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CONCENTRATION EFFECTS IN HPLC-SEC ANALYSIS OF PETROLEUM ASPHALTENES

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ABSTRACT

The effect of concentration on the HPLC-SEC elution profile of petroleum asphaltenes has been examined in order to investigate the possible determination of critical micelle or microstructure concentrations as observed by other more laborious methods. It was observed in one case that above a certain threshold concentration (TC) the profile became constant in shape. TC was found to be in the range of previously reported CMC values by calorimetric titration. However, according to micellization theory the profile should start changing beyond the threshold concentration. Hence the association process of asphaltenes may even commence below the previously reported CMC possibly through a step-wise mechanism. A large dependence of the observed concentration effect upon wavelength of the UV-vis diode array detector is also reported. Therefore caution should be exercised when applying HPLC-SEC in deriving physicochemical information on heavy self-associating petroleum fractions.

INTRODUCTION

The use of size exclusion chromatography in the characterization and fractionation of petroleum products

such as asphalt and residua is abundant in the literature (1-5). Although the SEC technique in this area is recognized to give only relative results depending almost entirely on the calibration standards used, molecular weights based upon these results are frequently reported in the literature (5).

Especially the very heavy part of the crude oil such as the asphaltenes have a significant complexity which may affect the elution of the different compounds. Asphaltenes are briefly defined as the solid organics precipitating upon treatment of the crude oil with an excess of hydrocarbon such as n-heptane (6).

Self-association of asphaltenes in solution has been covered in a vast number of works employing different methods (7). These works have basically had the goal of determining the molecular weight of the asphaltenes (8).

Asphaltenes in solution have been shown to exhibit self-assembly, micellar and colloidal behavior in a number of works (9-13). This micellar behavior is seen in the apparent existens of a critical micelle concentration above which asphaltene "monomers" associate and form larger "micelles" (10,11,13). Based on our knowledge of asphaltene composition the critical micelle concentration should however be regarded as hypothetical, as the participating "monomers" in these mixed micelles are polydisperse in both structure and molecular weight. Hence a uniform micelle is probably non-existing (14). The magnitude of asphaltene CMCs reported in the literature is dependent upon the solvent and CMC is generally found in the range of 1 to 10 g/L depending also on the origin of the asphaltenes investigated (14).

Recent work on fractionated asphaltenes has however shown that only a small part of the asphaltenes actually participates in the association, whereas up to 60 % of

the asphaltene consitutents remain unassociated when separated from the bulk asphaltenes as indicated by HPLC-SEC (15). The knowledge of the degree of association of a heavy feedstock may be of importance in the petroleum refining industry as a monomer is easy to process relative to a micelle.

Self-assembly of asphaltenes and other heavy petroleum products has been qualitatively investigated using HPLC-SEC indicating a small but significant change in the elution front as the concentration is changed (16). The association of the asphaltenes at and above a specific concentration has been monitored by both calorimetric titration (10) and surface tension measurements (11,13). This association has been related to the critical micellization concentration known from surfactants.

The present work was initiated in order to investigate whether the presence of a critical micelle or self-assembly concentration could be monitored by HPLC-SEC by examination of solutions of varying concentrations as the other methods previously used are tedious. Well characterized asphaltenes from Boscan and Kuwait oils were examined. The latter had previously been used in an extensive study of asphaltene association by calorimetric titration covering both effects of solvent and precipitation procedures for the asphaltenes (10). Due to the use of original samples in low supply only a few SEC measurements could be performed with these samples previously used in calorimetric investigations. These are used mostly as support for the results obtained using the Boscan asphaltenes. The chemical characterization of the particular asphaltenes has been reported elsewhere (15,17-20).

At this point it shall be emphasized that the term micelle is used arbitrarily due to the resemblance of the

observations to similar phenomena of reversed micelle formation in surfactant chemistry. Also the asphaltene micelles are more likely very complex aggregates of molecules, however needing a specific critical concentration in order to dominate the properties of the solution. Sheu et al. (12) have recently in detail explained the micellar resemblance of asphaltenes in solution.

EXPERIMENTAL.

Asphaltenes were separated according to a modified IP 143 procedure (18, 19) at ambient temperature followed by a thorough washing of the precipitated material to ensure removal of co-precipitated components. As precipitants n-heptane and mixtures of 10%toluene in n-heptane were used for respectively Kuwait and Boscan asphaltenes. Also a Kuwait asphaltenes precipitated at 43°C in n-octane was examined. The addition of toluene to the precipitant was used to prepare more complex asphaltenes as low molecular weight species are dissolved in the precipitant (19). Asphaltene solutions of increasing concentration in toluene were prepared by weight (+/-0.01 mg) in sealed vials and left overnight to ensure solution equilibrium.

The chromatographic equipment consisted of a Hewlett-Packard 1090 HPLC with a diode array detector with 8 wavelengths. Freshly distilled toluene was used as eluant at a flow rate of 2mL/min at 30°C. The column was a Phenomenex Phenogel 5 um, 10^4Å, 30 cm 7.9 mm i.d. The DAD wavelengths examined were 305, 340, 380, 410, 420, 450, 500, and 575 nm. Only a few of these responses are reported herein. 50 uL sample solution was injected automatically.

Calibration was performed by examining polystyrene standards (PS) of known molecular weight in order to establish the non-excluded volume. log MW(PS) = 7.4848 - $0.7836t_R$.

RESULTS AND DISCUSSION.

Several non-size effects are known to affect SEC of petroleum derived components such as polyaromatic hydrocarbons, and hence the elution volume cannot be directly correlated to the molecular size of the individual compounds (21). The tailing of SEC chromatograms of petroleum products often reported (22) can be due either to adsorption through charge-transfer interactions with the stationary phase or to other non-size effects where even large PAHs elute later than a non-excluded compound (21). This is also seen in the present case where a significant tailing can be observed beyond the non-exclusion volume of ca. 7.0 min (calculated from MW of toluene) as determined by the polystyrene standards.

In the present work the possible association of petroleum products into micro or micelle-like structures may also affect the retention behavior. The investigation of micellar solutions by size exclusion chromatography has been reported to be complex in nature. This is caused by the dilution effects in the column as the sample propagates through it. This dilution affects the dynamic equilibrium involved in the micellization process especially close to the critical micelle concentration (CMC). In order to exist micelles have to be in equilibrium with a monomer concentration equal to CMC. As micelles and monomers will have different elution velocities through the column, they will be separated and hence micelles must dissociate in order to reestablish the local monomer

concentration of CMC. Hence the chromatographic profile becomes complex. For simple systems in aqueous solution, however, the determination of CMC and the equilibrium constants has been possible by SEC. The theory for SEC of aqueous micellar solutions has been reviewed by Birdi (23). The above effects are expected to increase the threshold concentration relative to the "true" CMC. It is assumed that the dilution effect may be minimized by using a singel column configuration as employed herein.

Chromatograms of the Boscan asphaltenes recorded at 305 nm are given in Figure 1 as a function of concentration between 2.6 and 20.2 g/L. A disitinct increase in the total exclusion peak at 3.5 min is observed while the peak at 4.6 min is relatively constant. A quantitative measure is given in figure 2 where the ratio of absorbances at 3.5 and 4.6 min. are plotted versus concentration for two detector wavelengths. The relative size of the 3.5 min peak becomes constant above ca. 8 g/L. The constant profile observed above 8 g/L is in contradiction to the expected change beyond a certain concentration as seen in aqueous SEC of micellar solutions (23). This may indicate that asphaltenes do associate below CMC. However, it could as well indicate that the dilution effect is minimized above this threshold value.

A Kuwait n-heptane asphaltene was also examined but as only a very small or no total exclusion peak (3.5 min) was found a similar anlaysis could not be performed. Chromatograms at 305 and 575 nm of this asphaltene at a concentration of 10 g/L are given in Figure 1. The three concentrations examined were 5.64, 10.44, and 20.09 g/L which are all above the CMC (3.24 g/L) in toluene at 25.02°C of this specific sample determined by calorimetry (10). Only minor changes can be detected in the normalized chromatograms supporting the findings of constancy for the Boscan asphaltenes. The changes were seen to be

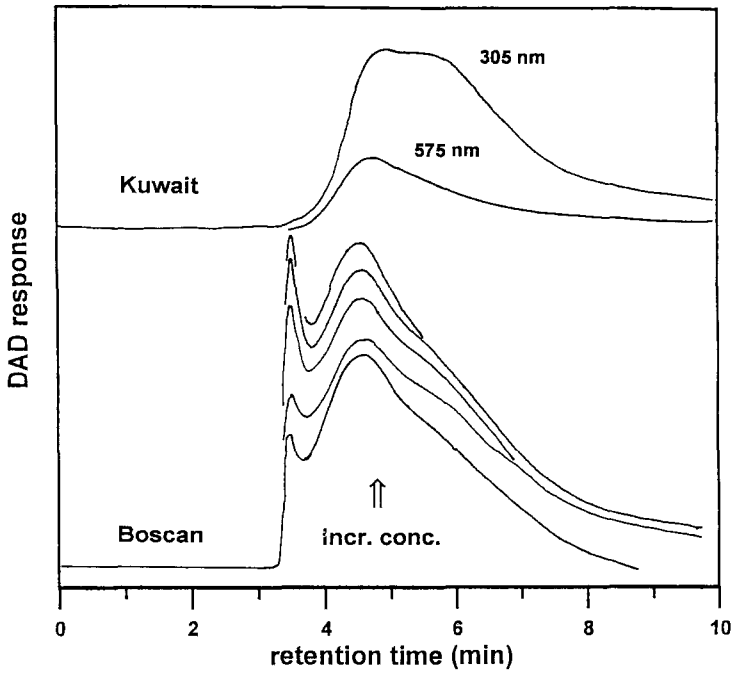

Figure 1. SEC Chromatograms of Boscan asphaltenes at 305 nm, concentrations 2.6, 5.0, 10.4, 16.0 & 20.4 g/L. Also given Kuwait n-C7 asphaltenes 10 g/L at 305 and 575 nm.

a small increase in response at short retention times and less at longer. The normalized area of the eluting front between 3.3 and 6 min was, however, found to increase about 8 % at all detector wavelength for 10 g/L where as a significant wavelength effect was observed for 20 g/L solution. In the latter case the response increased from 8 to 30 % between 305 and 500 nm as seen in Figure 3, where the 10 and 20 g/L responses are given relative to the 5 g/L response as a function of detector wavelength. Standard deviations on areas were less than 2 %. As can

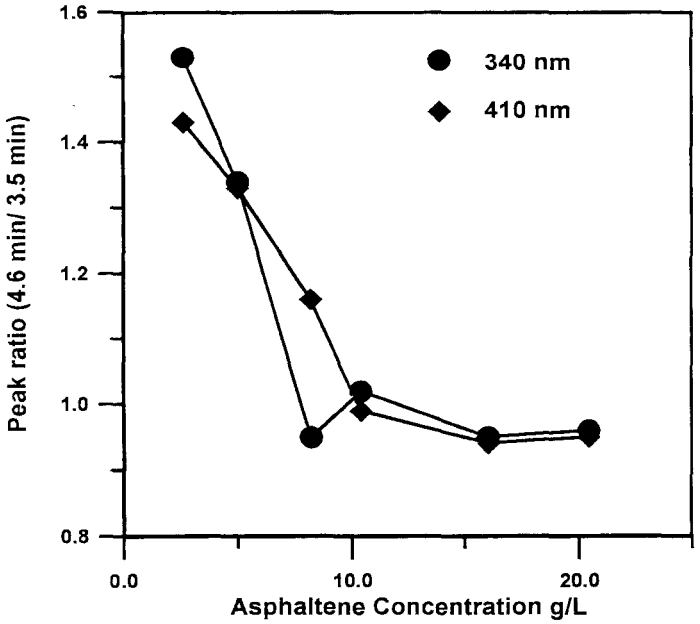

Figure 2. Ratio of detected absorbances at 4.6 to 3.5 minutes at 340 and 410 nm versus concentration of boscan asphaltenes in injected sample.

be seen the observations are significantly dependent upon the detector wavelength. The detector response could be caused by association of smaller molecules with larger ones that absorb light at higher wavelengths, hence rendering these even larger, giving them a shorter elution time. Also the species absorbing light at higher wavelength are more complex and may be the true associating species in asphaltenes. This is confirmed by the chromatograms as the bimodal type seen in Figure 1 dominates at low detector wavelengths, whereas only the 4.7 min peak with a significant tailing is observed at longer wavelengths for the Kuwait derived asphaltenes.

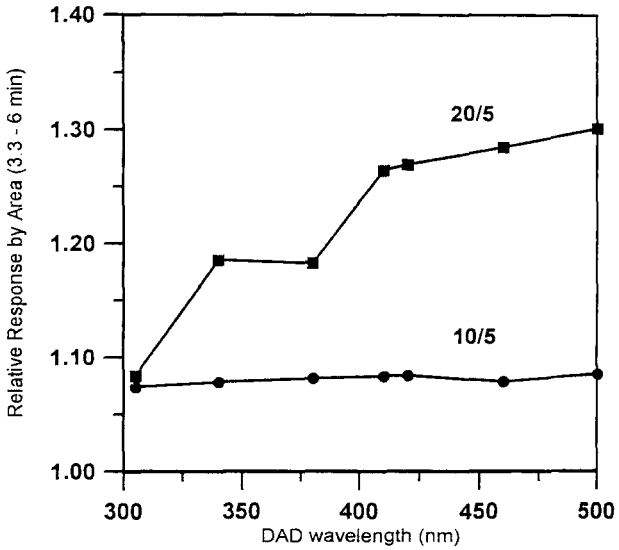

Figure 3. Relative response of main front of chromatograms versus detector wavelength. Response calculated for 10 and 20 g/L relative to signals for 5 g/L. Areas from integrator.

For the heavier Kuwait asphaltenes obtained by precipitation in n-octane at elevated temperature two concentrations were examined at both side of CMC (1.55 g/L) as detected previously by calorimetry (24). Note that this asphaltene sample has a lower CMC than the one precipitated at 20°C by n-heptane from the same crude oil. In Figure 4 the two normalized chromatograms recorded at 305 nm and 500 nm between 3.3 and 9.0 min are subtracted giving a differential indication of the changes observed. As can be seen for the 305 nm response a significant relative increase in the low retention time side is observed hence a significant association is taking place between 1.22 and 4.33 g/L of this asphaltene

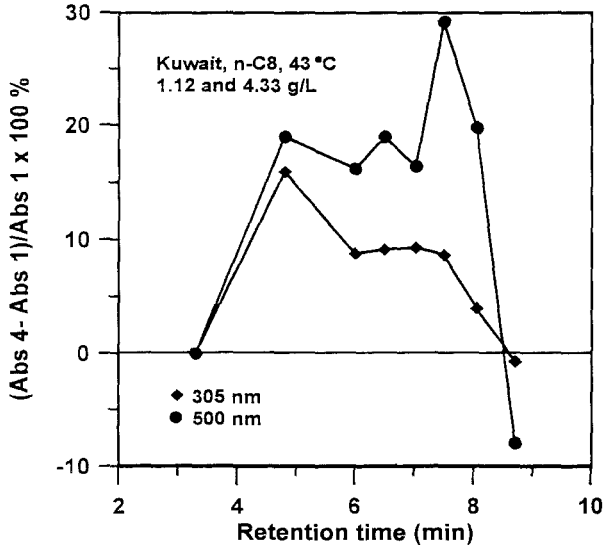

Figure 4. Differential chromatograms for Kuwait asphaltenes injected in concentrations of 1.12 and 4.33 g/L. Signal were normalized with respect to the injected concentrations. Two detector wavelengths investigated.

in toluene. This is in good agreement with the above CMC value. However, for the 500 nm response a fairly constant increase is observed through out the chromatogram with a maximum at about 7.4 min. Again the result is significantly dependent upon the detector setting. These normalized chromatograms were constructed from points taken from the integration program and the specific concentration of the injected solution. Note that the relative error on the 500 nm signal is comparably larger as the detector response at this wavelength is one fifth of the response at 305 nm.

For the Boscan asphaltene the total integrated DAD signal at various wavelength was examined as a function of concentration and found to obey Beer's law. This was

also found for the Kuwait n-heptane asphaltene. Hence the changes observed in the chromatograms are strictly related to the association or rearrangement of asphaltene constituents. However the findings herein does not provide evidence of a specific critical concentration above which association starts, but rather indicate the step-wise association leading to a certain limit above which association in some way is constant or in equilibrium.

Qualitative concentration effects has also been reported by Jenning et al. (16) for two asphalts injected in concentrations between 1 and 7 g/L, where the intensity of the high molecular weight side of the chromatogram increased and apparently became constant. In the work of Donaldson et al. (2) a concentration effect between concentrations as high as 30 and 70 g/L toluene was reported for an asphalt but apparently without a limiting concentration. In asphalts, however, other non-asphaltene types are present to a large extent, which may affect the actual result of the analysis. The current author has also observed an increase in the total exclusion peak of both Kuwait and Boscan asphaltenes as these are solvent extracted removing compounds with low molecular weight (15, 20). In that case the true concentration of the associating compounds is increased relatively when the injected solution concentration is kept constant as the content of complex molecules in the asphaltenes increases. And therefore the apparent CMC is expected to decrease as observed with the Kuwait asphaltenes used in this work (10).

The picture of the asphaltene self-assembly emmerging from the present HPLC-SEC study is very complex. Not only is a CMC obscured by dilution effects and changes in monomer-micelle equilibria but the difference in response also makes the conclusions

difficult. The origin of the asphaltene and the pretreatment of this may also have a large effect as observed for the different samples investigated herein.

The concentration region investigated herein is often used in characterization of asphalts which may contain large quantities of asphaltenes, hence it is evident that care must be taken in the interpretation of the obtained data as molecular weights, colloidal indices and other SEC derived magnitudes will be concentration dependent. Also it is important to emphasize the dependence of the result obtained by different DAD wavelengts as is evident from Figure 3. This significant wavelength dependence has also been reported by others (25).

CONCLUSION

The possibility of observing an asphaltene concentration effect in HPLC-SEC that could be related to previoulsy reported critical micellization processes in these petroleum fractions has been investigated. Although a strong asphaltene origin dependence is evident and no unambigious trends could be deduced we may conclude as follows. A threshold concnetration (TC) is observed above which no further changes takes place in the chromatogram. This cannot be related directly to the micellization where association starts above the critical micelle concentration (CMC). TC values are however well in agreement with the range of CMC magnitudes for asphaltenes reported in the literature. This was confirmed by examination of asphaltenes with known CMCs from investigations by calorimetric titration. Although the HPLC-SEC procedure is fast compared to other methods applied in association studies of asphaltenes the methods is hampered by the dilution effects in the column. More

quantitative work as presented herein compared to other of the above mentioned methods are however needed before definite conclusions can be drawn.

This work also confirmed that chromatograms of heavy pretroleum products are highly dependent upon both concentration and wavelength. This should be kept in mind when using HPLC-SEC for analystical determination of physicochemical properties of petroleum products to avoid ambigeous results.

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DETERMINATION OF HEXADECOIC AND OCTADECANOIC ACIDS IN STEARIN FOR INDUSTRIAL USE BY REVERSED-PHASE ION SUPPRESSION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A rapid and simple high-performance liquid chromatographic (HPLC) method was developed for the simultaneous determination of hexadecoic and octadecanoic acids in stearin. The samples are dissolved in ethanol without any additional pretreatment. HPLC was carried out on a MicroPak MCH-5 column (15cm×4mm I. D.) using water (pH 2. 5 with perchloric acid) - acetonitrile (10/90 V/V) as the mobile phase with UV detection at 210nm.

INTRODUCTION

Stearin for industrial use, made from animal and vegetable fats, consists chiefly of a mixture of octadecanoic and hexadecoic acids. It is widely used in manufacture of surfactant, cosmetics, rubber and plastics, etc[1].

The quality grade of stearin was defined by the acid, saponification, iodine values and solidfying point[2], which can only represent the total amount of hexadecoic and octadecanoic acids, as well as that of impurities, but can not directly give the respective amount of hexadecoic and octadecanoic acids. In recent years it was found that not only the total amount of hexadecoic and octadecanoic acids, but also the ratio of octadecanoic to hexadecoic acid has a conspicuous influence upon the technological process, products quality and economic profits.

Higher fatty acids are traditionally separated and determined using derivatization followed by either gas or liquid chromatography[2-3]. The derivatization step is usually time-consuming and requires several steps prior to injection. HPLC coupled with variable wavelength detection at 210nm provided a technique capable of direct determination of hexadecoic and octadecanoic acids in stearin without chemical derivatization.

EXPERIMENTAL

Reagents and Chemicals

Acetonitrile was HPLC grade (Institute for Fine Chemical Engineering of Huaiyin Plastic Product Factory, Huaiyin, Jiangsu, PRC; WHO Collaborating Center for Research in Human Reproduction, Tianjing, PRC).

Hexadecoic acid and Octadecanoic acid were GC grade (Shanghai No. 1 Reagent Factory, Shanghai, PRC).

Ethanol was analytical grade (Nanjing Chemical Reagent Factory, Nanjing, Jiangsu, PRC).

Water was distilled twice.

Stearin samples were provided by Nanjing Jinling Chemical Plant.

A stock solution of hexadecoic acid (10mg/mL) and octadecanoic acid (10mg/mL) in ethanol was prepared.

Apparatus and Chromatographic Conditions

The instrument used for HPLC separations was a Varian VISTA 5060 equipped with a Varian UV-100 variable wavelength UV detector (Varian Instrument Division, Walnut Creek, CA, USA) set at 210nm with a sensitivity of 0.025 AUFS.

Chromatograms were recorded on a Yokogawa Hokushin Electric Type 3066 pen recorder (Sino-Japanese No. 4 Meter Factory, Chongqing, Sichuan, PRC) and a Shimadzu C-R1B integrator (Shimadzu, Tokyo, Japan).

The separation was performed on a MicroPak MCH-5 Column (15cm×4mm I.D.; Varian Instrument Division). The mobile phase was water (pH2.5 with perchloric acid) - acetonitrile (10/90 V/V) at a flow - rate of 1.5 mL/min. The water is adjusted to a pH of 2.5 with perchloric acid to suppress ionization of the acids and provide better retention. The column temperature was maintained at 50℃ to increase the solubility of these higher molecular weight acids in the mobile phase.

Procedure

Weigh out 0.5 gram of samp! accurately into a 100-mL volumetric flask and diluted to volume with ethanol. Take 1 mL of sample solution and filter through a syringe filter with 0.5 μm micropore. 10 μL aliquots of the filted solution were injected to the HPLC column.

The standard substances were dissolved the same way as the samples.

Calibration Curve

Standards at concentrations of 2-10mg/mL of hexadecoic and octadecanoic acids were prepared by serial dilutions of the stock solution with ethanol. A

calibration graph for hexadecoic and octadecanoic acids were obtained by mesurements of peak heights.

RESULTS AND DISCUSSION

Chromatogram and Calibration Graph

Typical HPLC chromatograms for the hexadecoic and octadecanoic acids in standard and stearin sample are given in Figure 1. Hexadecoic and octadecanoic acids were clearly separated.

The quantitation was based on a calibration by series of dilution from primary standard. A linear regression analysis of the relationship between peak height versus amounts of standards was carried out within the range 20-100μg in 10μl injection volume. The results obtained were: $y = 2.598x - 0.202$ for hexadecoic acid and $y = 1.605x - 0.146$ for octadecanoic acid, with correlation coefficients of 0.9991 and 0.9990 respectively, where y equaled peak height (cm) and x equaled final staredard concentration (mg/mL).

Analysis of Industrial Samples

Table 1 gives results obtained by the proposed method in comparison with the iodine values. The results showed that the amounts of hexadecoic and octadecanoic acids in stearin is consistant with the quality grade and the iodine value. On one hand the amount of hexadecoic acid in the grade 1 and 2 is higher than that in the grade 3, and the amount of octadecanoic acid is reversed, the amount of hexadecoic acid in the grade 1 is smaller than that in the grade 2, and the amount of octadecanoic acid is reversed, on the other hand the total amount of hexadecoic and octadecanoic acids in the grade 1 (iodine value = 2) is a bit higher than that in the grade 2 (iodine value = 4), much higher than that in the grade 3 (iodine value = 8). The amount of impurities in stearin for industrial use, which are chiefly unsaturated higher fatty acids such as oleic, linoleic and linolenic acids, gives

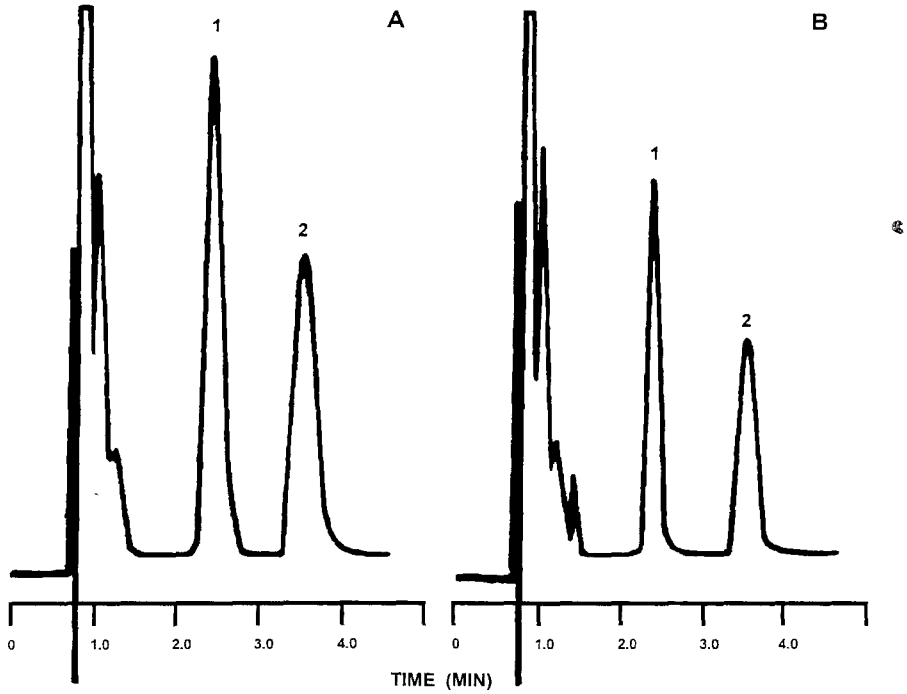

FIGURE 1. Chromatograms obtained from (A) standard, and (B) stearin (grade 3). Peaks identified are (1) hexadecoic acid, (2) octadecanoic acid.

TABLE 1

Analysis of Hexadecoic and Octadecanoic Acids in Stearin for Industrial Use

Quanlity grade	Hexadecoic acid* H	Octadecanoic acid* O	Total H+O	Rotio O/H	Iodine Value
1	53.70±0.69	46.24±0.70	99.94	0.86	2
2	58.35±0.65	40.47±0.75	98.82	0.69	4
3	31.83±0.78	61.76±0.55	93.59	1.94	8

* Each value in precent (m/m) represents the mean of the five samples ± standard diviation.

expression to the unsaturated value that was traditionally measured by adding iodine.

CONCLUSION

In this paper, an attempt was made to present a new method for determining the hexadecoic and octadecanoic acids in various types of strearin. The simplicity and short time of the analysis make it a convenient alternative over earlier methods.

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DETERMINATION OF SURFACTANT SODIUM LAURYL ETHER SULFATE BY ION PAIRING CHROMATOGRAPHY WITH SUPPRESSED CONDUCTIVITY DETECTION

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ABSTRACT

A method for the determination of the anionic Steol CS-330 surfactant is described. CS-330 is a complex mixture of oligomers due to the various sizes of fatty alcohols and the number of moles of the ethoxylation. The main component of CS-330 is sodium lauryl ether sulfate (SLES). Since a SLES molecule has a hydrophilic sulfate head and a hydrophobic alkyl ethoxyl tail, it is very difficult to separate these molecules with conventional reverse phase chromatography or ion exchange chromatography. This work uses ion pairing chromatography with suppressed conductivity detection. The separation of oligomers in CS-330 is achieved. SLES does not have UV-absorbing chromophores, therefore an optical detector is not very sensitive. Suppressed conductivity detection technique significantly increases sensitivity and a quantitation limit of 56.60 ppm is achieved.

INTRODUCTION

Recently, there has been considerable interest in using surfactants to remediate subsurface contamination, e.g., to immobilize contaminants for subsequent in situ treatment, to release contaminants from mineral surfaces, or to redistribute immobile

organic phases into the mobile aqueous phase. [1], [2], [3] Steol CS-330 is one of the surfactants under consideration for this application. ManTech Environmental has developed high performance liquid chromatography and supercritical fluid chromatography methods for the determination of various anionic and nonionic surfactants, such as T-MAZ (a registered trademark of PPG Industries, Inc.) ethoxylated sorbitan fatty acid esters, [4], [5] Dowfax (a registered trademark of Dow Chemical Company) 8390 monoalkylated disulfonated diphenyl oxide, sodium dodecylbenzene sulfonate, octylphenol polyether alcohols and polyethoxylated nonylphenols. [6]

Steol (a registered trademark of Stepan Company) CS-330 is an industrial chemical. CS-330 is derived from fatty alcohols, ethoxylated to an average of 3 moles, and sulfated via a continuous SO_3 process. This ionized surfactant consists of a saturated alkyl group, ethoxyl groups and a polar head, $-OSO_3$ Na (Figure 1a). As shown in Figure 1a, CS-330 can be a complex mixture of oligomers due to the various parent fatty alcohol and the number of moles of the ethoxylation. Because these molecules are amphipathic, i.e., contain both hydrophilic and hydrophobic moieties (Figure 1a), they are surface-active compounds and concentrate at oil-water interfacial regions. The major component of CS-330 is sodium lauryl ether sulfate (Figure 1b). According to the manufacturer, the content of sodium lauryl ether sulfate in CS-330 is 27.5 - 29.5% in weight.

Because of the amphipathic character of CS-330 molecules, it is very difficult to separate these molecules with conventional reverse phase chromatography or ion exchange chromatography. With reverse phase chromatography, the molecules cannot be retained by the column because the alkyl, ethoxyl and sulfate groups do not

(a) $(H_mC_n(OCHCH)_l)OSO_3^- Na^+$ n = 12, 13, 14, 15, ...

m = 2n+1

l = 1, 2, 3, 4, 5,

(b) $(H_{25}C_{12}(OCHCH)_3)OSO_3^- Na^+$

Figure 1 (a) The molecular formula of molecules in CS-330.

(b) The molecular formula of sodium laureth sulfate.

have strong enough interactions with a C_{18} reverse phase column to be retained. With ion exchange chromatography, CS-330 molecules are retained in the ion-exchange resins for a very long time due to the hydrophobic nature of the alkyl group. Reverse phase ion pair chromatography is ideal for the separation of ionic organic compounds, such as sodium laureth sulfate. A reverse phase column is used and an ion pairing reagent is added to the eluent. This reagent adsorbs onto the neutral, hydrophobic resin forming an ion-exchange surface on which the organic ions are differentially retained and separated. [7], [8], [9] Sodium laureth sulfate does not have UV-absorbing chromophores and therefore, an optical detector is not very sensitive. Suppressed conductivity detection significantly increases sensitivity and a quantitation limit of 56.60 ppm is achieved.

MATERIALS AND METHODS

CS-330 was from Stepan Company (Northfield, IL,U.S.A.), tetrabutylammonium hydroxide (55% aqueous solution, TBAOH) from Southwestern Analytical Chemicals,

Inc. (Austin, TX, U.S.A.), acetonitrile from Burdick and Jackson (Baxter Healthcare Corporation, Muskegon, MI, U.S.A.). 18 MΩ water was obtained from a Millipore Milli-Q system (Marlborough, MA, U.S.A.).

Instrumentation included a Waters (Waters Associates, Milford, MA, U.S.A.) 6000A HPLC pump, a Waters 717 autosampler, and a Waters 431 conductivity detector. Separations were accomplished using a Dionex (Dionex Corporation, Sunnyvale, CA, U.S.A.) IonPac NS1 column (4 mm x 250 mm) and a NG1 guard column. A Dionex anion micro membrane suppressor (AMMS-MPIC) and 25 mM sulfuric acid solution were used to suppress the background conductivity of the mobile phase. The mobile phase was 50% acetonitrile and 5 mM TBAOH in water with a pH value of 11.8. A silica based reverse phase column cannot be used with this strong basic eluent because a silica based column usually has an operating pH range from 4 to 7.5. Organic polymer packings, such as IonPac NS1 column, have a wide pH range (0 to 14) and they are ideally suited for the separation of molecules in CS-330. The injection volume was 400 µl at an eluent flow rate of 1.0 ml/min. Data acquisition and processing was accomplished with a Waters Maxima 820 chromatography workstation, which included a system interface module and an NEC PowerMate SX/16 computer.

RESULTS AND DISCUSSION

The eluent used in the ion pairing chromatography must maintain the analytes in their ionic states so that the ion pairing reagents can form ion pairs with the sample ions. The 50% acetonitrile and 5 mM TBAOH eluent has a pH value of 11.8 and the cation (TBA^+) can form an ion pair with the sample anion ($ROSO_3^-$). The conductivity

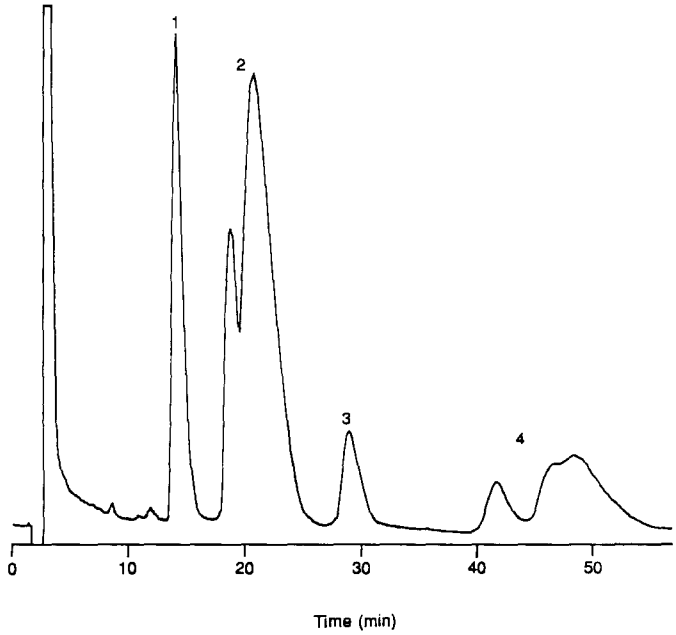

Figure 2 Chromatograms of Steol CS-330

Injection volume: 400 µl; concentration : 800 ppm. Detection: suppressed conductivity; Mobile phase: 50% acetonitrile and 5 mM TBAOH; Flow rate: 1 ml/min.

TABLE 1 Peak Area/Peak Height and Average Relative Response

Concentration	Peak Area/Peak Height [a]				Relative Response [b] (peak area)			
(ppm)	1	2	3	4	1	2	3	4
60	61	195			0.30	1.00		
80	54	172			0.32	1.00		
160	54	178	94		0.33	1.00	0.10	
200	63	180	106	372	0.33	1.00	0.10	0.31
320	56	189	98	408	0.32	1.00	0.11	0.34
800	66	220	105	460	0.32	1.00	0.10	0.34
1600	76	240	122	496	0.32	1.00	0.10	0.35

[a] Data of peak area and peak height were averaged from three injections.
[b] Relative response of the peak area was averaged from three injections.

TABLE 2 Analytical Precision and Detection Limit

Peak Group	Concentration		Detection Limit (ppm)
	160 ppm	640 ppm	
1	n = 3	n = 3	16.98
	$\bar{x}$ =163.91	$\bar{x}$ = 671.16	
	SD = 5.66	SD = 2.18	
	RSD = 3.5%	RSD = 0.3%	
2	n = 3	n = 3	13.05
	$\bar{x}$ = 164.01	$\bar{x}$ = 665.92	
	SD = 4.35	SD = 9.73	
	RSD = 2.7%	RSD = 1.5%	

n: Number of standard solutions analyzed
$\bar{x}$: Mean solution concentration (ppm)
SD: Standard deviation
RSD: Percent relative standard deviation (= 100 x (SD / $\bar{x}$))
Detection limit was calculated as three times the standard deviation of the mean (3 x SD).

background of this eluent is 650 μS due to the high concentration of TBAOH. With such a high conductivity background, the ratios of signal to noise by a conductivity detector are poor. The micromembrane suppressor removed cations in the column eluent after the oligomer separation was accomplished, reducing the background conductance to 92 μS.

Figure 2 shows the chromatogram of the oligomer separation of CS-330. The concentration of CS-330 in the chromatogram was 800 ppm with 400 μl injection. The peak areas were integrated as four groups, from 13.05 to 16.95, 17.46 to 26.44, 26.78

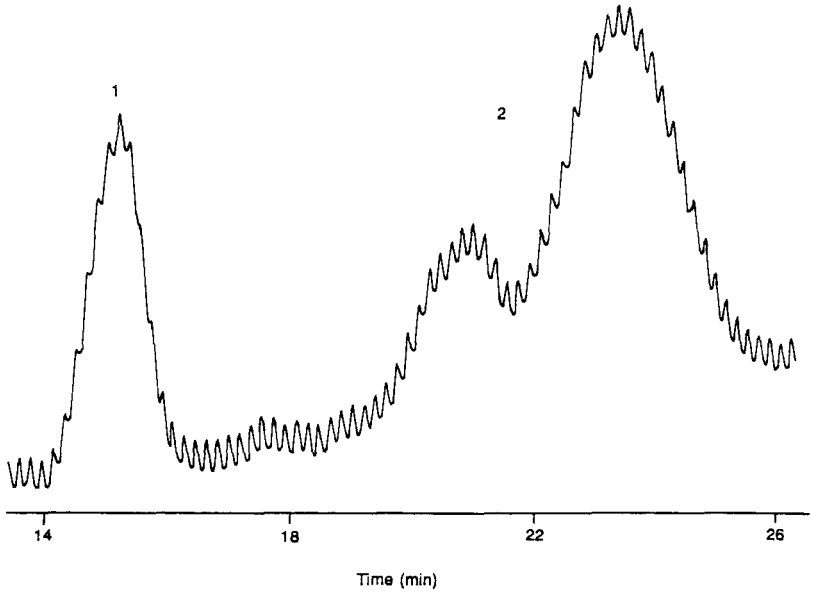

Figure 3 Chromatogram of Steol CS-330.

Injection volume: 400 µl; concentration : 60 ppm. Detection: suppressed conductivity; Mobile phase: 50% acetonitrile and 5 mM TBAOH; Flow rate: 1 ml/min.

to 33.05 and 38.81 to 56.80 minutes. Peak 3 and peak group 4 were not detectable at concentrations below 160 ppm. The ratio of the peak area to the peak height (TABLE 1) shows that peak group 4 is a very broad peak due to the long retention of 49 minutes. The averaged relative response of peaks and peak groups 1, 2, 3 and 4 is 0.32 : 1.00 : 0.10 : 0.33 (TABLE 1), which indicates that the peak 3 has the lowest response. Since peak 3 has the lowest response and peak group 4 is the broadest, it was determined to use peak 1 and peak group 2 to quantify CS-330.

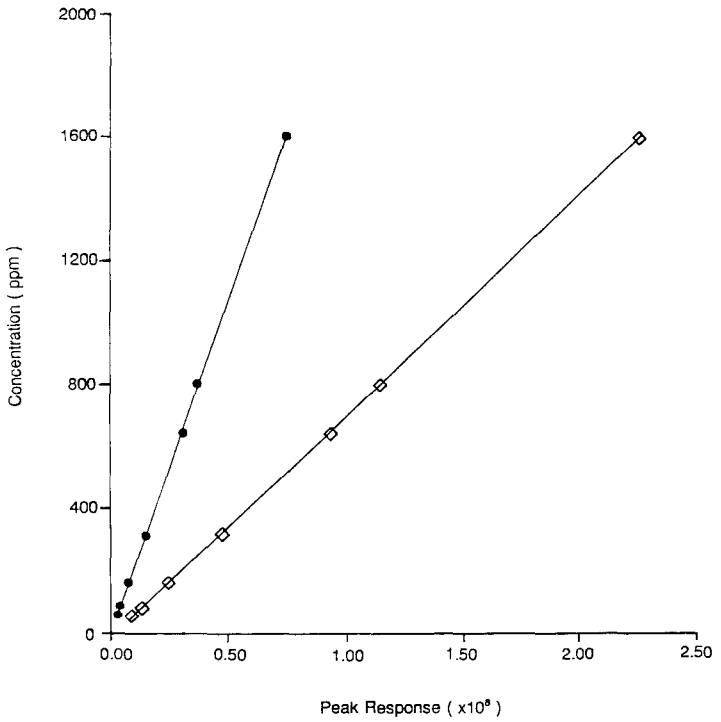

Figure 4 The calibration curves of the peak 1 (●) and peak groups 2 (■), in which quadratic curves were used to fit the data.

CS-330 was analyzed quantitatively with a good degree of precision and accuracy (TABLE 2). CS-330 standards in the concentration range from 60 to 1600 ppm were analyzed four times, and 160 and 640 ppm standards were used to determine the detection limit. The detection limits were 16.98 ppm for the peak 1 and 13.05 ppm for the peak group 2, calculated as three times the standard deviation of the mean. From the detection limit of 16.98 ppm for the peak 1, a quantitation limit of 56.60 ppm was estimated, calculated as ten times the standard deviation of the mean. Figure 3 shows the chromatogram of 60 ppm standard, which demonstrates that CS-

TABLE 3 Average Peak Area, Relative Standard Deviation, Calculated Concentrations and Relative Error

Concentr. (ppm)	Ave. Peak Area (x 10^{-7})[a]				Cal. Concentr.		Relat. Error (%)	
	1	(%RSD)	2	(%RSD)	1	2	1	2
60	0.3156	(6.5)	1.0389	(4.4)	59	63	-1.15	4.73
80	0.4001	(6.3)	1.2358	(4.8)	80	76	-0.06	-4.66
160	0.8057	(3.2)	2.4525	(3.2)	164	164	2.44	2.51
200	0.9644	(0.8)	2.9545	(2.2)	198	197	-1.01	-1.18
320	0.1613	(3.6)	4.8988	(1.9)	334	331	4.26	3.46
800	3.9986	(0.7)	12.337	(0.6)	852	856	6.47	6.95
1600	7.9483	(0.2)	24.589	(0.7)	1749	1742	9.29	8.88

[a] The peak areas were averaged from three experimental data.
RSD: Relative Standard Deviation = 100 x (Standard Deviation / Average Peak Area)

330 can be easily quantified at this concentration, confirming the estimated quantitation limit of 56.60 ppm.

Four standards of each concentration (60, 80, 160, 200, 320, 800 and 1600 ppm) were analyzed. A standard from each concentration was randomly selected to generate calibration curves for the peak 1 and peak group 2. Figure 4 shows the calibration curves of the peak 1 and peak group 2, in which a quadratic equation was used to fit the data. The three standards of each concentration, which were not used in the calibration curves, were used as unknowns and their concentrations were calculated independently with the calibration curves of the peak 1 and peak group 2 as shown in TABLE 3. As demonstrated by the relative error, the calculated concentrations are in a good agreement with the known concentrations. These results demonstrate that both the peak 1 and the peak group 2 can be used independently to

quantify CS-330 concentration. In a routine analysis, one can choose to use either one of the groups for quantification.

To insure that no components of CS-330 with high molecular weights were retained in the column, the column eluent was monitored for 90 minutes and no peak was found after 55 minutes with area counts higher than 315600, which corresponds to the lowest standard of 60 ppm for the peak 1 in the calibration curve (TABLE 3).

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DISCLAIMER

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QA/QC REQUIREMENTS

All QA/QC aspects of this work were performed in accordance with the requirements of the Quality Assurance Program Plan of the ManTech Environmental Research Service Corporation.

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QUANTITATIVE LIQUID CHROMATOGRAPHY, THERMOSPRAY/TANDEM MASS SPECTROMETRIC (LC/TSP/MS/MS) ANALYSIS OF SOME TRANQUILIZERS OF THE THIOXANTHENE GROUP IN WHOLE-BLOOD

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ABSTRACT

A LC/TSP/MS/MS selected reaction monitoring (SRM) method in the daughter ion scan mode can be succesfully applied for the quantitative determination of members of the Thioxanthenegroup like Chlorprothixene,Flupenthixol,Thiothixene and Zuclopenthixol in whole-blood following a BondElut extraction.Detection limits as low as 100pg for Chlorprothixene varying to 2ng per injection for the other compounds were found (corresponding to 0.5 to 10ng per mL whole-blood).Sensitivity of the proposed method is of the same order of chromatographic methods with other detectors or a RIA method.

INTRODUCTION

Members of the Thioxanthenegroup like Chlorprothixene,Flupenthixol,Thiothixene and Zuclopenthixol are difficult to gaschromatograph.They all have in common

large Retention Indices(1) causing on standard columns considerable analysis times.In order to overcome this problem we studied the use of liquid chromatography for the separation of the mentioned compounds,combined with thermospray tandem mass spectrometry for the selective and quantitative analysis of this type of drugs(2),like we did with a number of Benzodiazepines(3),several Explosives (4) and some representatives of the Methadone, Butyrophenone and Diphenylbutylpiperidine group(5).

EXPERIMENTAL

Materials:

Pure substances of the following drugs Chlorprothixene, (CAS 113-59-7);Flupenthixol,(CAS 2709-56-0);Thiothixene,(CAS 5591-45-7)and Zuclopenthixol,(CAS 53772-83-1) were donated by the representatives of their producers Pfizer and Lundbeck.For structures see Figure 1.Blood used was outdated tranfusion blood and was frozen until used.Water was purified by the Milli Q/Organex System (Millipore).Acetonitrile,chloroform,dichloromethane and methanol were of HPLC and glass distilled grade (Rathburn).All other reagents were of analytical grade.Extractions were done by BondElut Certify columns(Varian)

Apparatus:

HPLC:A Waters 600-MS programmable pump,equipped with a U6K injector,was used to pump 0.6mL/min of a mixture

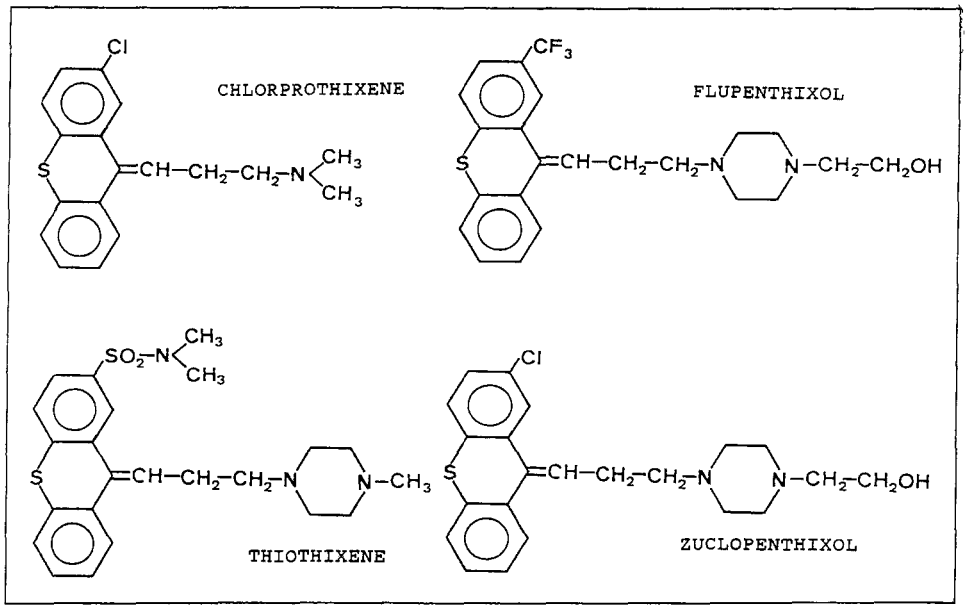

FIGURE 1
Structures of the compounds under investigation.

(85:15) of acetonitrile and 50mM ammoniumacetate in water through a Hewlett-Packard HPLC cartridge column 5µm Asahipak ODP-50 4.0 x 125 mm .Post-column,an extra 0.6mL/min of 50mM NH4Ac in water was added by a Waters 590-MS isocratic pump for ionizing enhancement in thermospray applications.

MS:A Finnigan MAT TSQ 700 tandem quadrupole mass spectrometer,coupled to a DEC station 2100 was used.The liquid chromatograph was connected to the mass spectrometer by the Finnigan MAT TSP-2 interface.The operating

conditions of the interface,such as the repeller voltage,vaporizer temperature,source temperature and ionic strength of the eluent were all optimized for the different drugs and are given in Table 1.

MS/MS experiments in the daughter ion mode(6,7) were done with the triple-stage quadrupole mass spectrometer (Q1,Q2,Q3).In these MS/MS experiments,the $[M+H]^+$ quasi-molecular ion was chosen as precursor ion and selectively transmitted by Q1 for further collisional dissociation in Q2.Argon was used as the collisiongas with a collision chamber pressure of 2.5 till 4.0 mTorr (see Table 1).Varying collision offset voltages were applied to Q2.The collision activated dissociation(CAD) daughter ions thus obtained,were then analysed by scanning with the third quadrupole(Q3) over the mass range m/z 40-500(FullScan).In order to obtain optimum selectivity for the different drugs,not the FullScan but the Selected Reaction Monitoring(SRM) technique(2,6) was applied.In this case,only one special ion was allowed to pass the third quadrupole(Q3).Then selectivity is extraordinarily increased(8),as was prospected for the sensitivity,by extremely suppressing the noiselevel. Collision offset voltage,argon pressure and MSMSC factor(a correction factor for increasing the transmission of ions in the MS/MS mode) were all optimized,and the most intense fragment ion in the MS/MS spectrum was chosen for SRM experiments(see Table 1).

TABLE 1

HPLC/MS/MS Parameters and Detection Limits SRM method (S/N ≈ 3) for Reference Solutions of some members of the Thioxanthene group.

TSP conditions:Repeller,70 V;Vaporizer temperature, 130-135°C;Source temperature,200°C;Filament off.
MS conditions:Multiplier Voltage,1500V;Dynode power, 15 kV;Scantime,1.20 sec;MSMSC factor 0.

Substance SRM m/z's	Collision Offset(V)	pArgon (mTorr)	R.T (min)	Dect.Lim. On Column
Chlorprothixene 316 - 271	-17.5	3.5	4.50	0.1ng
Flupenthixol 435 - 265	-35.0	4.0	3.15	2.0ng
Thiothixene 444 - 335	-15.0	2.5	2.50	5.0ng
Zuclopenthixol 401 - 128	-17.5	3.0	3.40	2.0ng

Reference solutions:

Stock solutions of the different substances were prepared once a week by dissolving 10mg of the pure substances in 10mL of methanol. From these,diluted solutions of 1,10,100 and 1000ng per mL of the different compounds were prepared by addition of methanol;10µL were injected in the chromatograph.All reference solutions were stored in glass vials with teflon coated silicone rubber-lined crimp caps.Whole-blood was spiked by adding a quantity of the drug in methanol to blank

blood,taking care that the amount of methanol did not exceed 2%.The blood was spiked with Chlorprothixene in the concen-tration range of 1 to 200ng per mL whole-blood with seven different concentrations.

Sample treatment:

The extraction procedure is the one we use in this laboratory in routine determinations of a general unknown in blood by HPLC with UV detection.Extractions were done with BondElut Certify 3cc columns(Varian). Preconditioning of the column was done with 2mL of methanol followed by 2mL of a 0.1 M phosphate buffer of pH 6.0.Care was given to wetting of the column until the prepared blood sample was brougth on column.The preparation of the blood sample was done by diluting in a polypropylene tube of 1mL of blood with 6mL of 0.1M phosphate buffer of pH 6.0.After vortexing and sonification the solution was centrifugated and the clear solution transferred to the column.Then the column was rinsed with water,followed by 1mM acetic acid(pH 3.3), afterwards the column was dried by suction.Elution was done first by 2mL acetone:chloroform 50:50,giving an acidic fraction,followed by elution with 3mL of freshly prepared ethylacetate:ammonia solution 98:2, giving the neutral and basic fractions used in the experiments Of the last fraction the solvent was evaporated at 40°C under a gentle stream of nitrogen.The extract is dis-

solved in 50µL methanol and an aliquot of this solution (10µL) is injected into the chromatographic system.

RESULTS AND DISCUSSION:

In Table 1 the thermospray and MS/MS parameters are given.In optimizing the thermospray parameters,it was found that moderately low repeller voltages could be used in all experiments and that a rather high vaporizer temperature was very benificial in terms of signal to noise ratio.Application of lower temperatures of the vaporizer gave less noise, but also a far lesser signal. Variations of the collision gas pressure were of relatively little importance regarding sensitivity of the SRM method,but the values given for the voltages of the collision offset and the MSMSC factor are of utmost importance.Slight variations of these parameters give undesirably large changes in sensitivity.

In Table 2 a comparison is given for the detection limits in the FullScan and the SRM(daughterion) mode.In contrast to the findings with the Benzodiazepines(3),the explosives(4) and some members of the Methadone,Butyrophenone and the Diphenylbutylpiperidine group(6) the sensitiviy of the SRM method was in the same order as was the FullScan method.So no enhancement of sensitivity by using the SRM method was found, only selectivity was improved.We found that the detec-

TABLE 2

On column Detection Limits in ng for FullScan and SRM (S/N ≈ 3) for Reference Solutions for some members of of the Thioxanthenegroup.		
Substance	FullScan	SRM
Chlorprothixene	0.25	0.1
Flupenthixol	1.0	2.0
Thiothixene	1.0	5.0
Zuclopenthoxol	1.0	1.0

tionlimit in the SRM mode was moderately influenced by other ions in the original Q1 spectrum being present together with the nearly always "dominating" protonated molecular ion(3,4,5,8).Furthermore it appeared,that although the parameters for the collision activation process are chosen with utmost care;the ideal collision activation process for analytical quantitative purposes, giving only one dissociation product from the starting product $[M+H]^+$ could not be adjusted.Even when varying the parameters for the collision activated dissociation process very carefully,the protonated molecular ion gave always rise to quite a lot of dissociation products. In our view the piperazine group plays in these processes an important role.Because of these two interacting processes the dectionlimits in the SRM mode cannot surpass the detection limits of the FullScan mode. The detection limits found for these members of

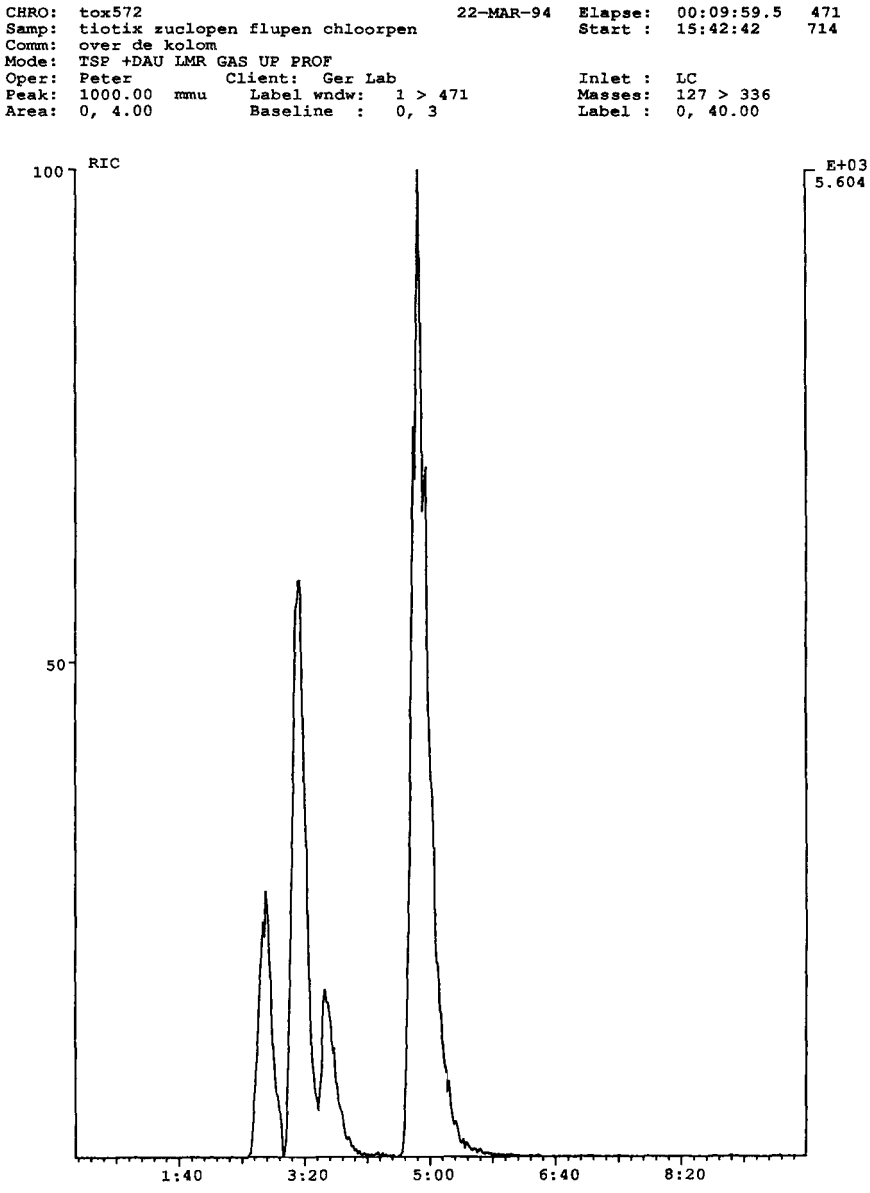

FIGURE 2 .
Chromatogram of the four thioxanthenes.For chromatographic conditions see Text.For mass spectrometric conditions see Text and Appendix 1.Sequence of elution:Thiothixene,Flupenthixol,Zuclopenthixol and Chlorprothixene On column about 10ng of each compound.

the Thioxanthene group were of the same order as the values usually found in litterature(1) using other analysis methods.The extraction method,as described in sample treatment, was checked for Chlorprothixene as model compound,as it can be thought that the other Thioxanthenes have more or less the same extraction properties.Spiked whole-blood samples,in the range of 1 to 200ng per mL blood were extracted by the BondElut method. For reference solutions and spiked blood extracts calibration curves could be constructed,with slopes of 0.25 and 0.24 counts.µl.10^4.ng^{-1} respectively and with intercepts of -2.12 and 1.01 counts.10^4 in this concentration range. From the slopes a recovery of 95 % could be calculated.The regression coëfficients of 0.982 and 0.996 were found.A standard deviation of 7.5% was found for spiked blood samples at a concentration of ≈10ng/mL (n=6).In Table 1 the values for the $[M+H]^+$ and the preferential ions in the MS/MS spectra are given. All the selected m/z values from the Table are tested for interference with the possible presence of ions from extracted blood.No such interferences were found.In Figure 2 a chromatogram of a separation of the four thioxanthenes is given using the procedure described in Appendix 1.About 10ng of each compound was injected.

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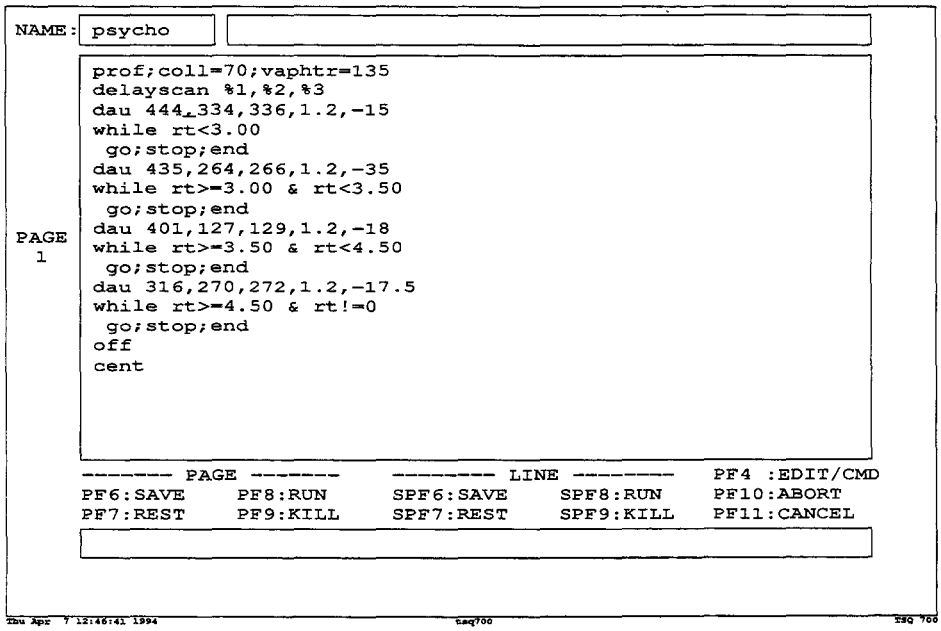
APPENDIX 1.



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ANALYSIS OF METALLOPORPHYRINS USING CYCLODEXTRIN STATIONARY PHASES WITH PHOTODIODE ARRAY UV DETECTION

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Abstract

A method for the high-performance liquid chromaographic separation of metalloporphyrins using cyclodextrin column with photodiode array UV detection is described. The isocratic separation of hemin, protoporphyrin IX, Mn protoporphyrin IX, cobalt protoporphyrin IX, Sn protoporphyrin IX, Zn protoporphyrin IX was achieved in less than 10 min using β-cyclodextrin stationary phases and a mobile phase consisted of a mixture of 28 ml of 5 mM ammonia solution and 90 ml of acetone (v/v, 28:90). The apparent pH of the mobile phase was 7.9. The chromatographic behavior of tin-protoporphyrin IX is markedly different from the transition metals-protoporphyrin IX in cyclodextrin column. The effects of ammonia concentration and the composition of the mobile phase were studied to optimize the separation of the compounds. The concentration of ammonia solution in the mobile phase affects the solvent strength and selectivity of the mobile phase.

Introduction

Porphyrins are the metabolites of heme biosynthesis. Different porphyrins represent different intermediate metabolites in the heme biosynthetic pathway. Zn-protoporphyrin is formed in heme biosynthesis and its concentration in body fluid is markedly different in lead poisoning and iron-deficiency anemia. Anemia is the major disorder related to low serum concentrations of vitamin B-12 (cyanocobalamin). Several

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other conditions manifest themselves as low serum vitamin B-12 content, including normal near-term pregnancy, vegetarianism, partial gastrectomy / ileal damage, oral contraception, parasitic competition, pancreatic deficiency, treated epilepsy, and advancing age (1-2). The biosynthesis of vitamin B-12, a member of the corrin family, shares the same precursors, 5-aminolevulinic acid and succinoyl CoA, of heme biosynthesis and is derived from uroporphyrinogen after a few step-wise reactions. Vitamin B-12 consists of a porphyrin-like ring system in which two of the four substituted pyrrole rings are connected directly with one another rather than through a methine bridging group (3). Other metalloporphyrins are linked to different biological functions and sources (4-5). Analysis of metalloporphyrins is useful in the diagnosis of disorders of heme biosynthesis and the other related manifestations. There are a few methods available for the determination of zinc-protoporphyrin (6-10) and other selected metalloporphyrins (11-15). The separation of metalloporphyrins by high-performance liquid chromatography (HPLC) is preferred because of its stronger separation capability. The lack of volatility of metalloporphyrins has made gas chromatographic work difficult. Studies of selected demetallated porphyrins and porphyrins in reversed-phase liquid chromatography on C_{18} columns have demonstrated its separation ability (6-10, 14-17). The demetallation procedure for metalloporphyrins yields an incomplete reaction and degradation of substituents on the porphyrin ring (15). Also, the separation of metalloporphyrins under acidic conditions results in demetallation. The present paper describes a novel HPLC method with photodiode array UV detection for the simultaneous determination of some common metalloporphyrins, namely, protoporphyrin complexes of Sn, Co, Mn, Fe, Zn together with some important porphyrins, protoporphyrin and coproporphyrin. The solvent strength is studied to characterize the separation performance .

Materials and methods

Protoporphyrin, coproporphyrin, hemin and metalloporphyrins (Sn, Co, Mn, Fe and Zn ion complexes) were purchased from Porphyrin Products, Inc. (Logan, UT). Acetone (HPLC quality) was purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). Ammonia solution was obtained from Sigma Chemical Co. (St. Louis, Mo). All other reagents were of analytical grade.

Apparatus

A modular HPLC system equipped with a Rheodyne 7125 injector fitted with a 20-μl sample loop was used. Separations were made on a β-cyclodextrin cyclobond I^{TM} column (25 cm x 4.6 mm I.D.). The column was a product from Advanced Separation Technologies (Whippany, NJ, USA) The detection system included a Waters Model 990 photodiode array detector equipped with a 8 μl flow cell attachment. All the measurements were recorded with the Waters 990 data processing system.

Preparation of standards

An amount of 150 nmol of each of the porphyrin and metalloporphyrins of Fe, Co, Zn, Sn and Mn ions were dissolved in 1 ml of 1 M ammonia solution. The dissolution was complete with sonication. The compounds were stable under refrigeration.

Chromatographic conditions

The mobile phase consisted of a mixture of 23.7% of 5mM ammonia solution and 76.3% of acetone by volume. The pH of the mobile phase was 7.9. The separation of metalloporphyrins and the demetallated porphyrins was carried out using isocratic elution

at a flow rate of 1 ml/min at ambient temperature. The injection volume was 2 μl. The UV absorbance of the elution profile of analytes was recorded at 400 nm for all measurements.

Results and discussion

The simultaneous determination of five metalloporphyrins has not been reported before. The present study reports the isocratic separation of five metalloporphyrins and the related porphyrins by liquid chromatography. The retention behavior of the metalloporphyrins on β-cyclodextrin column was studied with a wide range of solvent-based selectivity. Various combinations and compositions of traditional HPLC solvents, such as tetrahydrofuran, methanol, acetonitrile, acetone, ethanol, propanol, pyridine, with different aqueous buffer solutions including phosphate and acetate solutions, were used to develop the HPLC method but with little success. A β-cyclodextrin column was used with modifications of the earlier method for the separation of porphyrins (18). Although the elution strength and selectivity of the mobile phase is strong for demetallated porphyrins, metalloporphyrins are retained for a long period of time without practically any resolution.

Stability of β-cyclodextrin stationary phases and metalloporphyrins, as well as the solubility of porphyrins are of important considerations for developing the HPLC methods. Among the solvents tested, pyridine, 1M ammonia solution or 1M NaOH solution could readily dissolve the porphyrins. Acidic solution dissociates the metallated complex to form the corresponding metal ions and porphyrin ligand. Fortunately, the three alkaline solvents mixed with other HPLC solvents apparently show little effects on the stability of β-cyclodextrin stationary phases. After a series of experiments, we found that a mixture of acetone and pyridine as the mobile phase displayed a weak solvent

strength on the metallated porphyrins and other porphyrins on β-cyclodextrin column, and there was practical no separation among metalloporphyrins. Subsequently, NaOH solution was used in place of pyridine. The overall resolution among metalloporphyrins was improved but the selectivity of the mobile phase was dissatisfactory.

In a similar approach, NH_4OH was used in the mobile phase. Ammonia hydroxide solution allowed a good dissolution of the porphyrins and the pH of the resulting mobile phase was readily changed to the desired value. The variation of NH_4OH concentration in the mobile phase significantly affects the solvent selectivity (Figure 1). Due to the weak absorptivity, a larger amount of Fe-protoporphyrin IX was required to produce an equivalent peak size. The solvent selectivity of the mobile phase for Mn-, Co- protoporphyrin and coproporphyrin is limited at higher concentration of NH_4OH. And the resolution among coproporphyrin, cobalt protoporphyrin IX and manganese protoporphyrin IX were practically unaffected by the change of NH_4OH solution. The elution strength of the mobile phase for the three analytes was apparantly the same. However, the mobile phase selectivity for other metallated porphyrins (Zn, Fe) was improved at lower concentration of ammonia hydroxide solution and peaks of metalloporphyrins were better resolved at lower concentration of NH_4OH ($< 26\%$). The retention time of the two analytes was significantly changed with the concentration of NH_4OH in practice.

It is worth noting that Tin-protoporphyrin IX behaved quite differently from the other metalloporphyrins in the β-cyclodextrin column. Its chromatographic behavior was quite predictable in a linear manner at a higher concentration of NH_4OH ($> 26\%$). The compound was retained for relatively longer period of time when NH_4OH concentration was $< 26\%$. When NH_4OH concentration went below 24%, Sn-protoporphyrin IX was

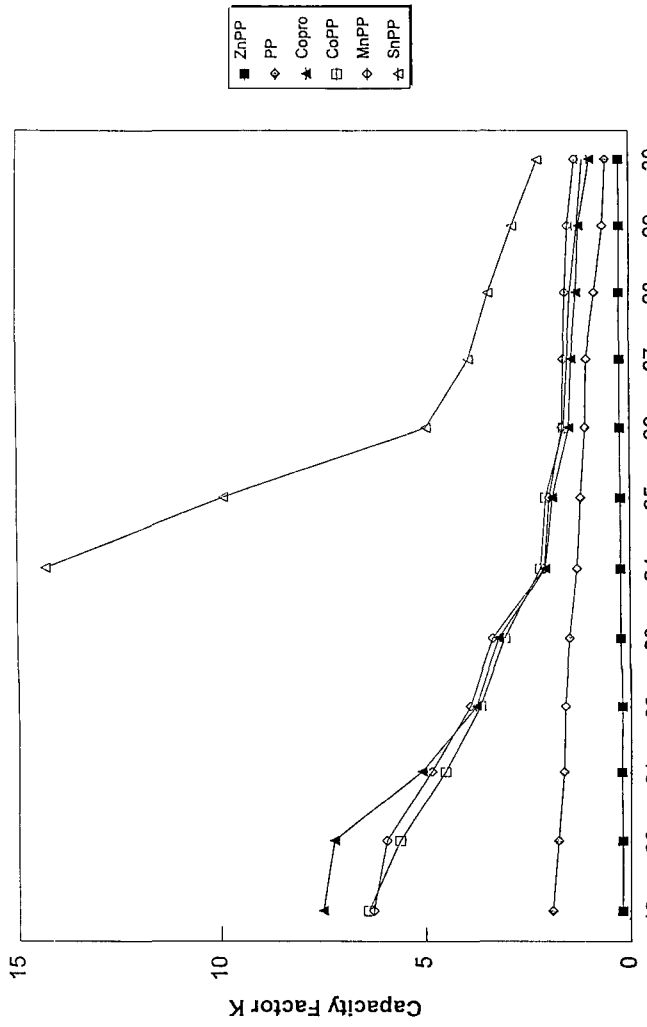

Fig. 1. Effects of NH_4OH concentration (% v/v) on the capacity ratio of the porphyrins.

Table I Absorption maximum and separation parameters of metalloporphyrins & porphyrins

Porphyrin	Retention time		Wavelength of maximum absorption	Colour of solution	Capacity Factor K	
	(90:25)*	(90:30)*			(90:25)*	(90:30)*
ZnPP	1.70	1.69	400 nm	Light Brown	0.14	0.13
PP	3.21	2.20	404 nm	Dark Brown	1.15	0.48
Copro	6.48	2.95	408 nm	Purple Red	3.35	0.98
Uro	37.13	32.10	409 nm	Bright Red	23.90	20.54
CoPP	5.52	4.04	423 nm	Bright Red	2.71	1.71
MnPP	5.46	3.80	373 nm	Dark Brown	2.66	1.55
SnPP	29.50	27.52	403 nm	Purple	18.80	17.47
FePP	10.21,38.56	4.00,27.94	400 nm	Dark Green	5.85,24.8	1.68,17.75
Air	1.49	1.49				

*Mobile phase composition (% v/v) = Acetone : 2 mM NH_3

*The composition of mobile phases cannot cleanly separate the porphyrins. There are some overlapping of peaks. However, the resolution among peaks can be improved by changing the percentage composition of NH_4OH by volume in the mobile phase.

practically retained in the column. The retention time of Sn-protoporphyrin IX was generally longer than that of any other porphyrins used in this study, and peak broadening of Sn-protoporphyrin IX was noticeable. With Sn ions, protoporphyrin IX seemingly interacts more strongly with the β-cyclodextrin stationary phases.

Effort has been made to use diluted base for dissolution of metalloporphyrins and the preparation of the mobile phase. The experimental results show that the β-cyclodextrin stationary phases are quite stable under this condition in all our experiments. Previous study has shown the effects of alkaline injection solvents on the elution order of metalloporphyrins (19). However, with the diluted base of fixed alkalinity as the injection solvent, the elution order of metalloporphyrins and porphyrins could well be predicted without incidents.

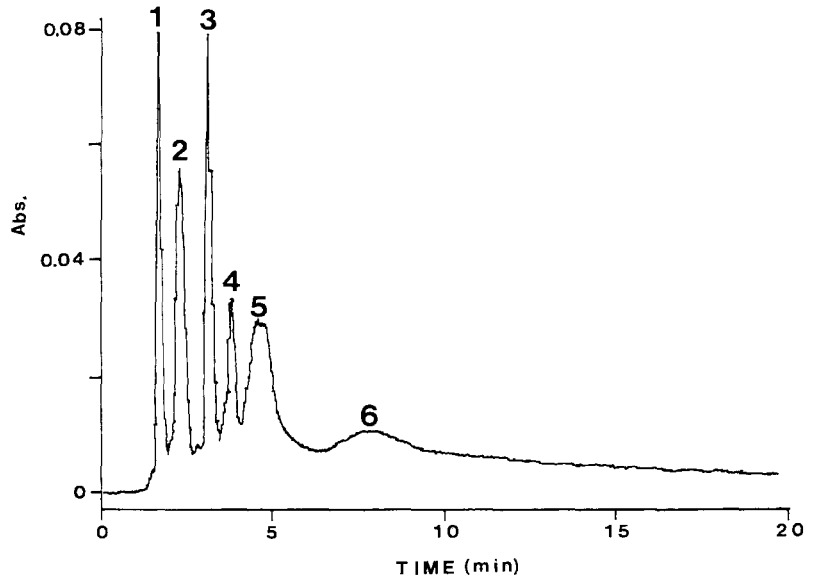

Fig. 2. Chromatogram of metalloporphyrins and porphyrins. See chromatographic conditions for experimental details. Peaks : 1, Zn-protoporphyrin IX; 2, protoporphyrin IX; 3, Coproporphyrin; 4, Co-protoporphyrin IX; 5, Mn-protoporphyrin IX; 6, Sn-Protoporphyrin IX.

The absorption characteristics of the metalloporphyrins and porphyrins used in the present study were listed in Table I. Under the present chromatographic conditions, all analytes display an absorption maximum close to 400 nm. An amount of sub-nano gram of each porphyrin, except Fe-protoporphyrin, is sufficient for quantitative analysis in each experiment. A typical chromatogram of the porphyrins and metalloporphyrins is shown in Figure 2. The HPLC method is sensitive and efficient, and it is unique in the way that a relatively strong base is used as the component in the mobile phase for isocratic separation of the porphyrins on the β-cyclodextrin column. The present method is useful for the analysis of metalloporphyrins and some important porphyrins.

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HPLC WITH ELECTROCHEMICAL AND FLUORESCENCE DETECTION OF THE OPA/2-METHYL-2-PROPANETHIOL DERIVATIVE OF FUMONISIN B_1

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ABSTRACT

The o-phthalaldehyde (OPA) derivative of fumonisin B_1 was prepared in the presence of 2-methyl-2-propanethiol (tert-butyl thiol). A hydrodynamic voltammogram for the derivative indicated that the optimum voltage for maximum electrochemical response was +0.7 V. The electrochemical response of the OPA/tert-butyl derivative was unstable. However, the fluorescence response was found to be stable for over an hour after an initial 30 minute reaction time. The minimum detectable limit (MDL) of the OPA/tert-butyl derivative using fluorescence detection was 30 ng/ml as compared to 250 ng/ml for electrochemical detection.

INTRODUCTION

Fumonisins are mycotoxins produced by the fungus Fusarium moniliforme, which are common fungal pathogens of corn (1). Two metabolites from Fusarium moniliforme cultures, identified as fumonisins B_1 (FB_1) and B_2 (FB_2), have cancer-promoting activity (2). Intravenous injection of FB_1 in horses induces

the neurotoxic disease leukoencephalomalacia (3,4). Fumonisin B_1 has also been implicated in inducing pulmonary edema in swine (5) and causing hepatocarcinogenic and hepatotoxic effects in rats (6).

The molecular structure of fumonisin B_1 contains a primary amine moiety (FIGURE 1) which can be utilized to form highly sensitive fluorescent derivatives. A sensitive method commonly used for quantitating fumonisin B_1 is that of Shephard et al. (7), which uses HPLC with fluorescence detection after derivatization with OPA in the presence of the reducing agent 2-mercaptoethanol. To remove interfering compounds when forming the OPA derivative, cleanup is necessary before derivatization and analysis by HPLC. The cleanup involves solvent extraction of the original sample followed by passing the extract through a strong anion exchange (SAX) column prior to derivatization and HPLC analysis. The OPA derivative with 2-mercaptoethanol is unstable, making it necessary to time the reaction of OPA with the sample to obtain reproducible results.

Allison et al. (8) obtained more stable derivatives of primary amines (amino acids) by reaction with OPA in the presence of tert-butyl thiol instead of 2-mercaptoethanol. This OPA/tert-butyl derivative was stable up to 4 hours; the background fluorescence was overcome by using electrochemical detection.

We have compared the sensitivities of fluorescence and electrochemical detection for the OPA/tert-butyl derivative of fumonisin FB_1 and looked at the possibility of improving an existing analytical method for trace amounts of fumonisin B_1 by determining the derivative's stability.

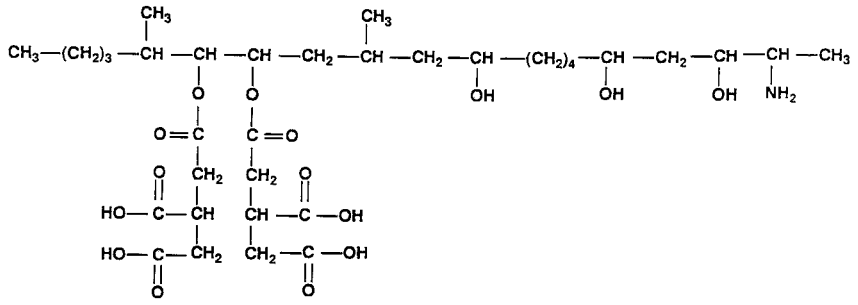

FIGURE 1. Structure of Fumonisin B_1.

EXPERIMENTAL

Instrumentation

The high performance liquid chromatography (HPLC) system consisted of a Waters Model M 6000A pump (Waters Chromatography Division, Milford, MA, USA) and an Altex Model 100A pump (Beckman Instruments, Fullerton, CA USA). A guard column (Upchurch Scientific, Catolog # C-135B, Dark Harbor, WA USA) with a 2 μm frit and containing 100 mg of C_{18} column packing was placed between the injector and the column. The column was a 250 X 4.6 mm 5 μm Supelco reversed-phase C_{18} column (Supelco, Bellefonte, PA USA). A Shimadzu Model RF-535 fluorescence detector was used with a Shimadzu Model C-R3A integrator (Shimadzu Scientific struments, Columbia, MD USA). A BAS LC-4B amperometric electrochemical detector (Bioanalytical Systems, West Lafayette, IN USA) with a dual glassy carbon electrode (GCE) and a Ag/AgCl reference electrode was used with a Model 3390 Hewlett-Packard integrator (Hewlett-Packard Company, Avondale, PA USA). A TG-5M(BAS)0.005 in. thick red gasket separated the stainless steel auxiliary electrode from the glassy carbon electrodes. The first electrode was maintained

at +0.4 V to remove background noise. The voltage of the second electrode or working electrode was varied from +0.5 to +0.9 V. The electrochemical detector was operated at 100 nA.

Reagents

The 100 mM borate buffer was prepared by dissolving 38.137 g of sodium tetraborate decahydrate (Fluka Chemical Company, 99% purity, Ronkonkoma, NY USA) in 1 L of deionized H_2O and adjusting the pH to 9.5 with dilute HCl.

The o-phthalaldehyde/tert butyl thiol (OPA/tert-butyl) reagent was prepared by first dissolving 27 mg of OPA (Sigma Chemical Company, 99% purity, St. Louis, MO USA)in 2 ml of methanol. The solution was transferred to a 10 ml serum bottle and then 20 μl of tert-butyl thiol (Aldrich Chemical Company, Milwaukee, WI USA) and 4.5 ml of borate buffer was added. This OPA-thiol reagent was stable for 3 days when stored in a sealed serum vial at 4°C.

The fumonisin B_1 standard (FB_1) (CSIR, 99% purity, Pretoria, South Africa) was used as received in a 10 mg vial. A stock standard of FB_1 was prepared by quantitatively transferring the FB_1 from the vial to a 100 ml volumetric flask using acetonitrile/water (50:50 v/v) to give a final concentration of 100 μg/ml. The stock standard is stable for 6 months when stored at 4°C. Working standards were made up from this stock standard in acetonitrile/water (50:50 v/v).

Precolumn Derivatization and HPLC

For the precolumn derivatization, 40 μl of the FB_1 standard and 40 μl of the OPA/tert-butyl reagent were reacted from 2 to 120 min at room temperature (27°C). A 50 μl aliquot of the reacted solution was injected into the HPLC.

The mobile phase used was acetonitrile/pH 7 KH_2PO_4 (45:55 v/v) with 1 mM of Na_2EDTA. The flow rate was 1.5 ml/min.

For optimum response, the fluorescence detector had the excitation and emission wavelengths set at 345 nm and 435 nm, respectively, with both the attenuation and gain at 10.

RESULTS AND DISCUSSION

The literature indicated that one of the most sensitive methods available for the analysis of fumonisins was HPLC analysis with fluorescence detection after derivatization with OPA in the presence of 2-mercaptoethanol. However, several problems exist with this method, including an unstable fluorescent derivative and a high fluorescence background even after solvent extraction of samples and additional cleanup with solid phase extraction (SPE) on a strong anion exchange (SAX) column. The OPA/tert-butyl derivative of fumonisin B_1 was investigated with the objective of obtaining a more stable fluorescent derivative. Also, electrochemical detection was evaluated to determine if high sensitivity could be obtained for fumonisin B_1 and hence, eliminate the high background fluorescence seen when fluorescence detection is utilized.

The mobile phase used for isocratic HPLC was acetonitrile/ pH 7 KH_2PO_4 with 1 mM Na_2EDTA. Several ratios of acetonitrile/ pH 7 KH_2PO_4 were tried with a final ratio of (45:55 v/v) selected since this could be used successfully to resolve the fumonisin B_1 peak using fluorescence detection.

Various quantities and ratios of standard FB_1 and OPA/tert-butyl reagent were tried in forming the derivative of fumonisin B_1, with equal amounts of each (40 μl) giving the optimum results.

The electrochemical detector took up to a day to equilibrate enough to give a stable baseline. Even though a great effort was taken to de-gas the mobile phase by filtering daily through 0.45 μm filters and sparging with nitrogen to keep out oxygen, the electrochemical detector was not very easy to operate. However, after equilibration, good HPLC chromatograms for FB_1 could be obtained with electrochemical detection (FIGURE 2).

A hydrodynamic voltammogram, comparing the detector response with applied voltage, showed the optimum voltage to give maximum response for the electrochemical detector to be +0.7 V.

The fluorescence detector on the other hand was quick to stabilize and easy to operate and consequently good chromatograms could be obtained after only a 10 to 15 min warm-up period (FIGURE 3). The retention times corresponding to fluorescence and electrochemical detection was 13.46 and 13.55 min, respectively. The peak for the FB_1 derivative was determined by comparing chromatograms of standard FB_1 at different concentrations to derivatization of blanks containing no FB_1. This made it possible to distinguish between the FB_1 derivative peak and the artifact peaks in the chromatograms.

Sensitivity studies were done on the OPA/tert-butyl derivative of FB_1, using both the electrochemical and fluorescence detectors. As the level of the OPA/tert-butyl derivative dropped below 1 μg/ml, longer periods of time were necessary for the electrochemical detector to equilibrate and stabilize, whereas this was not a problem with the fluorescence detector. The fluorescence detector could detect approximately 30 ng/ml of FB_1 compared to only 250 ng/ml for the electro-

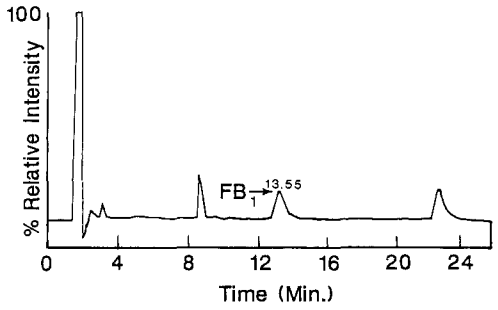

FIGURE 2. HPLC of the OPA/tert-butyl derivative of a 1 μg/ml fumonisin B_1 standard using electrochemical detection.

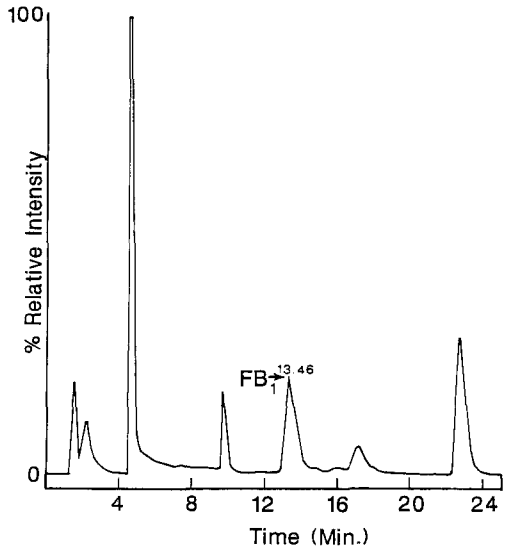

FIGURE 3. HPLC of the OPA/tert-butyl derivative of a 1 μg/ml fumonisin B_1 standard using fluorescence detection.

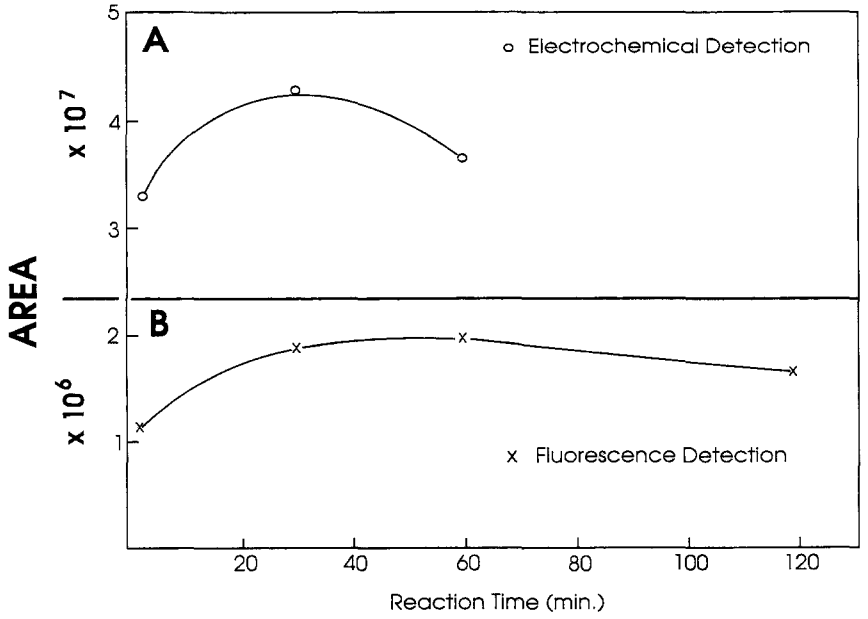

FIGURE 4. (A) Detector response vs reaction time of the OPA/tert-butyl derivative of fumonisin B_1 using electrochemical detection. (B) Detector response vs reaction time of the OPA/tert-butyl derivative of fumonisin B_1 using fluorescence detection.

chemical detector. Detector sensitivity was determined as twice background.

The electrochemical response for the OPA/tert-butyl derivative of FB_1 was not stable (FIGURE 4A).

The fluorescence response for the OPA/tert-butyl derivative was stable for at least an hour after an initial reaction time of 30 min (FIGURE IVB). Increasing the reaction time for the OPA/tert-butyl derivative from 2 min to 30 min resulted in a 63% increase in fluorescence response (FIGURE 4B).

CONCLUSION

Our study indicates that fluorescence detection is more sensitive than electrochemical detection for the OPA/tert-butyl derivative of FB_1. Also, a more stable derivative is formed when FB_1 is reacted with OPA in the presence of 2-methyl-2-propanethiol instead of 2-mercaptoethanol.

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APPLICATION OF HPLC EQUIPMENT WITH RAPID SCAN DETECTION TO THE IDENTIFICATION OF DRUGS IN TOXICOLOGICAL ANALYSIS

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ABSTRACT

The paper reports on an application of HPLC equipment with rapid scan detection, in which the UV-spectra recorded by the rapid scan detector are used for drug identification. A dBase database was set up, utilizing the retention data of a drug substance in a neutral and an acidic HPLC solvent together with the related UV spectrum. The identification of an unknown drug is supported by a home made search program, comparing the retention times first and then examining the maxima of UV spectra.
The data base presented here contains more than 200 entries, and data of additional substances are continuously attached.
It provides a fast and low cost possibility for the assay of drugs and medicaments in biological matrices and is especially used in emergency analysis.

INTRODUCTION

Much work has been done on the subject of assay and quantitative determination of drugs in biological matrices [1-3]. However, especially for the so called "general unknown analysis" there are still many problems left in the fields of forensic toxicology and analysis in emergencies. The increasing number of drugs to be encountered plays an important role in case of an intoxication with an unknown substance. During the last few years the number of available pharmaceutical products, narcotics, pesticides etc. has enormously increased. Therefore, a fast analysis leading to a reliable result has become a challenging procedure in case of intoxication.

HPLC analysis is well suited to meet these demands, and in case of a rapid scan detector the retention as well as the UV-spectrum can be used to identify a substance. Moreover, there are mathematical methods available for detecting peak overlays (proof of purity) and estimating spectra identities [4,5].

It is well known that it is very difficult to find the same retention data with measurements in different laboratories even in case of identical conditions. Therefore, it becomes necessary to develop home made data bases. This was done by us using the widespread dBase IV computer program, which now is available for a very low price.

The subject of this paper is to show that composing data sets from substances of toxicological relevance by combination of their retention data in two different eluents and the related UV spectra permits a sufficiently fast identification in toxicological analysis already by use of a home made dBase program. This low cost, but

high performance setup has been used by us since some month routinely in emergency analysis.

EXPERIMENTAL

Materials and Instrumentation

Two different isocratic HPLC setups (I and II) are used. Both of them, however, include an HPLC pump LC 1100 and an injection valve C6W from GAT(Gamma Analysentechnik Bremerhaven, Germany).
First, the setups differ in the detectors:

LC I: variable wavelength detector LCD 500 (GAT) at detection wavelength 254 nm, equipped with an C-R6A integrator (SHIMADZU)

and

LC II: rapid scan detector PHD 601 (GAT) at a detection wavelength range from 200 to 360 nm in steps of 1 nm; spectra are recorded by an IBM compatible personal computer with software delivered by the detector manufacturer.

The reference spectra were recorded from methanolic solutions of the substances.
A three-dimensional plot of the absorption as a function of wavelength and time as shown in Fig. 4 can be used to tune the detector for the highest sensitivity.
Second, the eluents for LC I and LC II are composed in the following way:

LC I : CH_3OH // 0.5 mol/l CH_3COOH //0.5 mol/l NH_4COOCH_3
(105 : 60 : 10 v/v/v) [6]

LC II: 156 g CH_3CN + 344 g phospate buffer solution pH=2.3
(4.8g 85 % H_3PO_4 and 6.66 g KH_2PO_4 in 1l H_2O) [7]
(All solvents: "BAKER ANALYZED" HPLC Reagent grade)

In both cases pre- (3.2 X 30mm) and main column (3.2 X 150 mm) are filled with ODS material (SI 100, 7 µm spheric), which was prepared in the former east German Central Institute of Organic Chemistry of the Academy of Sciences (Zentralinstitut für Organische Chemie der Akademie der Wissenschaften der DDR, ZIOC).
This material was investigated by EIGENDORF, who recommended it especially for the general unknown analysis [8].
The flow rate is to be chosen due to the retention time of the standard, normally between 0.5 and 1.0 ml/min.
Sample preparation is done by liquid-liquid extraction or solid phase extraction, the latter with Chem-Elut columns (Analytical-International) or Extrelut columns (MERCK).
From our experience, methanolic extracts from blood, urine or stomach contents are especially well suited for HPLC analysis.

Methods

Relative retention data were obtained by reducing the retention data of the investigated toxicological relevant substance to the retention time of 5-(4-Methylphenyl)-5-phenylhydantoin (MPPH) [9], which serves as standard substance.
UV spectra were recorded by LC II and stored on disk together with the chromatograms they were obtained from.

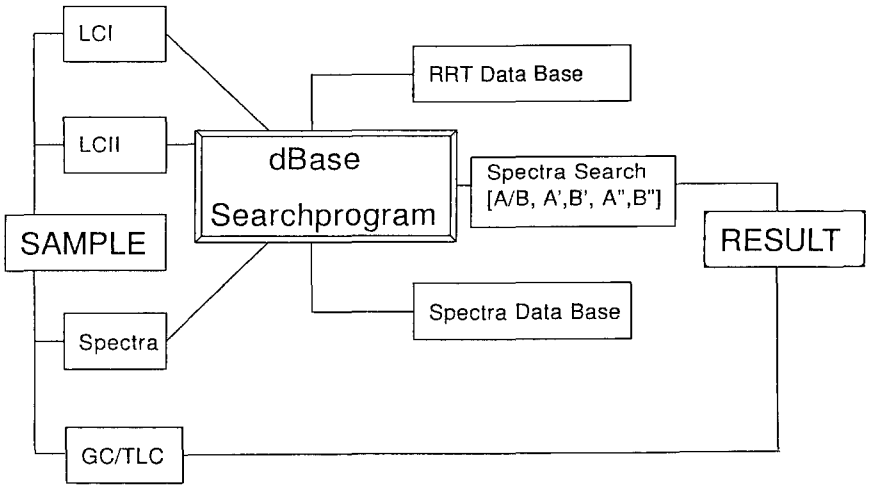

FIGURE 1: FLOW SCHEME OF SUBSTANCE IDENTIFICATION IN CASE OF AN ACCIDENTAL INTOXICATION

The chromatograms are used for the quantification of the unknown substance.
As can be seen from Figure 1 and mentioned before, access to the data base is provided by a home made dBase program [10], by which the following data of the references can be compared with the data of the unknown for identification:

A: relative retention data in LC I and LC II

B: relative retention data in LC II and spectra

C: relative retention data in LC I ,LC II and spectra

Concerning the identification by UV spectra [11-14], a pre-selection is done by comparing the UV maxima. The

TABLE 1
COMPILATION OF SOME OF THE RELATIVE RETENTION TIMES (RRT) AND ABSORPTION MAXIMA (AM) OF TOXICOLOGICAL RELEVANT SUBSTANCES AS MEASURED WITH SETUPS SPECIFIED IN THIS PAPER (LCI, LCII)

SUBSTANCE	LCI	LCII	AM1 [nm]	AM2 [nm]	AM3 [nm]	AM4 [nm]
Acetylsalicylsäure	0,39	0,47	231	298	0	0
Ajmalin	0,42	0,20	242	286	0	0
Aminophyllin	0,33	0,22	215	266	0	0
Bromhexin	1,53	0,48	208	242	311	0
Bromoprid	0,33	0,22	211	271	306	0
Butaperazin	0.27	0,46	238	273	0	0
Carbamazepin	0,78	0,58	208	233	282	0
Chinidin	0,44	0,17	207	245	312	340
Chlordiazepoxid	1,44	0,20	240	304	0	0
Chlorprothixen	2,07	0,82	202	225	264	324
Clenbuterol	0.00	0,24	207	240	295	0
Clonazepam	0,83	0,91	210	245	306	0
Coffein	0,37	0,21	225	267	0	0
Detajmium	0,29	0,15	240	284	0	0
Diazepam	1,94	1,58	227	279	310	0
Doxylamin	0,41	0,25	209	269	0	0
Ethosuximid	0,36	0,28	213	240	0	0
Flunitrazepam	0,87	1,19	213	248	308	0
Furosemid	0,43	0,64	227	266	338	0
Haloperidol	0,73	0,41	215	241	0	0
Hydrochlorothiazid	0,27	0,22	211	268	312	0
Levomepromazin	1,33	0,62	202	247	301	0
Metamizol	0,30	0,18	236	255	0	0
Methaqualon	1,00	0,92	222	262	301	313
Methocarbamol	0,40	0,27	218	269	0	0
Nicotin	0,28	0,09	254	0	0	0
Nitrazepam	0,87	0,69	211	255	303	0
Oxazepam	1,12	0,71	223	310	0	0
Parathionmethyl	1,90	4,44	270	0	0	0
Phenacetin	0,55	0,36	243	0	0	0
Phenazon	0,39	0,24	238	258	0	0
Phenprocoumon	1,89	4,07	280	305	0	0
Procain	0,42	0,20	216	290	0	0
Promazin	1,00	0,45	247	298	0	0
Propaphenon	1,00	0,52	204	244	300	0
Propranolol	0,63	0,28	210	225	286	312
Pyrithyldion	0,43	0,28	204	300	0	0
Salicylamid	0,40	0,33	230	296	0	0
Talinolol	0,57	0,26	238	282	0	0
Temazepam	1,25	1,18	226	308	0	0
Tetracain	0,62	0,33	222	309	0	0
Thebain	0,33	0,18	217	282	0	0
Thiopental	1,23	1,42	232	282	0	0
Triamteren	0,34	0,22	211	245	277	355
Verapamil	0,64	0,56	225	274	0	0
Zolpidem	0,44	0,24	203	233	292	0
Zopiclon	0,43	0,19	212	300	0	0
p-Nitrophenol	0,57	0,56	221	313	0	0

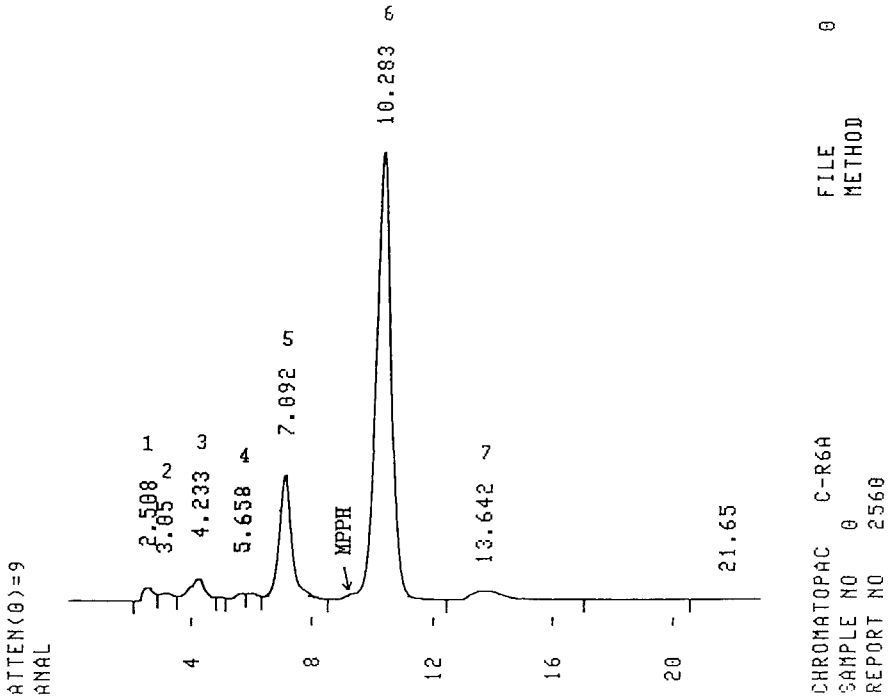

FIGURE 2: CHROMATOGRAM OF AN EXTRACT FROM SPE OF BLOOD IN AN EMERGENCY ANALYSIS (LC I, 220 nm). NOTE THE OVERLAY WITH THE STANDARD

range can be chosen, in which the experimental data must correspond to the stored data, i.e. a difference between the experimental and the stored spectrum of up to 9 % can be tolerated for the UV-spectra.

Once a number of substances are found similar to the unknown by the program, the further comparision is done by hand, inspecting the UV-spectra of the computer proposals for congruence with the spectrum of the unknown. During this step of manual search often also the first and second derivatives of the spectra are considered.

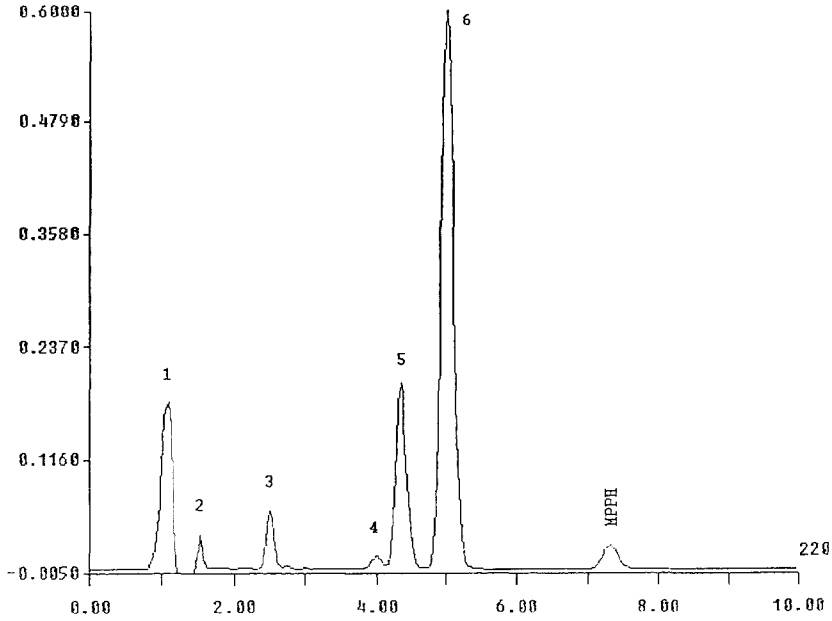

FIGURE 3: LC II CHROMATOGRAM OF AN EXTRACT OF SPE OF BLOOD IN AN EMERGENCY ANALYSIS (254 nm)

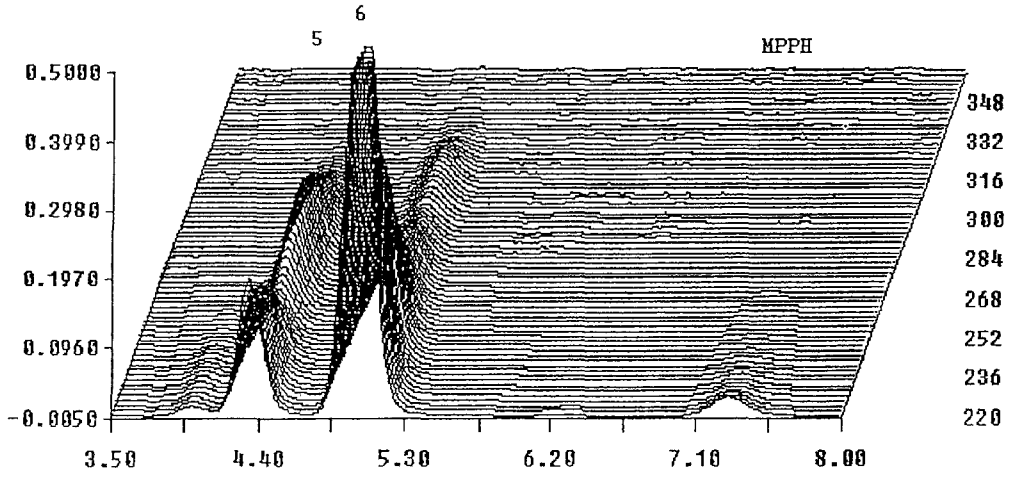

FIGURE 4: PART OF A THREE-DIMENSIONAL PLOT OF THE CHROMATOGRAPHIC INVESTIGATION OF THE BLOOD EXTRACT IN THE RAPID SCAN MODE (LC II)

YOUR INPUT WAS:

LCI	Tolerance [%]	from	to
0,26	10	0,23	0,29
0,32	10	0,29	0,35
0,44	10	0,40	0,48
0,60	10	0,54	0,66
0,74	10	0,67	0,81
1,08	10	0,97	1,19
1,43	10	1,29	1,57

YOUR INPUT WAS:

LCII	Tol. [%]	from	to	AM1 [nm]	from	to	AM2 [nm]	from	to	AM3 [nm]	from	to
0,15	10	0,14	0,17	219	210	228	275	266	284	0	0	0
0,21	10	0,19	0,23	235	226	244	0	0	0	0	0	0
0,34	10	0.31	0,37	0	0	0	0	0	0	0	0	0
0 54	10	0,49	0,59	235	226	244	0	0	0	0	0	0
0,59	10	0,53	0,65	208	199	217	235	226	244	280	271	289
0,69	10	0,62	0,76	225	216	234	310	301	319	0	0	0

SUBSTANCE	LCI	LCII	AM1 [nm]	AM2 [nm]	AM3 [nm]	AM4 [nm]
CARBAMAZEPIN	0,78	0,58	208	233	282	0
OXAZEPAM	1,12	0,71	223	310	0	0

FIGURE 5: RESULTS OF COMPUTER SEARCH BY THE dBase SEARCH PROGRAM

The results of the HPLC analysis should be validated by results of other methods (i.e. GC or TLC), whenever possible.

RESULTS AND DISCUSSION

Some of the relative retention data from LC I and LC II , as well as the absorption maxima in the range from 200 to 360 nm as obtained by rapid scan detection are shown in Table 1. The data base set up by us contains more than 200 data by now and will be continuously expanded.

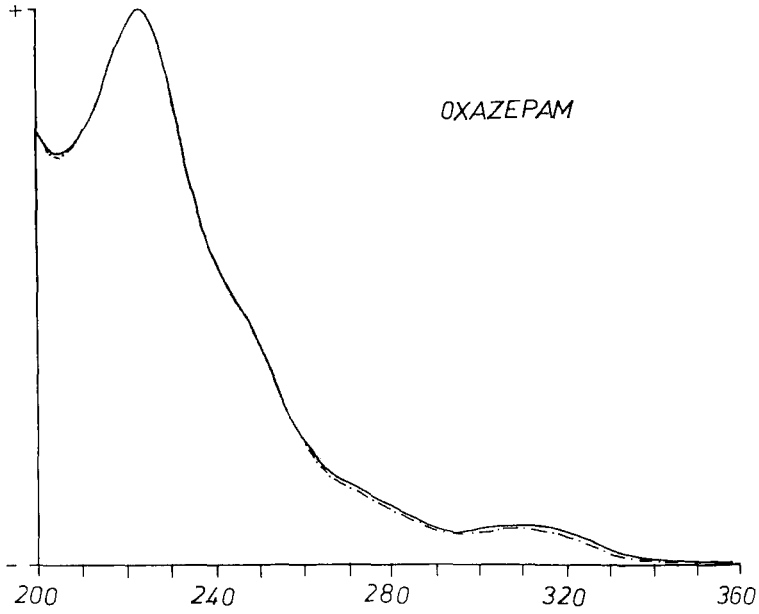

FIGURE 6: COMPARISON OF PEAK 6 (FIG. 3) WITH DATA BASE SPECTRUM FOR OXAZEPAME

The use of our method is now demonstrated by discussing an example of an analysis in an emergency:
A person was found unconscious with unknown cause. No indications for a special intoxication were reported. HPLC investigation of extracts from the persons blood resulted in the chromatograms given in Fig. 2 - Fig. 4. As mentioned before, the relative retention times and UV absorption maxima of the significant peaks were used for the computer search in the data base. Figure 5 shows the results of the search.
The proposal of the computer search could be verified by comparing the spectra as shown in Figure 6 and Figure 8,

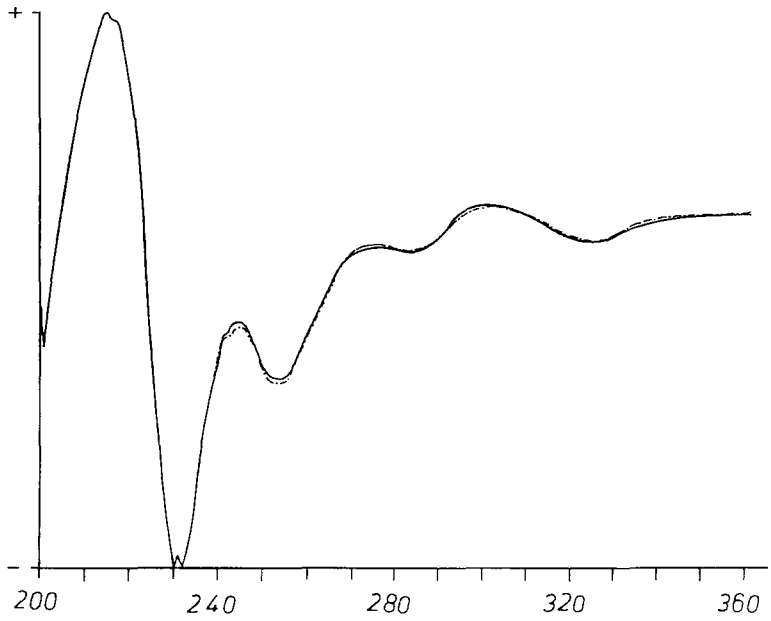

FIGURE 7: FIRST DERIVATIVE OF THE SPECTRA OF FIGURE 6

and the first derivatives of the spectra as shown in Figure 7 and Figure 9,respectively.
The excellent agreement of the spectra of the unknown substances with the substances proposed by the computer search is accomplished by the results of an enzymatic immunoassay (EMIT, Syva), which indicated the presence of benzodiazepines and tricyclic antidepressants.
Of course, the results are not always so unidirectional. Often about ten substances are included in the computer proposal, which have to be distinguished then by a more exhaustive spectra search. The HPLC investigation should anyhow be accomplished by another analytical method.

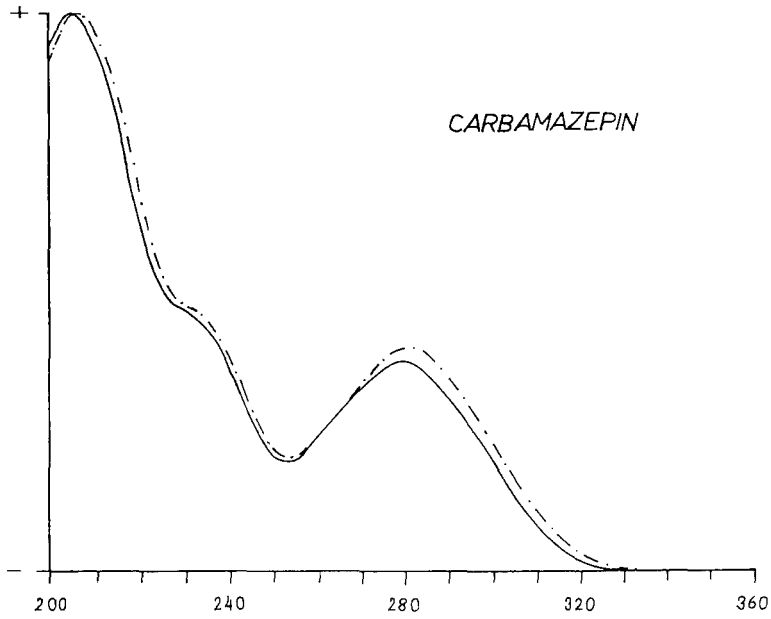

FIGURE 8: COMPARISON OF PEAK 5 (FIG. 3) WITH DATA BASE SPECTRUM FOR CARBAMAZEPINE

CONCLUSIONS

Provided that it is done by experienced personnel, HPLC emergency analysis as described above takes only about two to three hours, after which qualitative results are provided, having more than just temporarily character, because information from spectra are included. The method is fast and fairly simple, though some experience is needed. It can be highly recommended in those emergency cases, in which fast immunological drug screening does not suffice. It is amazingly less time consuming than classical methods of substance identification, which is of course especially meaningful in emergencies. Careful measurement of the absorption maxima of the references

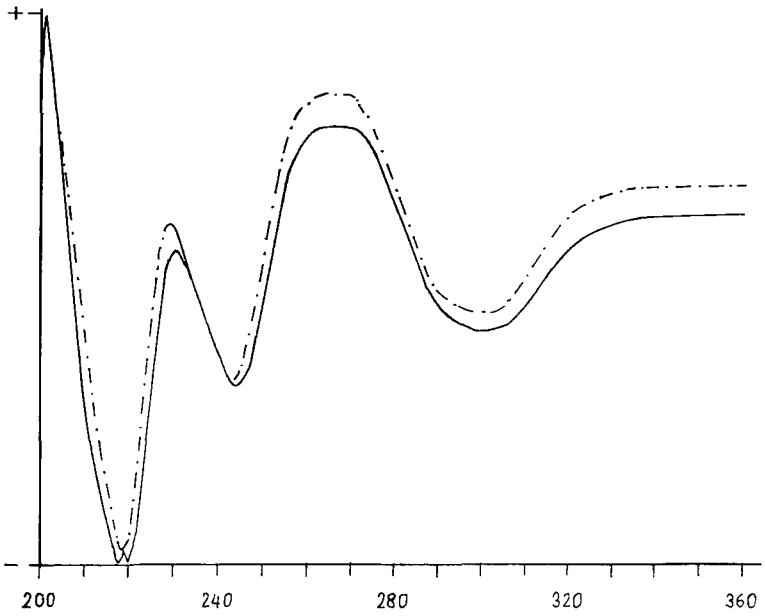

FIGURE 9: FIRST DERIVATIVE OF THE SPECTRA OF FIGURE 8

as well as the unknown substance is crucial to select reliable information from the data base.
There is of course more sophisticated equipment available (i.e. GC/MS, GC/HPLC). Considering limited fundings, however, the measurement of HPLC retention data and UV spectra by use of a rapid scan detector and data processing by use of standard software might be a low cost bypass, providing nearly the same information.

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ION-PAIR ISOLATION AND LIQUID CHROMATOGRAPHIC DETERMINATION OF ALBENDAZOLE, OXFENDAZOLE, OXIBENDAZOLE, AND THIABENDAZOLE RESIDUES IN MILK

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ABSTRACT

A liquid chromatographic [HPLC] method for the determination of Albendazole (ABZ), Oxfendazole (OFZ), Oxibendazole (OXBZ), and Thiabendazole (TBZ) residues in milk at levels below 3 ppb has been developed. Samples were deproteinized with acetonitrile, defatted with hexane partition, and evaporated to constant volume. Following addition of acidified aqueous octane-1-sulfonate solution, the target benzimidazoles were selectively extracted as ion pairs into chloroform and analyzed on a reversed-phase C_{18}, 5 μm, column. Overall recoveries were found to be 93.5 ± 2.6% for OFZ, 90.0 ± 1.3% for OXBZ, 85.7 ± 3% for ABZ, and 74.0 ± 2.6% for TBZ. Limited data on the depletion of OXBZ and ABZ in sheep milk were also generated.

INTRODUCTION

Benzimidazoles have become an integral part of the animal producing industry for the prevention and control of internal worm parasites. Among the benzimidazoles most widely used in farm animals are ABZ, OFZ, OXBZ, and TBZ. As some members of the benzimidazole class of anthelmintics have shown teratogenic and embryotoxic effects in a variety of animal species [1], quantitation of residues remaining in tissues and milk of treated animals intended for human consumption is of major interest.

There have been many multiresidue methods for analyzing benzimidazoles in animal matrixes but only two [2, 3] are available for milk. They are both useful HPLC procedures, the former one [2] utilizing a modern matrix solid-phase dispersion methodology for effective extraction and cleanup whereas the latter employing classic liquid-liquid partitions based on the weakly basic nature of the target residues, and solid-phase extraction. These methods, although useful for monitoring benzimidazole residues in milk, either lack the sensitivity required for trace residue analysis [2] or are time, labor and materials intensive [3].

This paper deals with the development of a sufficiently sensitive, accurate, precise, and rapid method for the determination of ABZ, OXBZ, OFZ, and TBZ residues in cow and sheep milk. Using a new ion-pair extraction approach, many of the limitations of the extraction and cleanup procedures outlined above are overcome.

EXPERIMENTAL

Instrumentation

HPLC was carried out on Gilson system consisting of a Model 805 manometric module, a Model 305 piston pump, a Model HM/HPLC dual-beam variable-wavelength spectrophotometer set at 292 nm, and a model N1 variable-span recorder (Villiers-le-Bel, France). A Model TC 831 HPLC-Technology column oven (Macclesfield, UK), set at 40 °C, permitted temperature regulation. Injections were made on a Hichrom, 250x4.6 mm, stainless-steel column, packed with Nucleosil 120 C_{18} 5-μm, through a Rheodyne 7125 sample injector equipped with a 100-μl loop.

Chemicals

Octanesulfonate sodium salt and HPLC grade methanol were obtained from Merck-Schuchard (Munchen, Germany). Benzimidazole standards included ABZ and OXBZ which were obtained from Smith and Kline (Westchester, PA, USA), and OFZ and TBZ which were purchased from Hoechst (Frankfurt, Germany) and Merck (Rahway, NJ, USA), respectively. All other reagents and solvents used were of analytical reagent grade. Deionized water was distilled before use.

Standard Solutions

Stock solutions of the individual benzimidazoles (100 μg/ml) were prepared by dissolving 10 mg of each standard in 10 ml dimethylsulfoxide and diluting to 100-ml volume with methanol.

Aliquots of these stock solutions were mixed and diluted with mobile phase to give mixed standard working solutions in the range 10-500 ng/ml.

Mobile Phase and HPLC Conditions

A 0.01 M ammonium phosphate buffer was prepared by dissolving 1.15 g $NH_4H_2PO_4$ in 950 ml deionized water, adjusting to pH 7.0 with 50% NH_4OH solution, and diluting to 1 l final volume.

Mobile phase was prepared by combining 600 ml methanol with 400 ml ammonium phosphate buffer, and passing through 0.45 μm filter before use. The mobile phase was degassed using helium and delivered at a rate of 0.7 ml/min. Recordings were made at chart speed of 2 mm/min and 0.020 a.u.f.s. sensitivity setting.

Extraction and Cleanup Procedure

A 5-ml sample was transferred to a 25-ml centrifuge tube and accurately weighed.. A volume (15 ml) of acetonitrile was added, and the tube was vortexed for 15 s and centrifuged for 3 min at 1000g. The clear supernatant liquid formed was decanted into another 50-ml tube, rinsing inner walls of first tube with additional 3 ml of extracting solvent and combining the extracts. A volume (10 ml) of hexane was added to the extracts, and partitioning was effected by vortexing for 15 s at high speed. After discarding the top layer, the remaining bottom layer was decanted into a 50-ml evaporating flask and rotary-evaporated under vacuum to constant volume at 40 °C to be further mixed with 5 ml chloroform and 3 ml of 0.005 M octane-sulfonate in 0.1% phosphoric acid. Following vigorous shaking, flask

content was decanted into a 10-ml centrifuge tube, vortexed for 5 s, and centrifuged for 10 min at 1000g. A 4-ml volume of the bottom layer was pipetted into another 10-ml tube and evaporated to dryness with gentle nitrogen stream at 40 °C. The residue remaining was redissolved in 500 µl of mobile phase, and a 100-µl aliquot was used for HPLC analysis.

Calculations

Calibration curves were constructed by running 100-µl aliquots from the series of working solutions and plotting the recorded peak heights versus quantity of each benzimidazole injected. The concentrations of ABZ, OXBZ, OFZ, and TBZ in samples were calculated by reference to calibration curves and multiplication by appropriate dilution factor.

RESULTS AND DISCUSSION

The extraction of milk with ethyl acetate caused a persistent emulsion. Emulsion formation was also observed when chloroform was used. To eliminate this problem, acetonitrile was used as an extracting solvent. Treatment of samples with 3 volumes of acetonitrile was effective in precipitating milk proteins, since clear extracts were consistently taken after centrifugation.

Some purification of the extracts was effected by partitioning them with hexane. Further purification was initially attempted by evaporating the extracts to constant volume, adding a pH 10 phosphate buffer, and partitioning the analytes into diethylether.

Although the weakly basic nature of the target benzimidazoles favors such a partition, a significant loss of OFZ recovery (<65%) was noted.

To increase the recovery of OFZ, a different purification scheme was tested. Benzimidazoles were first converted, under acidic conditions, to ion pairs with octanesulfonate anions to be further extracted with chloroform. This ion-pair extraction enhanced the recovery of OFZ, and resulted also in efficient cleanup.

The effectiveness of the cleanup procedure permitted chromatographic analysis of milk samples under isocratic conditions. Using the ammonium phosphate/methanol mobile phase, OFZ was eluted in 6.9 min, TBZ in 7.8 min, OXBZ in 11.5 min, and ABZ in 16.6 min. Peak heights, although quite reproducible at a given mobile phase flow rate, varied with it at different extent for each analyte. By gradually reducing the flow rate from 1.0 to 0.5 ml/min, peak heights of OFZ, TBZ, OXBZ, and ABZ were increased 8%, 6%, 14%, and 24%, respectively.

Due to absence of any interfering peaks in samples chromatograms (Figure 1), concentrations as low as 1 ppb for OFZ, TBZ and OXBZ, and 3 pp. for ABZ could be readily determined (peak to noise ratio, 3/1). Regression analysis of the data obtained by running a series of mixed standard working solutions showed the response to be linear for all compounds in the range examined ($y=0.51+3.14x$, $r=0.9996$ for OFZ; $y=1.04+5.01x$, $r=0.9990$ for TBZ; $y=0.89+3.40x$, $r=0.9983$ for OXBZ; $y=0.94+1.05x$, $r=0.9989$ for ABZ,

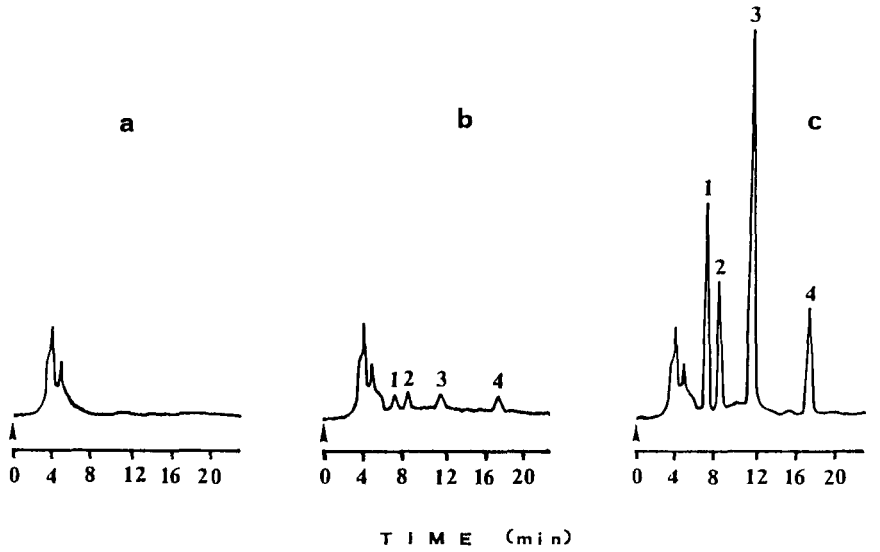

FIGURE 1. Typical chromatograms of (a) a blank milk sample, (b) a milk sample fortified with 1 ppb of OFZ (1), TBZ (2), OXBZ (3), and 3 ppb of ABZ, and (c) a milk sample fortified with 18 ppb of OFZ (1), 9 ppb of TBZ (2), 31 ppb of OXBZ (3) and 29 ppb of ABZ (4). Conditions: mobile phase, methanol-0.01 M ammonium phosphate buffer, pH 7.0 (60:40, V/V); column 250x4.6 mm, C_{18} (5 μm); temperature, 40 °C; flow rate, 0.7 ml/min; wavelength, 292 nm; recorder sensitivity, 0.020 a.u.f.s.; chart speed, 2 mm/min; injection volume, 100 μl.

where y represents peak height in mm and x the quantity in ng of the compound injected).

The accuracy and the precision of the method were studied by spiking milk samples at 6 fortification levels with mixed standard working solution, and analyzing 5 replicates. Least-squares and regression analysis of the data presented in Tables 1 and 2 showed that the relationship between "added" and "found" was

TABLE 1

Precision and Accuracy Data for the Determination of OFZ, and TBZ in Milk

	OFZ		TBZ	
Concn. added, ppb	Mean concn[a] found, ppb	Mean rec., %	Mean concn[a] found, ppb	Mean rec., %
15	13.7 ± 0.6 (4.2)[b]	91.1	12.0 ± 1.0 (8.3)[b]	80.0
60	58.6 ± 3.0 (5.1)	97.7	44.6 ± 4.0 (8.9)	74.3
120	110.6 ± 2.5 (2.3)	92.2	89.2 ± 1.8 (2.0)	74.3
180	168.0 ± 6.4 (3.8)	93.3	131.2 ± 4.4 (3.3)	72.9
240	219.0 ± 4.0 (1.8)	91.3	178.7 ± 1.1 (0.6)	74.4
300	283.4 ± 3.8 (1.3)	94.5	222.8 ± 3.9 (1.7)	74.3

[a]Mean of 6 replicates ± SD.
[b]Values in parenthesis represent RSD %.

TABLE 2

Precision and Accuracy Data for the Determination of OXBZ, and ABZ in Milk

	OXBZ		ABZ	
Concn. added, ppb	Mean concn[a] found, ppb	Mean rec., %	Mean concn[a] found, ppb	Mean rec., %
15	13.0 ± 1.0 (7.7)[b]	86.7	12.7 ± 0.6 (4.6)[b]	84.4
60	54.0 ± 3.2 (5.8)	90.0	49.4 ± 2.5 (5.1)	82.3
120	108.4 ± 4.4 (4.1)	90.3	100.4 ± 2.9 (2.9)	83.7
180	163.2 ± 6.3 (3.9)	90.7	152.4 ± 4.5 (2.9)	84.7
240	215.3 ± 3.5 (1.6)	89.7	209.0 ± 2.6 (1.3)	87.1
300	270.0 ± 1.9 (0.7)	90.0	254.2 ± 4.7 (1.8)	84.7

[a]Mean of 6 replicates ± SD.
[b]Values in parenthesis represent RSD %.

adequately described, for all compounds, by a linear regression ($y=0.054+0.935x$, $r=0.9988$ for OFZ; $y=0.076+0.740x$, $r=0.9991$ for TBZ; $y=0.099+0.900x$, $r=0.9992$ for OXBZ; $y=-1.43+0.857x$, $r=0.9991$ for ABZ). Therefore, the slopes of these regression lines could be used as estimates of the overall recovery for OFZ (93.5±2.6%), TBZ (74.0±2.2%), OXBZ (90.0±1.3%), and ABZ (85.7±3.0%) determination in milk.

Since other antibiotics or drugs might interfere with the analysis, an interference test was evaluated. Several compounds that are, frequently, added in feeds and/or used for treatment of mastitis, such as penicillin G, cloxacillin, furazolidone, nitrofurazone, oleandomycin, ampicillin, sulfathiazole, erythromycin, streptomycin, chlortetracycline, tetracycline, and oxytetracycline were dissolved in mobile phase at 1 ppm level and submitted to HPLC. It was found that none of the compounds tested interfered with the analysis.

To validate the method with real samples, a trial was undertaken to quantitate residues in milk of two dairy sheep each given orally a single dose of 10 mg OXBZ/kg of body weight and 20 mg ABZ/kg of body weight, respectively. The control milk samples, which had been collected before treatment, and all other samples taken during the trial at 12 h milking intervals were stored at -25 °C, until analyzed. The analysis data, presented in Table 3, showed that both compounds could be detected in milk for up to 48 h after dose. Extractable metabolites could also be seen in all positive chromatograms of the milk samples analyzed (Figure 2). The chromatographic behavior of these metabolites indicated a substantial increase in polarity over the parent compounds.

TABLE 3

Levels of OXBZ and ABZ Residues in Sheep Milk After Single Dosing with the Respective Benzimidazole

Hours after dosing	Concn of residue found[a], ppb	
	OXBZ	ABZ
12	13.6	1805.0
24	4.6	135.3
36	2.0	35.3
48	2.5	7.0
60	<1.0	<3.0
72	<1.0	<3.0
84	<1.0	<3.0

[a]Values corrected for recovery.

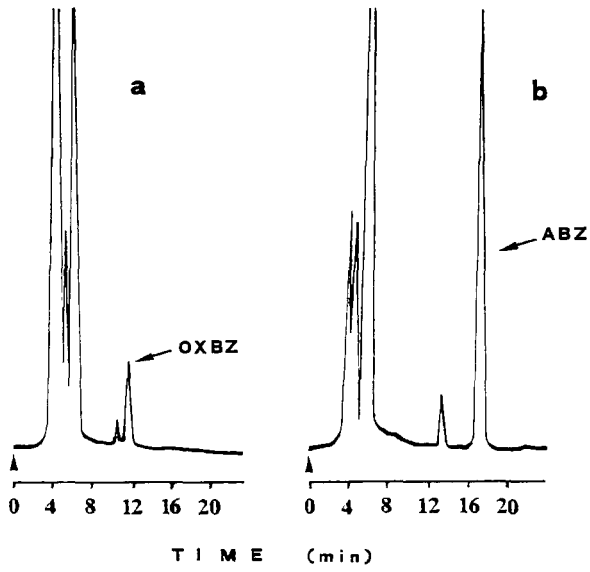

FIGURE 2. Chromatograms of milk samples from a sheep dosed with OXBZ (a) and a sheep dosed with ABZ (b). HPLC conditions as in Figure 1.

In conclusion, the results of the present study show that the developed HPLC procedure offers acceptable recoveries, high precision, increased sensitivity, and minimal background interference. It uses small sample size without any need for several pH adjustments, back washings, additional solvent partitioning steps, and evaporation of large volumes of extracting solvents. This results in considerable savings in terms of time, labor and materials requirements. One analyst can easily process 6 samples in 2 h. These advantages make the procedure an attractive alternative to published methods.

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DETERMINATION OF CEFTIZOXIME IN HUMAN ABSCESS FLUID BY PAIRED ION REVERSED-PHASE HPLC

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ABSTRACT

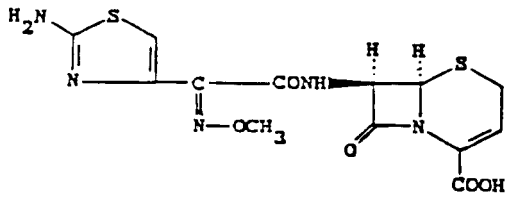
An ion paired reversed - phase high performance liquid chromatographic assay for ceftizoxime in human abscess fluid using barbital as the internal standard is described. Sample preparation consists of precipitating the proteins in diluted abscess fluid with methanol, and centrifuging. Decanted supernatant after evaporation to dryness is reconstituted with the HPLC mobile phase for injection. The HPLC separation was carried out on an octyl C_8 column using a mobile phase composed of potassium phosphate buffer, tetrabutylammonium dihydrogen phosphate IP reagent and 20% methanol (v/v). UV detection was at 254 nm and the concentration range for the assay was 2.0μg/mL to 50.0μg/mL. The results of the full validation of the assay gave coefficients of variation for inter-assay variability of 8.4-12.6% and intra-assay variability of 4.3 - 9.3% for three levels of concentration. This isocratic HPLC method is relatively simple, rapid and shows good reproducibility for the determination of ceftizoxime in human abscess fluid.

INTRODUCTION

Ceftizoxime (FK749), [(6R,7R)-7-[(Z)-2-(2-amino-4-thiazolin-4-yl) -2-methoxy-iminoacetomido]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid] (Figure 1) is a broad spectrum, semi-synthetic, third generation cephalosporin antibiotic (1) that is administered parenterally. Ceftizoxime possess activity against both aerobic and anaerobic, gram positive and gram negative microorganisms and it is widely used in the treatment of bacterial infections including abscesses.

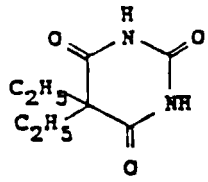
Various analytical methods including HPLC have been used to determine cephalosporin drugs in different biological matrices. Ceftizoxime concentrations have been determined in human plasma (2), serum (3,4,5), urine (6,7), blister fluid (8) and rat serum, bile and urine (9). However, an assay to determine the ceftizoxime concentrations in human abscess fluid was necessary and none of the available methods was found suitable. Some of the difficulties in the previous methods were the lack of an internal standard or the use of another cephalosporin drug as the internal standard. Thin layer chromatographic methods even with fluorescence detection were not considered satisfactory. HPLC methods frequently applied for determination of cephalosporins in aqueous dosage forms did not perform well in the presence of abscess-matrix. The method by Moore et al (10) using microbore HPLC column and mass spectrometry was only applied to aqueous media. This method could not possibly be applied to clinical assays due to matrix interference and high costs of operation and instrumentation. Accordingly, the method developed by us is the first HPLC assay suitable for determination of cephalosporins in abscess fluid. In this method an octyl (C-8) column with an ion pairing mobile phase system, very similar to that used for assaying iothalamate in plasma was used (11). The main difference was a more freely available ion-pairing reagent tetrabutylammonium dihydrogen phosphate used instead of dodecyltriethylammonium phosphate Q-12 ion- pairing reagent.

A complete assay validation, reported in this paper was performed. This was followed by determination of ceftizoxime in a set of clinical samples.



(A) CEFTIZOXIME

$C_8H_{13}N_5O_5S_2$
MW.383.40



(B) BARBITAL

$C_8H12N_2O_3$
MW 184.2

FIGURE 1. Chemical structures of (A) ceftizoxime and (B) 5,5 diethyl barbituric acid (barbital, internal standard).

EXPERIMENTAL

Apparatus

The HPLC system used consisted of a Waters M-510 HPLC pump, a model 712 WISP autoinjector, and a model M441 variable UV detector (Waters Assoc., Milford, MA). An HP 3392A-integrator (Hewlett

Packard, Avondale,PA) was used for collecting the chromatographic data. The detector wavelength was 254 nm and the absorbance was set at 0.05 aufs. Separation was achieved with an Ultrasphere, octyl (C-8), 5μm particle size, 4.6 mm (i.d.) x 25cm reversed-phase column (Beckman Instruments Inc., San Ramon, CA).

Reagents

All solvents were of HPLC grade [Fischer Scientific Co., Fair Lawn, NJ]. Tetrabutylammonium dihydrogenphosphate [(Ion pair reagent, 1M solution), Aldrich Chemical Company Inc., Milwaukee, WI 53233], potassium diacid phosphate, potassium mono acid phosphate [Fischer Scientific Co., Fair Lawn,NJ], ceftizoxime sodium [Fujisawa Pharmaceutical Co., Osaka, Japan], and barbital [Sigma Chemicals Co., St. Louis, MO], were used as received. Deionized distilled water was obtained from Barnstead Nanopure purification system (Barnstead Co., Boston, MA). For blank samples, human abscess fluid from patients who were not administered ceftizoxime was used and the clinical samples assayed for ceftizoxime were obtained from patients at UC San Francisco-Long Hospital, who were administered ceftizoxime (1g, iv, Q8) prior to surgery.

Mobile Phase

The mobile phase was composed of 20% methanol, and 10mM IP reagent in 40 mM potassium phosphate. This was prepared by dissolving 6.44 g KH_2PO_4, 7.04 g K_2HPO_4 and 10 mL of 1M ion-pairing reagent solution in 1 L of deionized water and the pH was adjusted to 7.0. The prepared buffer was filtered through a 0.22μm filter and mixed with 250 mL of methanol. The mixture was degased by sonication under vacuum. The isocratic flow rate of the mobile phase was 1 mL/min.

Sample preparation

Calibration curve samples were prepared by spiking 1:2 diluted abscess fluid with ceftizoxime and the internal standard (barbital). The proteins in these samples, quality assuarance (QA) controls and clinical samples (50 μL aliquots) were precipitated out with 500 μL aliquots of HPLC grade methanol. Samples were vortexed for 20 seconds and centrifuged for 10 minutes at 2500 rpm. The supernatants were concentrated under nitrogen and residues were reconstituted in running buffer (500μl). Twenty five to thirty microliter samples were injected onto the column for analysis.

All clinical samples, QA samples and stock solutions of compounds were stored at -80°C until analysis. Fresh stock solutions of ceftizoxime were prepared every week. Spiked samples for calibration curves were prepared immediately prior to the assay and frozen controls at three different drug concentrations were prepared every week. The frozen control samples were prepared in human abscess fluid which were relatively ceftizoxime free, and were spiked with separately prepared drug solutions.

RESULTS

Typical chromatograms of the internal standard in blank abscess fluid and the abscess fluid spiked with ceftizoxime and internal standard, are presented in Figure 2A and 2B respectively. The mean retention times of ceftizoxime and barbital are 16.2, and 18.0 minutes respectively. (However, fluctuations of these retention times are observed due to the variation of temperature, pH and column performance). The concentrations used for the calibration curve are 2.0, 5.0, 10.0, 20.0, 35.0 and 50.0 μg/mL.

A typical calibration graph used for the calculation of ceftizoxime concentration is presented in Figure 3. Linear regression of peak height ratio vs. drug - concentration gives typical coefficient of determination (r^2) of 0.999.

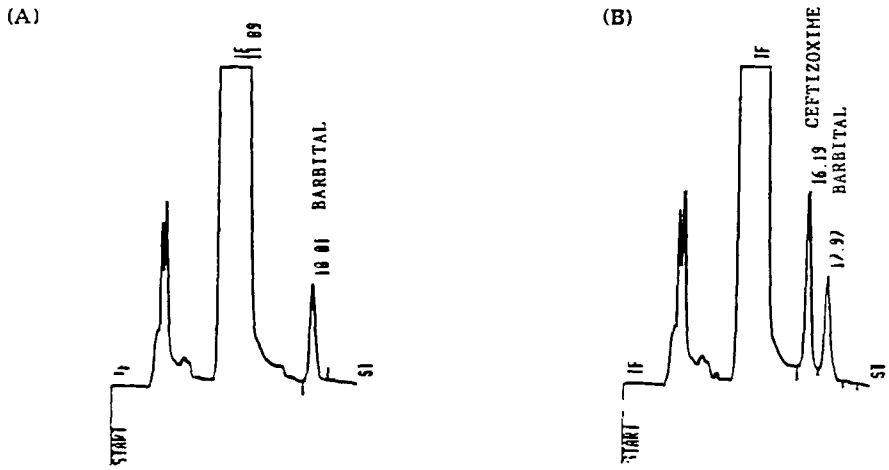


FIGURE 2 (A) Chromatogram of blank abscess fluid with internal standard. (B) Chromatogram of abscess fluid spiked with ceftizoxime and the internal standard.

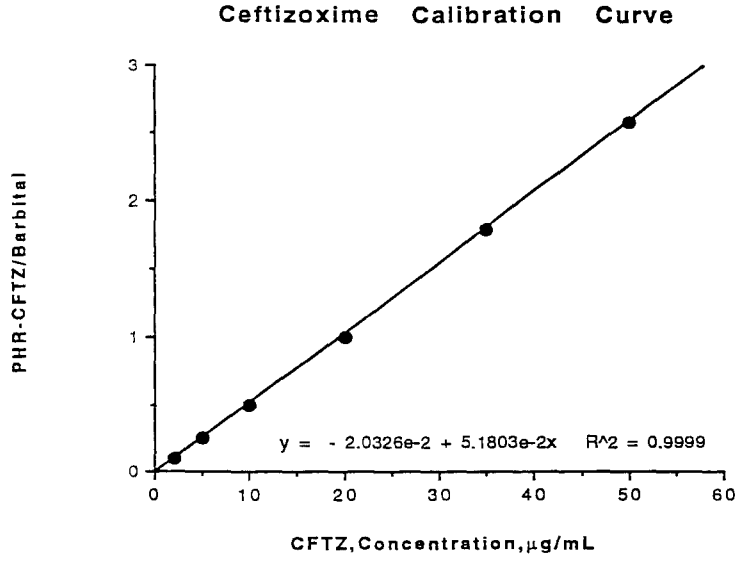


FIGURE 3. Calibration curve of ceftizoxime in abscess fluid.

Variability Studies

Inter-assay and intra-assay variability was studied using frozen controls at three concentrations, low, medium and high. Four to six samples from each concentration were assayed for both inter-assay and intra-assay studies. For inter-assay variability, six calibration curves on six different days were used and one calibration curve was used for the intra-assay variability study. The range for the coefficients of variation was 8.44 % to 12.60 % for the inter-assay study. Coefficient of variation ranged from 4.25 % to 9.31 % for the intra-assay study (Table 1).

Recovery

Assay recovery was measured by comparing the peak height ratios of ceftizoxime to barbital, at three different drug- concentrations in abscess fluid and in aqueous drug solutions spiked at the same concentration. The internal standard was added to the abscess fluid samples only after the supernatant was decanted from the precipitated proteins. Both sets of samples were then evaporated and reconstituted in running buffer for injection. The mean % recovery was calculated as follows:

$$\% \text{ Recovery} = \frac{\text{peak height ratio of ceftizoxime in abscess fluid}}{\text{peak height ratio of ceftizoxime in water}} \times 100$$

The recovery for low (6.0μg/mL), medium (16.0μg/mL) and high (40.0μg/mL) controls were 87.0%, 74.1% and 74.4% respectively. The mean recovery of the ceftizoxime was 78.5 %.

Stability

The stability of ceftizoxime during storage at room temperature for 24 hours was evaluated in abscess fluid, plasma and in distilled -

TABLE 1
Inter-Assay and Intra-Assay Precision of Ceftizoxime in Abscess Fluid

		CONCENTRATION* (µg/mL)		
		LOW [6.0]	MEDIUM [16.0]	HIGH [40.0]
Inter-Assay				
	Mean	5.78	16.43	38.60
	SD	0.72	1.39	3.86
	%CV	12.60	8.44	10.04
Intra-Assay				
	Mean	5.93	16.16	38.55
	SD	0.25	1.22	3.59
	%CV	4.25	7.55	9.31

*Each value represents mean of n=6

deionized water (Table 2). Six samples each from three controls were used for this stability study. Further, the stability of ceftizoxime during three freezing and thawing cycles was investigated. These results showed that there was some loss of ceftizoxime up to 10.6% for the lowest concentration, after the third cycle (Table 3).

DISCUSSION

This is the first method reported for the determination of ceftizoxime concentrations in abscess fluid. The stability of the drug in abscess fluid was unsatisfactory due to degradation of the drug. This decomposition was observed in abscess fluid, plasma and in distilled - deionized water. The manufacturers of ceftizoxime sodium for injection (Fujisawa Pharmaceutical Co.,) stated that the drug solutions are stable in most of parenteral fluids for 24 hours at room temperature and 96 hours under refrigeration (5°C) at 100mg/mL concentration (12). However, the

TABLE 2
Storage Stability (24 Hours at RT) of Ceftizoxime in Abscess Fluid, Plasma and DI water

	Percent Ceftizoxime Remaining*		
Conc.,µg/mL	Abscess Fluid	Plasma	DI Water
6.0	74.64	70.07	72.40
16.0	83.15	78.39	92.72
40.0	92.50	78.89	94.95

*Each value represents mean of n=5

TABLE 3
Freeze - Thaw Stability of Ceftizoxime in Abscess Fluid

	Percent Change CFTZ Conc.*	
Conc.,µg/mL	From Cycle 1 to 2	From Cycle 1 to 3
6.0	-9.51	-10.65
16.0	1.77	-0.77
40.0	-1.95	-6.28
Mean	**-3.23**	**-5.90**

* Each value represents mean of n=5

recent studies by A.B. Lesko et al revealed that the ceftizoxime sodium is stable in both NaCl solution and in 5Xdistilled water for 48 hours at RT and for seven days in NaCl solution and 5 days in 5Xdistilled water under refrigeration (5°C) at 20mg/mL and 40mg/mL concentrations (13). According to our experience the drug solutions were not stable in the range of 2.0-50.0 µg/mL for more than five days in plasma, in abscess fluid and in distilled - deionized water even at -20°C. Our results showed that the drug percent declined for these concentrations in the range of 7-26%, 21-30% and 5-28% at room temperature for 24 hours in abscess fluid, plasma and distilled-deionized water respectively (Table 2). Further, freeze-thaw stability results in abscess fluid showed that the

mean drug percent change, was in the range of -3.23 to -5.90% (Table 3). In order to maintain the stability of the drug in clinical samples, QA samples and stock solutions, they were stored at -80°C for 5 days.

The inter-assay and intra-assay variability results were satisfactory. The assay method has a lower limit of quantitation of 2.0μg/mL. The recovery of the drug during extraction was satisfactory and within acceptable limits.

Using this method, various abscess samples from the abdomen, pancreas, brain, gall bladder, thigh, cysts, and nodes from eighteen subjects were analysed for the ceftizoxime concentrations. The full range of concentration for these samples was 2.18-30.66 μg/mL.

The validation of this assay was carried out according to the suggestions offered by the conference on "Analytical Methods Validation; Bioavailability, Bioequivalence and Pharmacokinetics studies" (14). The IP-RP-HPLC method described here is simple, rapid, and reliable for the determination of ceftizoxime concentrations in various abscess fluids and hence is useful for clinical drug studies.

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QUANTITATIVE DETERMINATION OF P-COUMARIC ACID IN ECHINACEA PURPUREA PRESS JUICE AND URGENIN. A VALIDATED METHOD

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ABSTRACT

Echinacea purpurea species have been studied by various authors over the last decade. The starting material was the fresh or dried plant, or an ethanolic extract in which cichoric acid and derivatives were one of the major constituent groups. In our search for a suitable marker for the Echinacea press juice we found that cichoric acid was not the major compound but p-coumaric acid. The amount of p-coumaric acid was therefore determined in Echinacea press juice and Urgenin® and the method was validated.

INTRODUCTION

Urgenin®, which is used for benign prostate hyperplasia, consists of 35% Echinacea press juice and 65% Sabal serrulata extract. E. purpurea

has immunostimulating properties (1) and S. serrulata exhibits a specific anti-androgenic activity by inhibition of 5 α-reductase (2).

In order to standardize the plant preparation and investigate its stability a suitable marker had to be found in the Echinacea press juice and in Urgenin®.

The Echinacea press juice has been studied by various authors over the last decade (3,4,5). The starting material was the fresh or dried plant or an ethanolic extract in which cichoric acid and derivatives were one of the major constituents. In the Echinacea press juice, however,no cichoric acid nor its analogues seemed to be present. Therefore we isolated the main component from the press juice and identified it by NMR and MS as p-coumaric acid.

We developed an analytical HPLC method to quantify p-coumaric acid in the press juice and in the drug Urgenin®. Prior to use, the method was validated.

EXPERIMENTAL

Apparatus

A Hewlett Packard liquid chromatograph, which was equipped with a 20 µl sample loop (manual injection), a HP 1050 series pump and a HP 1040M Series II diode array detector was used. The chromatographic data were processed using HP Chemstation software.

HPLC Conditions

A RP-18 column (Superspher, 5 µm, 250 mm*4mm, LiChroCart, Merck) was used. The mobile phase consisted of a mixture of acetonitrile

(A) and 0.1 M H_3PO_4 (B). Gradient elution was used: within 30 minutes, the composition of the mobile phase changed from 15% acetonitrile (85% 0.1 M H_3PO_4) to 30% (70% 0.1 M H_3PO_4). The flow rate was 0.7 ml/min. The detector was set at 310 nm with a bandwidth of 4 nm and as reference wavelength 380 nm with a bandwidth of 10 nm.

Reagents

The p-coumaric acid was obtained from Fluka (Switzerland, Buchs). Acetonitrile was Labscan for HPLC, phosphoric acid was from J.T. Baker, N.V., (Holland, Deventer). Water used in the chromatographic mobile phase was distilled, deionized (Millipore System) and filtered through a 0.45 µm nylon 66 membrane filter (Alltech). The solvents were degassed by helium before and during the run.

Standard Solutions

The standard solutions were freshly prepared daily by dissolution of p-coumaric acid in a mixture of acetonitrile/water (1/9). The standard solutions were prepared by dilution of stock solutions of approximately 0.25, 0.5, 1, 2.5 and 5 mg/ml. All standard solutions were filtered through a 0.45 µm disposable polyamide (nylon 66, Filterservice) filter before injection.

Sample and Sample Preparation

The Echinacea press juice and Urgenin® were preparations obtained from Madaus s.a. (Germany, Köln).

A 1 ml sample of Echinacea purpurea press juice and Urgenin®, prepared by Madaus, was taken and filtered over a disposable polyamide (nylon 66, Filterservice) filter of 0.45 µm and collected in a vial. The filtrate was injected.

ANALYTICAL RESULTS AND DISCUSSION

Method Validation

Linearity

In order to examine the linearity of the absorbance as a function of the concentration of p-coumaric acid, five solutions with different concentrations of p-coumaric acid, varying from 2.5 ng/µl to 50 ng/µl, were injected twice a day. From every calibration curve the least squares line ($y=a+b*x$), standard error of a (s_a) and b (s_b), the correlation coefficient (r), the significance of the regression coefficient ($\alpha=0.05$) (6) and analysis of variance were performed. The results are presented in Table 1. A linear relationship between the absorbance and the injected concentration of p-coumaric acid was obtained, however, the intercept in some cases significantly differs from the zero point (0,0). The correlation coefficient indicates only a linear positive tendency, but is nevertheless often used to prove the linearity (7).

Analysis of variance (ANOVA) which implies the lack of fit was applied to examine the linearity (8). In the ANOVA, the total sum of squares can be broken up in the sum of squares due to the regression and the sum of squares about the regression or the residual sum of squares. The latter can be broken up in the pure error sum of squares (SS_{PE}) and the lack of fit sum of squares (SS_{LOF}). The SS_{PE} is due to the

TABLE 1

Evaluation of the Linearity

curve	a s_a	b s_b	r	regr. coeff. S/NS	F p
1	13.903	147.630	0.99996	S	2.345
	9.531	0.356			0.190
2	33.805	147.882	0.99997	S	6.680
	11.519	0.377			0.034
3	44.542	146.667	0.99992	S	0.478
	17.624	0.646			0.711
4	61.540	145.868	0.99988	S	20.53
	21.208	0.789			0.003
5	16.000	146.099	0.99989	S	1.136
	20.179	0.758			0.419
6	49.776	143.509	0.99978	S	1.240
	11.060	0.401			0.387

variability within each group of replicate measurements, the SS_{LOF} is due to the variability of group averages about the regression line. The ANOVA of the first curve is given in Table 2, the probability level (p) is the probability that there is no lack of fit.

Sensitivity and limit of detection

The sensitivity (S) is defined as the slope of the calibration curve (9). The range over which the sensitivity can be considered to be constant has lower and upper limits. The lower limit will be the detection limit. For the upper limit no generally accepted definition could be found.

S=146.480±0.401 mAU/ng

TABLE 2

Analysis of variance of Curve 1

Source	SS	Df	Mean Square	F	p
Model	64613347	1	6461335		
Residual	3009.149	8	376.144		
LOF	1759.100	3	586.367	2.345	0.190
PE	1250.049	5	250.010		
Total	64616356	9			

The Kaisers detection limit was calculated (7). If a field blank is unobtainable the lowest detectable instrument signal, y_L, is given by: $y_L=a + k^*s_{y/x}$, in which a is the intercept, $s_{y/x}$ is the standard error of the estimate. Kaiser suggested k=3. The corresponding concentration is: $c_L=k^*s_{y/x}/b$, in which b is the slope.

y_L=199.418 mAU

c_L=1.15 ng/µl

Precision

Five different samples of Echinacea purpurea press juice were injected and the amount of p-coumaric acid was determined. This was repeated on 3 different days to examine the intermediate precision (10) in addition to the repeatability (11). The repeatability expresses the precision of a method under the same operating conditions or a short period of time (within-day). The repeatability is expressed as the

TABLE 3

Repeatability - Intermediate Precision in Echinacea Press Juice and Urgenin®

	Sample	
Parameter	Echinacea press juice	Urgenin
Repeatability		
s	0.61	0.13
s^2	0.38	0.02
RSD	3.92%	2.06%
Intermediate precision		
s	0.99	0.16
s^2	0.99	0.03
RSD	6.38%	2.60%
ANOVA		
F	1.647	0.683
F(critical)	3.885	3.982
p-value	0.233	0.525

TABLE 4

Comparison of the Slope of the Calibration Curve with the Slope of the Standard Addition Curve (α=0.05)

Sample	Echinacea press juice		Urgenin	
Experiment	1	2	1	2
t	2.047	1.0	-0.845	0.146
t_{tab} (d.f.=10)	2.228	2.228	2.228	2.228
S/NS	NS	NS	NS	NS

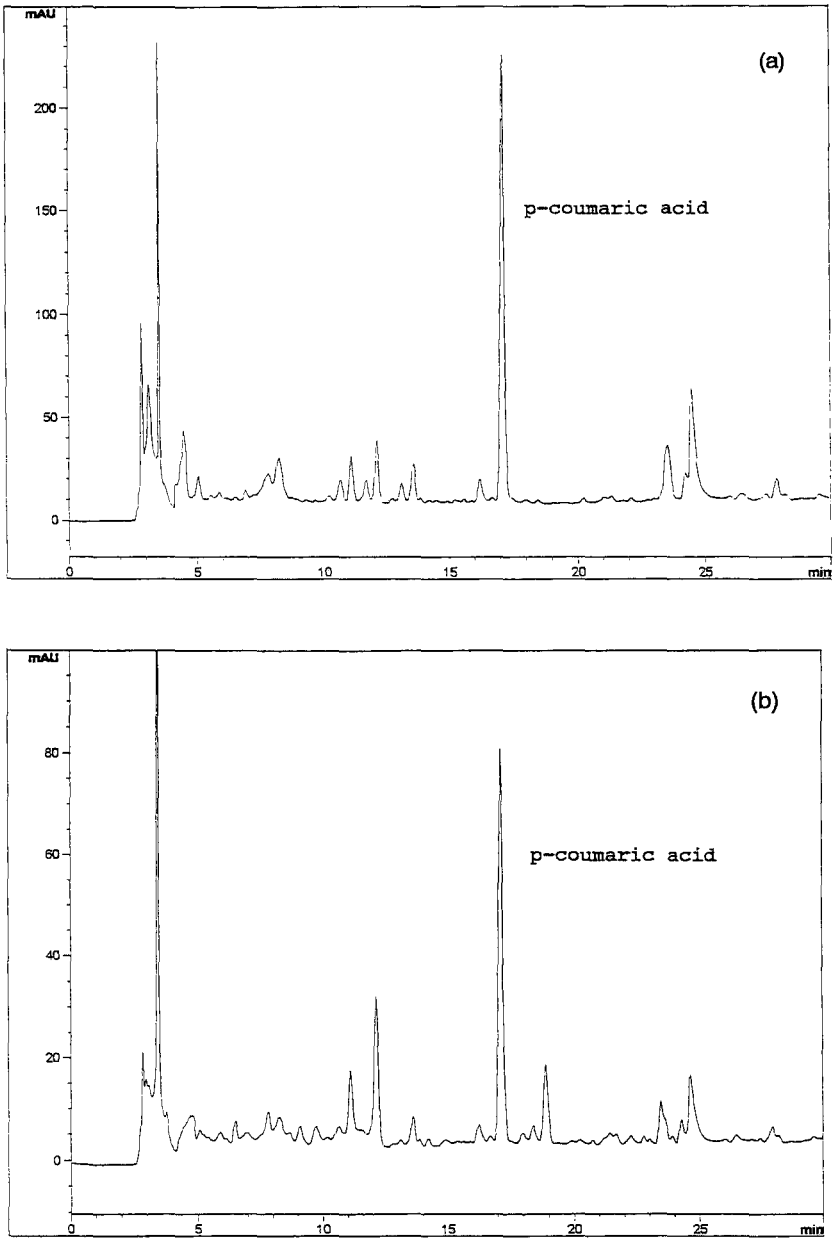

FIGURE 1: typical chromatogram of Echinacea press juice (a) and Urgenin® (b)

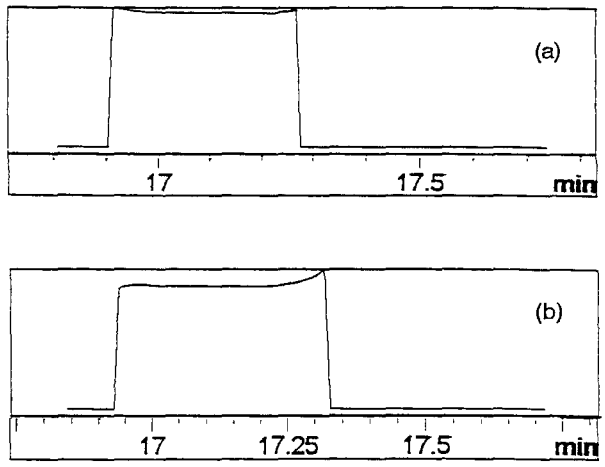

FIGURE 2: shows the absorbance ratio (280nm/330nm) in Echinacea press juice (a) and Urgenin® (b).

repeatability standard deviation (s), the repeatability variation (s^2) and the residual standard deviation (RSD). The intermediate precision i.e. within laboratory variations, different days, different analysts, etc. is expressed as s, s^2, RSD. To investigate whether or not there is a significant difference between the results of the different days a single-factor ANOVA was performed. The results are summarized in Table 3. They indicate that no significant differences between the amounts of p-coumaric acid are found in the samples on different days.

Accuracy

The accuracy can be examined by comparison with another method or by the standard addition method when dealing with plant preparations. Therefore, we preferred to determine the accuracy by the standard

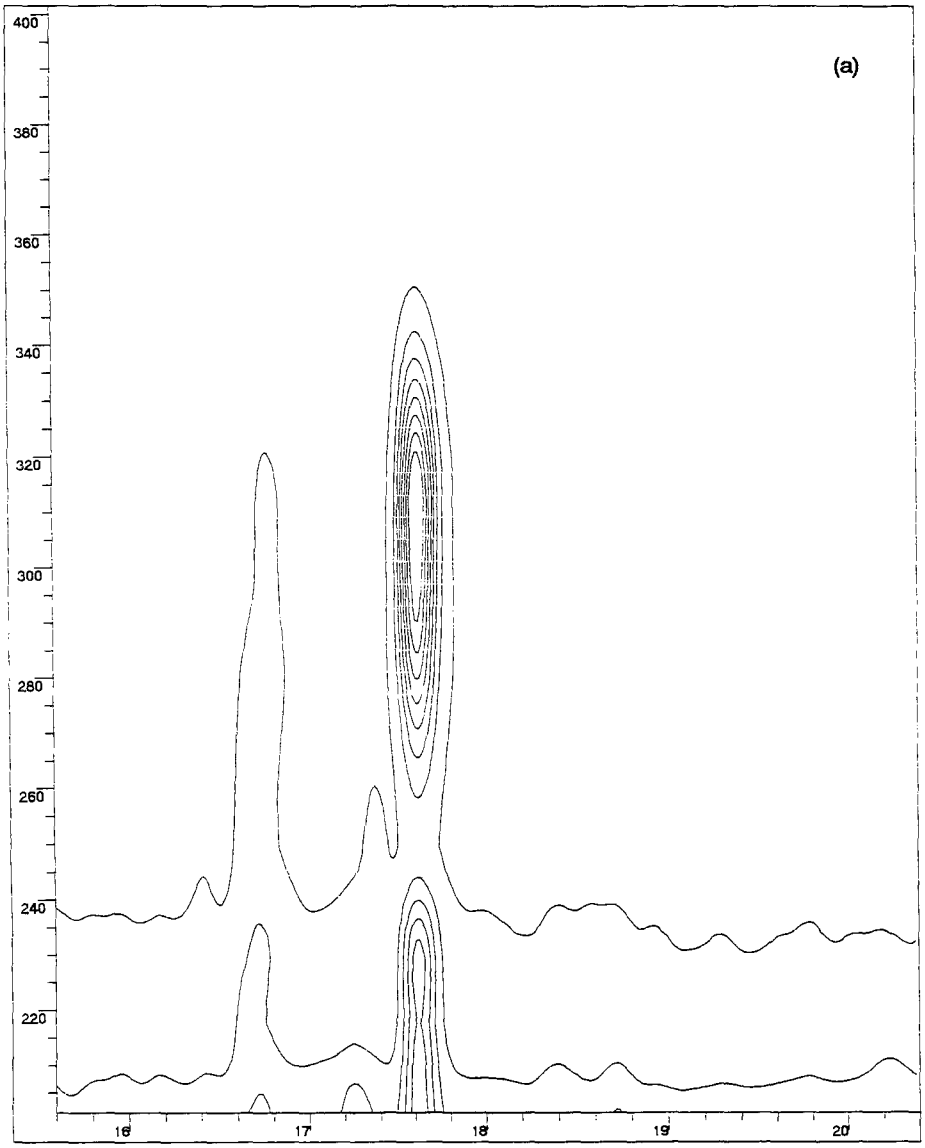

FIGURE 3: shows a contour plot of p-coumaric acid in Echinacea press juice (a) and Urgenin® (b).

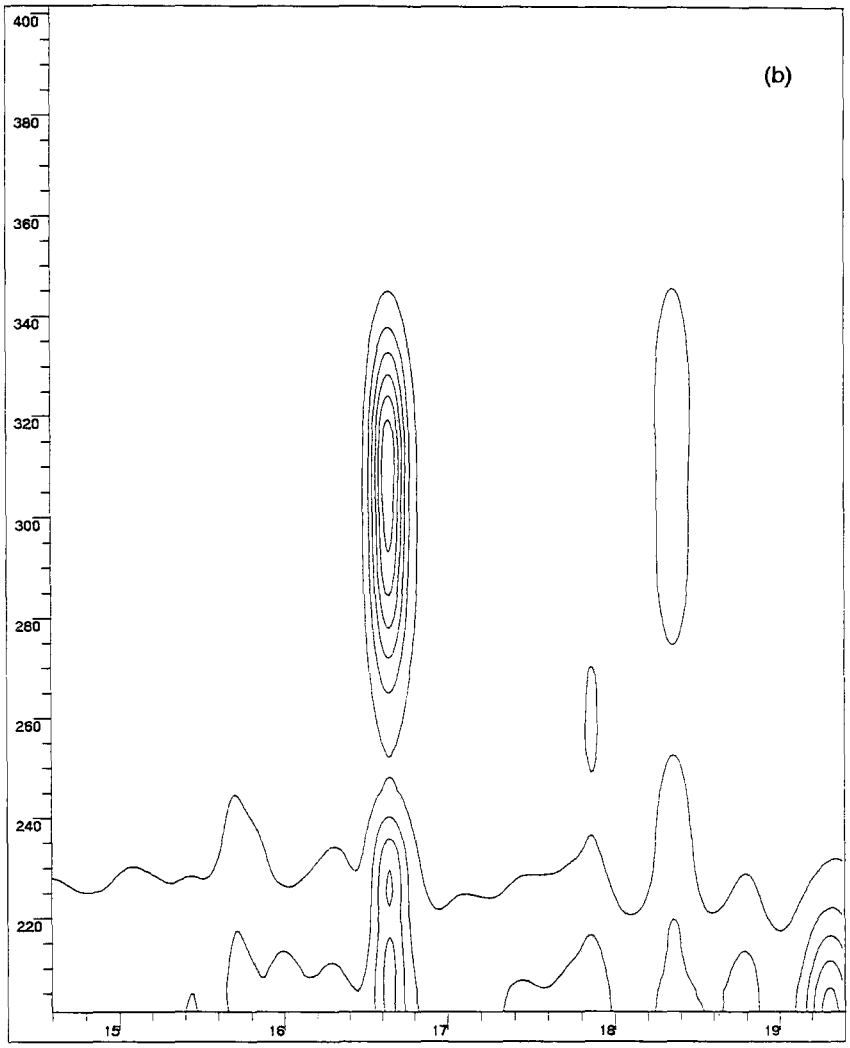

FIGURE 3 (continued)

addition method. Thus, known amounts of p-coumaric acid were added to the sample at 4 different concentration levels (2.5, 5, 15 and 20 ng/µl were added to the sample). The experiment was done twice. We calculated the recovery and compared the slope of the standard addition curve with the slope of the calibration curve (12). The recovery varied in all cases between 96.6% and 101.5%. The results of the comparison of the two curves are given in Table 4.

Selectivity

An analytical determination of a compound is defined as selective, when, with a certain probability and accuracy, the determined compound can be distinguished from related substances, impurities, etc (11). An important tool in the evaluation of the selectivity is the peak purity. We have chosen two visual methods, viz. the absorbance ratios and the contour plot. Although these methods seem to be rather rough, we think that major co-eluting peak(s) can be detected when their UV spectrum is different from that of the major component.

Typical chromatograms of the Echinacea press juice (a) and Urgenin® (b) are given in Figure 1.

The absorbance ratio technique is based on plotting the ratio of absorbances at two wavelengths over the elution profile. The absorbance ratio of a pure compound is constant. Figure 2 shows the absorbance ratio (280nm/330nm) of p-coumaric acid in Echinacea press juice (a) and Urgenin® (b), and indicates the absence of a major co-eluting impurity.

In a contour plot the data are presented as concentric isoabsorptive lines in the (Absorption,time) plane, so that all the data can be observed simultanuously. Major co-eluting peaks, with different UV-spectrum will disturb the normally symmetrical concentric lines of a peak. Figure 3

TABLE 5

Determination of p-Coumaric Acid in Echinacea Press Juice and Urgenin®

Batch Echinacea purp. press juice	Mean amount of p-coumaric acid ng/µl (s)	Batch Urgenin	Mean amount of p-coumaric acid ng/µl (s)
Ia	15.92 (0.25)	Ib	6.07 (0.12)
IIa	16.74 (0.08)	IIb	5.77 (0.14)
IIIa	23.34 (0.68)	IIIb	8.44 (0.19)

shows a contour plot of p-coumaric acid in Echinacea press juice (a) and in Urgenin® (b). In Figure 3, no major impurity is observed.

Analytical Results

The results of the determination are given in Table 5. The amount of p-coumaric acid found in Urgenin® ranges from 90 to 110% of the nominal amount found in the Echinacea press juice, which are the currently accepted limits of the registration authorities for plant drugs in Belgium.

CONCLUSION

As for synthetic pharmaceuticals, medicines of plant origin should be carefully analysed so that standardisation of these drugs can be done. One should, however, take into account that small variations in the

extract composition are inevitable and, therefore, the used analytical methods should be thoroughly validated prior to use on a large scale.

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LIQUID CHROMATOGRAPHIC ASSAY FOR DEXTROMORAMIDE IN HUMAN PLASMA

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ABSTRACT

Dextromoramide is a narcotic analgesic drug which has been said to be clinically useful where rapid onset and short duration of action is required. The present communication describes a modification of previous high performance liquid chromatographic methods for determining plasma dextromoramide concentrations. The method described is sensitive, accurate and precise, with intra-assay CV's of 4.1%, 4.1% and 4.2% and between-assay CV's of 2.9%, 2.4% and 3.9% at concentrations of 10, 100 and 1000 μg/L, respectively. It has a limit of quantitation of 5 μg/L with a chromatographic run time of 8 min. Pharmacokinetic studies in 2 patients given 5mg of dextromoramide intravenously are presented as applications of this method. These studies showed a bi-exponential decay of dextromoramide in plasma over 24h with terminal half-lives of 3.7 and 23.5h which resulted from variability in plasma dextromoramide clearance and distribution volumes.

INTRODUCTION

Dextromoramide tartrate [Palfium®, (+)-1-(3-methyl-4-(4-morpholinyl)-1-oxo-2,2-diphenylbutyl)pyrrolidine] is an older narcotic

analgesic drug [1] described as having a potency equal to or greater than morphine [1,2]. It has a rapid onset of action with a duration similar to morphine [2]. It is eliminated primarily by hepatic metabolism to 2'-hydroxy-dextromoramide metabolite [3,4]. Tolerance and addiction properties have also been described [5]. Hence the clinical application of this drug in this institution has typically been restricted to short-term usage, such as in "break-through" pain in cancer patients treated with morphine, in post-operative pain, etc.

Previous methods for the determination of dextromoramide in plasma have been primarily directed at either the drug-abuse situation [6], or forensic testing [7, 8] or pharmaceutical product testing [FH Faulding & Co Ltd, personal communication], limited clinical pharmacology testing [7], or animal doping analysis [9]. Hence, these methods were not considered completely appropriate for pharmacokinetic studies where greater analytical sensitivity, as well as appropriate precision and accuracy, may be required.

This communication reports a refinement of the previous methods to improve analytical sensitivity, as well as reliability for kinetic studies. By way of example, an application of this method is presented in 2 patient studies where 5 mg of dextromoramide was administered intravenously and blood sampled over the ensuing 24 h.

MATERIALS & METHODS

Stock Solutions

A 1.0 g/L dextromoramide (base concentration) stock solution was prepared by dissolving dextromoramide tartrate (F.H. Faulding & Co Ltd, Salisbury, South Australia) in glass distilled water. This solution was serially diluted to give concentrations of 100, 10 and 1.0 mg/L.

Desipramine HCl (internal standard, Geigy Pharmaceuticals, Lane Cove, NSW, Australia) was prepared in a similar manner to give 1.0 mg/L. The sodium chloride, sodium hydroxide and hydrochloric acid were Univar grade (Ajax Chemicals, Auburn, New South Wales, Australia), and orthophosphoric acid was Analar grade (BDH, Kilsyth, Victoria, Australia).

Plasma Extraction

Each assay was calibrated by adding appropriate volumes of the dextromoramide stock solutions to a series of 1.0 ml dextromoramide-free heparinized plasma aliquots to give final concentrations of 0, 100, 250, 500, 1000, 2000 µg/L. These 1.0 ml calibration standards, as well as quality control samples (described below) and patient specimens were spiked with; 200 µl of 1.0 mg/L of the internal standard, 200 µl of sodium hydroxide (1.0 mol/L) and, following a brief vortex mixing, 8 ml of extracting solvent, 95:5 mixture of cyclohexane (BDH, Hypersolv grade) and iso amyl-alcohol (BDH, Analar grade) in 15 ml disposable borosilicate glass screw-capped tubes. This mixture was shaken by vortex mixing for 1 min, followed by centrifugation at 1000 xg for 10 min. The organic layer was transferred to a 15 ml conical borosilicate glass tube containing 150 µl of 0.05 mol/L HCl. The mixture was again vortex mixed for 60 sec and centrifuged at 1000 xg for 10 min. The aqueous phase was transferred to an autosampler tube and 50 µl injected for chromatographic separation.

Quality control was assessed in each run by analysing aliquots from 3 separate plasma pools which had been previously spiked from a separate dextromoramide stock solution to give concentrations of 10, 100 and 1000 µg/L. Patient blood samples were drawn into lithium heparin blood collection tubes as described below and the plasma fraction separated by centrifugation at 1000 xg for 10 min. Specimens were stored at -20°C prior to analysis.

Chromatography

The mobile phase consisted of a 40:60 (v:v) mixture of acetonitrile (BDH Hypersolv, Far UV grade) and NaCl (0.1 g/L) with H_3PO_4 (0.114 ml/L) (pH=2.9) in glass distilled water. This mixture was filtered (0.2 μm, Millipore, part number GVWP-04700) under vacuum before use, degassed continuously with helium and pumped (model P4000, Spectra Physics Analytical, San Jose, California, USA) at 2.0 ml/min via an autosampler (Spectra Physics, model AS3000) through a 10 μm phenyl column (30 cm x 3.9 mm, part WAT27198, Millipore/Waters, Milford, MA, USA) maintained at 50°C. Compounds separated by this system were quantified by UV detection (Spectra Physics, model UV2000) at a wavelength of 215 nm and range of 0.05 AUFS. The output to a dual-pen chart recorder was plotted at both 10 and 100 mV at a chart speed of 0.25 cm/min. The retention time of the internal standard and dextromoramide peaks were 5.25 and 7.5 min, respectively, with baseline separation. Samples were quantified using the peak height ratio of dextromoramide to internal standard.

Statistical Considerations

The method was validated within a single run by assaying 6 replicates at 10, 100 and 1000 μg/L. Between-run performance was assessed by considering the run-to-run (n=5) reproducibility of these 3 controls, ie., at 10, 100 and 1000 μg/L. The performance of each assay run was controlled by reviewing both the coefficient of variation (CV%) of the concentration-corrected peak height ratio for each calibration standard (ie., the apparent slope of the calibration curve indicated by each calibration standard), as well as by the concentration derived from the calibration curve for each of the 3 quality control samples. Runs were accepted when the CV% of the concentration-corrected calibration standard values was <10% and each of the controls within 10% of their respective target concentrations.

The limit of quantitation was determined by guaging that dextromoramide concentration which could be measured with a CV% of <15%. For samples at this very low concentration the back extraction into HCl was amended to 100 µl and range on the detector attenuated appropriately.

Patient Studies

Ethics Committee support was obtained to invite patients, admitted for various medical conditions, who required narcotic analgesia (other than dextromoramide) as part of their therapy. Inclusion criteria were; age >18 and <80 years, renal or hepatic function less than 50% above upper limit of normal, no known dextromoramide allergy, ability to understand and complete standard consent criteria, adequate venous access, no evidence of anaemia, and where survival was likely to exceed one week . On the day of the study, other analgesic medication was with-held if ethically acceptable and, following the insertion of an indwelling venous catheter, the 5 mg dose of dextromoramide was administered over 5 min into an arm vein. Blood samples were drawn into lithium heparin blood collection tubes, pre-dose, and at 5, 10, 15, 20, 30, 45 min and 1, 1.5, 2, 3, 4, 6, 8 and 24 h post-dose. Samples were then held on ice for up to 30 min before separating the plasma fraction by centrifugation at 1000 xg for 10 min. This plasma was stored at -20°C until assayed.

RESULTS & DISCUSSION

Table 1 shows the accuracy and precision of the method described and suggests that acceptable data were observed both within and between analytical runs for pharmacokinetic purposes. Within-run CV%'s ranged from 4.1 to 4.2%, and between-run ranged from 2.4 to 3.9%. Figure 1 shows a range of chromatograms, including a dextromoramide-spiked calibration standard extract, a

TABLE 1.

Precision and Accuracy Data of the Method Described in the Text.

Dextromoramide concentration	n	Measured concentration (mean ± SD)	Accuracy (%)	C.V. (%)
With-in run:				
10 µg/L	6	10.33 ± 0.42	+3.3	4.1
100 µg/L	6	106.6 ± 4.46	+6.6	4.2
1000 µg/L	6	1063 ± 44.7	+6.3	4.2
Between-run:				
10 µg/L	5	10.4 ± 0.29	+3.6	2.9
100 µg/L	5	102.1 ± 2.42	+2.1	2.4
1000 µg/L	5	979.8 ± 38.0	-2.1	3.9

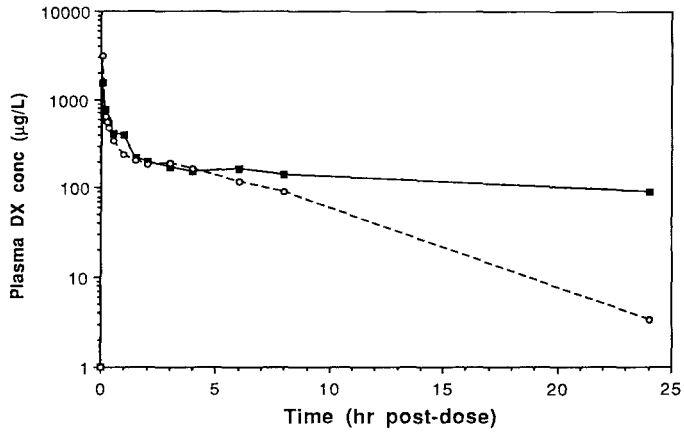

FIGURE 1. This shows chromatograms generated using the method described. Peaks A and B are desipramine (internal standard) and dextromoramide, respectively. The panels represented are; (a) a dextromoramide-spiked calibration standard, (b) dextromoramide-free plasma extract, (c) an extract from a dextromoramide-treated patient, and (d) a dextromoramide QC sample.

dextromoramide-free extract, a patient dextromoramide plasma extract, and a quality control sample plasma extract. The peak retention times were 5.25 and 7.5 min for the internal standard and dextromoramide, respectively, with a chromatographic run-time of approximately 8 min. In pilot studies from a range of other patients taking dextromoramide, as well as the present study patients, no chromatographic interferences have been noted from other concurrent medications (including; amiodarone, dexamethosone, digoxin, enalapril, flunitrazepam, haloperidol, indomethicin, lactulose, metoclopramide, metoprolol, morphine, nifedipine, paracetamol, propantheline bromide, prednisolone, omeprazole, oxycodone, ranitidine, simvastatin, warfarin). The advantage of testing the method using patient specimens was that it considers clinically relevant concentrations as well as potential interfering metabolites of these drugs.

The limit of quantitation for the method described was found to be 5μg/L with a CV of 14%, which represented adequate sensitivity for the proposed kinetic study. One could envisage extending this limit further, if required, by extracting a larger plasma sample volume (eg., 2 ml) and injecting more of the extract for HPLC separation, thereby potentially achieving a limit of around 1-2 μg/L.

Figure 2 presents the plasma dextromoramide concentrations observed in the 2 patient studies. These data, plotted on a semi-logarithmic scale, suggest a bimodal plasma dextromoramide profile following intravenous administration. The initial α-half-lives of 0.26 and 0.32 h were similar in these 2 patients; however, the terminal β-phases had apparent differences in slope, suggesting half-lives of 3.7 and 23.5 h, respectively. This latter difference appeared to result from variability in plasma clearances of 2.2 and 0.77 L/h and apparent distribution volumes of 11.8 and 26.1 L. These preliminary data, which will be pursued in further clinical studies, are consistent with the apparent variability in the trough dextromoramide concentration data of Rop and coworkers [7] who recorded undetectable whole blood

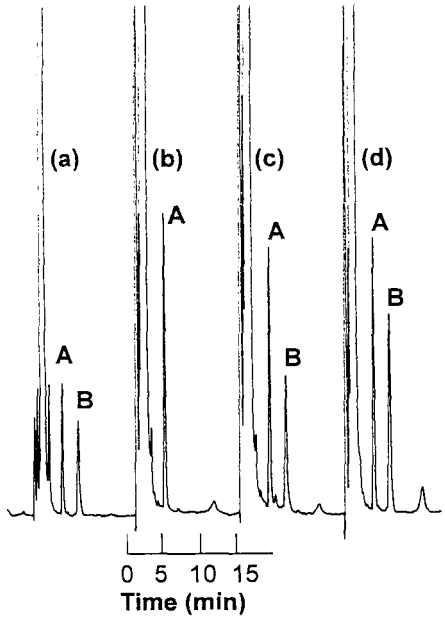

FIGURE 2. This shows the bi-phasic log-linear plasma dextromoramide concentration (μg/L) versus time profile for 2 patients given 5 mg of dextromoramide intravenously over 5 min.

concentrations of dextromoramide in one of 3 cancer patients on chronic intrvenous dextromoramide therapy and could potentially relate to a genetically determined biomodal distribution in metabolism. However, this latter issue remains speculative at this stage but will be persued further in subsequent clinical studies.

In summary, the analytical method presented provides a sensitive and reliable HPLC-UV method which has improved upon the previously described methods for the determination of dextromoramide in human plasma specimens.

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ANALYSIS OF ERYTHROMYCIN A AND ITS METABOLITES IN BIOLOGICAL SAMPLES BY LIQUID CHROMATOGRAPHY WITH POST-COLUMN ION-PAIR EXTRACTION

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ABSTRACT

The development of a selective and sensitive method for analysis of erythromycin A (EA) and its metabolites in biological fluids (urine and plasma) is reported here. A mobile phase consisting of acetonitrile - 2-methyl-2-propanol - 0.2 M phosphate buffer pH 9.0 - water (3:19:5:73, v/v) at 1.5 ml/min enables the separation of the main component (EA) from all of its potential metabolites on a 250 x 4.6 mm I.D. poly(styrene-divinylbenzene) (PLRP-S 8 μm, 1000 Å) column at 70°C. To improve the sensitivity of the method, a post-column ion-pair extraction with the strongly fluorescent 9, 10-dimethoxy-anthracene-2-sulphonate was used. The ion-pairs were extracted in chloroform for on-line fluorescence detection. Analytical recoveries for erythromycin and its metabolites after extraction of the plasma with tertiary butyl methyl ether were better than 90 %. The calibration curves of EA and its potential metabolites in plasma and urine were linear over the concentration range studied. The detection limits were 12.5 ng/ml plasma and 50 ng/ml urine. The method allows the detection of all the erythromycin metabolites in human plasma and urine.

INTRODUCTION

The major problem in the determination of the macrolide antibiotic erythromycin in biological fluids using liquid chromatography (LC), is the detection of this substance at the low concentration levels present in plasma and urine. Erythromycin is UV transparent at wavelengths above 220 nm, except for a weak chromophore at 280 nm. Below 220 nm, where the molar absorptivity is comparatively higher, additional problems occur, such as interference of species co-extracted from biological samples. Tserng and Wagner [1] determined erythromycin and erythromycin propionate in plasma and whole blood using fluorimetry after addition of the fluorescent ion-pair reagent 2-(stilbyl-4″)-(naphtho-1′, 2′ : 4,5) - 1,2,3 triazole- 2″, 6′ disulfonic acid, sodium salt and extraction. Tsuji [2] used the same detection reagent but in a LC method using a reversed-phase silica-based column (RP-18) and post-column extraction, with considerable loss of efficiency. After administration of erythromycin ethyl-succinate Tsuji was able to detect anhydroerythromycin ethyl succinate, erythromycin A enol ether ethyl succinate and erythralosamine in serum samples. The limit of detection was less than 0.01 μg/ml. Stubbs et al [3] used UV detection at 200 nm and obtained a limit of detection of 0.25 μg/ml and 1 μg/ml for erythromycin in serum and urine, respectively. Detection of metabolites was not reported. Most published LC methods employed electrochemical detection. Chen and Chiou [4] obtained a detection limit for erythromycin A of 5-10 ng/ml in plasma . Anhydroerythromycin A was also detected, but presence of N-demethylerythromycin A in plasma samples was not reported. According to work by Duthu [5], N-demethylerythromycin A was undetectable by the dual coulometric electrode detector used. Nilson et al. [6] reported a method using a polymeric stationary phase and amperometric detection. Only the main compound was detected at plasma concentrations down to 0.2 μmol/l. Stubbs et al. [7] used amperometric detection to detect anhydroerythromycin A, the main degradation product formed during the storage of biological fluids, with a sensitivity limit of 0.1 μg/ml. Recently Kato et al. [8] developed a method for analysis of erythromycin in plasma and whole blood using a reserved-phase Asahipak C-18 polymer-based column with an alkaline mobile phase. The detection limit was 0.1 μg/ml for erythromycin A. So far, no paper has reported the detection of N-

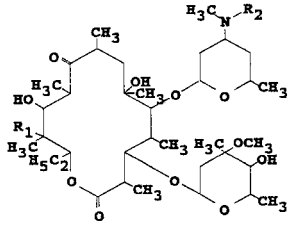
demethylerythromycin in plasma by LC. However, it has been shown that N-demethylerythyromycin A is an important metabolite of erythromycin, formed by demethylation in the liver [9]. Attempts to do quantitative TLC of erythromycin metabolites have been described elsewhere [10].

Because of the availability of low dead volume connections and a sandwich-type phase separator [11], causing negligible additional band broadening, it is now possible to develop a selective and sensitive method for analysis of erythromycin A and its metabolites in biological fluids. As described by Tsuji [3], a post-column ion-pair extraction system coupled to a fluorescence detector was used in the method proposed here. In order to separate erythromycin A from its metabolites, the previously developed method for analysis of erythromycin A and its related substances [12] on wide-pore poly(styrene-divinylbenzene) was further investigated.

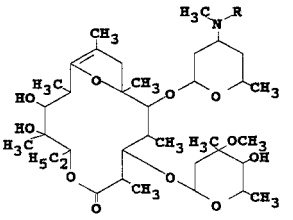
EXPERIMENTAL

Samples and Sample Solutions

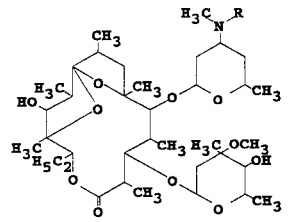
An erythromycin A house standard (EA-HS, 94.7 % pure) was obtained by crystallization of a commercial sample [13]. Reference substances for N-demethylerythromycin A (dMeEA) [14], anhydroerythromycin A (AEA) [15] and erythromycin A enol ether (EAEN) [16] were prepared from EA following described methods. Anhydro-N-demethylerythromycin A (AdMeEA) [13] and N-demethylerythromycin A enol ether (dMeEAEN) [17] were prepared from dMeEA according to the methods used to prepare AEA and EAEN. A commercial sample of josamycin (UCB, Brussels, Belgium) was used as internal standard (IS). The structures of erythromycin A, its metabolites and of josamycin are shown in Figure 1. Sample solutions in methanol-water (1:1) were prepared at a concentration of 1 mg/ml, except for EAEN and dMeEAEN where 0.1 mg/ml solutions were used. These sample solutions were injected during the preliminary LC development work using the UV detector. More dilute solutions (10 μg/ml) were injected when fluorescence detection was used.



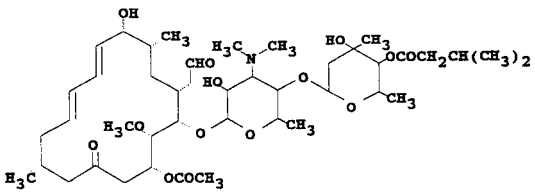
	R_1	R_2
Erythromycin A (EA)	OH	CH_3
Erythromycin B (EB)	H	CH_3
N-demethylery-thromycin A (dMeEA)	OH	H



	R
Erythromycin A enol ether (EAEN)	CH_3
N-demethylerythromycin A enol ether (dMeEAEN)	H



	R
Anhydroerythromycin A (AEA)	CH_3
Anhydro N-demethylerythromycin A (AdMeEA)	H



Josamycin

FIGURE 1 : Chemical structures of erythromycin , its metabolites and josamycin.

Solvents and Reagents

2-Methyl-2-propanol 99.5% and dichloromethane were purchased from Janssen Chimica (Beerse, Belgium) and distilled before use. 2-Methoxy-2-methylpropane (Janssen Chimica) was used without purification. Acetonitrile, LC grade S and methanol LC grade, were from Rathburn (Walkerburn, UK). Chloroform was distilled before use (Belgolabo,Overijse, Belgium). Water was distilled twice in glass apparatus. Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, citric acid monohydrate, potassium carbonate and disodium edetate (EDTA) were of pro analysi quality from Janssen Chimica. Sodium 9,10-dimethoxyanthracene-2-sulphonate 97% (DAS), sodium naphthalenesulphonate and bromocresol purple were purchased from Aldrich (Bornem, Belgium). Ammonium 8-anilino-1-naphthalenesulphonate and methyl orange were obtained from Merck (Darmstadt, Germany).

Equipment

Figure 2 shows a schematic diagram of the assembly of the analytical equipment used for on-line ion-pair extraction, phase separation and fluorescence detection.

The mobile phase was delivered by a Merck-Hitachi, L-6200 intelligent pump. For injection of the samples a Valco injector model CV-6-UHPa-N60 (Houston, TX, USA) equipped with a fixed loop of 100 µl was applied. The stationary phase, PLRP-S 8µm, 1000 Å (Polymer Labs, Church Stretton, Shropshire, UK) was packed in a 250 mm x 4.6 mm I.D. stainless steel column and kept at 70°C using a water bath. The detector used in the development of the mobile phase was a Waters Model 420 UV detector (Milford, MA, USA) set at 215 nm. In the system with ion-pair extraction finally used, a Merck-Hitachi F-1050 fluorescence spectrofluorometer with variable excitation and emission wavelengths setting was used. Chromatograms were recorded on a Hewlett-Packard HP 3396A integrator (Avondale, PA, USA). For delivery of the reagent solution, a Milton Roy minipump (Laboratory Data Control, Riviera Beach, FL, USA) was used, equipped with a pulse dampener and a Bourdon manometer as described previously [18]. A 250 x 4.6 mm stainless steel column packed with glass beads was used as an

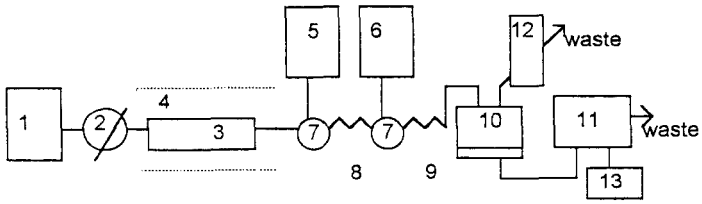


FIGURE 2 : Scheme of the assembly of the equipment used for on-line ion-pair extraction and fluorescence detection. 1: eluent pump, 2: injector, 3: column, 4: heating device, 5: reagent pump, 6: extraction solvent pump, 7: T-piece, 8: reaction coil, 9: extraction coil, 10: phase separator, 11: fluorescence detector, 12: back pressure regulator, 13: integrator.

additional pulse dampener. A second Merck Hitachi, L-6200 intelligent pump delivered the organic extraction solvent. Mixing and extraction coils were of stainless steel capillary coiled to a helix with a diameter of 45 mm. A sandwich-type phase separator (Vrije Universiteit, Amsterdam, The Netherlands) with a 40 µl groove volume [11] was connected to a SSI back pressure regulator (State College, PA, U.S.A) for adjustment of the flow of organic solvent through the fluorescence detector.

Urine and Plasma Samples

For urine, no extraction procedure was needed. Urine was centrifuged at 2500 *g* for 5 min and an aliquot of the supernatant (100 µl) was injected directly on the system. For spiking experiments with plasma, rabbit blood (1.0 ml) was collected in a tube containing 0.5 ml of 10 % m/v EDTA solution and centrifuged to separate the plasma. Plasma (1.0 ml) was spiked with EA and metabolites. 250 µl of internal standard solution (IS) (0.004 mg of josamycin/ml methanol-water 1:1) and 60 µl of saturated solution of K_2CO_3 were added. Extraction was done with 5 ml of 2-methoxy-2-methylpropane and 4 ml of the ether layer was evaporated under reduced pressure. The residue was reconstituted with 200 µl of methanol-water (1:1) and an aliquot of 100 µl was injected. Real human blood samples (200

µl) were collected, after pricking the index finger, into a tube containing 100 µl of 10 % m/v EDTA solution. The tube was weighed before and after taking blood. IS (50 µl) (0.02 mg/ml methanol-water 1:1) was added and the mixture was centrifuged to separate the plasma. To 200 µl of plasma, 20 µl of saturated K_2CO_3 solution and 1.0 ml of 2-methoxy-2-methylpropane were added. After centrifugation, 0.8 ml of the ether layer was evaporated and the residue was reconstituted with 200 µl of methanol-water (1:1). Here too, a 100 µl aliquot was injected.

RESULTS AND DISCUSSION

Development of Mobile Phase

The previously described LC method [11] for analysis of erythromycin A and related substances on wide-pore poly(styrene-divinylbenzene) (PLRP-S 8µm, 1000 Å) was taken as a starting point. This method used as mobile phase a mixture of 2-methyl-2-propanol - acetonitrile - 0.2 M potassium phosphate buffer pH 9.0 - water (165:30:50:755, v/v) , at a flow rate of 2.0 ml/min. This mobile phase was investigated for its selectivity towards EA and its potential metabolites (dMeEA, AEA, EAEN, AdMeEA and dMeEAEN). The pH and the amount of 2-methyl-2-propanol (t-BuOH) were the parameters most influencing the selectivity. As can be seen in Figure 3, AdMeEA and EA were not separated at a pH < 8.0. Good selectivity was obtained at higher pH. Finally pH 9.0 was adapted. As we wanted EAEN to be eluted within 40 min, the initial amount of 2-methyl-2-propanol had to be adapted. As shown in Figure 4, the capacity factor of EAEN decreased significantly with increasing the amount of 2-methyl-2-propanol. An amount of 19 % gave good separation and a sufficiently short analysis time. The flow rate was decreased to 1.5 ml/min, in view of the use of an on-line phase separator. Smaller volumes of aqueous phase are easier to separate and the volume of organic phase consequently can be reduced. A typical chromatogram of a mixture of reference compounds of EA and its metabolites recorded at 215 nm, is shown in Figure 5.

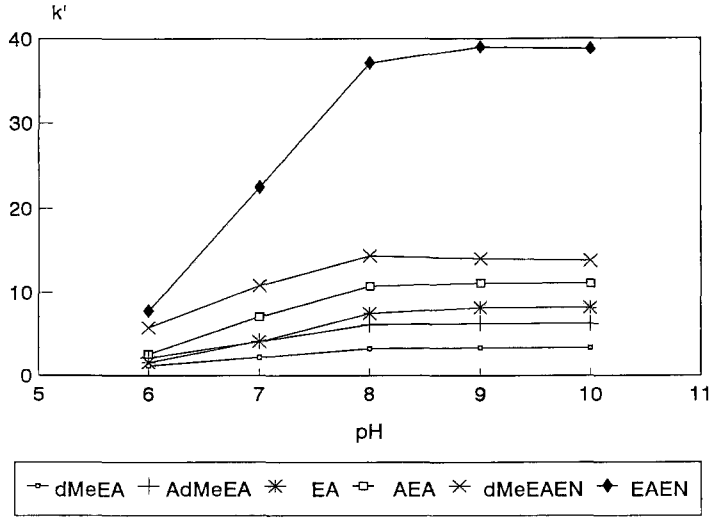


FIGURE 3 : Effect of the pH of mobile phase on the mass distribution ratios (k') of erythromycin A and its metabolites.

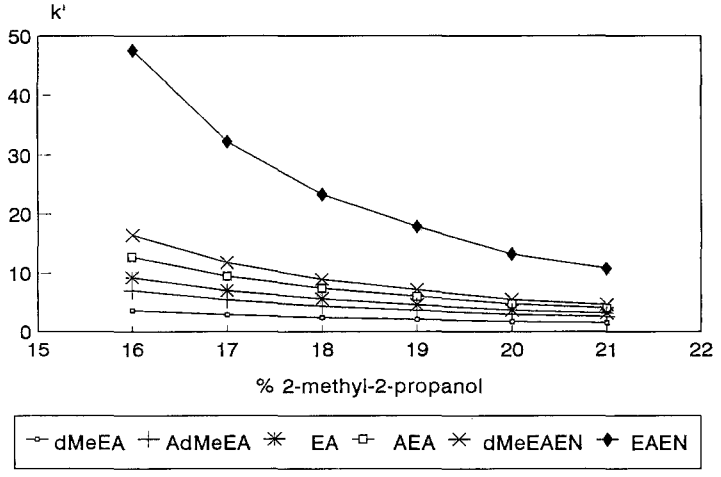


FIGURE 4 : Influence of the concentration of 2-methyl-2-propanol (t-BuOH) on the mass distribution ratios (k') of erythromycin A and its metabolites.

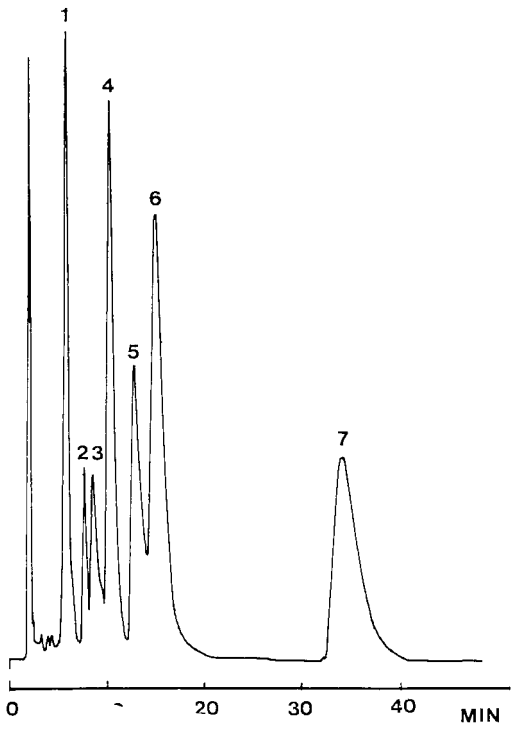


FIGURE 5 : Typical chromatogram of a mixture of reference compounds of erythromycin A and its metabolites. Column: PLRP-S (8 µm 1000Å) at 70 °C. Mobile phase: acetonitrile - 2-methyl-2-propanol - 0.2 M phosphate buffer pH 9.0 - water (3:19:5:73, v/v) at 1.5 ml/min. Detection: UV 215 nm. 1. dMeEA, 2. Impurity of 6, 3. AdMeEA, 4. EA, 5. AEA, 6. dMeEAEN, 7. EAEN.

Ion-pairing Reagent and Extraction Solvent

In acidic medium, compounds containing tertiary or secondary amino groups easily form ion-pairs with strongly UV-absorbing or fluorescent counter-ions. By adding a non-miscible organic solvent after the chromatographic separation, these ion-pairs can be extracted and subsequently detected. In our study, a number of reagents described in the literature was evaluated as counter-ion for erythromycin

and their sensitivity was compared. The reagents tested were dissolved in 0.1 M citric acid solution at a concentration of 0.5 x 10^{-5} M. Chloroform was used as extraction solvent. With methyl orange and bromocresol purple [19], the limit of detection was 1 μg, which is not sensitive enough for detecting serum levels of erythromycin. Using sodium naphthalenesulphonate [1], the ion-pair could not be extracted. With two other fluorescent dyes, ammonium 8-anilino-1-naphthalenesulphonate [20] and DAS [21], erythromycin could be detected down to 10 ng and 5 ng respectively. The fluorescent reagent used by Tsuji [2] was not available for evaluation. DAS was chosen as the fluorescent reagent for further work. Maximum signal height for DAS was obtained at excitation and emission wavelengths of 365 nm and 450 nm, respectively. On replacing chloroform by dichloromethane as the extraction solvent, the signal height decreased by 20 %. Hexane was too apolar as extraction solvent. Moreover, organic solvents with high density are preferred because of the ease of separating the layers in the sandwich phase separator.

Influence of Concentration and Flow Rate of DAS Reagent

The derivatization reagent DAS was dissolved in 0.1 M citric acid. The influence on the signal area of the concentration of DAS in the 0.1 M citric acid solution was investigated. The peak area of erythromycin after injection of 1 μg on column was recorded at every condition. Figure 6 shows that on decreasing the DAS concentration from 10^{-4} M to 0.5 x 10^{-5} M, there was an increase in the peak area, which can be explained by the fact that at higher reagent concentration, the blank fluorescence signal was increased, which masks the sensitivity for detection of the ion-pair. The concentration of the citric acid solution had no effect on the signal area in the concentration range studied (0.025 M to 0.2 M). The flow rate of the reagent solution was investigated in the range 0.5 to 0.9 ml/min. 0.7 ml/min was chosen because flow rates lower than this were less sensitive for the detection of ion-pairs and flow rates higher than 0.7 ml/min would only increase the flow rate of extraction solvent required, and hence the total flow rate, which makes the operation of the sandwhich phase separator less efficient.

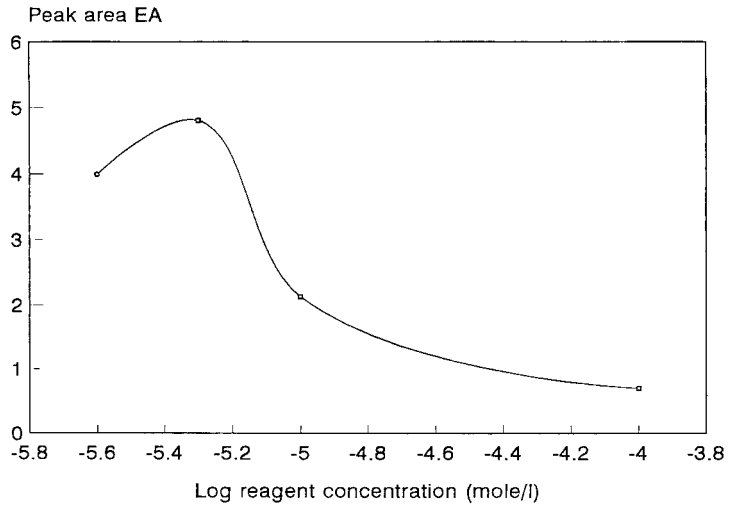


FIGURE 6 : Effect of concentration of fluorescence reagent on peak area (EA).

Influence of Dimensions of Reaction and Extraction Coils

The influence of the internal diameter of the extraction coil on the extraction was evaluated using a reaction coil of 1 m x 0.02 inch. Changing the internal diameter from 0.01 to 0.02 inch had little effect on the resolution, but the peak area increased with a factor 1.7. The repeatability also improved much, because of fewer problems associated with the separation of the organic layer in the phase separator. A further increase in internal diameter (0.02 to 0.03 inch) was not suitable because the resolution decreased and the peak area decreased with a factor 0.9. The length of the reaction coil was investigated for 1 and 1.5 m. No change in signal area was seen. Finally a uniform combination of 1.5 m x 0.02 inch I.D. was chosen for both reaction and extraction coils.

Influence of Total Flow Rate of Extraction Solvent and Flow Rate Through Detector

The effect of different total flow rates of chloroform (0.75, 1.0, 1.25, 1.5, and 1.75 ml/min) on the peak area of EA (amount injected, 1 μg) was recorded. The

flow rate of chloroform through the detector was kept constant at 0.5 ml/min. This resulted in different separation effeciencies (i.e. total flow rate of chloroform/flow rate of chloroform through detector). It was seen (Figure 7) that decreasing the total flow rate of chloroform caused an increase in the peak area due to the concentration effect. A flow rate of 1.5 ml/min was finally chosen because the risk of water leakage towards the detector excluded the use of lower flow rates of chloroform. Variation of the flow rate of chloroform through the detector (0.1, 0.2, 0.3, 0.4, 0.5 and 0.75 ml/min) did not influence the peak area. Resolution and number of theoretical plates were found to be optimal at 0.5 ml/min.

Linearity, Repeatability and Limit of Detection for Urine Samples

Calibration curves were constructed by spiking human urine to yield five different concentrations of 2.0 to 26.0 μg of EA per ml and 1.0 to 12.0 μg of metabolite per ml. Each concentration was injected three times. Calibration curves were linear and yielded the following equations, with y = peak area, x = concentration in μg/ml and r = correlation coefficient. For EA, $y = 6767 x + 358$, $r = 0.9974$; for dMeEA, $y = 5697 x + 164$, $r = 0.9963$; for AdMeEA, $y = 11882 x - 17$, $r = 0.9981$; for AEA, $y = 10582 x - 167$, $r = 0.9991$; for dMeEAEN, $y = 12637 x - 37$, $r = 0.9988$ and for EAEN, $y = 7887 x + 277$, $r = 0.9969$.

The repeatability was tested by injecting six times a urine sample spiked with EA (20 μg/ml, amount injected 2 μg). The relative standard deviation (RSD) value on the peak area of EA was 0.9 %.

The limit of detection was found to be 4 ng on column at a signal-to-noise ratio of 3. Figure 8 shows a chromatogram of blank urine and urine spiked with EA and its metabolites.

Recovery, Linearity and Limit of Detection for Plasma Samples

Rabbit plasma was utilized to investigate the recovery, linearity and sensitivity of the method. The developed method was found to be applicable on human plasma.

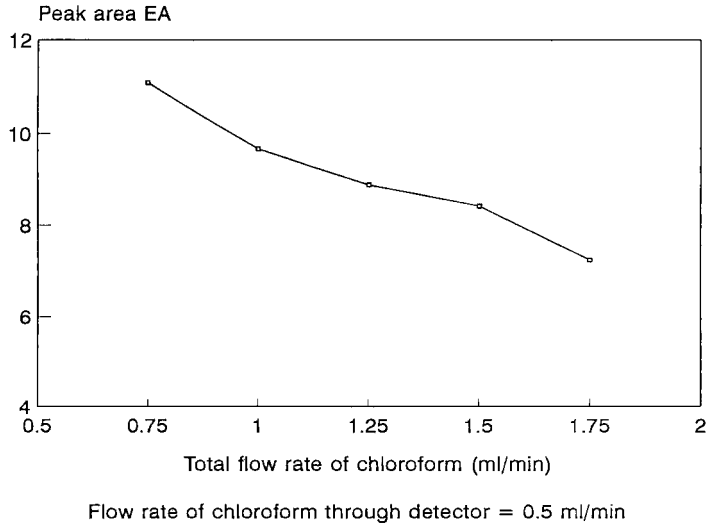


FIGURE 7 : Influence of total flow of extraction solvent on peak area.

Other macrolides such as troleandomycin, midecamycin and josamycin were chromatographed in order to find a suitable internal standard. Troleandomycin and midecamycin could not be used because they were co-eluted with some metabolite of EA. Josamycin was chosen because it was eluted in the area between dMeEAEN and EAEN.

Plasma samples, spiked to a final concentration of 10 μg/ml with EA, dMeEA or AEA were taken through the sample preparation procedure described under experimental. The absolute recovery of EA and its metabolites was better than 90 % and for josamycin (IS) it was about 84 %.

The calibration curves for EA and some of its metabolites in plasma were linear over the range of concentration studied (0.25 to 2.0 μg/ml). The following data were obtained (number of analysis n = 9). For EA, $y = 3\,x + 0.12$, $r = 0.9847$; for dMeEA, $y = 2.5\,x + 0.19$, $r = 0.9872$ and for AEA, $y = 4.7\,x + 0.17$, $r = 0.9818$, where y = ratio peak area/peak area of the IS, x = amount injected in micrograms and r = correlation coefficient.

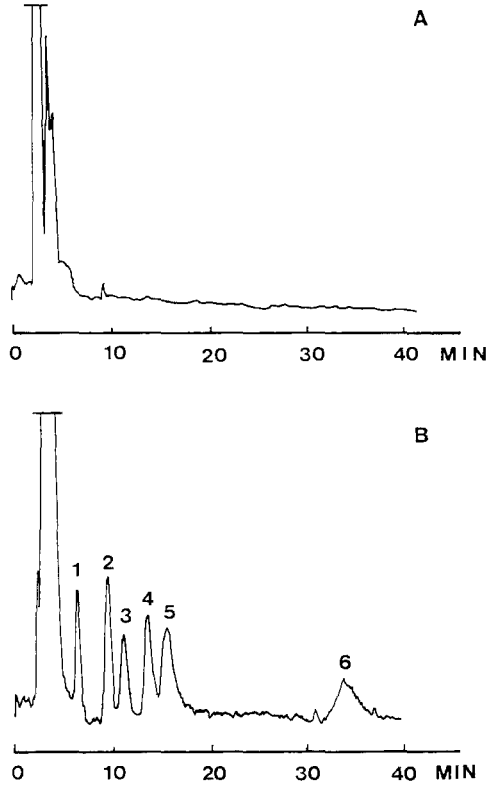


FIGURE 8 : Typical Chromatograms of blank urine (A) and urine spiked with 25 ng each of EA and its metabolites (B). 1. dMeEA, 2. AdMeEA, 3. EA, 4. AEA, 5. dMeEAEN, 6. EAEN.

The limit of detection in plasma was 5 ng on column at a signal-to-noise ratio of 3. Typical chromatograms obtained with blank rabbit plasma and plasma spiked with EA and some of its metabolites are shown in Figure 9.

Application to Real Urine and Plasma Samples

To test the clinical applicability of this method, plasma and urine samples from several healthy male volunteers receiving one oral dose of enrobed microgranules

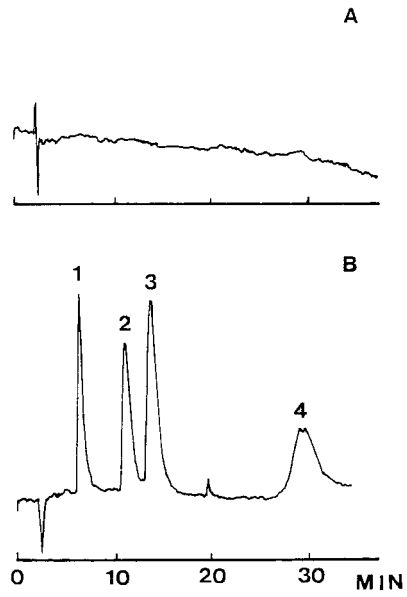


FIGURE 9 : Typical chromatograms obtained with blank rabbit plasma (A) and rabbit plasma spiked with 100 ng each of EA, AEA and dMeEA (B). 1. dMeEA, 2. EA, 3. AEA and 4. IS.

containing 250 mg of erythromycin were analyzed. The chromatograms for urine and plasma samples from one healthy male volunteer taken 3h after administration are shown in Figure 10. In the urine sample, unchanged EA was present in high concentration. The main metabolite present was AEA, small amounts of dMeEA and AdMeEA were also present. EB which is present in commercial erythromycin was also detected. EB, unlike EA, is stable in acid conditions. In the plasma sample shown, the major metabolite dMeEA was also the major component. However, in plasma samples from other volunteers, EA was the major component. This is an indication for unequal metabolism of erythromycin in different organisms. Therefore, it is important to utilize a method which allows the detection of N-demethyl derivatives of erythromycin.

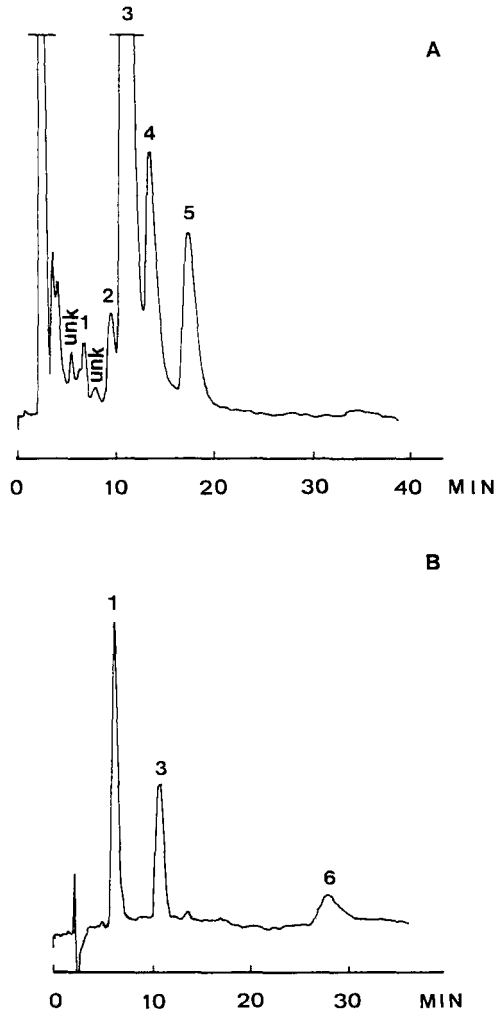


FIGURE 10 : Typical chromatograms of samples taken from a healthy male 3 h after oral administration of 250 mg of erythromycin: urine (A) and plasma (B). 1. dMeEA, 2. AdMeEA, 3. EA, 4. AEA, 5. EB, 6. IS, unk = of unknown identity.

CONCLUSION

The method described here is the only method reported in the literature which enables the separation and quantitation of EA from all of its potential metabolites present in biological samples. This is a suitable method for pharmacokinetic studies of erythromycin.

ACKNOWLEDGMENT

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DETERMINATION OF DELTAMETHRIN LEVELS IN WOOL BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method is described for the extraction and analysis of deltamethrin in wool after pour-on or plunge dipping application. Acetonitrile was used for extraction followed by simple clean-up on Florisil or C18 cartridges. Deltamethrin levels were determined by liquid scintillation spectrometry of [^{14}C]-deltamethrin and high performance liquid chromatography at 217-222 nm. Results showed good recovery and correlation between the two methods. This extraction procedure is relatively simple and rapid, it eliminates the need for prolonged liquid-liquid extraction and solid-phase steps thus making it more suitable for pharmacokinetic studies.

INTRODUCTION

Deltamethrin ((s)-α-cyano-3-phenoxybenzyl (IR,3R)-cis-3-(2,2-dibromo-vinyl)-2,2-dimethylcyclopropanecarboxylate; DM) is a particularly photostable synthetic pyrethroid (SP) and has been used extensively against many insect pests (1,2), its high potency proving very valuable in the treatment of the sheep body louse, Damalinia ovis. The lipophilic nature of DM has been utilized in the

development of formulations which were originally solvent, but latterly aqueous based, which facilitated SP diffusion in wool grease after topical application on the sheep's backline. These treatments have been widely used, albeit with little knowledge of their diffusion kinetics, for over a decade (3,4). During the course of a study to determine the diffusion kinetics of backline application of SP drugs, a simple and accurate assay procedure for quantitation of SP in wool was required.

Only limited information is available on extraction and analytical procedures necessary for the determination of SP content in wool or animal hair. These procedures generally involve the extraction of SP with non-polar solvents such as hexane or petroleum spirit followed by prolonged clean-up using liquid-liquid and solid-phase extractions (4-6). These multi-step procedures are time consuming and reduce SP recovery (unpublished observations). Acetonitrile (ACN) has been reported to be an effective extraction solvent for cypermethrin residues in vegetable and animal tissues (6); the relatively low fat solubility of ACN lowers the quantity of contaminating lipid extracted although the extracts can be frozen to further separate residual lipids (6). Gas liquid chromatography (6-9), normal and reversed-phase high performance liquid chromatography (HPLC) is then used to determine SP concentration of commercial formulations and vegetable or animal tissues (4, 7, 10).

This paper describes a method which uses ACN to remove DM from wool without prolonged extractions, followed by a simple clean-up procedure. This method has been utilized effectively in subsequent studies to define the pharmacokinetics of DM in wool following backline or plunge dipping application to sheep after shearing and of alphacypermethrin after long wool application.

MATERIAL AND METHODS

Instrumentation

The HPLC system consisted of a Millipore Waters (Milford, MA., USA) Model 510 pump, Model 710B automatic injector, with the elution profile

monitored by a Model 481 UV detector and peak analysis determined using a Model 730 data module. A 4 μ Nova-Pak C18 radial-pak cartridge contained in an 8 x 10 mm Radial Compression Module, preceded with a C18 Guard-Pak pre-column, was used to separate SP compounds. Assay of [^{14}C]-DM concentrations utilized a Packard 2000CA Tri-Carb scintillation spectrometer (Canberra Packard, Melbourne, Australia). Sample sonication was carried out in a Bransonic 220 bath (Branson, Shelton, CN., USA).

Reagents

Standard DM was provided by Roussel Uclaf (Paris, France). [^{14}C]-DM formulation was provided by Pitman-Moore (Sydney, Australia). The acetonitrile used for extraction and HPLC elution was of HPLC-grade (Mallinkcrodt, Melbourne, Australia), and petroleum spirit of Univar grade (AJAX, Sydney, Australia). Florisil Sep-Pak Cartridges were obtained from Millipore Waters, C18 Bond Elut from Analytichem International (Harbour, CA., USA) and C18 Maxi-Clean and Nylon 66 filters from Alltech Associates (Deer Field, IL., USA). The scintillant OCS was obtained from Amersham (Arlington Heights, IL., USA). All water used for dilution and elution was of HPLC-grade.

HPLC Conditions

The isocratic system utilized 83-88% ACN in water as the elution solvent, pumped at a flow rate of 1-2 ml/min with the eluent analysed for absorbance at a wavelength 217-222 nm. Injection volumes ranged between 10 and 200 μl and the total run time was 10 min.

Wool Samples

Shorn sheep were treated by pour-on or plunge dipping methods. Only the pour-on formulations contained [^{14}C]-DM, these having a specific activity of 1.25 MBq/ml and a final concentration of 10 mg DM/ml. The dose rate for the pour-on treatment was 1 ml per 4 kg bodyweight and the dip concentration was 15 μg

DM/ml. Wool samples were collected over 98 days from various sites around the fleece.

Extraction Procedure

Wool samples (1-5 g) were weighed into 25 or 50 ml glass stoppered Erlenmeyer flasks and ACN added at 10 ml/g wool. The mixture was sonicated for 5 min, the flasks covered with aluminium foil to prevent exposure to light and stirred intermittently overnight. The samples were again sonicated for 3 min, and then stored at 4°C.

Quantitation

Standard curves were prepared by spiking an ACN extract of untreated wool with 0.5-100 μg DM/ml. DM content in unknown wool samples was calculated by comparing peak area with that of the standard curve.

Sample clean-up

ACN extracts of wool were subjected to one of the following three alternative clean-up procedures:

1. Florisil clean-up: A Florisil Sep-Pak was conditioned with 25 ml ACN, then 1 ml ACN extract containing DM was passed through the Sep-Pak into an HPLC vial, and followed by elution with a further 3 ml ACN (final volume 4 ml).
2. Bond Elut: A C18 Bond Elut column was conditioned by successive washes with 5 ml ACN, 3 ml water then 3 ml 50% ACN/water. One ml of ACN extract containing DM was diluted with 1 ml water and applied to the column which was then washed with 3 ml water followed by 3 ml 50% ACN/water. These fractions were discarded and DM was then eluted with 4 ml ACN into an HPLC vial.
3. Maxi-Clean cartridges: Since these cartridges require more pressure than Sep-Pak or Bond-Elut columns for solvent elution a vacuum manifold was

used. A cartridge was conditioned with 10 ml ACN, 5 ml water and then 5 ml 50% ACN/water. One ml ACN extract containing DM was diluted with 1.0 ml water and applied to the cartridge, followed by a 5 ml water and 5 ml 50% ACN/water wash. These fractions were discarded and DM was then eluted with 4 ml ACN into an HPLC vial.

The collected ACN eluents were then analysed by HPLC for DM concentration.

The efficiency of ACN extraction of DM

The efficiency was determined using wool samples containing 1-1000 μg DM/g which were obtained from sheep previously treated with [^{14}C]-DM. After extraction with ACN, the wool was removed from the flask, the remaining solvent removed under vacuum and the wool blotted to dryness and placed in a scintillation vial. Petroleum spirit (10 ml) was added and the vial sonicated for 5 min and then allowed to stand in the dark overnight. Ten ml OCS scintillant was added and residual [^{14}C] content determined.

Recovery of DM from solid-phase extraction

For each of the alternative solid-phase clean-up procedures the recovery of [^{14}C]-DM in the ACN eluent was determined for a range of wool samples containing 1-1000 μg/g. After the final 4 ml elution the cartridges or columns were washed with 3 x 5 ml volumes of ACN and each washing was examined for [^{14}C] content. The solid-phase adsorbent material was also removed and assayed for [^{14}C] content.

Verification of HPLC Results

ACN extracts of wool obtained after pour-on treatment of sheep were analysed by both HPLC and liquid scintillation spectrometry. Aliquots (2-5 ml) of the of ACN extract of wool were added to scintillation vials followed by 10 ml OCS and allowed to stand in the dark for at least 2 hours to eliminate

TABLE 1

Typical Extraction Recoveries of DM from Wool Treated with [^{14}C]-Pour-on Formulation

Wool Sample	% Recovery
1	99.0
2	98.3
3	98.7
4	99.3
5	98.9
6	99.6
7	98.5
8	98.0
9	99.7
10	98.6
mean ± SD =	98.9 ± 0.5

luminescence before determination of [^{14}C] content. The results obtained were compared with those determined by HPLC using analysis of variance.

RESULTS AND DISCUSSION

Based on total [^{14}C] in wool samples, the recovery of DM from either clean or dirty wool samples using ACN was > 98% (n=100) (Table 1) although total recovery was probably closer to 100% since it was extremely difficult to remove all the extractant from the wool fibre. Recovery of [^{14}C]-DM in the fractions used for HPLC analysis was 100% from Florisil Sep-Pak while that from Bond Elut and Maxi-Clean was 96.4 and 96.0 respectively (Table 2). Complete recovery of DM from Florisil was probably due to its relatively high lipophilicity which facilitated

TABLE 2

Recovery of DM from Solid-Phase Extraction of Wool Treated with [^{14}C]-Pour-on Formulation

ACN Elution Volume (ml)	3	4	9
	% recovery		
Florisil Sep-pak	> 95	100	100
Bond Elut	> 80	96.4	96.4 - 96.6
Maxi-Clean	> 80	96.0	96.0 - 96.2

partioning into ACN, hence no [^{14}C]-DM remained on the solid-phase after elution.

HPLC separation of the ACN extract obtained from the Florisil eluent contained less contaminating peaks than that obtained from samples injected directly without clean up or those filtered through Nylon 66 filter (Fig. 1). With clean wool repeated use (up to 8 times) of a Florisil Sep-Pak was possible as no interfering peaks were observed. Florisil clean-up was suitable for most samples except those from particularly soiled wool or those containing less than 2 μg DM/g wool where large injection volumes were required. In these cases the relatively slower fractionation by Bond Elut columns was used. This provided separations with fewer interfering peaks and cleaner chromatograms than those obtained with Florisil (Fig. 2).

When wool samples from a related plunge dipping experiment were analysed by HPLC following clean-up with Florisil or Bond Elut, contaminating peaks co-eluted with the DM peak. When the dip formulation containing no DM was

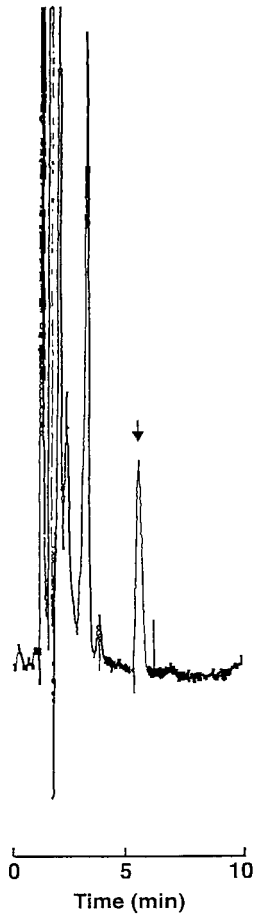

FIGURE 1. Typical elution of deltamethrin from wool extracts after pour-on treatment and Florisil clean-up. Mobile phase 86% acetonitrile in water, flow rate 2 ml/min, detection 217 nm. Injection volume 100 μl containing 9 ng deltamethrin (indicated by arrow).

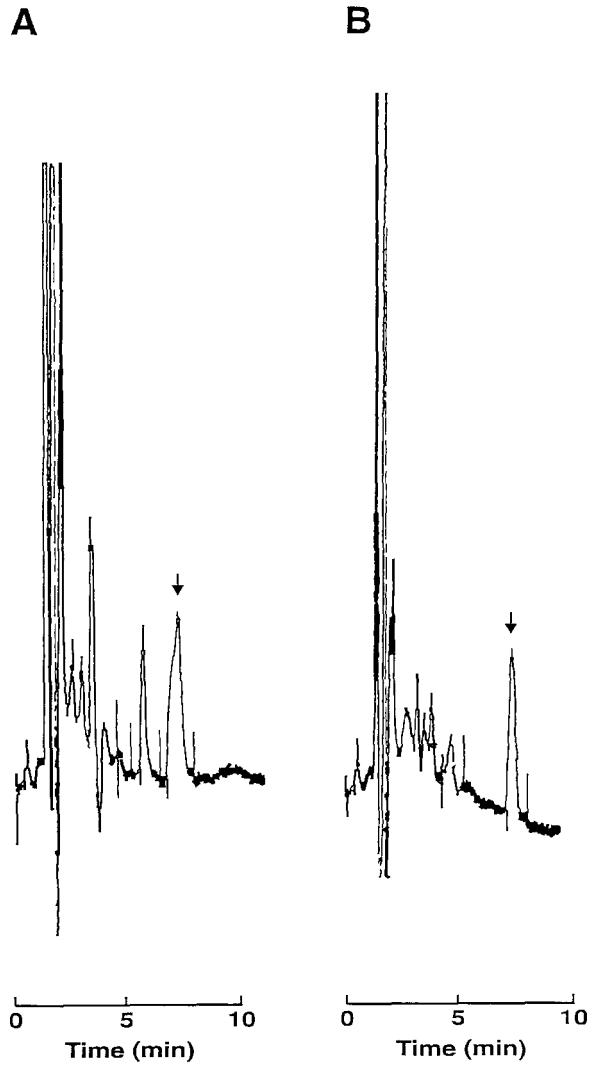

FIGURE 2. Elution of deltamethrin from soiled wool extracts after pour-on treatment and clean-up with Florisil (A) and Bond Elut (B). Mobile phase 87% acetonitrile in water, flow rate 1.5 ml/min, detection 217 nm. Injection volume 100 μl containing 9.5 ng deltamethrin.

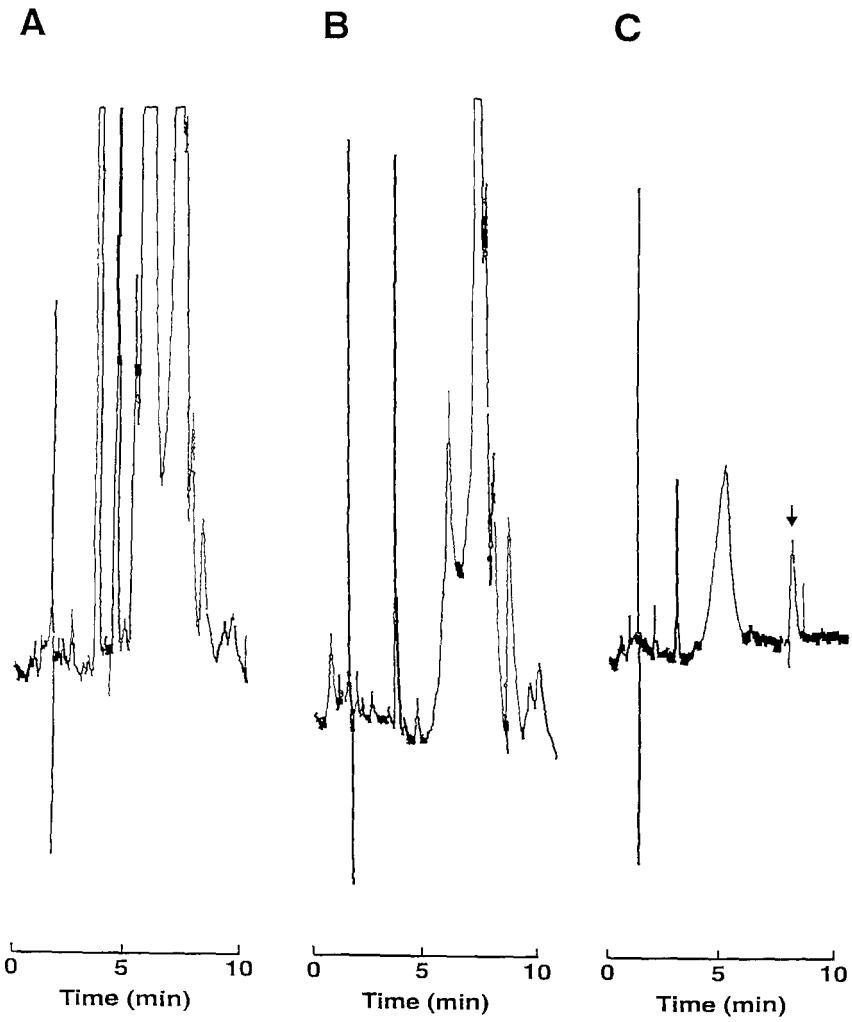

FIGURE 3. Elution of deltamethrin from wool extracts after plunge dipping and clean-up with Florisil (A), Bond Elut (B) and Maxi-Clean (C) chromatographic conditions as for Figure 2. Injection volume 10 μl containing 5.3 ng deltamethrin.

TABLE 3

Quantitation of Spiked Wool Extract by HPLC after Florisil or Bond Elut Clean-up

Spike Level µg/ml	Recovered µg/ml	% Error
98	103	+5.1
69	70	+1.4
37	36	-2.7
28.2	28.8	+2.1
14.2	14.3	+0.7
7.1	7.2	+1.4
3.4	3.5	+2.9
3.0	3.0	0
1.36	1.35	-0.7
0.70	0.71	+1.4
		Mean: +1.2

similarly examined the same interfering peaks were present. In this case Maxi-Clean cartridges minimised interference and provided clear chromatograms (Fig. 3). Regardless of the clean-up method used, occasional ACN washing of the HPLC column (after every 20-40 injections) ensured little increase in back pressure and minimal column deterioration. Standardisation used in this study for DM quantitation gave linear and reproducible curves and the correlation co-efficient exceeded 0.99. The amount of DM added to wool samples before extraction and those calculated from standard curves over a wide range were in good agreement (Table 3). For wool containing DM outside the range of the standard curve the ACN extract was diluted accordingly. Final calculations of DM content in wool were made by incorporating the dilution factors and the weight of wool samples extracted. The detection limit was 0.2 µg DM/g wool.

TABLE 4

Comparison of [^{14}C] and HPLC Quantition of DM from Wool Treated with [^{14}C] Pour-on Formulation

Wool Sample	[^{14}C]	HPLC after Florisil or Bond Elut Clean-up	% Error
	μg DM/g wool		
1	395	410	+3.8
2	224	221	-1.3
3	136	139	+2.2
4	64	63	-1.6
5	27	28	+3.7
6	17.9	17.8	-0.6
7	7.8	7.5	-3.8
8	1.9	2.0	+5.2
9	1.6	1.6	0.0
10	0.8	0.7	-12.5
			Mean: -0.5

Following pour-on application of [^{14}C]-DM, the concentrations of DM determined by [^{14}C] count and HPLC after Florisil or Bond Elut clean-up were in close agreement (Table 4). Analysis of variance of these results demonstrated no significant differences between the two methods for over 600 samples examined. The good correlation and reproducibility of the standard curves and the close agreement of the results obtained with [^{14}C] and HPLC quantitation negated the need for internal standards.

In this study ACN extraction provided significant time savings over the use of more polar solvents such as hexane since the latter often require subsequent

liquid-liquid partioning and solid-phase clean-up especially when reversed-phase HPLC is used (4, 6). The present method provides simple and rapid procedures for the analysis of DM from clean and contaminated wool. The use of this assay in describing the distribution kinetics of topically applied DM and other related SP compounds will be detailed in subsequent reports.

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CHROMATOGRAPHIC BEHAVIOR OF THE ANTHELMINTIC FENBENDAZOLE AND ITS MAJOR METABOLITE OXFENDAZOLE IN VARIOUS ION-PAIR LIQUID CHROMATOGRAPHIC SYSTEMS

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ABSTRACT

The chromatographic behavior of fenbendazole (FBZ) and oxfendazole (OFZ) in various reversed-phase liquid chromatographic (LC) systems has been investigated. The addition of negative and/or positive charged ion-pair reagents in the mobile phase has been examined, whereas the influence of mobile phase pH, mobile phase composition, and column temperature on retention and peak height has been evaluated. The observed behavior of the analytes during the various chromatographic processes has been discussed.

INTRODUCTION

Various LC systems for analyzing residues of the anthelmintic FBZ and its major metabolite OFZ (Fig. 1) in food of animal origin have been described. Most are ion-suppression systems [1-5], whereas a few are ionization enhancement systems [6-8]. Owing to the significant polarity difference of the compounds, excessive retention and peak broadness of the late eluted FBZ is noted in ion-suppression systems. In ionization enhancement systems, the retention time of FBZ is considerably shortened due to its protonation but severe peak tailing occurs as a result of the residual free silanol action on the stationary phase. Two approaches have been developed to overcome these problems. Some workers used, as an alternative to gradient elution, a weak elution strength ion-suppression mobile phase to isocratically analyze OFZ and, in succession, a much higher elution strength mobile phase to quickly elute FBZ [2-4]. Others suggested addition of pentanesulfonate and/or triethylamine pairing ions into an ionization enhancement [9] or ion-suppression [10] mobile phase, respectively, in order to better control the selectivity and elute the analytes in a single LC run without tailing. Both approaches are useful in LC analysis of these benzimidazoles but the variables affecting the chromatographic behavior of these compounds in such LC systems remain to be studied.

This paper reports on the chromatographic behavior of FBZ and OFZ in various LC systems. Using mobile phases containing or not negative and/or positive charged ion-pair reagents, the influence

A B

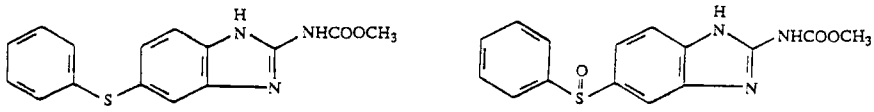

Figure 1. Structure of FBZ (A) and its major metabolite OFZ (B).

of pH, organic modifier content and column temperature on retention and peak height of the analytes is investigated.

EXPERIMENTAL

Instrumentation

LC was carried out on a Gilson system consisting of a Model 805 manometric module, a Model 305 piston pump, a Model HM/HPLC dual-beam variable-wavelength spectrophotometer set at 293 nm, a Model 831 column oven, and a model N1 variable-span recorder (Villiers-le-Bel, France). Injections were made on a Hichrom, 250x4.6 mm, stainless-steel column packed with Nucleosil 120 C_{18}, 5-μm, through a Rheodyne 7125 sample injector equipped with a 100-μl loop.

Chemicals

Octanesulfonate (OS) sodium salt, tetrabutylammonium (TBA) hydrogen sulfate, and LC grade acetonitrile and water were purchased from Merck-Schuchard (Germany). Standard OFZ was

donated from Hoechst (Germany) whereas standard FBZ was obtained from Riedel-de Haen (Germany).

Stock solutions of the individual benzimidazoles (ca. 100 μg/ml) were prepared by dissolving each standard in 10 ml dimethylsulfoxide and diluting to 100-ml volume with acetonitrile. Mixed working solutions were prepared by diluting appropriate aliquots of the stock solutions of FBZ and OFZ in the mobile phase used each time.

Chromatographic Conditions

The mobile phases used were all mixtures of acetonitrile and 0.01 M phosphate buffer (25:75, 35:65 or 40:60, v/v) in the pH range 2.2-6.5, containing or not OS and/or TBA as ion-pair reagents. Addition of the ion-pair reagents was carried out in the phosphate buffer so as their final concentration in the mobile phase to be 5 mM for TBA or 10 mM for OS addition, and 5 mM TBA plus 5 mM or 10 mM OS co-addition. Following addition of the tested pairing ion, the pH of the phosphate buffer was adjusted using 1 M phosphoric acid or sodium hydroxide solution. The mobile phase was degassed using helium and delivered at a rate of 1 ml /min.

The LC column was thoroughly equilibrated with mobile phase each time before use. Reproducible capacity factors (k′) could be obtained after passage through the column of at least 70 ml of mobile phase. When the mobile phase contained ion-pair reagents, passage of 150-ml volume was indispensable for column equilibration. On changing the mobile phase, successive column washing with at least 100 ml portions of water and acetonitrile was

found to be effective for removing the adsorbed pairing ions. Recordings were made at a chart speed of 5 mm/min and a recorder setting of 0.020 a.u.f.s.

RESULTS AND DISCUSSION

Influence of mobile phase pH and ion-pair reagent on retention and peak height of FBZ and OFZ

The effect of the mobile phase pH and ion-pair reagents on retention and peak height of FBZ and OFZ was investigated using the Nucleosil 120 C_{18}, 5 μm, stationary phase equilibrated at ambient temperature (~20 °C). The mobile phases used were all mixtures of acetonitrile and 0.01 M phosphate buffer (40:60, v/v) in pH range 2.2-6.5, containing or not OS and/or TBA reagents. Owing to the wide pH range of the mobile phases, a detection wavelength of 293 nm was selected to compensate for absorption differences between protonated and unprotonated OFZ; the absorption maximum of this compound undergoes a remarkable red shift (291.2 nm to 295.5 nm) at pH values higher than 3 (Fig. 2). Such a shift was not observed in the case of FBZ.

Effect of mobile phase pH and ion-pair reagent on retention

The effect of the mobile phase pH and pairing ion (type and concentration) on the capacity factors of FBZ and OFZ are shown in Figures 3 and 4, respectively. When the pH of the mobile phase ranged between 3.7 and 6.5, the capacity factors of both

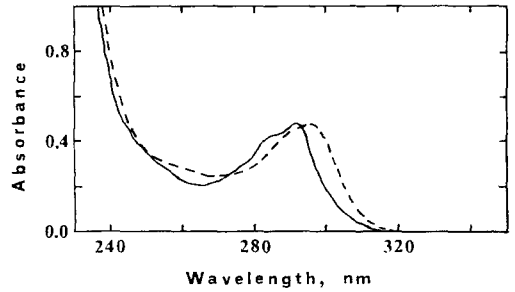

Figure 2. UV absorption spectra of standard OFZ (7.7 μg/ml) in 40% acetonitrile in 0.01 M phosphate buffer adjusted at pH 2.2 (full line) or pH 6.5 (broken line).

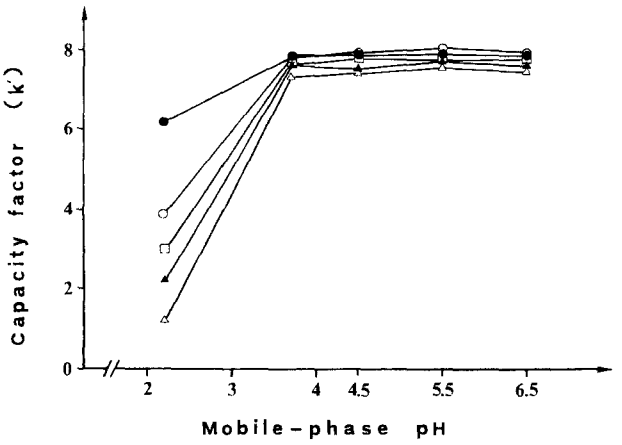

Figure 3. Influence of mobile-phase pH and pairing ion on the capacity factors of FBZ. Chromatographic conditions: stationary phase, Nucleosil 120 C_{18}, 5 μm; mobile phase, acetonitrile/0.01 M phosphate buffer (40:60, v/v) in pH range 2.2-6.5 (○) containing 10 mM OS (●), 5 mM TBA (△), 5 mM TBA + 5 mM OS (▲), and 5 mM TBA + 10 mM OS (□); column temperature, 20 °C; flow rate, 1 ml/min; wavelength, 293 nm.

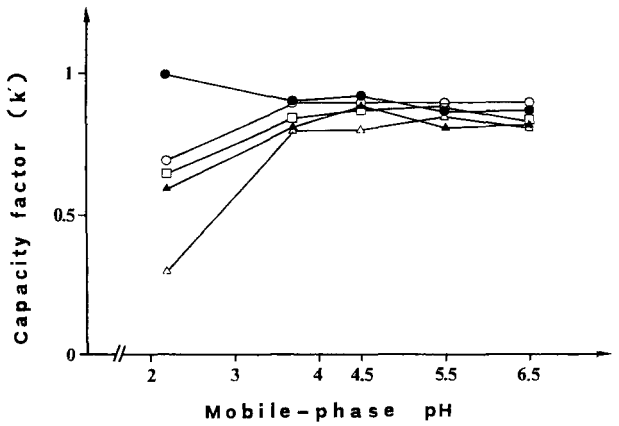

Figure 4. Influence of mobile-phase pH and pairing ion on the capacity factors of OFZ. Chromatographic conditions and curve symbols as shown in Figure 3.

benzimidazoles were not affected by pH value. Addition of negatively charged OS and/or positively charged TBA ions had also no considerable effect on retention due, obviously, to suppression of the ionization of FBZ and OFZ molecules in this pH range.

Decreasing the pH of the mobile phase to 2.2, protonation of FBZ and OFZ molecules occurs. As a result of it, the solubility of both analytes in the mobile phase was increased, thereby sharply reducing the column retention of the late eluted FBZ and slightly that of the early eluted OFZ. The retention decrease for FBZ varied with both the presence and the type of the ion-pair reagent being lower in the case of OS anions, more pronounced in absence of pairing ions, higher in presence of both OS anions and TBA cations, and arrived its maximum when only TBA cations were present in

the pH 2.2 mobile phase. OFZ exhibited a similar retention behavior in all cases except that of OS addition where a slight retention increase was noted instead.

The maximum retention noted in case of OS addition indicated ion-pairing of the OS anions and positively charged benzimidazoles to more hydrophobic forms. On the other hand, the minimum retention observed in case of TBA addition could be partly at least due to efficient masking of the negatively charged residual silanols by the TBA cations [11-12]. Electrostatic repulsion of the protonated benzimidazoles by the TBA cations adsorbed on to the octadecylsilica surface might also contribute to this effect [13-14].

The retention enhancement noted when the mobile phase in addition to TBA cations contained equal or higher concentration of OS anions was difficult to explain. The retention mechanism in such chromatographic systems has not yet been elucidated. The alkanesulfonate may interact with both the anti-tailing quaternary ammonium ions and solute ions; further, the two opposite charge surfactants may be co-adsorbed on to the column material [15-17]. Figures 3 and 4 indicate that the affinity of TBA cations to residual silanols is more pronounced than that to OS anions, as the anti-tailing effect of TBA is not reduced by the OS presence. These observations lend support to previous findings suggested by other workers [18]. Figures 3 and 4 also suggest that negatively charged counter ions capable to form ion pairs with solute cations are present even in case the concentration of the alkanesulfonate is not higher than that of the quaternary ammonium compound.

Therefore, the effect of variations in the concentration of the OS anions can be expected to be as in reversed phase ion pair chromatography; further enhancement of retention occurs when the concentration of OS anions is twice than that of TBA cations.

Effect of mobile phase pH and ion-pair reagent on peak height

The effect of the mobile phase pH and pairing ion (type and concentration) on peak height of FBZ and OFZ are shown in Figures 5 and 6, respectively. In absence of ion-pair reagents, broad and low height peaks were consistently taken for the late eluted FBZ at any mobile phase pH. At pH 2.2, where FBZ is in its protonated form., the distortion was more pronounced as severe peak tailing also appeared due, obviously, to strong silanophilic interactions with the stationary phase (Fig. 7).

Addition of negatively charged OS and/or positively charged TBA ions had a spectacular effect on both the height (Fig. 5) and the shape (Fig. 8) of FBZ peak. Even in cases of excessive column retention, peak shape was greatly improved, peak heights were markedly increased, and peak distortion was totally eliminated.

Influence of column temperature and organic modifier content on retention and peak height of FBZ and OFZ

The influence of column temperature and organic modifier content on retention and peak height of FBZ and OFZ was investigated using different mixtures of acetonitrile and 0.01 M phosphate buffer pH 2.2, containing 5 mM TBA and 5 mM OS. The

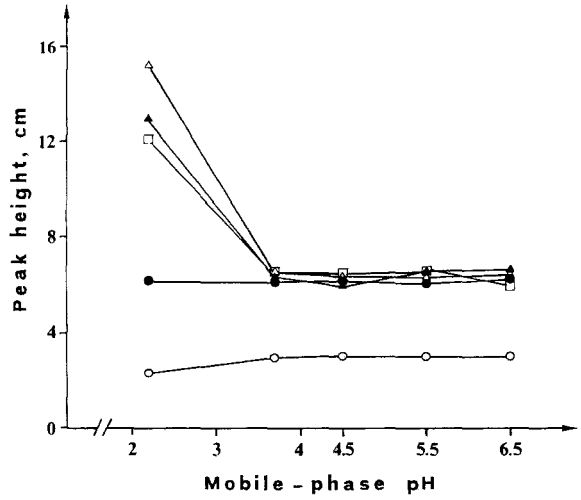

Figure 5. Influence of mobile-phase pH and pairing ion on peak height of FBZ. Chromatographic conditions and curve symbols as shown in Figure 3.

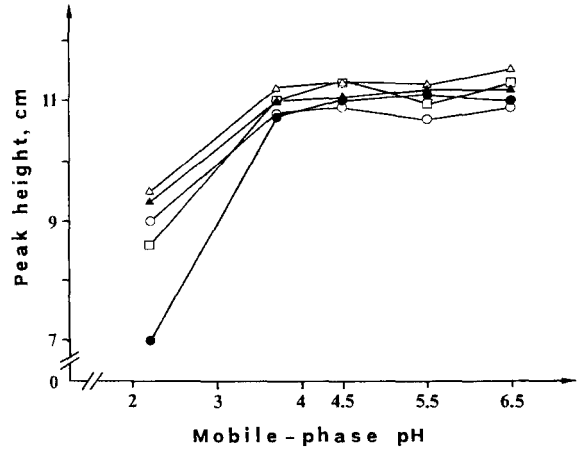

Figure 6. Influence of mobile-phase pH and pairing ion on peak height of OFZ. Chromatographic conditions and curve symbols as shown in Figure 3.

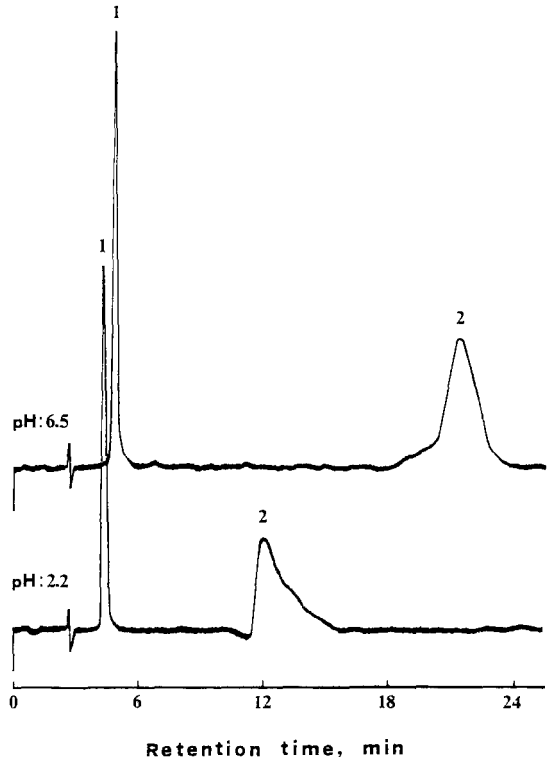

Figure 7. Typical chromatograms of standard solutions containing 0.6 μg/ml OFZ (1) and 1.1 μg/ml FBZ (2). Chromatographic conditions: stationary phase, Nucleosil 120 C_{18}, 5 μm; mobile phase, 40% acetonitrile in 0.01 M phosphate buffer adjusted at pH 2.2 or 6.5; column temperature, 20 °C; flow rate, 1 ml/min; wavelength, 293 nm; sensitivity, 0.02 a.u.f.s.; chart speed, 5 mm/min; injection volume, 100 μl.

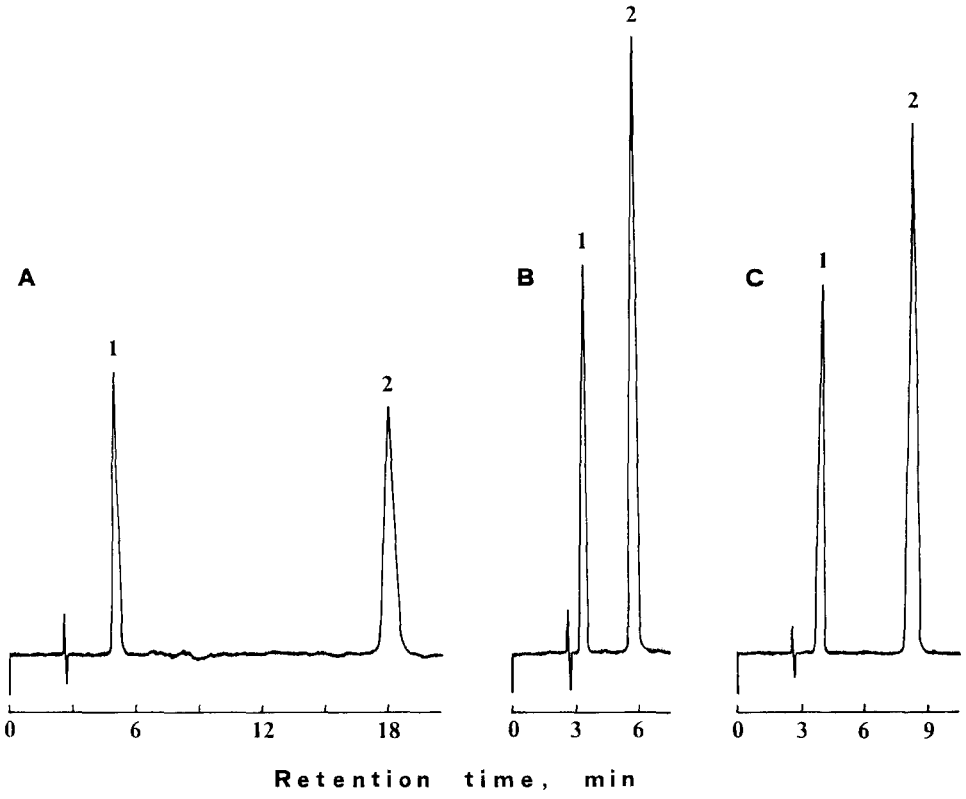

Figure 8. Typical chromatograms of standard solutions containing 0.6 μg/ml OFZ (1) and 1.1 μg/ml FBZ (2). Mobile phase, acetonitrile/0.01 M phosphate buffer (40:60, v/v) pH 2.2, containing 10 mM OS (A), 5 mM TBA (B), and 5 mM TBA + 5 mM OS (C). Other chromatographic conditions as shown in Figure 7.

changes in k′ values as a function of column temperature and organic modifier content for mobile phases containing 25%, 35%, and 40% acetonitrile are shown in Figure 9.

Increasing the concentration of acetonitrile in the mobile phase, decreased capacity factors were taken for both

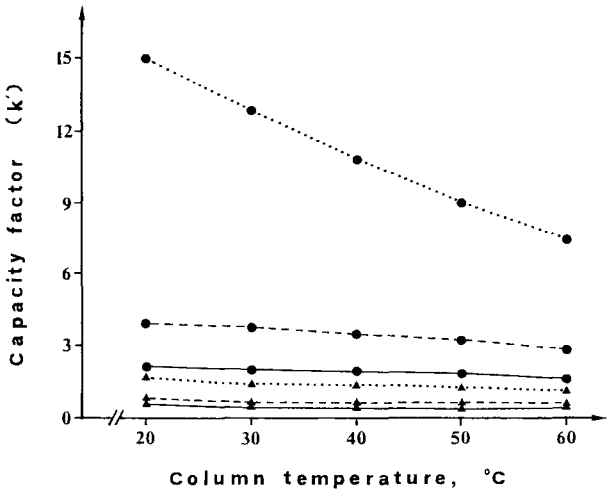

Figure 9. Influence of column temperature and organic modifier content on capacity factors of FBZ (●) and OFZ (▲). Mobile phase, 25% (dotted lines), 35% (broken lines), and 40% (full lines) acetonitrile in 0.01 M phosphate buffer pH 2.2 containing 5 mM TBA + 5 mM OS; column temperature ranged from 20 °C to 60 °C. Other chromatographic conditions as shown in Figure 3.

benzimidazoles at any column temperature. This reduction of retention, being more pronounced at 20 °C, had a significant effect on peaks of both analytes. Peak shape was considerably improved and peak heights were markedly increased when the mobile phase composition changed from 25% to 40% acetonitrile.

Increasing column temperature up to 60 °C for any mobile phase composition, a progressive but reasonable reduction of OFZ retention was noted. FBZ exhibited a similar behavior in case the mobile phase contained 35% or 40% acetonitrile whereas at lower (25%) concentration its retention was considerably affected by

column temperature; changing temperature from 20 °C to 60 °C, the retention decreases up to 50%. Increasing column temperature exerts also a beneficial effect on peak heights. This effect was more pronounced when the mobile phase contained 25% acetonitrile and moderate at 35% acetonitrile, a finding which suggests that control of temperature may be of help in specific ion-pair separations. It is of interest, however, to note that this effect was almost negligible when the mobile phase contained 40% acetonitrile.

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ANALYSIS OF DEZOCINE IN SERUM AND URINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND PRE-COLUMN DERIVATIZATION

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ABSTRACT

We present a reverse phase liquid chromatographic assay involving pre-column o-phthalaldehyde derivatization and fluorescence detection with the capability of measuring dezocine in human serum and urine for pharmacokinetic or forensic purposes. It features a simple liquid-liquid extraction scheme using hexane, conservative detection limits of 1 ng/ml, linearity to 1000 ng/ml, precision of 10% (CV%) in the low ng/ml region, approximate chromatography time of 25 minutes and the use of a new internal standard, WY-19083.

INTRODUCTION

Dezocine is a recently developed, parenterally administered, opioid analgesic for use in the management of postoperative pain. The pharmacology of the drug has been recently reviewed[1,2]. It has a clearly distinct receptor-binding profile compared to other narcotic agents, binding preferentially to mu_1 receptors over mu_2 receptors and weakly to kappa and delta receptors[3]. While dezocine is as potent as morphine when administered for clinical effect it is a significantly less potent respiratory depressant.[4,5]

Pharmacokinetically, dezocine is characterized by rapid distribution, rapid elimination and very low circulating blood levels[6,7]. There is an indication that dezocine may exhibit capacity limited elimination[6] and dosages, particularly in repeated administrations, should be monitored carefully.

Analyses for dezocine have been reported using gas chomatography with electron capture detection[7] and by liquid chromatography using electrochemical detection[8]. Both of these systems are highly sensitive owing to the need for measurement of dezocine levels down to concentrations of 1 ng/ml for pharmacokinetic studies. Our laboratory did not possess the cited equipment and to validate the blood concentration data in the literature it was necessary to develop and evaluate an additional method with comparable sensitivity. The analytical potential offered by the production and separation of fluorescent derivatives has been well reviewed[9] and the fact that dezocine possesses at least two functionalities which could be immediately exploited for that purpose suggested to us that a means existed offering the possibility of an assay with high sensitivity and precision. o-Phthalaldehyde has proved to be a particularly useful derivatizing agent due to the prevalence of biochemically important amines and the fact that, while its derivatization products are intensely fluorescent, the reagents are not fluorescent at all[10,11].

MATERIALS

Instrumentation.

Our chromatographic system utilized a model 6000A solvent delivery system, a model U6K injector with a 2 ml sample loop (Waters Assoc., Millipore Corp., Milford, MA) and a model FS 970 Kratos Fluorescence detector (Applied Biosystems Inc., Ramsey , NJ). The chromatographic column was a micro-Bondapak C18, 300 mm in length by 4.6 mm id., and packed with 10 micron particles (Waters Assoc., Millipore Corp.,Milford, MA). Two columns were placed in series to provide protection from contamination for the analytical column and additional retention to separate the internal standard peak from poorly retained products of derivatization. The mobile phase was 76% methanol in pH 4.0 sodium acetate buffer to which was added 10 ml of concentrated acetic acid for each 1000 ml of mobile phase. The typical flow rate for this system was 1.5 ml/min. The detector excitation wavelength was 260 nm and the emission filter was 489 nm as provided by the manufacturer. Data was output to a 10 mV Houston Instrument Co., model 500 strip chart recorder(Houston Inst. Co, Austin, TX).

Reagents.

Dezocine and the internal standard(WY-19083), depicted in Figure 1. were provided by the Astra USA (Westborough, MA) and by Wyeth-Ayerst Research (Princeton, NJ), respectively, and were stored in methanol solution at a concentration of 100 mcg/ml at -4 deg. C. Working standards were prepared in 60 gm/l bovine serum albumin Factor V(Sigma Chemical Co, St. Louis, MO) at concentrations of 1, 2, 5, 10, 20, 50, 100, 500 and 1000 ng/ml and stored at -4 deg C until use. Standards and patient serum and urine samples were extracted with 2% isoamyl alcohol in hexane. The o-phthalaldehyde/mercaptoethanol derivatizing reagent was purchased from the Pierce Chemical Co (Rockford, IL).

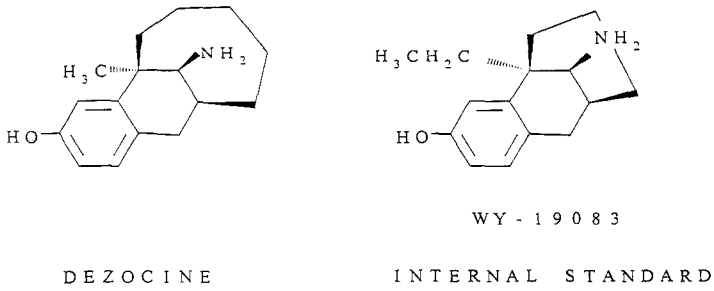

Figure 1. Molecular structure of dezocine and the internal standard(WY-19083).

Mercaptoethanol obtained from the Aldrich Chemical Co. (Milwaukee, WI) was used occasionally to restore the activity of the reagent.

METHODS

Procedure.

Two ml of standard, control or patient sample is added with a volumetric pipet to a 15 ml screwtop glass test tube containing 1 ml of internal standard (WY-19083, 50 ng/ml in 0.5 N NaOH). Ten ml of the extraction solvent(98% hexane/isoamyl alcohol) is added to each tube and rotated for ten minutes on a rotary mixer. The extraction tubes are centrifuged for 5 minutes to separate the liquid fractions and the hexane layer is removed to a 12 ml glass conical screw top centrifuge tube. The organic solvent was evaporated under nitrogen in a water bath at 45 deg C. When dry, 0.1 ml of o-phthalaldehyde derivatizing reagent is added to each tube, vortexed to mix, capped, and allowed to sit overnight. On the next day the tubes are uncapped and the derivatizing reagent is removed by evaporation to dryness in a water bath under nitrogen, again at 45 deg C. Each tube is reconstituted with 0.1 ml of HPLC grade methanol just prior to injection of 20 microliters into the liquid chromatograph.

RESULTS

Derivatization.

o-Phthalaldehyde has provided the basis for a variety of analytical procedures, notably for amino acids, drugs and tumor markers[9]. The presence of a primary amine is the necessary structural feature. The likely derivative is shown in Figure 2 for dezocine and analogously for the internal standard. The rate of the reaction and the stability of the products can be limiting features in the use of the reagents[11]. An informal evaluation showed that product formation increased over the first four hours after addition of the o-phthalaldehyde reagent and decreased only slightly over the next three days. As a result, it was convenient to perform extraction of twenty or more samples over approximately six hours, derivatize our samples overnight and perform the chromatography on the next day. Occasionally, a noticable decrease in product formation would occur. If so, 20 microliters of mercaptoethanol was added to the o-phthalaldehyde working bottle (20 ml) and the derivatization efficiency would return.

Extraction.

Several solvents were evaluated for their ability to extract dezocine: ethyl acetate, hexane and methylene chloride. In our view hexane provided the cleanest extracts and adequate recovery.

Chromatography.

While it was possible to elute dezocine more rapidly using a single analytical column, the application of this arrangement to the larger scale pharmacokinetic studies involving runs of twenty to fifty serum extracts per day proved damaging to column performance. Our best results were obtained after placing a previously used column of the same type at the head to act as a guard column. Though retention times were extended, better separation was obtained for the internal standard relative to polar serum derivatization products and for dezocine from

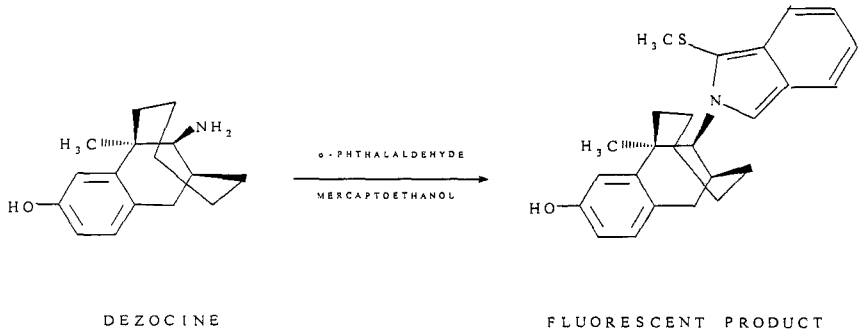

Figure 2. The expected derivative formed from dezocine by o-phthalaldehyde.

later baseline disruptions. Figure 3 represents the appearance of standards in bovine serum albumin and a pre- and post-dose subject sample. Wy-19083 elutes in approximately 16 minutes and dezocine elutes in 24 minutes. On rare occasions a late eluting peak from a previous injection would interfere with the calculation of the internal standard peak height. It was necessary to allow the late peak to elute before reinjecting.

Linearity.

Linearity was evaluated by assaying bovine serum albumin solutions containing dezocine in concentrations ranging from zero to 1000 ng/ml. Standard curves, such as depicted in Figure 4, were found to be satisfactory.

Detection Limits.

A 1 ng/ml standard was run with each standard curve. It was always possible to detect a dezocine peak originating in this sample. Injection of quantities larger than 20 microliters is possible, but our experience was that, if done consistently, column performance would be degraded and either cleaning or replacement would become necessary.

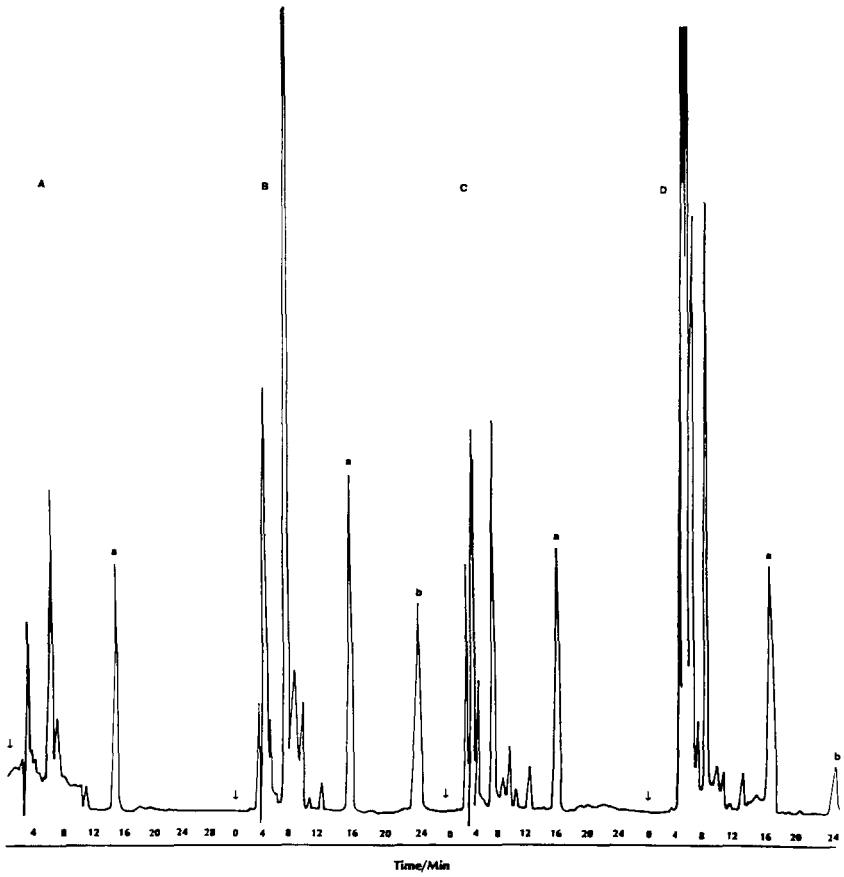

Figure 3. LC chromatograms showing A) a blank standard from bovine serum albumin, B) a 50 ng/ml standard from bovine serum albumin, C) a serum sample drawn prior to receiving dezocine and D) a serum sample drawn 20 minutes after a 20 mg IV injection of dezocine.

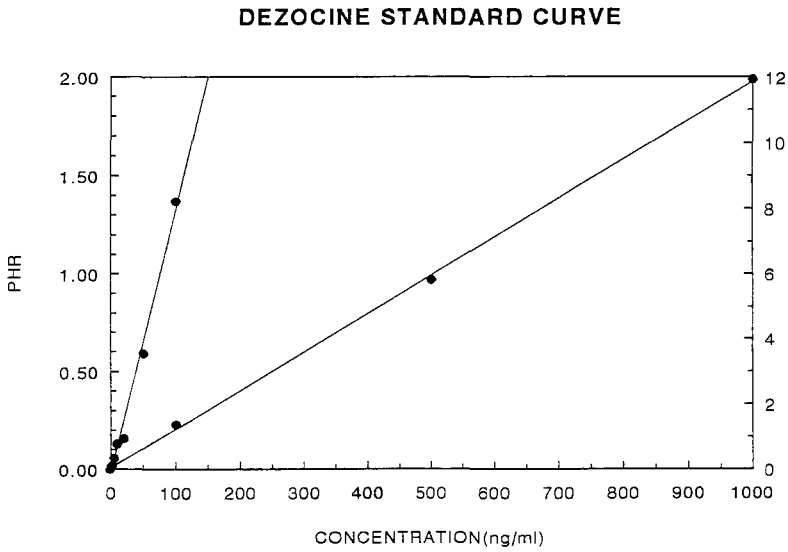

Figure 4. A standard curve demonstrating the linearity of the LC assay from 1 to 1000 ng/ml.

Precision.

Within-run precision was evaluated at approximately 27 ng/ml by combining equal amounts of the 1, 2, 5, 10, 20, 50 and 100 ng/ml standards. Fourteen out of sixteen samples exhibited satisfactory chromatographic performance with a mean value of 27.92 ng/ml and a standard deviation of 3.05 for a coefficient of variation of 10.9%. Day-to-day precision obtained over a ten week period gave a mean of 27.55 ng/ml with a standard deviation of 4.79 for a coefficient of variation of 17.4%(Table 1). The range of these values was 20.0 ng/ml to 36.8 ng/ml with 6 of 10 values within 3 ng/ml of the mean value.

Application.

The assay has been used for the determination of dezocine pharmacokinetics. Figure 5 is a concentration versus time plot of serum levels of dezocine in a

TABLE 1

Precision Studies

	Within-run	Run-to-run
N	14	10
MEAN	27.92	27.55
STD DEV	3.05	4.79
CV(%)	10.9	17.4

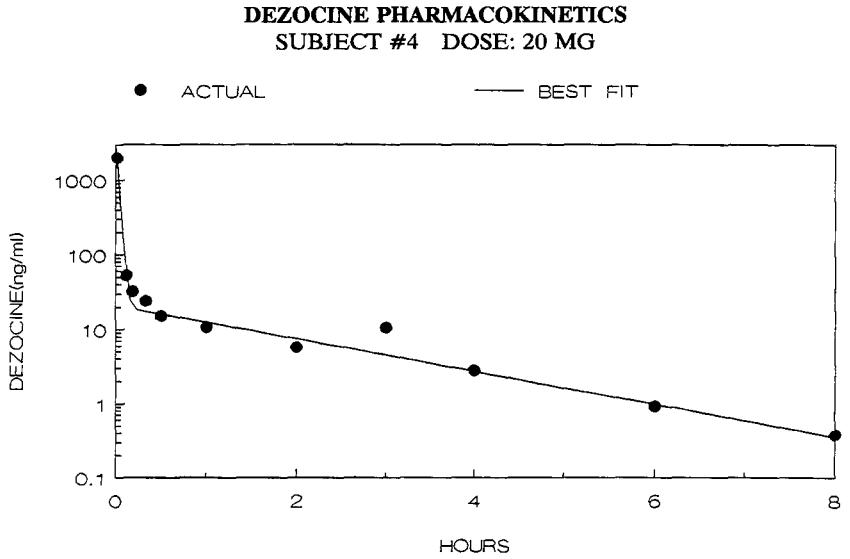

Figure 5. A plot of serum dezocine concentration of serum from a human subject with time after a 20 mg IV injection.

subject that received 20 mg of drug intravenously over thirty seconds into the right arm. Blood was first drawn from the left arm thirty seconds after administration. The serum concentration at this time was 2008 ng/ml. Seven minutes after administration the serum level was 53 ng/ml. Sixty minutes after administration the serum level was 10.6 ng/ml. Clearly, dezocine is rapidly distributed after iv administration. The data was fit to a two compartment open model and the value obtained for the distribution phase half-life was 1 minute. The half-life in the elimination phase was 1.4 hours.

Urine Testing.

The same assay has been applied to urine testing. The same subject whose data is presented in Figure 5 also had urine collected. Concentrations found in urine exceeded that of serum over the eight hour blood sampling period (Table 2). A total of 0.2612 mg(1.3 % of the dose) was excreted unchanged in eight hours.

DISCUSSION

Analysis of dezocine requires careful sample preparation and special detection systems. Analytical approaches to this time have involved gas chromatography with electron capture detection[7] and liquid chromatography with electrochemical detection[8]. We describe a straight forward approach that involves a single hexane extraction at a basic pH, overnight derivatization with o-phthalaldehyde and mercaptoethanol and liquid chromatography with fluourescence detection. We have found detection limits to 1 ng/ml and linearity over 4 orders of magnitude. The assay has been used for serum and urine pharmacokinetic studies[12] and has recently been effective in an *in vitro* evaluation of dezocine plasma protein binding[12]. Because dezocine has a very rapid distribution, concentrations of drug in the serum quickly reach levels of 10 ng/ml

TABLE 2

Dezocine Urine Excretion

Time of Collection (minutes)	Urine Volume (mls)	Dezocine Conc (ng/ml)	Amount (mg)
0-125	144	1126	0.1621
126-180	65	336	0.0218
181-245	80	243	0.0194
246-360	280	116	0.0325
361-495	300	85	0.0254
496-715	250	20	0.0050
716-955	730	5	0.0037
956-1435	355	2	0.0007

and place considerable demands on the analytical system. We have demonstrated precision on the order of 10% within-run and 17% from run to run. These standard deviations of 3 and 5 ng/ml are acceptable considering the levels measured. Since dezocine is a narcotic substance and has the potential for abuse, the capability of measuring very low serum levels expands the window of detectability. It would appear that dezocine could be detected in serum samples as long as 12 hours after a single dose and potentially longer given repeat administrations. Dezocine can be detected unchanged in the urine for 24 hours by this method and expands the window further. It has been reported that dezocine is metabolized principally via conjugation routes in monkeys and rats[13] and, since these are likely to also be important metabolic routes in man, it is probable that

hydrolysis of the urine would aid both in screening and confirmation tests and permit even greater possibility of detection.

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AN AUTOMATED ANALYTICAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURE FOR IOPAMIDOL SOLUTIONS USING A BENCHMATE WORKSTATION

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ABSTRACT

A flexible compact robot system for sample work-up and preparation of calibration standards for HPLC analyses is presented. The equipment consisting of a BenchMate workstation and a conventional HPLC system was used to analyse aqueous solutions containing iopamidol, a contrast medium. The analytical procedure was characterized with respect to sensitivity, selectivity and linearity of the detector response. The use of the workstation yielded excellent data with respect to accuracy and intra-assay precision. The application could be used after few modifications to determine other compounds in solution or microcrystalline suspensions. The automatization of this kind of analysis saved man-hours (ca. 25%) and could reduce the costs of glassware and of solvents. An additional advantage is the reduced individual exposition to solvent vapour and other chemicals.

INTRODUCTION

Within the framework of drug research and development compounds are characterized toxicologically and pharmacokinetically. The formulations, e. g. solutions, microcrystalline suspensions, test-substance diet mixtures, lactose premixes and tablets, administered to the test animals are monitored for correct concentration to validate the preparation process. Additionally, stability and homogeneous distribution of the active ingredient in certain formulations are investigated. Numerous compounds occur in similar concentrations in formulations of the same type. Such samples are usually analysed by HPLC. Furthermore quality control units checking similar features of developmental and final market formulations to be administered in man work within the same field.

In modern laboratories the equipment for HPLC is fully automated and is often computer-controlled leading to time saving and unattended operation. However, the preparation of calibration standards and sample work-up is still done manually and consequently is tedious and time consuming. Exposition to the laboratory personnel to potentially toxic compounds and solvents is unavoidable in many cases.

There is a current trend for an increased automation in analytical laboratories, replacing many manual operations (1-3). This trend applies in particular to laboratories with a high sample turnover, as such robot systems are expensive, space consuming and require highly trained personnel. An alternative is the BenchMate Workstation (4) which is less expensive, takes up little room and is easy-to-use. Some fully or partially automated applications were reported for the determination of drugs and/or their metabolites both in plasma samples (4,5), in pharmaceutical formulations (6) and in samples obtained from in-process control (7). Analytical procedures could be automated yielding accurate and repeatable data.

The aim of our experiments was to develop generally usable procedures which should be able to replace the manual preparation of calibration standards and manual work-up of similar samples with different compounds. For the first experiments iopamiol dissolved in aqueous solutions was chosen. Iopamidol is a monomer iodine containing contrast medium for uroangiography (8,9).

EXPERIMENTAL

Instrumentation

The HPLC equipment consisted of two Model 510 pumps, a Model 680 automated gradient controller (all by Waters, Eschborn, FRG), a Perkin-Elmer ISS-100 autosampler and a Spectroflow 773 UV-detector (Kratos, Karlsruhe, FRG). The detector was connected via an interface to a mainframe computer (VAX 4000-300, Digital Equipment, Munich, FRG) for data acquisition and evaluation. The chromatograms were evaluated with ACCESS*CHROM Release 1.8 (Perkin-Elmer). UV spectra were measured with a Perkin-Elmer LC-480 diode array detector. Finally, a Model B220 BenchMate Workstation (Zymark Corporation, Inc., Hopkinton, USA) was used for dilution both of the samples and of the calibration stock solutions.

Materials

Iopamidol, L-N,N'-Bis(2-hydroxy-1-hydroxymethylethyl)-5-(2-hydroxypropionylamino)-2,4,6-triiodoisophthalamide (see Figure 1), was synthesized in the laboratories of SCHERING AG (Bergkamen, F.R.G.). SOLUTRAST-300 ampoules with 300 mg iodine/ml corresponding to 612.4 mg iopamidol / ml were used for calibration, for the preparation of quality control samples and for preparation of the solutions administered to the test animals.

Liquid chromatographic grade acetonitrile, water and phosphoric acid were purchased from Merck (Darmstadt, FRG).

Chromatographic conditions

The chromatographic conditions were developed in-house. Chromatographic columns of 12.5 cm x 4.6 mm i.d. were packed with Spherisorb ODS II (5 µm) by M & W Chromatographie Technik (Berlin, FRG). The analyte was eluted with a

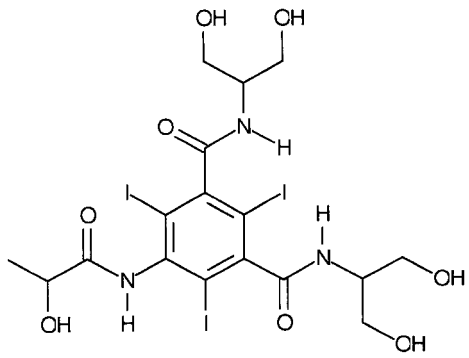

Figure 1: Structural formula of iopamidol.

gradient system. A linear gradient was used, from mobile phase A consisting of acetonitrile/water/phosphoric acid (10/990/0.2 by volume) to mobile phase B consisting of acetonitrile/water/phosphoric acid (220/870/0.2 by volume) in 10 min, the flow rate being 1.0 ml/min. Subsequently, the column was eluted with B for one min and then with A for 5 min. The column was operated at ambient temperature. The absorbance of the effluent was monitored at 240 nm. The UV spectrum of iopamidol and a representative chromatogram are shown in Figure 2 and 3, respectively.

Sample work-up (final method)

The quality control samples contained the active ingredient dissolved in physiological saline. The concentrations ranged within 0.1 and 200 mg iodine / ml.

In summary, the solutions were diluted with physiological saline to 50 µg iodine / ml. Volumes of 10 µl were injected onto a reversed phase column.

For example, in case of 2 mg iodine/ml samples, the workstation added 3.5 ml of physiological saline to a manually pipetted sample aliquot of 0.5 ml. The content of the tube was mixed by cycling (drawing and dispensing the tubes' content

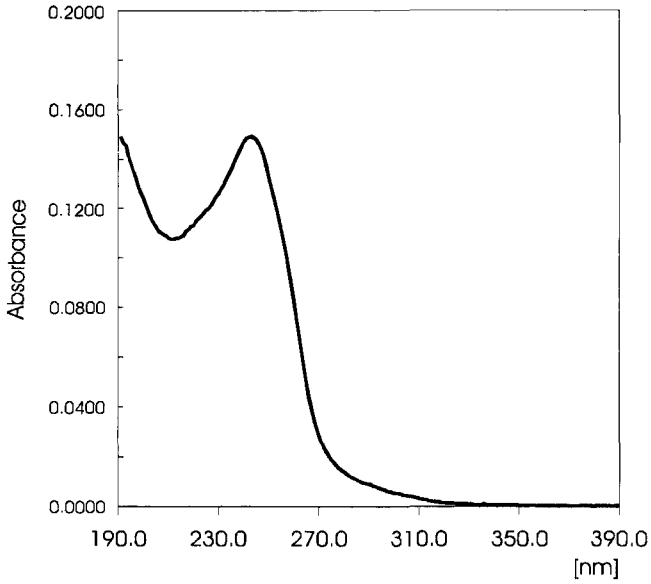

Figure 2: UV spectrum of iopamidol

The spectrum was measured on-line following chromatography of 612 µg iopamidol. The spectrum was taken at peak maximum and corrected by the background at a retention time which was shorter by 0.3 min than that of the peak maximum.

into and out of a cannula) four times. Then the solutions were volumetrically diluted with physiological saline to 4 ml, followed by another cycling. After dilution, the workstation's syringe was purged with 3 ml physiological saline. The sample work-up was followed by further dilution steps in case of higher concentrations. The BenchMate commands applied for 2 mg iodine / ml samples and the commonly used setup parameters are summarized in Table 1.

Calibration and evaluation

The six point calibration was performed by injecting amounts in the relative proportion of 100/75/50/25/12.5/6.25. 100 corresponded to 1,200 ng iodine. The

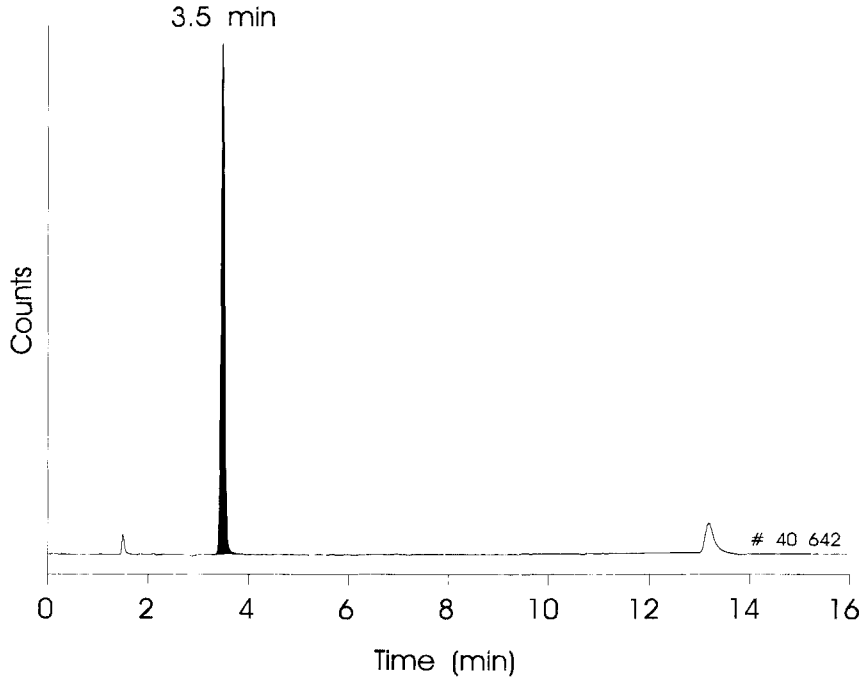

Figure 3: Chromatography of a quality control sample with 0.1 mg iodine/ml. 10 µl was injected. The peak at 3.5 min was based upon the analyte, the peak at the later retention time was related to matrix ingredients.

workstation diluted aqueous stock solutions containing 300 mg iodine/ml with physiological saline by appropriate factors. Each of the six dilutions was injected once. The calibration range was 75 - 1,200 ng iodine per injection, the volume injected 10 µl.

ACCESS*CHROM was used for evaluation of the chromatograms. The peak areas of the calibration standards were correlated with their amounts injected. The data were modelled with an unweighted linear regession (model: $y = K_0 + K_1 \cdot x$). The results were given in % of intended concentration of the samples. The intended value was always given in mg iodine / ml. The conversion factor (mg iodine -> mg iopamidol) accounts for 2.041.

TABLE 1

Listing of BenchMate Commands and Setup Parameters Applied for the Work-up of a 2-mg Iodine /ml Sample

Zymark BenchMate 2.5

Zymark BenchMate Procedure : 2MG/ML_500NG/10MML

Step 1 : Add 3.5 ml of LOESUNGSMITTEL
Step 2 : Mix by cycling 3 ml in tube 4 times
Step 3 : Dilute (volumetric) 1:5 into LOESUNGSMITTEL making 4 ml
Step 4 : Mix by cycling 3 ml in tube 4 times
Step 5 : Wash syringe with 3 ml of LOESUNGSMITTEL
Step 6 : END

SETUP PARAMETERS - 1

SAMPLE TUBE
Initial Volume: 0.00 ml

FLOW RATES
Aspirate: 0.50 ml/sec
Dispense: 1.00 ml/sec
Internal Std: 0.12 ml/sec
Mix: 1.50 ml/sec
Filter: 0.10 ml/sec
Air Push: 0.15 ml/sec

DISPENSING
Liquid driven: Y Y=YES N=NO

AUTOWASH PARAMETERS
Reagent Vol: 1.00 ml
Sample Vol: 0.20 ml

GRAVIMETRIC PARAMETERS
Gravimetric on: Y Y=YES N=NO
Tolerance: 10%
Tare Sample Tubes: N Y=YES N=NO

RACK PARAMETERS
LV Tube Numbering: Y Y=YES N=NO
Reset to Sample 1: N Y=YES N=NO

SETUP PARAMETERS - 2

LC PARAMETERS
Inject Load Vol: 0.50 ml
Calibrate every: 0 samples
Calib Replicates: 1
Calibration Reagent: 1
Calib Wash Vol: 0.00 ml

NAME REAGENTS
Reagent 1 : LOESUNGSMITTEL ; Density : 1.0000

NAME INTERNAL STANDARD
Standard : INTERNAL STD ; Density : 1.0000

BenchMate Table Setup

THE ESTIMATED TIME FOR ONE SAMPLE IS: 5.5 MINUTES.
NO FILTERS ARE USED.
NO SPE COLUMNS ARE USED.

Samples will be processed starting at Rack 1 position 1,
through position 50, then Rack 2 positions 1 through 50
or until an empty position is encountered.
Final tubes are in Racks 3 and 4.

Experimental characterization of the HPLC procedure

The analytical procedure was characterized with emphasis on limits of detection and quantitation, selectivity, linearity, accuracy, precision and ruggedness (10).

The instrumental limit of detection was determined at a signal-to-noise ratio of 2, and the linearity by injecting increasing amounts of iopamidol within the range of 75 to 3,000 ng iodine. The linearity was checked graphically to about twice the highest point of calibration in order to assure, that the calibration range does not border on the range of linearity. Accuracy and intra-assay precision were obtained by analysis of quality control samples. The inter-assay precision was estimated based upon the results of routinely analysed samples. The values of the pooled standard deviation over different concentration levels were calculated according to Scheffé (11). The squared values of the standard deviation or coeffcient of variation weighted with the degrees of freedom were summed. The square root of the resulting sum of squares divided by a sum of degrees of freedom yieided a pooled standard deviation (S.D.p). The over-all relative standard deviation (r.S.D.p.) was calculated by division of the over-all accuracy by S.D.p.

Finally, the acceptance criterion for the limit of quantitation was a coefficient of variation of $\leq$ 10% and a bias of $\leq$ 10% of expected concentration obtained following analysis of a series of real samples with decreasing concentrations.

Furthermore, the method's ruggedness was tested by investigating the influence of the dilution solvents, i. e. physiological saline and mobile phase A, and the number of dilution steps on the accuracy.

RESULTS

Characterization of the final HPLC method

The UV absorption of the column effluent was measured by a photo diode array detector yielding the spectrum shown in Figure 2. Optimal UV absorption was observed in the range of the detection wavelength used.

The system was selective for the analyte investigated as no other interfering peaks were observed. The instrumental limit of detection was 2.4 ng iodine per injection. Including an injection volume of 10 µl, the method's limit of detection was then 240 ng iodine/ml. Of course, the sensitivity could still be enhanced by factors of at least 10 by increasing the injection volumes. The linearity of the detector response was tested between 75 and 3,000 ng iodine per injection (i.e. 7.5 - 300 µg iodine/ml) and could be verified.

To determine the final method's accuracy and intra-assay precision, four quality control samples containing 0.1 - 100 mg iodine/ml were analysed yielding concentrations from 98 to 106% (see Table 2). The values of the relative standard deviation amounted to 0.4 - 1.8%. The over-all values for accuracy and precision (r.S.D.p) accounted for 102% and 1%, respectively. The limit of quantitation was defined at the lowest concentration level tested, 0.1 mg iodine/ml, because accuracy and intra-assay precision did not exceed the limits of acceptance. However, the real limit of quantitation is supposed to be lower taking into account that samples can be injected onto the HPLC column without dilution. The estimate on the basis an injection of 200 ng iodine dissolved in an injection volume of 10 µl is then 2 µg iodine/ml.

Influence of the dilution solvent on the accuracy:

The individual data of the sample work-up and the corresponding results are summarized in Table 3.

First, mobile phase A was used for the dilution of the calibration solutions and quality control samples. At the lower concentration levels, 0.1 - 2.0 mg iodine/ml, the means of the analytical results ranged from 101% to 103%. However, the higher levels of 10, 50 and 100 mg iodine/ml yielded 119% ± 0.6, 154% ± 2 and 154% ± 5. The sample work-up was performed five times. The bias exceeding 50% of expected concentration indicated that the apparatus was inaccurate where dilution factors of higher than 200 were used for dilution with mobile phase A.

For a second experiment, the solutions were diluted with physiological saline. On the average, the analytical results of the solutions with 0.1, 0.5, 2.0, 10, 50

TABLE 2

Accuracy and Intra-assay Precision of the Determination of Iopamidol in Physiological Saline

The number of replicates was five. 10 µl was injected per sample.

Intended concentration of the sample [mg iodine/ml]	**Portion injected** [ng iodine]	**Accuracy** [% of intended concentration]	**intra-assay Precision** [% of intended concentration] (standard deviation)
0.1	500	103.4	1.0
0.5	500	98.1	1.8
2.0	500	100.1	1.4
10.0	500	103.0	0.4
50	500	104.2	0.5
100	500	102.1	0.9
		Mean value: 101.8	S.D.p: 1.1

TABLE 3

Results from Testing Ruggedness Owing to Different Methods of Sample Work-up Made Manually or by a BenchMate Workstation

Concentration of the quality control samples [mg iodine/ml]	**Manual dilution** Aliquot pipetted [ml]	**Manual dilution** Final volume [ml]	**Automated dilution** - 1st step Aliquot pipetted manually [ml]	**Automated dilution** - 1st step Final volume [ml]	**Automated dilution** - 2nd step Aliquot pipetted automatically [ml]	**Automated dilution** - 2nd step Final volume [ml]	**Automated dilution** - 3rd step Aliquot pipetted automatically [ml]	**Automated dilution** - 3rd step Final volume [ml]	**overall Dilution factor**	**Analytical results** [% of intended concentration] (Mean ± standard deviation and number of replicates)
0.1	-	-	0.5	1.0	-	-	-	-	2	103 ± 1 (4)
	-	-	0.5	1.0 * (*eluent A)	-	-			2	103 ± 2 (5)
0.5	-	-	0.5	5.0	-	-	-	-	10	98 ± 2 (5)
	-	-	0.5	5.0 * (*eluent A)	-	-			10	101 ± 1 (5)
2.0	-	-	0.5	4.0	0.8	4.0	-	-	40	100 ± 1 (5)
	-	-	0.5	4.0 * (*eluent A)	0.8	4.0			40	102 ± 0.3 (5)
10	-	-	0.25	5.0	0.5	5.0	-	-	200	106 ± 2 (5)
	1.0	5.0	0.5	4.0	0.8	4.0	-	-	200	103 ± 0.4 (5)
	-	-	0.1	4.0	0.8	4.0	-	-	200	102
	-	-	0.5	5.0 * (*eluent A)	0.2	4.0	-	-	200	119 ± 0.6 (5)
50	-	-	0.5	5.0	0.5	5.0	0.5	5.0	1,000	112 ± 2 (5)
	1.0	25	0.5	4.0	0.8	4.0	-	-	1,000	104 ± 0.5 (5)
	-	-	0.02	4.0	0.8	4.0	-	-	1,000	101
	-	-	0.5	5.0 * (*eluent A)	0.09	9.0	-	-	1,000	154 ± 2 (5)
100	-	-	0.25	5.0	0.5	5.0	0.5	5.0	2,000	111 ± 1 (5)
	0.5	25	0.5	4.0	0.8	4.0	-	-	2,000	102 ± 0.9 (5)
	-	-	0.01	4.0	0.8	4.0	-	-	2,000	100
	-	-	0.25	5.0 * (*eluent A)	0.09	9.0	-	-	2,000	154 ± 5 (5)

and 100 mg iodine/ml were 103%, 98%, 100%, 106%, 112% and 111% of the intended values, respectively. The bias values at the high concentration levels were evidently lower than in the previous experiment. Thus, physiological saline should be preferred with the purposes of sample work-up. At the 50 and 100 mg iodine / ml levels, however the deviation was always higher than 10% and not yet acceptable.

Influence of the number of dilution steps on the accuracy:

For the samples of the previous experiment with 10, 50 or 100 mg iodine / ml, aliquots of 0.25 or 0.5 ml pipetted manually were diluted automatically by 3 serial steps by factors of 200 up to 2,000. The number of dilution steps was considered to be a possible source of the bias in the results. Consequently, the samples with more than 10 mg iodine/ml were prediluted manually by factors of 5 up to 50 in a third experiment to save one automated dilution step. The mean analytical results were 103%, 105% and 102% for the samples with 10, 50 and 100 mg iodine/ml, respectively, indicating acceptable accuracy values and the precise processing of the BenchMate Workstation. To confirm the results, the initially pipetted aliquot was reduced from 250, 500 and 250 µl to 100, 20 and 10 µl for the samples with 10, 50 and 100 mg iodine/ml, respectively. The dilution of the quality control samples was performed once by two steps. The over-all dilution factors remained constant at 200, 1,000 and 2,000. The results accounted for 102%, 101% and 100% of intended concentration indicating that the BenchMate worked exactly by using 2 dilutions steps.

DISCUSSION AND CONCLUSIONS

The results of the over-all validation indicate the excellent accuracy and repeatability of the analytical method. The sensitivity is sufficient. The manual method could be automated nearly completely. The preparation of the calibration stock solutions, the allocation of sample aliquots and the transfer of diluted solutions into autosampler vials remained to be done manually. Inspite of that, the saving in time accounted for ca. 25%. Problems may occur when 3 dilutions steps

are used. However, within the framework of method development the problematic BenchMate operation can be tested.

The BenchMate procedures developed for the experiments described here have already been modified for routine analyses of other compounds, i. e. other contrast media and various steroids. Microcrystalline suspensions need to be dissolved manually to prevent a possible loss in recovery. Recently, the automation of a method for analysis of test-substance diet mixtures provided for the administration to mice and rats has been tried. The BenchMate workstation added internal standard solution and extraction medium to aliquots of the test-substance diet mixture. The extraction was performed by cycling or vortexing. Because the methanolic extract can not be filtered efficiently before incorporated into the workstation's cannulas and obstructions have occured. However, partial automatization could be achieved by use of solid phase extraction. The extract were prepared conventionally as described earlier (12) and cleaned by a BenchMate solid phase extraction procedure.

In summary, the automation of our HPLC procedures yielded apparent savings in costs for routine analyses. The workstation is compact and easy-to-handle, and has found acceptance by those using it.

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RETENTION OF SOME SIMPLE ORGANIC CATIONS ON AN ANION EXCHANGE COLUMN

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ABSTRACT

The retention behavior of a homologous series of phenyl-substituted alkylammonium ions on a styrene-divinylbenzene copolymeric anion exchange column with acetonitrile - aqueous solution mobile phase was investigated. As the concentration of eluent anion increased, the retention of eluite cation was increased. This relationship showed good linearity in log-log coordinates. The methylene selectivity of members of a homologous series of eluite cations was also studied.

INTRODUCTION

Ion exclusion chromatography has been used for the separation of ionic from non-ionic species (1), especially for the separation of weak organic acids with cation exchange resins in the hydrogen form (2-8). Ion

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exclusion separation is based on Donnan exclusion, i.e., electrostatic interaction between the eluite and the charged functional groups in the stationary phase. When cations are introduced onto an anion exchange column, they pass quickly through the column and elute faster than neutral solutes because they are excluded from, or repelled by the positively charged resin surface because of its Donnan potential. Non-ionized solutes can penetrate the resin network and are retained by partition or other forces.

There are several factors which play a role in the retention process other than Donnan exclusion in ion exclusion chromatography. These are sorption on the surface of the matrix of the ion exchange resin (1,7-9), size (steric) exclusion (6-7,10), hydrophobic interaction (5-6), and hydrogen bonding (5). Many experimental conditions contribute to the retention of eluites in ion exclusion. These include the temperature (11), pH of mobile phase (12-13), and ionic strength of eluent (12-14).

In this paper, the retention behavior of a homologous series of phenylalkylamines on a styrene-divinylbenzene copolymeric anion exchanger was investigated. The effect of eluent ion concentration on the retention of eluites under conditions of ion exclusion chromatography was observed. A homologous series of eluites was chosen to evaluate solvophobicity on retention. Benzyl alcohol was chosen to investigate the dependence of a nonionized solute on eluent ion concentration.

EXPERIMENTAL

Equipment

The liquid chromatograph consisted of a Waters M-6000A solvent delivery pump (Waters Associates, Milford, MA), a Rheodyne model 7125 injector (Cotati, CA) with a 200 μL sample loop (Alltech Associ-

ates, Deerfield, IL), a Kratos Spectroflow 773 variable wavelength UV-VIS detector (Ramsey, NJ), and a Houston Instrument model 4511 strip chart recorder (Austin, TX). A Hamilton PRP-X100 column (150 x 4.1 mm) (Hamilton, Reno, NV) packed with 10 μm, spherical poly(styrene-divinylbenzene) trimethylammonium exchanger was used. To control the column temperature, a FIAtron (Madison, WI) CH-30 column heater and TC-50 controller were used. The column was thermostated at the desired temperature for 1 hour prior to injection.

An Alltech solvent filtration kit and 0.45 μm membrane filter were used to filter the eluent solutions. An Alltech series 6000 syringe filter (0.45 μm) and a 10 mL Luer-lox glass syringe (Curtin Matheson Scientific, Inc., Houston, TX) were used to filter the injection solvent. An Orion Research model 601 A/digital Ionalyzer (Orion Research, Inc., Boston, MA) was used to measure pH. The deionized water was prepared by using a Sybron-Barnstead 60209 water purification system (Boston, MA). An ultrasonic water bath (L&R Manufacturing Industry, Kearny, NJ) was used to degas the mobile phases.

Reagents

Benzyl alcohol was analytical grade and was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). Benzylamine, 2-phenylethylamine, 3-phenyl-1-propylamine, and 4-phenyl-1-butylamine were of 98%+ purity and were purchased from Aldrich Chemical Co. (Milwaukee, WI). Sodium nitrate and sodium chloride were ACS grade and were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Sodium bromide was Mallinckrodt (St. Louis, MO) analytical-reagent grade. Hydrochloric acid was ACS grade and was purchased from Curtin Matheson Scientific, Inc. (Houston, TX). Acetonitrile was HPLC grade and was also obtained from Curtin Matheson Scientific, Inc.

Chromatographic Conditions

The mobile phase flow rate was 1.0 mL/min. The detector wavelength was set at 254 nm with sensitivity 0.005 AUFS. The injection volume was 10 μL. To inject the eluite a Hamilton 50 μL microsyringe was used. The eluite solution was injected three times and average values of k′ were calculated. To determine t_o, 5-10 μL of deionized water was injected. The column temperature was controlled at 35°C.

Procedure

In a typical procedure, the necessary amount of sodium nitrate to produce the desired eluent anion concentration was weighed into a 500 mL volumetric flask. A volume of 5 mL of 0.1 M HCl solution was added to it to get the desired pH (pH = 3.0). The solid was dissolved and the solution was made up to 500 mL by adding deionized water. Then the solution was filtered with a 0.45 μm membrane filter. From this solution the necessary volume was taken to prepare the mobile phase. The mobile phase was prepared by mixing 10 volume % of acetonitrile with 90 volume % of nitrate anion solution. Bromide and chloride ion solutions were prepared in the same way. Eluent anion concentrations ranged from 30 to 300 mM in the aqueous solution prior to mixing with acetonitrile. The change in volume on mixing was assumed insignificant. After mixing the mobile phase was degassed with an ultrasonic bath.

In a typical procedure for preparing eluite solution, 20 μL of benzylamine was dissolved in 1 mL of acetonitrile, and 5 μL of this stock solution was taken and diluted to 2 mL with mobile phase, and the mixture was vortex mixed for 10 s.

RESULTS AND DISCUSSION

The pH of the eluent solution was adjusted to pH = 3.0, where the phenylalkylamines used as eluites remained in their ammonium cation

form. It was expected that all the cations would be repelled by the ammonium group on the column resin, thus showing short retention times. Indeed, short retention occurred when the mobile phase had no eluent ion added except 1mM H^+ and 1mM Cl^- to keep the pH 3.0 even though the mobile phase was 90 % water and only 10 % acetonitrile. All the phenylalkylammonium ions eluted earlier than deionized water. Retention volumes less than the void volume (v_o) were attributed to the eluites' inability to occupy all of the volume used by the deionized water because of the repulsion between eluite ion and the resin functional group (15).

When inorganic ions were added to the mobile phase, the retention volumes of the phenylalkylamines increased. Figure 1 shows the linear relationship between the log of the capacity factors of benzylammonium, 2-phenylethylammonium, 3-phenyl-1-propylammonium, and 4-phenyl-1-butylammonium ions and the log of the concentration of nitrate ion in the aqueous fraction of mobile phase. Similar curves were obtained with bromide and chloride eluent anions.

The retention of phenylalkylammonium ions increased with an increase in the salt concentration from 30 to 300 mM. Similar dependencies were observed in earlier studies. Slais (14) observed an increase in k' of aromatic acid anions as the concentration of ammonium sulfate in the mobile phase increased. Jandera et al (16) showed that the retention of aromatic sulfonic and carboxylic acids on a reversed phase column was increased by adding strong electrolytes to the mobile phase in the absence of an ion pair forming substance. The retention volume increased in the order of the size of the anion or cation used in the mobile phase. They also found linear relationships between the concentration of Na_2SO_4 and the k' of aromatic sulfonic acids in log-log coordinates. They obtained the empirical equation that describes the influence of salt concentration, c, on the capacity factor, k': $\ln k' = A +$

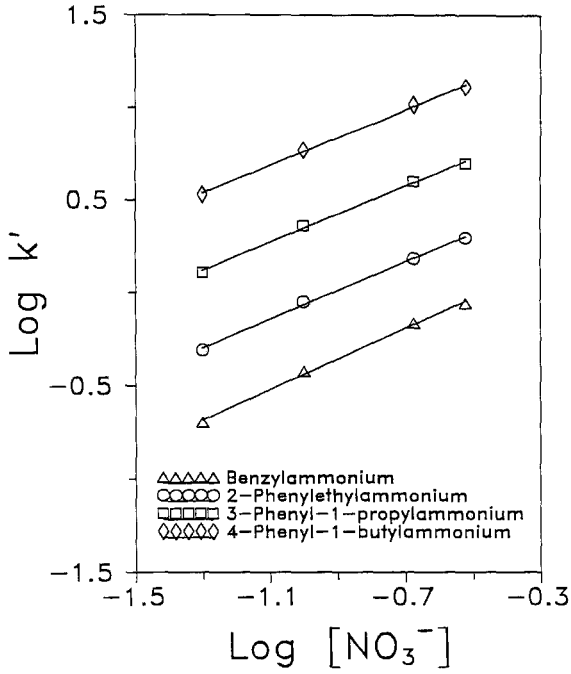

FIGURE 1. Relationship between the log of the capacity factor of phenylalkylammonium ions and the log of nitrate ion concentration in the aqueous fraction of the mobile phase.

B ln c, where A and B are constants depending on the eluite, eluent salt, solvent and column used. An explanation of the meaning of these constants was not given. The linear relationship between log k' of phenylalkylammonium eluites and the log of the concentration of salt was consistent with the empirical equation obtained by Jandera et al (16).

These results for the phenylalkylammonium eluites were similar to ion pair reversed phase liquid chromatography results although a hydrophobic ion pairing reagent was not added. The reversible formation

of an ion pair between eluent anion and eluite cation is a possible interpretation of these results. As would be expected from an ion pairing equilibrium the k' of ammonium ions increased as the concentration of eluent anion increased.

The retention of phenylalkylammonium ions was also influenced by the kind of salt in the mobile phase. Figure 2 presents the log k' of 4-phenyl-1-butylammonium ion as a function of the log of eluent ion concentration in the mobile phase. The k' of 4-phenyl-1-butylammonium ion increased in the order $Cl^- < Br^- < NO_3^-$. The order is related to the ion exchange strength of the eluent ion (17), i.e., the size of anion (18). This result also can be interpreted in terms of ion pairing. As the size of the anion increased, pairing strengthened and solvophobic interaction between the ion pair and the mobile phase increased.

Another possible interpretation is that the eluent ion in its double layer produces a blocking effect. As the concentration of eluent ion increased, the accessible functional group volume is decreased because of the electrostatic interaction between eluent ion and the functional group of the anion exchange column. Therefore, the eluite cation experienced less repulsion, and consequently longer retention. The blocking effect was in the order $NO_3^- > Br^- > Cl^-$. This is consistent with ion exchange strength, the interaction between eluent ion and functional group of the stationary phase (17).

There was an increase in retention of phenylalkylammonium ions as the carbon number in the alkyl group increased. This is again an example of a homologous series of eluites eluting in the order of increasing length of their aliphatic chains and it is attributed to sorption by the resin due to the solvophobicity of the organic ammonium ions.

Figure 3 depicts the plot of log k' vs. carbon number of the alkyl group in the phenylalkylammonium cations for different inorganic eluent

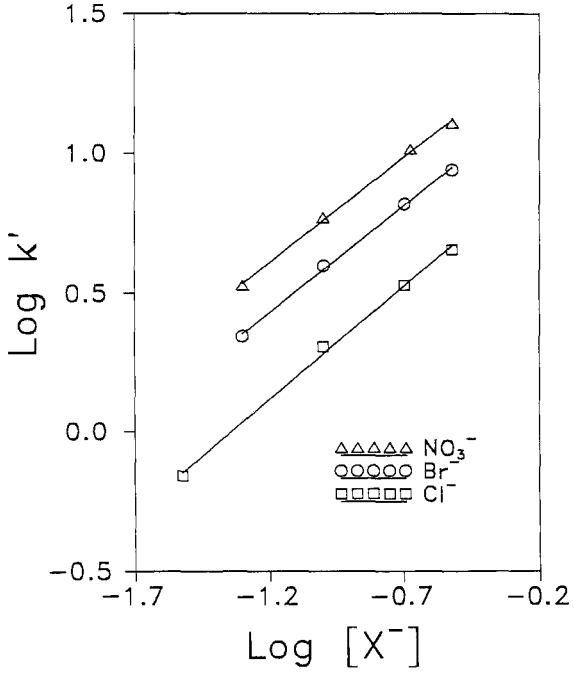

FIGURE 2. Relationship between the log of the capacity factor of 4-phenyl-1-butylammonium ion and the log of the inorganic ion concentration in the aqueous fraction of the mobile phase.

ions of 0.100 M concentration in the aqueous fraction of the mobile phase. The slope of this plot is log α_{CH_2}, where α_{CH_2} is the selectivity between nearest homologous ammonium cations. At the 95% confidence level, these three lines are parallel. Thus, the selectivity was not affected by changing the type of eluent ion.

Figure 4 presents the relationship between log k' and the carbon number of the alkyl group in phenylalkylammonium cations for different nitrate ion concentrations. The slope shows that this increase is

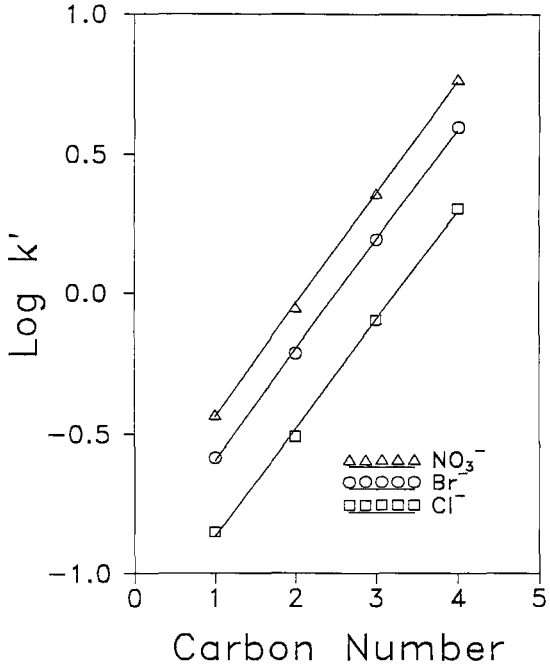

FIGURE 3. Relationship between the log of the capacity factor and the carbon number of the alkyl group in phenylalkylammonium ions with different inorganic eluent ions: 0.100 M of eluent ion in the aqueous fraction of the mobile phase.

systematic, and these three lines are parallel at the 95% confidence level. Thus, the selectivity between two homologous eluites was not affected by the concentration of the eluent ion. These results are consistent with those for phenylalkylcarboxylate anions in ion exchange chromatography (17).

Even though it is not shown, the retention of benzyl alcohol was little affected by the kind and concentration of eluent. As the concentration of eluent ion increased, the k′ of benzyl alcohol increased a small,

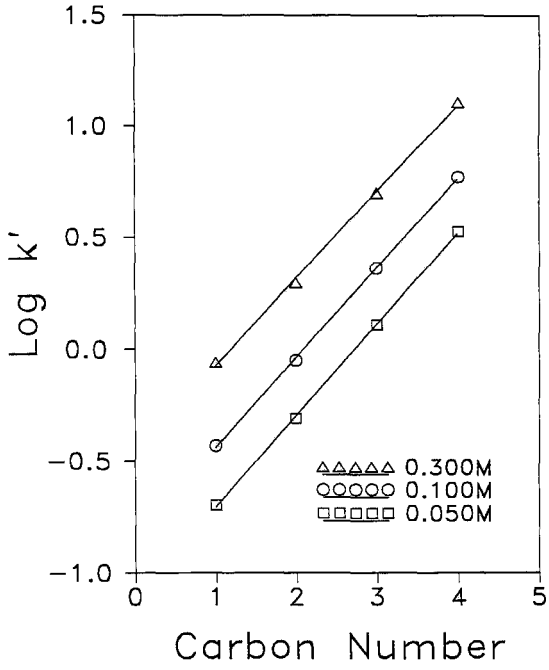

FIGURE 4. Relationship between the log of the capacity factor and the carbon number of the alkyl group in phenylalkylammonium ions with different nitrate ion concentrations in the aqueous fraction of the mobile phase.

almost insignificant, amount. This is thought to be due to salting out (19).

CONCLUSION

The retention of organic cations on an anion exchange column has been interpreted as being the result of a combination of ion exclusion and solvophobic interaction. As the eluent ion concentration increased, the retention of eluite cations increased. The extent of retention

depended on the concentration and kind of inorganic eluent ion. However, the methylene selectivity among members of a homologous series of eluite cations was not affected by the nature or concentration of eluent ion.

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ANALYSIS OF POLYETHERS BY ISOCRATIC HPLC WITH UNIVERSAL DETECTORS. III. A STUDY ON REPRODUCIBILITY

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ABSTRACT:

Liquid chromatography under critical conditions (LCCC) on ODS-columns in methanol-water separates ethoxylated fatty alcohols (FAE) and block copolymers of ethylene oxide (EO) and propylene oxide (PO) according to the length of the hydrophobic block. The elution behaviour of polyethylene glycols (PEG), polypropylene glycols (PPG) and fatty alcohols on different columns, in different mobile phase compositions, and at different temperatures is studied and the long-term reproducibility evaluated. It is shown, that - depending on the length of the blocks - the best separation need not be achieved exactly at the critical point of adsorption.

INTRODUCTION:

The analysis of non-ionic surfactants, such as ethoxylated fatty alcohols (FAE) and block copolymers of ethylene oxide (EO) and propylene oxide (PO), which are in widespread use for various applications [1], is complicated by the distributions of molar mass (MMD), chemical composition (CCD) and functionality (FTD).

A full characterization of these materials requires the determination of the distributions mentioned above. This can only be achieved by a combination of different chromatographic techniques, such as HPLC [2-19], GC [20-23], and SFC [11,24]. Recently, matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) [25] has been combined with chromatography and yields valuable informations.

As has been pointed out in preceding papers [26-29], three different chromatographic modes can be applied to the analysis of nonionic surfactants:

Size Exclusion Chromatography (SEC) [30] separates according to molecular dimensions, provided, that adsorption effects can be excluded.

Normal phase HPLC [2,3,12,13,15,16] separates according to polar groups (EO-chain, OH-groups).

Reversed phase HPLC [7-11,17,18,26,29] separates according to hydrophobic groups (alkyl groups, PO-chain, in mobile phases with a high water content the EO-chain, too).

None of these techniques - if used alone - provides all required informations, even though multiple detection can enhance the analytical power of one-dimensional chromatography. The ultimate goal - the characterization of a sample in the form of a 3-dimensional map - can, however, only be reached by two-dimensional chromatography using two of the techniques mentioned above [31,32].

The most feasible approach involves a separation according to the non-polar part - the alkyl group or the poly(propylene oxide) chain - using liquid chromatography under critical conditions (LCCC) [31-34] for poly(ethylene oxide) (PEO) in the first dimension, and subsequent analysis of the pure homologous series thus obtained by SEC [28], normal phase HPLC [35], SFC or MALDI-TOF-MS [36].

In such a two-dimensional method, the problems with quantification can be solved, which arise from the dependence of response factors on molar mass and chemical composition, and from different preferential solvation of both blocks, as has been pointed out in previous communications [28,29,37-41].

In LCCC all members of a homologous series elute at the same volume, because entropic and enthalpic terms compensate each other (critical point of

adsorption). Hence the corresponding block (EO) becomes chromatographically invisible, and the separation occurs exclusively according to the length of the hydrophobic block.

In previous papers [26,27,29,39] we have shown, that this can be achieved on ODS columns in methanol-water at compositions between 80:20 and 90:10 (w/w).

As has been shown by several authors [42-49], the properties of alkyl bonded phases can be quite different, depending on the nature of the matrix (silica or polymer based) and on the surface density of the alkyl groups. Moreover, the properties of a column can change, when it is used for longer time in some mobile phases.

Hence we have studied the elution behaviour of polyethylene glycols (PEG), polypropylene glycols (PPG), fatty alcohols and FAE with respect to

1. the mobile phase composition corresponding to the critical point of adsorption for PEG
2. the reproducibility of the separation on different columns
3. the long-term reproducibility on the same column
4. the effect of operating temperature
5. the best composition of the mobile phase for different types of surfactants

In order to achieve a sufficient separation of homologous series with the same alkyl end group or the same number of PO-units, the difference of their elution volumes must be larger than the peak width of the PEG block. It should be shown, whether or not this is always the case exactly at the critical point of adsoption.

EXPERIMENTAL:

These investigations were performed using the density detection system DDS70 (commercially available from CHROMTECH, Graz, Austria), which has been developed in our group. This instrument has been described in full detail in previous communications [50-52]. The column box was thermostatted to 25.0°C and 30.0°C

using a Lauda MS-3 thermostat. The density detector cell was combined with a Bischoff 8110 RI detector and connected to a MS-DOS computer via the serial port. Data acquisition and processing was performed using the software package CHROMA [52], which has been developed for the DDS 70.

Several analytical and a semi-preparative column were used for these investigations. In the following text the columns will be denoted by the following abbreviations:

a) ODS2-10cm: Spherisorb ODS2 3 μm, 4.6*100 mm (PhaseSep)
b) ODS2-prep: Spherisorb ODS2 5 μm, 10 *250 mm (PhaseSep)
c) ODS2-3: Spherisorb ODS2 5 μm, 4.6*250 mm (PhaseSep)
d) ODS2-4: Spherisorb ODS2 5 μm, 4.6*250 mm (PhaseSep)
e) ODS B Spherisorb ODSB 5 μm, 4.6*250 mm (PhaseSep)
f) Merck Lichrospher 100 RP 18, 5 μm, 4.6*100 mm (Merck)
g) ACT-1 ACT-1, 10 μm, 4.6*150 mm (Interaction)

The flow rate was 0.5 ml/min on the 4.6 mm - columns and 2 ml/min on the 10 mm - column. Two JASCO 880 PU pumps were used: one for column a and one for columns b-g, which were connected to two Rheodyne 7060 column switching valves. Samples were injected using Rheodyne 7125 injection valves with a 50 and a 500 μl loop, respectively.

Fatty alcohols, polyethylene glycols, and polypropylene glycols were purchased from FLUKA or Aldrich and used without further purification. Methanol and water were HPLC grade (Merck, LiChroSolv). The mobile phases were mixed weight by weight and degassed in vacuo prior to use.

COMPARISON OF COLUMNS UNDER DIFFERENT CONDITIONS:

When looking for the critical point of adsorption for PEG, a comparison of different columns is rather easy: in this case it is sufficient to plot the elution volumes versus the molecular weight of the PEGs (or vice versa): under critical conditions the elution volumes should be constant.

In the analysis of PPG or fatty alcohols, a comparison of different columns requires a parameter, which is independent on column dimensions: the capacity factor k′ of a substance, which is given by its elution volume V_e and the void volume V_0:

$$k' = \frac{V_e - V_0}{V_0} \quad (1)$$

Within homologous series, ln k′ increases linearly with the number n of repeating units, such as methylene groups, EO- or PO-units [44,53].

$$\ln k' = A + B.n \quad (2)$$

(A and B are constants for a given polymer in a given mobile phase)

In a binary eluents, the capacity factors depend on the (volume or weight) fraction of the organic component in the mobile phase

$$\ln k' = \ln k'_w + S.x \quad (3)$$

wherein k'_w is the (extrapolated) capacity factor in pure water, and S is the so-called solute acceleration factor [44,54].

On a given stationary phase, the capacity factor of a given substance depends on the change in free energy ΔG°_{sorp}, when the molecule is adsorbed and on the volume ratio ϕ of the stationary and the mobile phase]:

$$\ln k' = -\frac{\Delta G^\circ_{sorp}}{RT} + \ln\phi \quad (4)$$

With the corresponding changes in enthalpy ΔH°_{sorp}, and entropy ΔS°_{sorp}, one may write

$$\ln k' = -\frac{\Delta H^\circ_{sorp}}{RT} + \frac{\Delta S^\circ_{sorp}}{R} + \ln\phi \quad (5)$$

It is obvious, that capacity factors will depend on temperature. This may be especially important at the critical point of adsorption, where the enthalpic and the entropic terms compensate each other.

RESULTS AND DISCUSSION:

In the first step of these investigations we tried to find the critical point of adsorption for polyethylene glycols on different columns and studied the influence of temperature. Then we analyzed fatty alcohols and polypropylene glycols on the same columns and evaluated the reproducibility of the separation. Finally we tried to optimize the conditions for each separation problem.

Polyethylene glycols:

First of all we compared several octadecyl-columns in the analysis of PEGs in methanol-water 80:20 (w/w) at a temperature of 25.0°C. In Figure 1 the elution volumes thus obtained are plotted versus the molecular weight of the PEGs.

At the critical point of adsorption all PEGs should be eluted at the same volume, regardless which molecular weight they have. As can be seen, there are considerable differences between the columns, even between col.3 and 4, which come from the same producer, but from a different batch.

Figure 2 shows the results obtained on the same column (col.4), but in different mobile phase compositions: Obviously, the critical point of adsorption is reached on this column rather at a mobile phase composition above 90:10.

Figure 3 shows the results obtained on the 25 cm columns at two different mobile phase compositions.

In Figure 4 the corresponding results from the short columns (10 and 15 cm, respectively) are shown.

It is evident, that for three columns (ODS2-4, ODS2 10 cm, and Merck LiChroSpher ODS) the critical point of adsorption for PEG is reached at a composi-

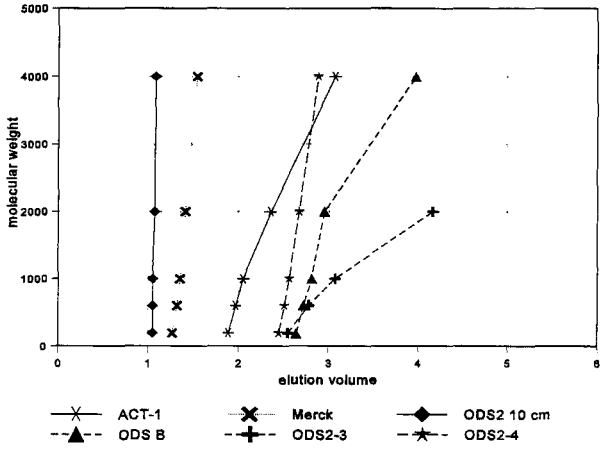

FIGURE 1:

Elution behaviour of polyethylene glycols on different columns in methanol-water 80:20 (w/w) at 25.0°C

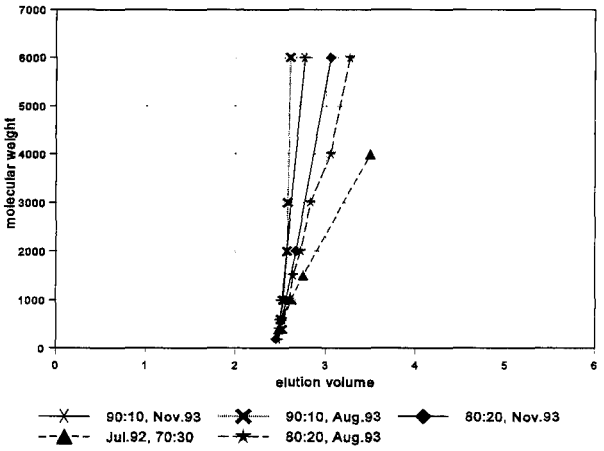

FIGURE 2:

Elution behaviour of polyethylene glycols on column ODS2-4 in methanol-water of different composition. Solid lines: new measurements, dashed lines: old measurements

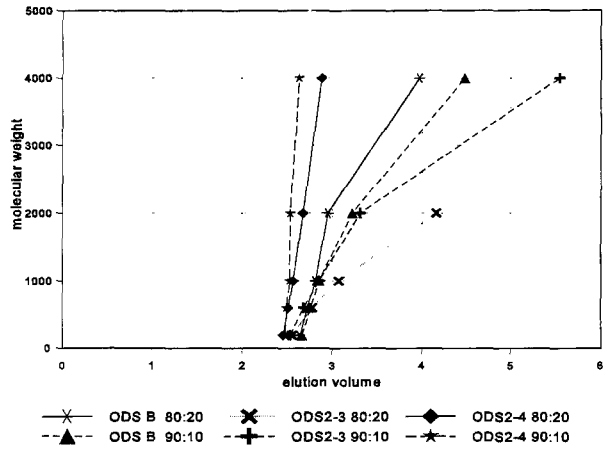

FIGURE 3

Elution behaviour of polyethylene glycols on different columns in methanol-water of different composition

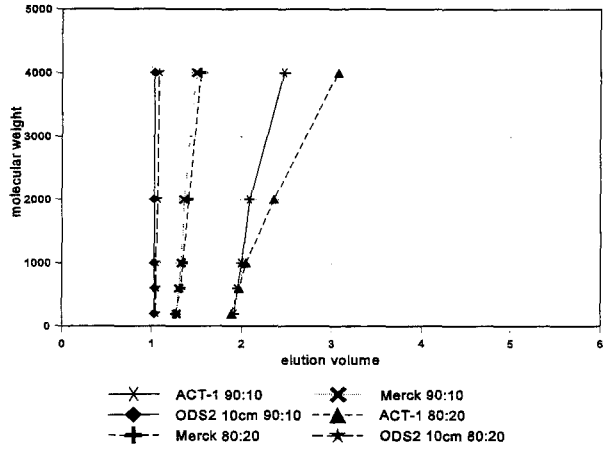

FIGURE 4

Elution behaviour of polyethylene glycols on different columns in methanol-water of different composition

tion very close to 90:10, on ODS B somewhat higher. The difference between 80:20 and 90:10 is, however, not dramatic: at 80:20 the difference in elution volumes is still quite small (at least for lower molecular weights). The semi-preparative ODS2-column, which is not shown in these figures, is almost identical with ODS2-4 (most probably it comes from the same batch). For two columns (ODS2-3 and ACT-1) no critical point of adsorption for PEG is observed in methanol-water.

When the measurements in methanol-water 80:20 were performed at a temperature of 30.0°C, no considerable effect was observed for ODS2-4, as can be seen from Fig.5. ODS B comes a little closer to the critical point of adsorption, while ODS2-3 is still far away.

Fatty alcohols:

Once the critical point of adsorption for PEG had been determined, we analyzed various fatty alcohols on different columns in methanol-water 90:10 (w/w). In Figure 6 the capacity factors thus obtained are plotted versus the carbon number of the alcohols. As can be seen, the differences between the columns are not very large.

In Figure 7, the results obtained on ODS2-10cm in methanol-water of different compositions are shown. The slopes of the lines can be used as a measure for the selectivity of the mobile phase. In a different representation of the same data, which is shown in Figure 8, it becomes clear, that no satisfactory separation is achieved in 100% methanol, while a composition of 90:10 will be suitable for higher alkanols ($n > 12$) and 85:15 or even 80:20 for lower alkanols.

Polypropylene glycols:

The next step was the analysis of polypropylene glycols up to molecular weights of 1200. The lower molecular samples (PPG 425, PPG 725) could not be analyzed in methanol-water 90:10, which would correspond to the critical point of adsorption for PEG, but in 80:20 a satisfactory separation was achieved.

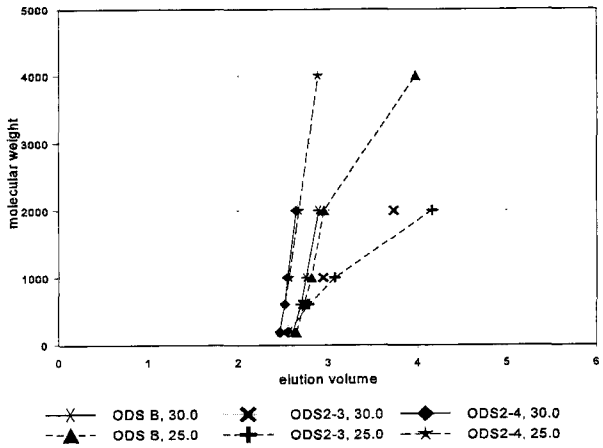

FIGURE 5:

Elution behaviour of polyethylene glycols in methanol-water 80:20(w/w) on different columns at different temperatures

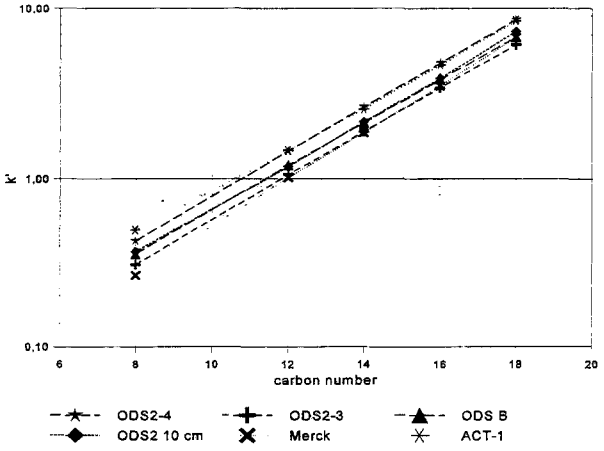

FIGURE 6:

Capacity factors of fatty alcohols on different columns in methanol-water 90:10 (w/w)

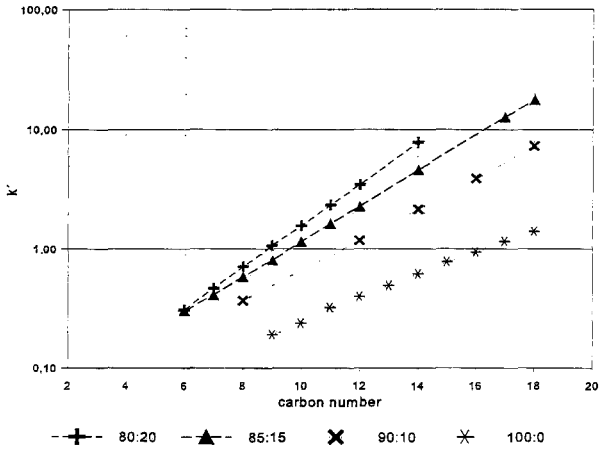

FIGURE 7:

Capacity factors of fatty alcohols on ODS2 10 cm in different mobile phase compositions

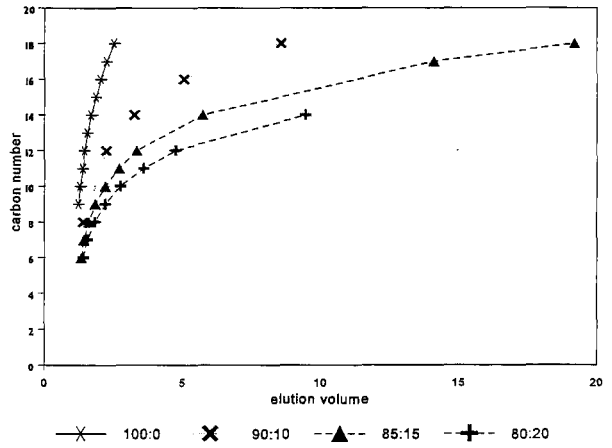

FIGURE 8:

Elution behaviour of fatty alcohols on ODS2 10 cm in methanol-water of different composition

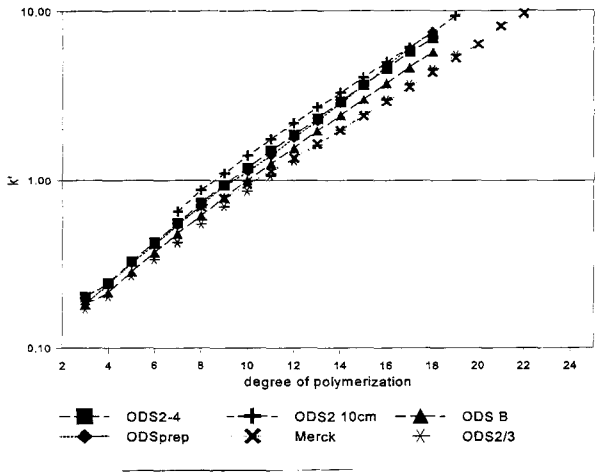

FIGURE 9

Capacity factors of polypropylene glycols on different columns in methanol-water 80:20

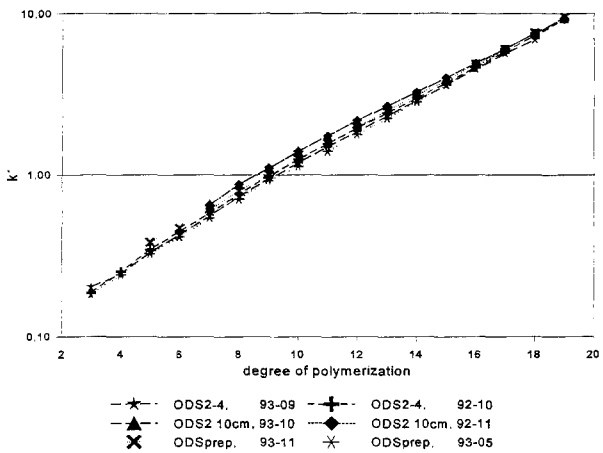

FIGURE 10

Reproducibility of capacity factors of polypropylene glycols on three different columns in methanol-water 80:20 (w/w) over a period of 6 and 11 months, respectively

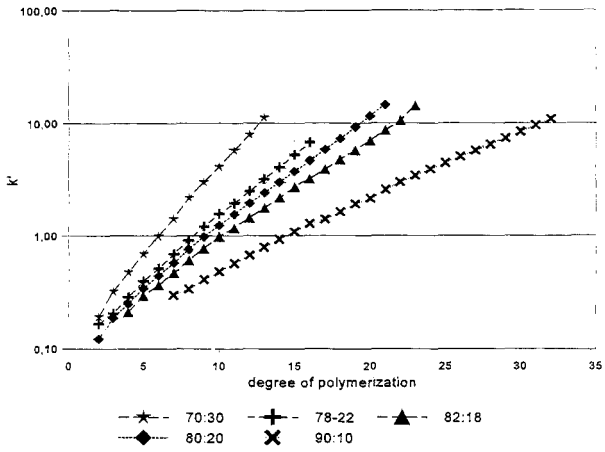

FIGURE 11.

Capacity factors of polypropylene glycols on ODS2-4 in different mobile phase compositions

In Figure 9, the capacity factors of polypropylene glycols on different columns in methanol-water 80:20 are plotted versus the degree of polymerization (the number of PO-units). Again the difference between the columns is not very large.

Figure 10 shows a comparison of data from three ODS2 columns of different dimensions. The data were obtained in the same mobile phase composition, but after a period of 6 or 11 months. As can be seen, not only the reproducibility between the columns is excellent, but also the long-term stability.

In Figure 11, the capacity factors of PPG on ODS2-4 in different mobile phase compositions are shown. The selectivity increases with decreasing water content, as expected.

In a different representation the elution behaviour of PPG is shown in Fig. 12: as already mentioned, 90:10 will only work well with PPG 1000 or 1200, for lower molecular weights 80:20 would be adequate. If the PEG-block of a copolymer is rather short, it might be reasonable to go even below this composition.

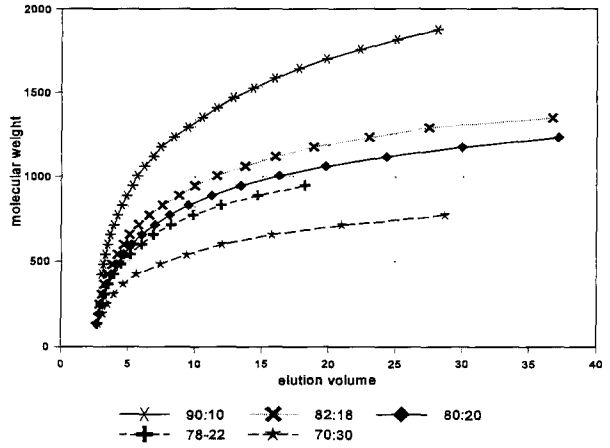

FIGURE 12.

Elution behaviour of polypropylene glycols on ODS2-4 in different mobile phase compositions

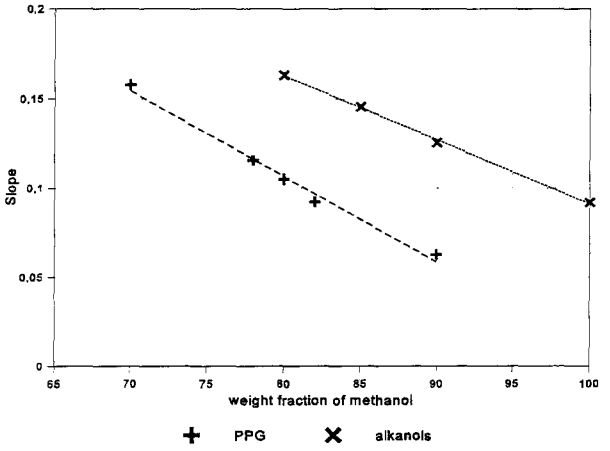

FIGURE 13

Selectivity of methanol-water for polypropylene glycols and fatty alcohols on ODS2

Optimization of the mobile phase:

From the plots shown in figures 7 and 11, we have calculated the slopes B of the regression lines in equation 2, which represent the selectivity of a mobile phase for fatty alcohols and PPG.

In Figure 13, the slopes thus obtained have been plotted versus the composition of the mobile phase. From this diagram one can very easily determine the composition of the mobile phase yielding the required selectivity.

In general, the optimum will depend on the relative lengths of both blocks. If the PEG-block is rather short, only the hydrophobic block determine the mobile phase composition, if it is longer, the best composition must be closer to the critical point of adsorption, as will be shown in further communications.

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THE BOOK CORNER

GAS CHROMATOGRAPHIC ENVIRONMENTAL ANALYSIS - PRINCIPLES, TECHNIQUES, INSTRUMENTATION, by Fabrizio Bruner, VCH Publishers, Weinheim, Germany, 1993, xii + 233 pp., DM 98.00; ISBN: 3-527-28042-1

This book is a welcome addition to the scientific literature, as it is one of the few books available on the applications of gas chromatography to environmental analysis specifically.

The volume consists of five chapters with good illustrations and figures. Each chapter ends with a list of references up to 1992.

The author discusses, in Chapters 1 and 2, the general principles and instrumentation required for gas chromatographic environmental analysis. Chapter 3 is dedicated to mass spectrometry and its usefulness and significance in environmental organic analysis. While Chapter 4 is devoted to the chromatographic analysis of volatile air and water pollutants such as hydrocarbons, sulfur gases, halocarbons, among others. Finally, Chapter 5 deals with sample preparation and analysis of organic micropollutants from complex matrices.

Dr. Bruner discusses, in detail, the techniques required for sample preparation, since he states that "it is useless to exploit even the most sophisticated apparatus when the injected sample does not represent the original one."

This book is highly recommended for analytical chemists in both industrial, academic contexts who are involved in research, in environmental analysis and also graduate students. It is also an excellent reference for governmental research centres, chemical companies.

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CAPILLARY ZONE ELECTROPHORESIS, F. Foret, L. Krivankova and P. Bocek
VCH Weinheim, Germany, 1993, xiv + 346 pages DM 228.00. ISBN: 3-527-30019-8

Capillary zone electrophoresis (CZE) is a powerful analytical technique which recently gained popularity among other separation modalities. It had proven its efficiency in separation and analysis of small ions and molecules and also macromolecules such as proteins, nucleic acid, viruses and cells among others. The book consists of 10 chapters which ends with a list of references upto 1992.

Topics covered include:

- Fundamental concepts and theoretical principles
- Phenomena accompanying electrophoresis
- Practice of capillary electrophoresis
- Instrumentation - principles, components and how to operate it
- Applications

The book is well illustrated as it contains 201 figures and 32 tables. The chapters are clearly presented in a concise format.

The book is recommended for graduate students, analytical chemists in both pharmaceutical and biotechnology industries as well as academic professionals.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 17(19), 4305-4310 (1994)

LIQUID CHROMATOGRAPHY CALENDAR

1994

NOVEMBER 10 - 11: 17th Int'l Conference on Chemistry, Biosciences, Pharmacy & Environmental Pollution, New Delhi, India. Contact: V. M. Bhatnagar, Alena Chem of Canada, P. O. Box 1779, Cornwall, Ont., Canada K6H 5V7.

NOVEMBER 11 - 12: 3rd Conference on Current Trends in Computational Chemistry, Jackson, Mississippi. Contact: J. Leszczynski, Jackson State University, Dept. of Chem., 1400 J. R. Lynch St., Jackson, MS 39217, USA.

NOVEMBER 13 - 16: 50th Southwest Regional Meeting, ACS, Fort Worth, Texas. Contact: H. C. Kelly, Texas Christian Univ, Chem Dept, Ft. Worth, TX 76129, USA.

NOVEMBER 13 - 18: Annual Meeting of the A.I.Ch.E., San Francisco, California. Contact: AIChE Express Service Center, 345 East 47th St., New York, NY 10017, USA.

NOVEMBER 14 - 17: 33rd Eastern Analytical Symposium, Somerset, New Jersey. Contact: Sheri Gold, EAS, P. O. Box 633, Montchanin, DE 19710, USA.

NOVEMBER 16 - 18: Envirosoft'94, 5th Int'l Conference on Development and Application of Computer Techniques to Environmental Studies, San Francisco, California. Contact: Computational Mechanics, Inc., 25 Bridge St., Billerica, MA 01821, USA.

DECEMBER 4 - 6: IBEX'94, International Biotechnology Expo & Scientific Conference, Moscone Center, San Francisco, California. Contact: Cartlidge & Associates, Inc., 1070 Sixth Avenue, Suite 307, Belmont, CA 94002, USA.

1995

MARCH 6 - 10: PittCon'95: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, New Orleans, Louisiana. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

APRIL 2 - 7: 209th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

APRIL 25 - 28: Biochemische Analytik '95, Leipzig. Contact: Prof. Dr. H. feldmann, Inst. fur Physiologische Chemie der Universitat, Goethestrasse 33, D-80336 Munchen, Germany.

MAY 28 - JUNE 2: HPLC'95, 19th International Symposium on Column Liquid Chromatography, Convention Center, Innsbruck, Austria. Contact: HPLC'95 Secretariat, Tyrol Congress, Marktgraben 2, A-6020 Innsbruck, Austria.

MAY 31 - JUNE 2: 27th Central regional Meeting, ACS, Akron Section. Contact: J. Visintainer, Goodyear Research, D415A, 142 Goodyear Blvd, Akron, OH 44236, USA.

JUNE 6 - 8: 28th Great Lakes Regional ACS Meeting, LaCrosse-Winona Section. Contact: M. Collins, Chem. Dept., Viterbo College, La Crosse, WI 54601, USA.

JUNE 14 - 16: 50th Northwest/12th Rocky Mountain Regional Meeting, ACS, Park City, Utah. Contact: J. Boerio-Goates, Chem Dept, 139C-ESC, Brigham Young Univ, Provo, UT 84602, USA.

JULY 9 - 15: SAC'95, The University of Hull, UK, sponsored by the Analytical Division, The Royal Society of Chemistry. Contact: The Royal Society of Chemistry, Burlington House, Picadilly, London W1V 0BN, UK.

JULY 7 - 8: FFF Workshop, University of Utah, Salt Lake City, UT. Contact: Ms. Julie Westwood, FFF Research Center, Dept. of Chem., University of Utah, Salt Lake City, UT 84112, USA.

JULY 10 - 12: FFF'95, Fifth International Symposium on Field-Flow Fractionation, Park City, Utah. Contact: Ms. Julie Westwood, FFF Researcvh Center, Dept. of Chem. Univ. of Utah, Salt Lake City, UT 84112, USA.

AUGUST 20 - 25: 210th ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 12 - 15: 5th International Symposium on Drug Analysis, Leuven, Belgium. Contact: Prof. J. Hoogmartens, Inst. of Pharmaceutical Sciences, Van Evenstraat 4, B-3000 Leuven, Belgium.

OCTOBER 18 - 21: 31st Western Regional Meeting, ACS, San Diego, Calif. Contact: S Blackburn, General Dynamics, P. O. Box 179094, San Diego, CA 92177-2094, USA.

OCTOBER 22 - 25: 25th Northeastern Regional Meeting, ACS, Rochester, New York. Contact: T. Smith, Xerox Corp, Webster Res Center, M/S 0128-28E, 800 Phillips Rd, Webster, NY 14580, USA.

NOVEMBER 1 - 3: 30th Midwestern Regional ACS Meeting, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr., Miami, OK 74354-3854, USA.

NOVEMBER 1 - 4: 31st Western Regional ACS Meeting, San Diego, California. Contact: T. Lobl, Tanabe Research Labs, 4450 Town Center Ct., San Diego, CA 92121, USA.

NOVEMBER 5 - 7: 30th Midwestern Regional Meeting, ACS, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr, Miami, OK 74354, USA.

NOVEMBER 29 - DECEMBER 1: Joint 51st Southwestern/47th Southeastern Regional Meeting, ACS, Peabody Hotel, Memphis, Tenn. Contact: P.K. Bridson, Chem Dept, Memphis State Univ, Memphis, TN 38152, USA.

DECEMBER 17 - 22: 1995 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1996

FEBRUARY 26 - MARCH 1: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

MARCH 24 - 29: 211th ACS National Meeting, New Orleans, LA. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

JUNE 16 - 21: "HPLC '96: Twentieth International Symposium on High Performance Liquid Chromatography," San Francisco Marriott Hotel, San

Francisco, California. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

AUGUST 18 - 23: 212th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

OCTOBER 16 - 19: 52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

OCTOBER 24 - 26: 52nd Southwestern Regional Meeting, ACS, Houston, Texas. Contact: J. W. Hightower, Chem Eng Dept, Rice Univ, Houston, TX 77251, USA.

NOVEMBER 6 - 8: 31st Midwestern Regional Meeting, ACS, Sioux Falls, South Dakota. Contact: J. Rice, Chem Dept, S. Dakota State Univ, Shepard Hall Box 2202, Brookings, SD 57007-2202, USA.

NOVEMBER 9 - 12: 48th Southeast Regional ACS Meeting, Greenville, South Carolina. Contact: H. C. Ramsey, BASF Corp., P. O. Drawer 3025, Anderson, SC 29624-3025, USA.

1997

APRIL 6 - 11: 213th ACS National Meeting, San Antonio, Texas. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 7 - 12: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1998

MARCH 29 - APRIL 3: 215th ACS National Meeting, St. Louis, Missouri. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 23 - 28: 216th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1999

MARCH 21 - 26: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 22 - 27: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2000

MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 20 - 25: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2001

APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 19 - 24: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2002

APRIL 7 - 12: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 13: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

The Journal of Liquid Chromatography will publish, **at no charge**, announcements of interest to liquid chromatographers in every issue of

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1. D. K. Morgan, N. D. Danielson, J. E. Katon, Anal. Lett., 18: 1979-1998 (1985)

Book:

1. L. R. Snyder, J. J. Kirkland, Introduction to Modern Liquid Chromatography, John Wiley & Sons, Inc., New York, 1979.

2. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in HPLC of Biological Macromolecules, K. M.

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8. Each page of manuscript should be numbered lightly, with a light blue pencil, at the bottom of the page

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