography (GC) with alkali flame ionization detection. Detection limits in the routine application of these fairly labour-intensive methods are between 0.01 and 0.1 ppb.

Solid-phase extraction is often considered to be the state-of-the-art technique for sample enrichment. However, in several respects, LC sample enrichment is clearly superior. The enrichment column is further miniaturized, control of the enrichment step is more accurate (owing to on-line detection) and elution of the material of interest is more selective (owing to higher efficiency and on-line detection).

With GC as a final analytical step, on-line coupled LC is a highly attractive sample preparation method. LC enriches the sample, provides some clean-up by the

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transfer of a small fraction and on-line coupling allows full automation, starting with the raw water from an LC autosampler. Miniaturization of the sample enrichment column is possible, as the whole sample material introduced into the LC system is transferred to the GC column.

The application of coupled LC–GC to the determination of atrazine in water was considered as a first example, testing a technique (concurrent eluent evaporation of water-containing eluents) that had never been used before. There are a large number of other applications that can be carried out along the same lines. Fully automated water analysis outside the laboratory (*e.g.*, in water treatment plants) is of interest to many authorities.

Removal of water from the enrichment column

Sample enrichment necessarily occurs through a reversed-phase system, *e.g.*, an ODS LC column. However, with two exceptions[3,4], only normal-phase LC has been coupled to GC, primarily introducing "easy" solvents such as alkanes and ethers [one of these exceptions involved the use of pure acetonitrile, the other the transfer of a 2-μl volume of methanol–water (80:20)]. To permit the use of *n*-hexane for transferring the sample from the enrichment column to GC, Boo and Krohn[5] and later Noroozian *et al.*[6] proposed displacement and evaporation of the residual water from the LC column in a gas stream and by vacuum (possibly with additional heating). Complete removal of water was essential in order to allow quantitative desorption of the components of interest with the water-immiscible *n*-hexane.

The removal of water from LC columns is demanding. Experimentally, we found that mechanical displacement by gas only removes *ca.* 15–25% of the water. Apparently, a channel is opened through the packing, through which the gas flows without displacing the rest of the water. The remaining water must be evaporated. However, under these conditions the enrichment column becomes a packed GC column. As the GC retention power of ODS LC columns is fairly low, there is a considerable risk of losing volatile components together with the water vapour. On the other hand, Noroozian *et al.*[6] showed that these losses are surprisingly small.

Coupling reversed-phase LC to GC

Of course, avoidance of the water evaporation step would be desirable. In fact, the sample material trapped on the reversed-phase column can be desorbed directly with an organic solvent as long as this solvent is water miscible. The problem, however, is the water carried into the GC system.

Theoretically, the transfer could be started exactly at the front of the organic desorption medium, although even in this instance a small amount of water is carried into the GC system, as water mixes into the organic solvent. However, as the solute material of interest is likely to move right at the front of the organic eluent, such cuts exactly at the interface between the water and the organic eluent are hardly feasible in practice. To obtain a quantitatively reliable method, the cut must occur slightly earlier, including some water in the fraction transferred. Transfer of water into the GC system is only one problem; another concerns the change in the water concentration from 100% to nearly 0% during transfer, calling for a corresponding adjustment of the GC conditions.

In the method described in this paper, the problem was solved by using an LC

eluent that gives atrazine some retention on the enrichment (LC) column. This moves the atrazine peak away from the breakthrough of the organic eluent system, *i.e.*, from the water. It provides a stable eluent composition and an efficient sample clean-up. On the other hand, retention of atrazine could only be achieved with a fairly high water concentration (40%) in the desorption medium (LC eluent).

Transfer by concurrent eluent evaporation

Recently we investigated several possibilities of transferring water and water-containing reversed-phase eluents to capillary GC. The use of retention gap techniques[7] (with or without partially concurrent eluent evaporation) is restricted to liquids that wet the pre-column wall. This requirement could not be met with water[8], but was possible with many commonly applied aqueous reversed-phase mixtures. However, a second requirement is more difficult to fulfil: water must evaporate at least as rapidly as the organic solvent in order to prevent non-wetting water from being left behind. The eluent mixture in our application, water–methanol (40:60) containing 5% 1-propanol, does not fulfil either of these two requirements[9], ruling out the use of retention gap techniques.

Transfer to GC by the concurrent eluent evaporation technique[10] through a loop-type interface[11] does not require wettability of the pre-column, nor is it important which solvent evaporates more rapidly. The eluent plug is pushed into the oven-thermostated capillary pre-column by the carrier gas. Flooding of the pre-column is prevented by using an oven temperature above the boiling point of the eluent at the inlet pressure, producing an eluent vapour pressure to stop the eluent flow.

On the other hand, concurrent eluent evaporation causes strong broadening of peaks up to elution temperatures considerably above the transfer temperature. This is a particularly severe problem with aqueous eluents because water does not produce any phase soaking (increase of retention power in the coated column by swelling the stationary phase[12]). With polar stationary phases, even a reversed-phase soaking effect may be observed[13], accentuating band broadening. As reconcentration just relies on cold trapping, peak broadening affects components with higher elution temperatures than when using well soaking, less polar eluents.

LC–GC transfer of atrazine occurred by concurrent eluent evaporation. As will be shown, the difference in oven temperature between transfer and elution was just about sufficient for complete reconcentration.

EXPERIMENTAL

Experiments were carried out on an LC–GC prototype instrument with a Phoenix 20 syringe pump, an alkali flame ionization detector (NPD-40) and a fully automated interface system from Carlo Erba (Milan, Italy).

LC enrichment and desorption

LC sample enrichment column. Of the commonly used reversed-phase LC packing materials, ODS phases exhibited the highest retention power (capacity factor) for atrazine. This was important for obtaining a maximum capacity of the LC enrichment column and for keeping the required column size minimal. As atrazine is highly retained, it forms a short band at the top of the LC column, which, in turn, is

a prerequisite for obtaining an efficient LC separation and sharp LC peaks (small fraction volumes). Finally, a high retention power allowed elution with an eluent containing a minimum of water. Among the 5-μm ODS materials tested, Spherisorb, Nucleosil, Hypersil and LiChrosorb had similar retention powers (columns from Stagroma, Wallisellen, Switzerland). We selected a Knauer 100 × 2 mm I.D. column packed with 5-μm Spherisorb ODS.

Sample introduction. A 10-ml volume of the water sample was introduced into the enrichment column through an injector loop of slightly larger internal volume. After passage of 10 ml of water through the LC column, shortly before complete discharge of the loop contents, the LC injection valve was returned from inject to load, feeding desorption medium. This ensured a sharp transition from water to eluent (water and eluent are mixed at the rear end of the sample plug in the sample loop), and this rear was discarded in this way. Further, the desorption medium entered at an accurately known time, which is important for an accurate determination of retention times during desorption.

Eluent flow-rate. The pump constantly delivered the eluent for desorption, selected as the strongest eluent mixture safely separating the atrazine peak from the breakthrough peak, namely methanol–water (60:40) with 5% 1-propanol. The flow-rate of 400 μl/min was optimized for desorption. Owing to the lack of an additional pump or a possibility of automatically changing flow-rates delivered by the pump, passage of the water sample through the enrichment column had to occur at the same flow-rate. As this led the sample enrichment to be time consuming, this step was

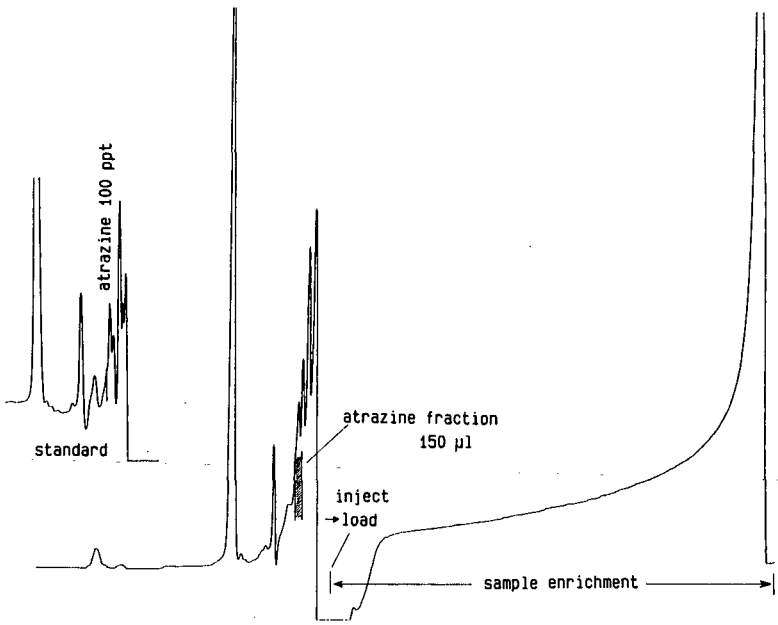

Fig. 1. Liquid chromatogram (UV detection at 220 nm) with initial sample enrichment (10 ml of tap water). At the point indicated, shortly before the content of the sample loop was completely transferred, the injection valve was switched, starting elution. The atrazine fraction is marked. At the left, part of the chromatogram of another tap water sample spiked with 100 ppt of atrazine is shown.

started on the next sample before the preceding GC analysis was completed. From the LC eluate, detected at 220 nm (Fig. 1), an atrazine fraction of 150-μl volume was transferred to the GC system.

Transfer to GC

The GC system involved an early solvent vapour exit in order to accelerate the discharge of the large volume of vapour produced by the aqueous eluent. A 2 m × 0.53 mm I.D. uncoated precolumn (phenyldimethylsilylated fused silica) was used to keep the pressure drop over this precolumn small. It was followed by a 3 m × 0.32 mm I.D. retaining precolumn taken from the separation column. The solvent vapour exit, positioned between the retaining precolumn and the separation column, was constructed of a glass press-fit Y-piece, connected to an electric three-way valve through a 30 cm × 0.32 m I.D. fused-silica capillary. The exit was automatically switched to a high resistance (2 m × 0.10 mm I.D. fused-silica capillary) 2 min after the pressure started to drop from the high transfer level towards the lower analysis level.

In order to overcome broadening of the atrazine peak, a GC column with a high retention power was selected, providing a high elution temperature, *viz.*, a 40 m × 0.32 mm I.D. column, coated with Carbowax 20M of 0.4-μm film thickness. In this way, the elution temperature of atrazine could be increased to 260°C.

The carrier gas (hydrogen) pressure delivered to the flow regulator was 1.0 bar. The flow regulator was in position 100, creating an inlet pressure of 0.5 bar at 112°C. During transfer, this pressure increased to 0.8 bar. Higher inlet pressures were avoided in order to keep the transfer temperature low. This change in the inlet pressure during transfer was sufficient for recognizing the end of eluent transfer and causing automatic actuation of the solvent vapour exit valve, as well as starting the GC run.

Under the conditions used, involving a high eluent evaporation rate and a carrier gas inlet pressure of 0.8 bar during transfer, the safe transfer temperature required to rule out excessive flooding was 112°C. This is *ca.* 10°C above the temperature listed[14], which was determined at a lower evaporation rate (strong cooling and a strong tendency towards delayed evaporation of aqueous eluents renders the minimum required column temperature dependent on the eluent evaporation rate).

A problem that occurred with one precolumn, but not with two others, taken from the same piece of deactivated fused-silica, must be examined more carefully in the future. On this one pre-column (2 m × 0.53 mm I.D., phenyldimethylsilylated fused silica), the eluent plug rushed at high speed through the whole uncoated precolumn, too rapidly to be warmed up and to build up the vapour pressure required to stop the liquid. Eluent penetrated into the retaining precolumn and some of it was lost through the solvent vapour exit. Such "shooting" liquid causes peak broadening (spreading of the solutes over the retaining precolumn) and losses in peak area (affecting all components equally). Oven temperatures had to be increased to 150–160°C to stop the liquid in an early part of the uncoated precolumn, *i.e.*, to a temperature far above the actual boiling point of the eluent. This problem could be due to poor wettability of the precolumn wall by the eluent, causing low friction of the moving liquid and poor transfer of heat through the capillary wall–liquid interface. On the other hand, the two other precolumns were not wetted by the eluent either, and even pure water could be concurrently evaporated at 125°C.

RESULTS AND DISCUSSION

Atrazine in water

Fig. 2 shows a full gas chromatogram obtained from 10 ml of tap water spiked with 100 ppt of atrazine. In the upper right corner, part of the gas chromatogram of another tap water sample, containing 15 ppt of atrazine, is shown. The peak that eluted after the atrazine, observed in all samples, was not identified, but is probably an artifact from the interface. The method proved to be simple and the analysis time per sample was about 35 min, with practically no work involved (owing to limitations of the autosampler, full automation was possible only when the sample volume was reduced to 2 ml).

Concurrent evaporation of aqueous eluents

This study also gave some insight into the LC–GC transfer of aqueous eluents by concurrent eluent evaporation, revealing two weak points. First, experiments clearly showed that a large difference in oven temperature between eluent transfer and elution of the solute of interest is required in order to obtain sharp peaks, when water is involved. Fig. 3 illustrates this for atrazine. A 15 m × 0.32 mm I.D. capillary column was used, coated with Carbowax 20M of 0.4-μm film thickness, which eluted atrazine at 238°C. Transfer of a fraction volume of 150 μl at 112°C did not lead to significant peak broadening (full chromatogram). At 120°C, however, broadening is already visible, and at 130°C it is very strong. When the fraction volume was increased to 250 μl, broadening was clearly visible even at the lowest transfer temperature.

Other organics in waters

The method described is highly specific for atrazine. The gas chromatograms are correspondingly "empty", just showing the atrazine peak. Such methods are of high

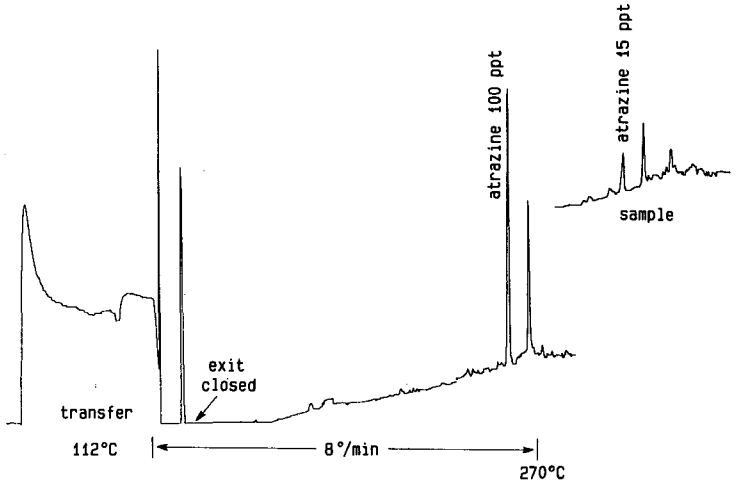

Fig. 2. Gas chromatogram of tap water spiked with 100 ppt of atrazine. Transfer (eluent evaporation) took 7.5 min. The solvent vapour exit was closed where marked. Right, part of the chromatogram of another tap water sample containing 15 ppt of atrazine.

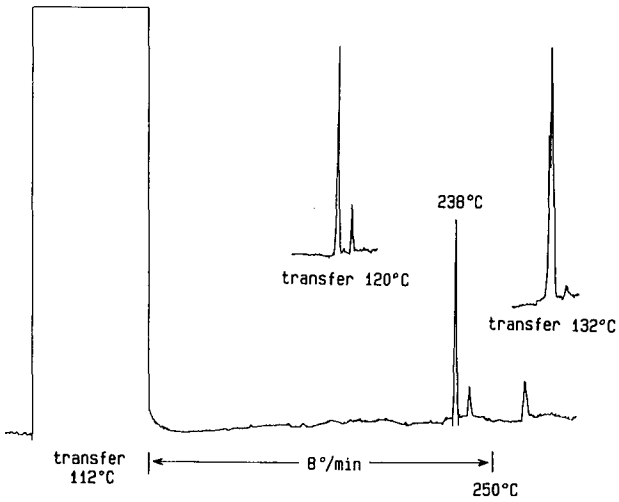

Fig. 3. LC–GC transfer of aqueous eluents requires large temperature differences between transfer and elution of the components of interest. Using a column from which atrazine was eluted at 238°C, transfer at 112°C resulted in negligible, at 120°C in clearly visible and at 132°C in strong peak broadening. Hence, in this instance the required temperature difference was 125°C. Experiments were carried out with 150-μl transfer volumes of methanol–water (60:40) eluent.

value for "dirty" samples, allowing efficient removal of interfering peaks. On the other hand, the method is not suitable for determining atrazine together with its first degradation product, desethylatrazine, or for the analysis of a whole "cocktail", *e.g.*, of triazine herbicides, as the fraction volumes would become very large. Nor is the method suitable for determining components eluted at GC temperatures below *ca.* 240°C owing to the problem concerning peak broadening by concurrent eluent evaporation.

The LC column used was far larger than required for retaining atrazine from 10 ml of water. A smaller LC column would allow elution with a smaller volume of eluent, as would the use of a stronger eluent containing less water. However, both options were discarded in view of the requirement of separating the atrazine peak from breakthrough. As long as conventional concurrent eluent evaporation is used, substantial enlargement of the LC fraction is impossible; with the GC column used in Fig. 2, an increase in the fraction volume above about 300 μl caused visible peak broadening. A further increase in the elution temperature of atrazine, for further improved reconcentration, is difficult.

The method described produced perfect results for atrazine. However, there are a large number of other organic components to be determined in water, and only a limited selection of them could be determined by the same technique. This calls for a complementary method, possibly transferring the LC column in backflushing; problems related to peak broadening, especially of components eluted at lower temperatures, can be solved using concurrent solvent evaporation with co-solvent effects[15].

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CHROMSYMP. 1574

ANALYSIS OF 5-FLUOROURACIL IN PLASMA BY PRECOLUMN DERIVATIZATION WITH 4-BROMOMETHYL-7-METHOXYCOUMARIN, FOLLOWED BY MULTI-DIMENSIONAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An assay for 5-fluorouracil (5-FU) has been developed that utilizes a double extraction with ethyl acetate, followed by precolumn derivatization with 4-bromomethyl-7-methoxycoumarin. The reaction mixture was quenched with 5% acetic acid, extracted with hexane, and analyzed by multi-dimensional high-performance liquid chromatography. Derivatized 5-FU was injected into a cyanopropyl column and a heart cut containing the analyte was then switched to an octadecyl column and quantitated by fluorescence detection. The assay had a limit of detection of 0.5 ng 5-FU/ml plasma and was linear to 20 μg/ml. It was shown to be free of interferences from the other anticancer agents commonly used in combination with 5-FU. This assay should have the sensitivity needed to measure the low levels that occur after low-dose, continuous infusion of 5-FU.

INTRODUCTION

5-Fluorouracil (5-FU, Fig. 1, I) is an antimetabolite which has been used in cancer chemotherapy for many years[1]. Although a large number of quantitative methods for 5-FU have been reported, including microbial assays[2], high-performance liquid chromatography (HPLC)[3–14] and gas chromatography with mass spectroscopic detection (GC–MS)[15–18], only GC–MS has enough sensitivity for determining the pharmacokinetic parameters for 5-FU following low-dose, continuous infusions. After the termination of a continuous intravenous infusion of 5-FU, the plasma concentration of 5-FU quickly decreases below the limit of detection of most assays (*ca.* 5–10 ng/ml), because 5-FU has an elimination half-life of 6–12 min[14,15]. Derivatization of 5-FU with 4-bromomethyl-7-methoxycoumarin (BrMmc, Fig. 1, II) allows the use of HPLC with the increased sensitivity of fluorescence detection[11–13]. Finn and Sadee[18] have discovered the existence of a second, much longer, β-

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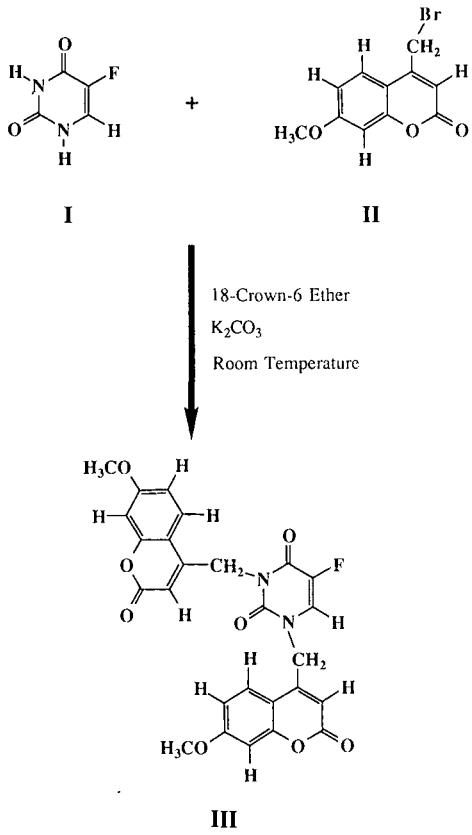

Fig. 1. Structure of 5-fluorouracil (I), 4-bromomethyl-7-methoxycoumarin (II), and the resulting 5-FU-$(Mmc)_2$ derivative (III).

elimination phase for 5-FU by GC–MS; however, their method of analysis was unable to quantify this parameter reliably. BrMmc was first synthesized by Secrist *et al.*[19] and used for the derivatization of thiouracil. Later[20], BrMmc was utilized for the derivatization of fatty acids. Iwamoto *et al.*[10,11] first reported the derivatization of 5-FU with BrMmc in acetone–acetonitrile with powdered potassium carbonate and 18-crown-6 ether as catalysts. Subsequently, it was found that the 18-crown-6 ether could be eliminated[12,13] by conducting the reaction in dimethyl sulfoxide (DMSO). This reaction proceeds quickly and quantitatively at room temperature. However, the full potential of this highly fluorescent derivative was not realized due to plasma interferences. Previously, the purification and identification of the 5-FU–BrMmc derivatization product [5-FU-$(Mmc)_2$, Fig. 1, III] was reported[21], along with preliminary evidence that multi-dimensional chromatography can provide the low detection limits needed to characterize the pharmacokinetics of 5-FU more thoroughly.

Using DMSO as the derivatization solvent gave satisfactory yields for 10 ng or more of 5-FU. However, attempts to derivatize 1 ng 5-FU or less with this reaction

system did not yield any detectable product. Returning to the original scheme of Iwamoto *et al.*[10,11], who used a 18-crown-6 ether catalyst and acetone–acetonitrile, it was possible to derivatize 0.2 ng 5-FU reproducibly. This reaction scheme, coupled with the multi-dimensional chromatography previously described[21], yielded an assay with a limit of detection of 0.5 ng/ml plasma and linearity up to 20 μg/ml on only 0.5 ml of sample.

EXPERIMENTAL

Chemicals

5-Fluorouracil (5-FU), prednisolone, prednisone, methotrexate, uracil, thymine and cytosine were purchased from Sigma (St. Louis, MO, U.S.A.). 4-Bromomethyl-7-methoxycoumarin (BrMmc), 18-crown-6 ether, and cyclophosphamide were obtained from Aldrich (Milwaukee, WI, U.S.A.). Injectable 5-FU (50 mg/ml, SoloPak Labs., Franklin Park, IL, U.S.A.), mitomycin C (0.5 mg/ml, Bristol Labs., Syracuse, NY, U.S.A.) and vincristine (1 mg/ml, DuPont Critical Care, Waukegan, IL, U.S.A.) were obtained from the University of Kansas Medical Center pharmacy. Adriamycin was obtained from Dr. S. Lindenbaum (University of Kansas) and 5,6-dihydro-5-fluorouracil was a gift from Dr. K. Chan (University of Southern California). Sodium acetate, glacial acetic acid, HPLC-grade solvents and potassium carbonate were purchased from Fisher Scientific (St. Louis, MO, U.S.A.). The potassium carbonate was powdered with a mortar and pestle and dried in a vacuum oven (30°C) overnight before use. All water was deionized and purified with a Milli-Q water system (Millipore, Bedford, MA, U.S.A.) before use. All glassware used for the extraction and derivatization was silanized with trimethylchlorosilane in chloroform.

Extraction of plasma

Initially, the extraction efficiencies of various solvents were compared by extracting 0.5 μg 5-FU from 0.5 ml phosphate buffered saline. Either 5 or 9 ml volumes of ethyl acetate, methyl-*tert.*-butyl ether (MTB), diethyl ether, chloroform and dichloromethane were used. After addition of solvent, the tubes were mixed for 2 min and centrifuged (1000 *g*) for 5 min. The solvent layer was removed, dried under nitrogen, and reconstituted in 50 m*M* KH_2PO_4 (pH 3.0). The recovery of 5-FU was assessed by HPLC with UV detection[7].

In the final assay procedure, plasma samples containing 5-FU (0.5 ml) were vortex-mixed with 100 μl 0.5 *M* KH_2PO_4 (pH 3.5) in screw-capped culture tubes (150 × 16 mm). Ethyl acetate (9.0 ml), previously saturated with water, was added, and the samples were mixed for 2 min. Following centrifugation (1000 *g*, 10 min) to achieve separation of the layers, the ethyl acetate was removed and the remaining aqueous layer was re-extracted with another 9 ml of ethyl acetate (water-saturated). The extracts were combined in silanized glass, conical centrifuge tubes and dried under nitrogen.

The extraction efficiency was determined by adding 250 ng 5-FU to 0.5 ml plasma and to the ethyl acetate extract from 0.5 ml blank plasma. The plasma samples were extracted as described above and derivatized with BrMmc, as described below. Following multi-dimensional HPLC, the recovery of 5-FU from plasma was expressed as the ratio of the peak heights from the two samples.

Derivatization of 5-FU with BrMmc

5-FU was derivatized with BrMmc, using a modification of the procedure previously reported by Iwamoto *et al.*[10,11]. 5-FU or dried plasma extracts were solubilized with 100 μl of ethyl acetate, containing 1 mg 18-crown-6 ether. This was followed by the addition of 5 mg potassium carbonate and 0.25 mg BrMmc in 400 μl acetone–acetonitrile (1:2, v/v). The samples were vortex-mixed after the addition of each compound. After 2 h at 75°C or 40 h at room temperature (22 ± 2°C) the reaction was quenched with 625 μl of 5% acetic acid in water and the mixture was extracted twice with 1-ml portions of hexane. The hexane extracts were discarded and the remaining aqueous layers were then ready for analysis by HPLC.

To determine the derivatization yield, 5-FU was derivatized in the presence of plasma extract and compared with previously isolated and purified 5-FU-$(Mmc)_2$[21]. 5-FU (250 ng) was added to a plasma extract, dried, derivatized as described above, and quantitated by multi-dimensional HPLC. The peak heights of these samples were divided by the peak heights from the molar equivalent of 5-FU-$(Mmc)_2$ to obtain the efficiency of derivatization.

Stability of 5-FU-$(Mmc)_2$

The stability of 5-FU-$(Mmc)_2$ in acetone–acetonitrile and buffer mixtures was investigated. A sample of 5-FU-$(Mmc)_2$ (50 ng) in 1 ml acetone-acetonitrile was mixed with 1.25 ml 10 m*M* acetic acid (pH 3.4), 10 m*M* sodium acetate (pH 5.0), or 50 m*M* potassium carbonate (pH 11.0) and kept in the dark at room temperature (22 ± 2°C). At various times, an aliquot of each mixture was removed and analyzed by multi-dimensional HPLC.

HPLC

The chromatographic system (Fig. 2) consisted of two Model 110 A pumps (Altex Scientific, Berkeley, CA, U.S.A.), a WISP 712 autosampler (Waters Assoc., Milford, MA, U.S.A.), an SLIC 1400 controller (Sys-Tec, New Brighton, MN, U.S.A.), a 6-port air-actuated switching valve (Rheodyne, Cotati, CA, U.S.A.), and a Model 535 fluorescence detector and C-R5A integrator (Shimadzu, Kyoto, Japan). The columns used were 150 × 4.6 mm I.D., packed in this laboratory[22] with 5-μm silica particles, bonded with cyanopropyl (CPS-Hypersil, Shandon Southern Products, London, U.K.) or octadecyl (ODS-Hypersil) side chains.

The CPS and ODS columns were eluted at 1.5 ml/min with methanol–10 m*M* sodium acetate, pH 4.75 (4:6, v/v) and methanol–10 m*M* sodium acetate, pH 4.75 (1:1, v/v), respectively. Both columns were jacketed and thermostated at 35°C with a circulating water bath (Haake, Saddlebrook, NJ, U.S.A.) to minimize fluctuations in retention due to periodic changes in the ambient temperature.

Samples (100 μl) were injected onto the CPS column with the WISP autosampler and a 2.5-min heart cut containing the derivative peak was switched onto the ODS column. The time of the cut was determined by injecting a standard solution of 5-FU-$(Mmc)_2$ onto the CPS column and monitoring the eluent with the UV detector (325 nm). The switching valve was actuated by the SLIC controller and immediately after the cut, the integrator was started. After further separation on the ODS column, the derivative was detected with the fluorescence detector (λ_{ex} = 325 nm, λ_{em} = 395 nm) and quantitated by peak height measurement.

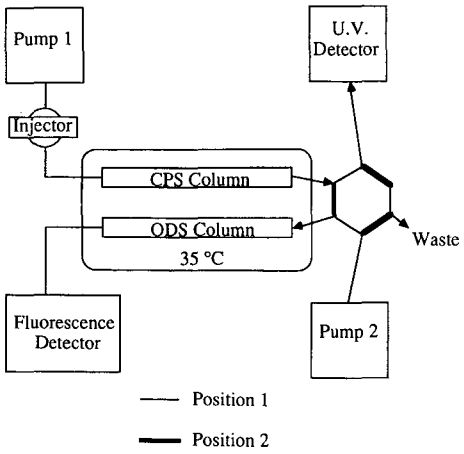

Fig. 2. Schematic drawing of the column switching HPLC system. The valve is in position 1 for normal operation. During the heart cut the valve is in position 2 and the eluent flows from the CPS column into the ODS column.

Standard curve

Linearity of the assay was established by generating standard curves of 5-FU from plasma over two concentration ranges. The low range was 0, 0.5, 1, 5, 10, 50, 100 and 200 ng 5-FU/ml, while the high range was 0, 0.5, 1, 5, 10 and 20 μg 5-FU/ml. Each solution was analyzed in triplicate with a reaction time of 40 h at room temperature. The 5, 10 and 20 μg/ml concentration levels were diluted 1:10 before injection to keep the peaks within the linear range of the fluorescence detector. For analysis of samples, a single standard curve that covered the expected concentrations of the samples was generated.

Rabbit pharmacokinetics

A preliminary pharmacokinetic profile of 5-FU in a rabbit was obtained. 5-FU (50 mg/kg) was administered to a New Zealand rabbit (4.3 kg) by intravenous bolus in the ear vein over 2 min. At 1, 4, 6.5, 10, 14.5, 18.5, 25, 28 and 43 min, and 1, 1.5, 2, 3 and 21.5 h after injection, blood samples (1.8 ml) were collected from the ear artery with a syringe, containing 0.2 ml of 0.1 *M* potassium oxalate. Plasma was obtained by centrifugation (1000 *g*, 15 min) and stored at −20°C until analyzed. The data were fit to a two-compartment model with bolus administration and first order elimination, using PCNONLIN (Statistical Consultants, Edgewood, KY, U.S.A.).

RESULTS AND DISCUSSION

Several assays for 5-FU have been reported[2–19], however, most of these have severe limitations. The most sensitive of these, GC-MS[15–18], requires expensive equipment and a high degree of technical expertise. The use of HPLC is less expensive and much simpler, however, it lacks the required sensitivity to determine the terminal β-elimination phase of 5-FU in plasma[18]. An assay that has the high sensitivity of

GC–MS and the ease of HPLC was required to characterize 5-FU pharmacokinetics further. The various stages of this assay were developed in a rational and systematic fashion. The optimization of the extraction and derivatization of 5-FU was required before multi-dimensional HPLC could be used to its fullest potential. The linearity, accuracy and reproducibility of the assay were established, once the assay development had been completed. Finally, the use of this assay for pharmacokinetic analysis was demonstrated.

Choice of extraction solvent

Extraction of 5-FU from phosphate buffered saline was investigated with a variety of solvents. The results are summarized in Table I and show that ethyl acetate and dichloromethane gave comparable extraction efficiencies. However, ethyl acetate was selected over dichloromethane, because it is less hazardous and yielded a cleaner organic layer after extraction (no precipitated plasma proteins in the upper layer after centrifugation). Further investigation revealed that acidification of the sample with 0.5 *M* KH_2PO_4 (pH 3.5)[10], followed by a double extraction of 0.5-ml samples with 9 ml ethyl acetate (water-saturated) gave the best results.

Because the derivatization reaction was dependent on the sample matrix, the determination of the extraction efficiency was performed while keeping the derivatization conditions constant. To achieve this, 5-FU was added to an extract from 0.5 ml blank plasma. The peak height of the derivative formed from 250 ng 5-FU, extracted from 0.5 ml plasma, was 21 619 $\pm$ 1869 μV ($\bar{x} \pm$ S.D.) and 23 275 $\pm$ 1170 μV ($\bar{x} \pm$ S.D.) from 250 ng 5-FU added to blank plasma extract. The recovery of 5-FU from 0.5 ml plasma with two 9-ml portions of ethyl acetate was calculated to be 92.9 $\pm$ 8.0% ($\bar{x} \pm$ S.D.). Since the standard curves were found to be linear, it is reasonable to assume that the recovery of 5-FU remains constant and is independent of concentration.

Derivatization kinetics

Initially, the effects of temperature and time on the derivatization reaction were determined with solutions of 5-FU in acetone–acetonitrile. 5-FU (100 ng) was derivatized at 75°C or at room temperature (22 $\pm$ 2°C), and the reaction was quenched at various times. It can be seen from Fig. 3a that the reaction reached a plateau after 15

TABLE I

RECOVERY OF 1 μg 5-FU/ml PHOSPHATE BUFFERED SALINE WITH VARIOUS EXTRACTION SOLVENTS

Solvent	*Volume (ml)*	*% Recovery*
Ethyl acetate	5	54
	9	77
MTB	5	23
	9	36
Diethyl ether	9	25
Chloroform	9	3
Dichloromethane	9	78

min at 75°C and 45 min at room temperature. However, when the derivatization of 5-FU from a plasma sample was carried out for 0.5 h at 75°C or 1 h at room temperature, the peaks were only 10% of the expected height. This led to further investigation of the derivatization of 5-FU extracted from plasma.

Human plasma was spiked with 200 ng 5-FU/ml, and 0.5-ml aliquots were extracted twice with 9 ml ethyl acetate. Following the evaporation of the solvent, the extract was derivatized at 75°C or room temperature and quenched at different times. When 5-FU extracted from plasma was derivatized, the reaction did not reach a plateau until 2 h at 75°C or 20 h at room temperature (Fig. 3b). This decrease in reaction rate could be attributed to several factors. Two of the most likely factors are either inhibition of the derivatization reaction by extracted plasma components or a decrease in the dissolution rate of 5-FU from the plasma residue. The latter seems to be the most probable, since the final yield of the reaction is the same for both 5-FU and the 5-FU extracted from plasma. Also, the solubilization of the extraction residue with 100 μl ethyl acetate before derivatization for 1 h at room temperature resulted in a 2-fold increase in peak height (data not shown). The chromatographic profile from

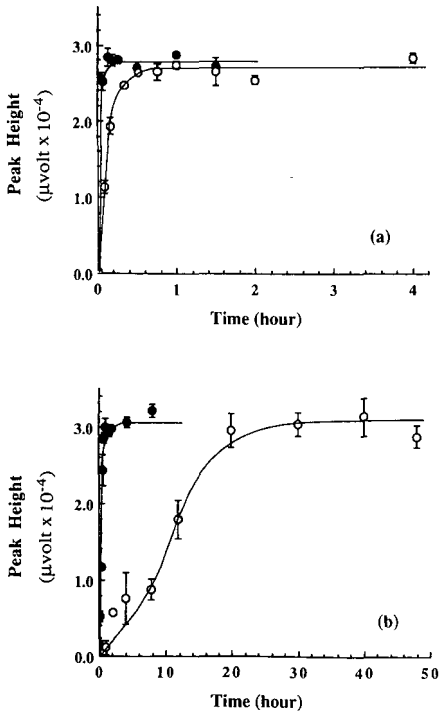

Fig. 3. Time profile for the derivatization of 5-FU with BrMmc. 5-FU in (a) neat solution (100 ng) or (b) extracted from plasma (200 ng/ml) was derivatized with 0.25 mg BrMmc in 500 μl acetone–acetonitrile (1:2, v/v), containing 0.1 mg 18-crown-6 and 5 mg potassium carbonate at (●) 75°C or (○) room temperature (22 ± 2°C). The reaction was quenched with 625 μl 5% acetic acid at various times and the product was analyzed by HPLC. The derivatization of 5-FU in neat solution is complete in 15 min at 75°C or 45 min at room temperature. The plasma extract extends the derivatization time to 2 h at 75°C or over 20 h at room temperature.

reversed-phase HPLC was significantly cleaner with derivatization at room temperature than at 75°C. Difficulty in maintaining the seal of some of the reaction tubes resulted in evaporation of the derivatization solvent from these tubes at 75°C. To alleviate these problems, the derivatization reactions were carried out in the dark at room temperature for 40 h. This time was chosen to insure that the reaction had gone to completion and because it was found to be the most convenient for scheduling the analysis of large numbers of samples. When the peak heights from 250 ng 5-FU, added to plasma extract, were compared with a molar equivalent of isolated 5-FU-$(Mmc)_2$, the extent of derivatization was calculated to be 92.5 ± 4.6% ($\bar{x}$ ± S.D.). Comparison of the peak heights from 5-FU added to plasma with those obtained with the 5-FU-$(Mmc)_2$ gives the absolute efficiency of the extraction and derivatization, which was calculated to be 85.9 ± 7.4% ($\bar{x}$ ± S.D.).

Stability of the derivative

To insure that there was no loss of the derivative when samples were stored in an autosampler, its stability was investigated at various pH values. The solutions in aqueous acetic acid or sodium acetate (pH < 5.0) showed no sign of degradation after 6 h (Fig. 4). However, in the potassium carbonate solution (pH 11.0) extensive degradation was seen with a half-life of the derivative estimated to be less than 50 min. Further investigation revealed that 625 μl of 5% acetic acid neutralized the potassium carbonate used as a catalyst in the reaction and kept the pH of the reaction mixture below 5.0. Repeated injection of a spiked plasma sample over 24 h showed no decrease in the resulting peak (data not shown), allowing the use of an autosampler.

Chromatography

The use of a single cyanopropyl (CPS) or octadecyl (ODS) HPLC column was investigated, but the late-eluted peaks and lack of baseline separation of 5-FU-$(Mmc)_2$ from other contaminants (Fig. 5a and b) led to the development of a multi-dimensional system (single-column HPLC would be adequate if one were working at the elevated

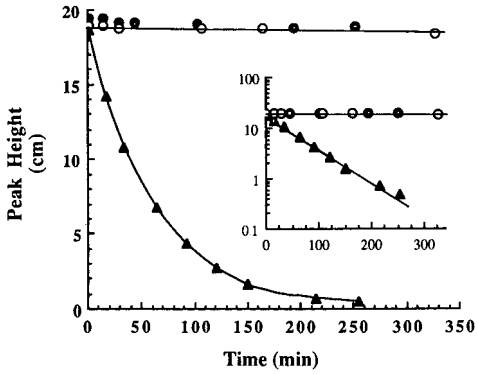

Fig. 4. Stability of 5-FU-$(Mmc)_2$ in the reaction mixture. A 5-FU-$(Mmc)_2$ solution (50 ng/ml) in acetone–acetonitrile (1:2, v/v) was diluted 1:1.25 with (○) 10 m*M* acetic acid (pH 3.4), (●) 10 m*M* sodium acetate (pH 5.0), or (▲) 50 m*M* potassium carbonate (pH 11.0) and analyzed by multi-dimensional HPLC. The 5-FU-$(Mmc)_2$ derivative is stable below pH 5.0. However, at pH 11.0, the half-life estimated from the semi-log plot (inset) is 50 min.

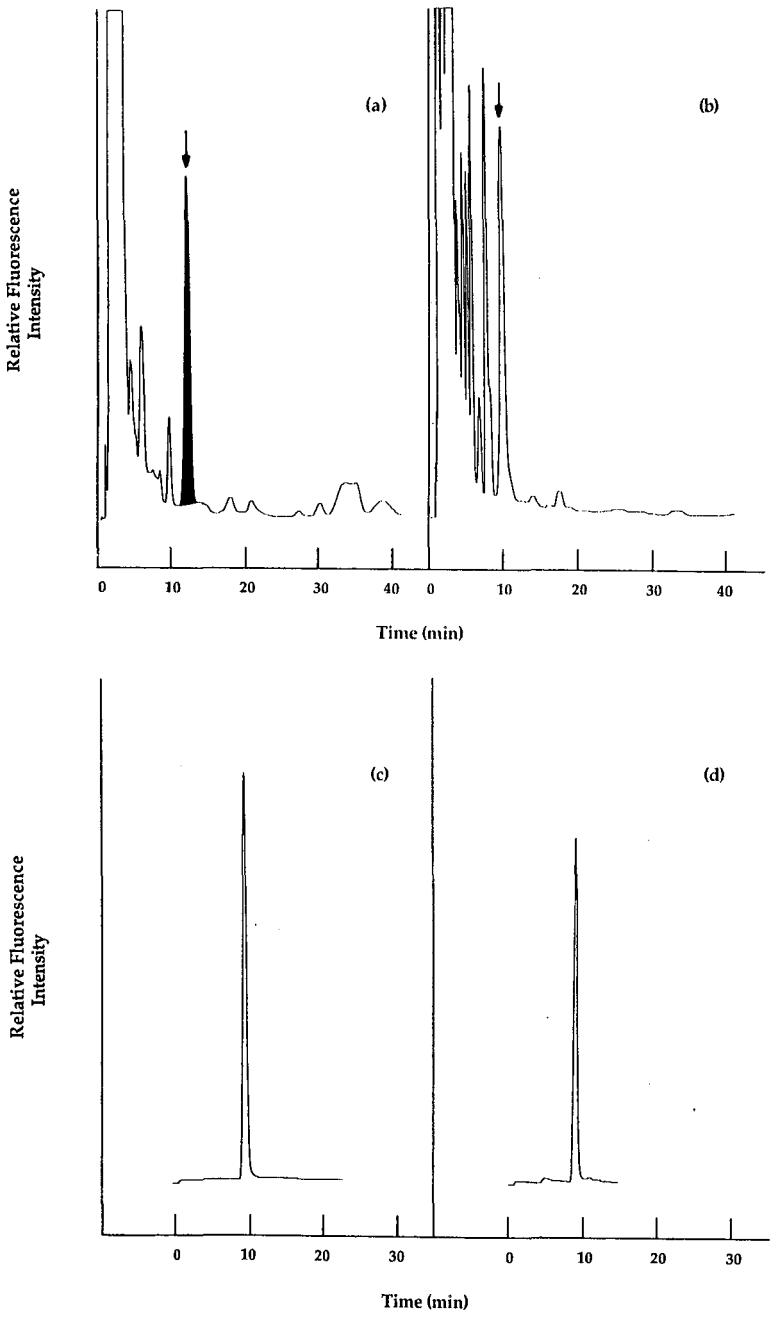

Fig. 5. Chromatograms from 5-FU-$(Mmc)_2$ or plasma samples. The arrows mark the analyte peak. The chromatograms from a patient sample containing 433 ng 5-FU/ml that was injected into (a) a CPS column eluted with methanol–sodium acetate (4:6, v/v) at 1.5 ml/min at 35°C, (b) an ODS column eluted with methanol–sodium acetate (1:1, v/v) at 1.5 ml/min at 35°C, or (c) the peak (filled in area) from the CPS column was switched to the ODS column. A chromatogram from a rabbit plasma sample (110 ng 5-FU/ml) after multi-dimensional HPLC is also shown in (d).

levels shown in Fig. 5a and b). Fig. 5c and d show the resulting chromatograms when multi-dimensional chromatography was used. The 5-FU-$(Mmc)_2$ peak was free of interfering peaks at concentrations of 110 and 433 ng/ml. The use of column switching with two different column packings improved the separation and shortened the analysis time. When the next sample is injected shortly after the heart cut is finished, the analysis time is about 15 min per sample. If either the CPS or ODS column were used alone, the late-eluted peaks extend the analysis time to more than 20 min.

5-FU is used in combination with many other anticancer agents. The most common of these drugs are adriamycin, methotrexate, vincristine, spirogermanium, prednisone, prednisolone, mitomycin C and cyclophosphamide. To insure that these drugs would not interfere with this assay, eight separate solutions, containing 110 ng 5-FU and 5 μg of one of these agents were derivatized. After quenching, the reaction mixtures were analyzed with the column switching system. There was no difference in the peak heights of the 5-FU derivative from the samples containing the other drugs when compared with 5-FU alone. Also, the chromatograms did not have any other peaks related to these other agents. Endogenous compounds with similar structures: 5,6-dihydro-5-fluorouracil (the major metabolite), uracil, thymine and cytosine were also tested. None of these caused any interference with the 5-FU-$(Mmc)_2$ peak. However, peaks believed to be the corresponding coumarin derivative for uracil and 5,6-dihydro-5-fluorouracil were found to be eluted before and after 5-FU-$(Mmc)_2$, respectively. Both of these other peaks were well separated from the analyte peak.

Assay validation

Representative standard curves for the high- and low-concentration ranges are shown in Fig. 6. The extraction and derivatization is linear from 0.5 ng to 20 μg 5-FU/ml plasma, although dilution of solutions containing more than 5 μg/ml is needed to remain in the linear range of the detector used in this study. Linear regression of peak heights *versus* concentration gave eqns. 1 and 2 for the high and low standard curves, respectively:

$$y = 8.7 \cdot 10^2 + 1.4 \cdot 10^2 x \qquad (r^2 = 0.999) \tag{1}$$

$$y = 1.3 \cdot 10^1 + 1.8 \cdot 10^2 x \qquad (r^2 = 0.999) \tag{2}$$

The units for the peak heights (y) are in μV and the concentrations (x) are in ng/ml. The slopes for the two curves are not significantly different (Student's t-test). The limit of detection was 0.5 ng 5-FU/ml (signal-to-noise ratio = 2), which is a great improvement over that reported previously with this reaction procedure[10–13].

The accuracy and precision of the assay (Table II) was determined, using the standard curve data. The error over the complete assay range was generally less than 6%. As expected, it was higher at the low concentration end of the two standard curves (*i.e.*, 0.5 and 1 ng/ml for the low curve and 500 ng/ml for the high curve). However, the accuracy could be improved by using a smaller concentration range than was used here. Although, it is preferable to use an internal standard to improve the reproducibility of the assay, a suitable compound with similar extraction, derivatization and chromatographic characteristics could not be found. Even without an internal standard, the relative standard deviation was acceptable with occasional scatter. At the 1000 ng 5-FU/ml level, two of the three determinations were within 10%

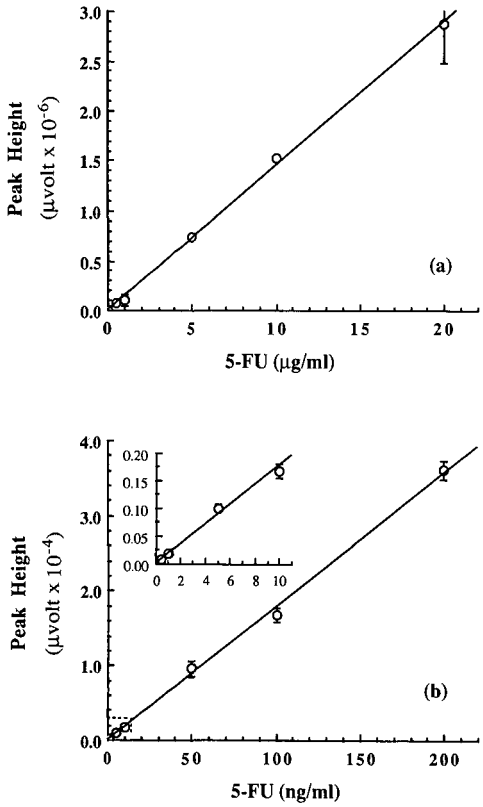

Fig. 6. Standard curves from spiked human plasma. Human plasma was spiked with 5-FU at varying concentrations and aliquots were analyzed, as described in the text. Points shown are the average of three determinations (±S.D.). (a) High-level standard curve of 0.5–20 μg 5-FU/ml. Samples containing more than 5 μg/ml were diluted 1:10 with acetone–acetonitrile–5% acetic acid (1:2:3, v/v/v) before analysis by HPLC. (b) Low-level standard curve of 0–200 ng 5-FU/ml. The points below 10 ng/ml are shown in the inset. The assay was linear to 20 μg 5-FU/ml plasma, with a limit of detection of 0.5 ng/ml.

of each other, however a third point caused the large standard deviation. It should be noted that even with the large standard deviation, the mean calculated concentration is in good agreement with the theoretical concentration. Also, subsequent experiments have shown (Table III) that the between-day reproducibility was acceptable. The between-day variability over the linear range of the assay was 10% for four concentrations over three days (Table III).

Rabbit pharmacokinetics

The utility of the assay was demonstrated by monitoring the plasma concentration of 5-FU in a rabbit after a bolus dose. One New Zealand rabbit (4.3 kg) was given 50 mg 5-FU/kg (225 mg) by intravenous bolus over 2 min. Blood samples (1.8 ml) were drawn at various times, and the plasma was analyzed after centrifugation to remove the erythrocytes. The data were fit to a two-compartment model. The experimental

TABLE II

ACCURACY AND PRECISION OF THE ASSAY OVER THE COMPLETE STANDARD CURVE RANGE

Concentration (ng/ml)			
Actual	*Calculated*	*%Error*	*%R.S.D.*[a]
0.5	0.6	20.0	20.0
1.0	1.2	20.0	6.0
5.0	5.8	16.0	8.0
10.0	9.5	−5.0	7.2
50.0	53.8	7.6	11.9
100	94.3	−5.7	5.5
200	201.3	0.7	3.6
500	594.8	19.0	15.4
1000	996.8	−0.3	34.7
5000	4801	−4.0	4.8
10000	10424	4.2	1.9
20000	19576	−2.1	13.3
	Mean	5.9	11.0

[a]Relative standard deviation.

data are shown (Fig. 7) along with the computer generated line. The semi-log plot (Fig. 7, inset) demonstrates that the elimination of 5-FU does not fit a two-compartment model, as others have recently reported[23]. The data suggest that the elimination profile may follow saturable Michealis–Menten kinetics. Non-linear pharmacokinetics of 5-FU has been demonstrated in humans[2,15,24], and further experiments are being conducted in this laboratory to define the pharmacokinetics of 5-FU in rabbits and humans better.

TABLE III

BETWEEN-DAY VARIATION OVER THE WORKING RANGE OF THE ASSAY

Concentration (ng/ml)					
Actual	*Calculated*			*Mean*	*%R.S.D.*
	Day 1	*Day 2*	*Day 3*		
1	1.6	1.6	1.0	1.4	21.4
10	11.2	11.4	9.0	10.5	12.4
100	91.6	101.5	101.8	98.3	5.9
1000	1087.0	1003.8	992.1	1027.6	5.0
				Mean	9.9

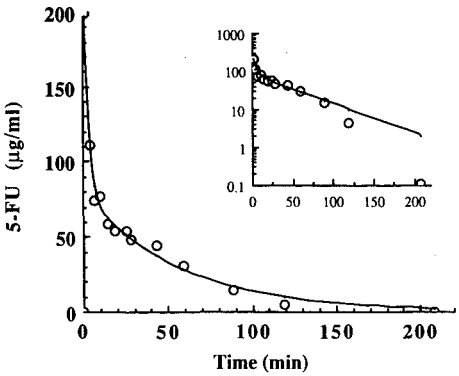

Fig. 7. Pharmacokinetic profile of 5-FU after a single intravenous dose in a rabbit. A rabbit was administered 50 mg 5-FU/kg (225 mg) by intravenous bolus over 2 min. Samples were drawn at various times and analyzed, as described in the text. The experimental data are shown along with the computer generated line for a two-compartment model. The semi-log plot (inset) shows that the data do not fit a two-compartment model and is more indicative of non-linear, saturable kinetics.

CONCLUSION

The resulting 5-FU assay described in this paper utilizes a double ethyl acetate extraction, followed by precolumn derivatization with BrMmc. Both, temperature and sample matrix were found to have profound effects on the derivatization reaction. The combined efficiency for the extraction and derivatization of 5-FU was more than 85%. The limit of detection was 0.5 ng/ml plasma, and the linear range of the assay extended over four orders of magnitude. This sensitivity and large range will be sufficient to further characterize the pharmacokinetics of 5-FU. Finn and Sadee[18] were able to measure a β-elimination phase of 5-FU on a few occasions. However, their assay could not do this on a routine basis. The present assay has the sensitivity to measure the low levels that occur during the terminal β-phase and the results of further pharmacokinetic studies being conducted in these laboratories will be published elsewhere. Since peaks for the uracil and 5,6-dihydro-5-fluorouracil derivatives were detected, adaptation of this method to uracil and 5,6-dihydro-5-fluorouracil determinations, in addition to 5-FU, should be straightforward.

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Cyclopentolate	2.47	OJ		2.3	CA-1	Praziquantal	complete resolution	CA-1
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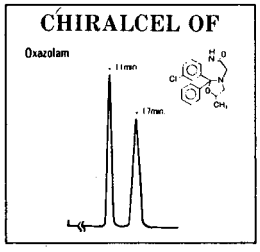
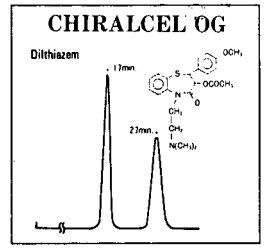
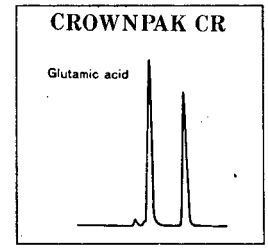
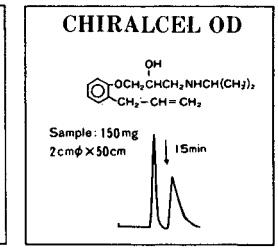
Ethiazide	1.54	OF				Sulconazole	1.68	OJ
Ethotoin	1.40	OJ	Mianserin	1.75	OJ	Suprofen	1.6	OJ
Fenoprofen	1.35	OJ	Nilvadipine	complete resolution	OT	Trimebutine	1.81	OJ
Glutethimide	2.48	OJ				Warfarin	1.96	OC









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