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OSMOTIC EFFECTS IN GEL PERMEATION CHROMATOGRAPHY WITH MULTICOMPONENT SOLVENTS

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ABSTRACT

Secondary separation mechanisms are important in a GPC-column when multicomponent eluents are used. The solvent will be at a lower potential in the injected sample than in the eluent, leading to a separation or redistribution of components by the osmotic pressure differences. The smallest components will dominate the separation due to the highest diffusion rates. When charged macromolecules are present, the Donnan equilibrium also contributes to the redistribution of salt. For some surfactant systems, the micellar size is very sensitive to salt concentration, which can give quite complex GPC-chromatograms. In order to discuss GPC-results from polymer-surfactant systems with repulsive interactions, these secondary separation mechanisms must be included.

INTRODUCTION

It is known that components can be separated by gel permeation chromatography, GPC, according to their molecular sizes. In practice, however, various types of interactions occur among the components of the chromatographic system, and as a result, secondary separation mechanisms take place. These interactions and separation mechanisms are important in polymer-surfactant systems applicable for chemical flooding of oil reservoirs. When polymers and surfactants are coinjected in order to improve the oil recovery, phase separation and incompatibility are crucial for the process(1,2). Polymer-surfactant interactions have been studied by a GPC-approach(3-6) that is based on preferential solvation of macromolecules in mixed solvents(7-9). The surfactant solution is used as the eluent and the polymer is dissolved in the eluent and injected into the column. Both associative and repulsive interactions may be present in polymer-surfactant systems depending on charges, hydrophobic groups, etc.(10).

Associative interactions lead to formation of a polymer-surfactant complex, that is usually larger than the pure polymer. This is seen in GPC-chromatograms as a lower elution time for the complex peak than for the polymer peak. Also a negative peak related to the amount of surfactant associated with the polymer occurs. Studies of polymer-surfactant association are described in detail elsewhere(4-6). When it comes to repulsive interactions, the chromatograms for polymer-surfactant systems are more complex(5,6), due to different separation mechanisms and effects of the components in the solvent. Micellar size is affected by salt and very different results can be obtained for surfactant solution in the absence and presence of salt, as will be presented.

In order to distinguish between different secondary separation mechanisms, binary polymer systems have been studied. The eluent is a solution of one of the polymers at an appropriate concentration. Phase separation in the polymer1 - polymer2 solution must be avoided. With low concentrations of polymer, interactions are of little importance, but as will be shown osmotic effects are important. Due to the mobile counterions, charged macromolecules like polyelectrolytes are expected to give larger osmotic effects. For polyelectrolytes, the Donnan equilibrium(11) for salt distribution will also be a main separation mechanism.

The present GPC-method can be compared with dynamic dialysis, where the membrane is omitted. Of the secondary separation mechanisms that have to be considered in these experiments are osmosis and Donnan effect on salt equilibrium. Both these effects are also important in membrane equilibrium. The results from polymer-polymer separation studies illustrate some of the effects seen in the more complex polymer-surfactant systems(5,6). Also chromatograms for pure surfactant solutions in solvents of surfactant with and without salt show large redistribution effects that give insight into the polymer-surfactant systems.

EXPERIMENTAL

Chemicals

Poly (ethylene oxide), PEO4, with average molecular weight of 4 000 g/mole was obtained from Merck. Dextrans, T500 and T10 were obtained from Pharmacia B, Uppsala Sweden, having an average molecular weight of 500 000 and 10 000. Poly(styrene sulfonic acid, sodium salt, 100% sulfonated), PSS500, with average molecular weight 500 000 from Polysciences, Inc.. These polymers were used as delivered. Xanthan, Xc 85-II F4, was produced by Bioferm Statoil, filtered and purified by precipitation with isopropanol and dried. Molecular weight range is $2\text{-}3\cdot10^6$. Sodium dodecylbenzene sulfonate, NaDDBS (Hard type),

D 0990, with Mw = 248.48 g/mole and 95 % active material from Tokyo Kasei Ltd., was used as delivered. NaCl p.a. delivered from Merck was used.

Chromatographic Instrumentation and Conditions.

The HPLC equipment consists of a 600E pump, a 715 Ultra Wisp Sample Processor, a 410 Differential Refractometer, all delivered from Waters. A NEC, APCIV^{TM}, Power Mate 486/33i integrator, a 991M photodiode array detector and a TI microLaser Plus complete the instrumental set-up. The samples were chromatographed on a Waters Ultrahydrogel^{TM} 250, 6μm GPC-column (7.8 x 300mm). Sample volumes have been 20-100μl. Flow rate was set at 0.5 ml/min. The column and the RI-detector was thermostated at 32°C.

Procedures.

The eluent was either a surfactant or a polymer solution depending on what system to be studied. Polymers were dissolved into the eluent at appropriate concentrations to avoid phase separation. All solutions were run through a 0.45μ Millipore filter prior to injection and degassed constantly with He-gas.

RESULTS AND DISCUSSION

The chromatograms in fig.1 show separation of two nonionic polymers, dextran T500 and poly(ethylene oxide) PEO4. Molecular weights are 500 000 and 4 000, respectively. The eluent is 0.60 wt% PEO4 (aq) and the concentration of T500 is 0.35 wt% in all chromatograms.

In a) T500 is dissolved in the eluent and injected. There is little interactions in the system, but a separation of the two polymers is clearly seen. The solvent in the sample is at a lower chemical potential than the solvent in the surrounding eluent. The separation is due to osmosis pressure difference and of course size exclusion. This will be more discussed later and is also illustrated by chromatograms b-d), where the same concentration, 0.35 wt% of T500 is dissolved in a solution with lower concentration of PEO4 than in the eluent, 0.56, 0.50 and 0.46 wt%, respectively. For c) and d) the concentrations were to low and negative peaks are observed. Estimations based on fig.1 indicate that 0.54 wt % of the eluent in the injected sample will provide osmotic equilibrium. Calculations, based on the Flory-Huggins model(12), gave that the chemical potential of water is unchanged if 0.6% PEO4 is replaced by 0.35% T500 + 0.48% PEO4. This is in reasonable agreement with the result in fig.1 where a

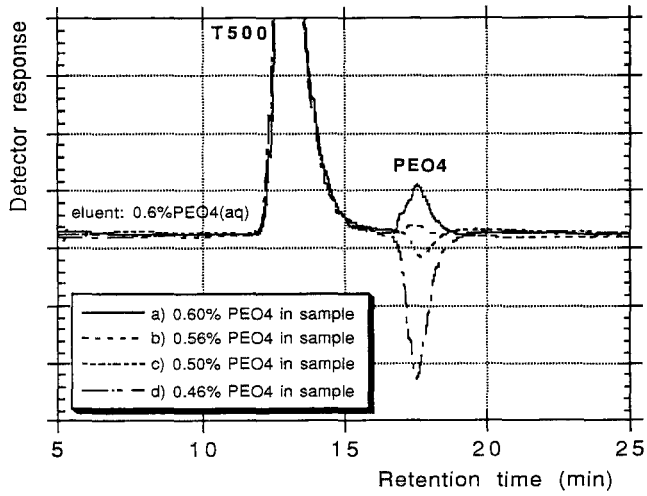

FIGURE 1. GPC-chromatograms where the eluent is 0.6wt% PEO4(aq), in a) 0.35% T500 dissolved in the eluent, in b) 0.35% T500 is dissolved in 0.56% PEO4, in c) 0.35% T500 is dissolved in 0.50% PEO4, and in d) 0.35% T500 is dissolved in 0.46% PEO4. The flow rate is 0.5 ml/min and an RI-detector is used.

concentration of 0.54% PEO4 was needed together with 0.35% T500 to remove osmotic pressure effects. The calculations gave the same result regardless of whether interaction parameters were included or not (interaction parameters were taken from ref.13). With the small concentrations used in the GPC-experiments the effect is dominated by the entropy of mixing and interactions are usually of marginal importance.

The osmotic separation mechanism is illustrated in fig.2. The column is equilibrated by the eluent, which is a nonionic polymer A dissolved in a solvent, s, (e.g.water). Region I represents pure eluent. Region II shows the injected volume of the sample. The sample is a solution of B (another nonionic polymer, $Mw_A < Mw_B$) dissolved in the eluent. It is assumed no net flow in the column.

The chemical potentials of the solvent, s, in the different regions I and II are:

in the eluent, I: $$\mu_S(I) = \mu_S^0 + RT \ln x_S(I) \approx \mu_A^0 - RTx_A \quad (1)$$

in the sample, II: $$\mu_S(II) = \mu_S^0 + RT \ln x_S(II) \approx \mu_A^0 - RTx_A - RTx_B \quad (2)$$

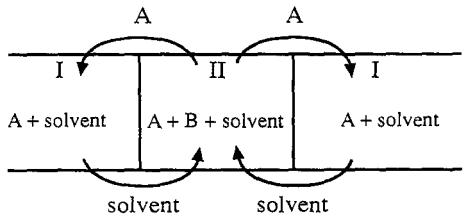

FIGURE 2. A schematic illustration of a GPC-column divided into two regions I and II. The injected sample B is dissolved in the eluent (A+solvent). No net flow in the column.

where x_A and x_B are the mole fractions of A and B, respectively. The chemical potential of the solvent in the sample (region II) is lower than in the eluent. Assuming no net flow the osmotic pressure difference, due to the lower chemical potential of the solvent in the sample, will provide a mainly entropic, thermodynamic driving force for the water molecules to diffuse into the sample to gain osmotic equilibrium. To maintain a constant volume of the injected solution, other components have to diffuse out of the sample. Here A is the smallest component, thereby having higher diffusion rate than B, and is transported away from the sample region. From fig.1 it is seen that by reducing the concentration of A in the sample region (here PEO4) the redistribution of the two nonionic polymers is reduced. It is possible to find an equilibrium concentration of A in the sample that removes the osmotic separation.

If the solvent in fig.2 contains salt, the salt ions will dominate the diffusion from region II to region I, due to the higher diffusion rate. This is experimentally shown in fig.3, where two dextrans, T500 and T10 with molecular weights of 500 000 and 10 000, respectively, are studied in the absence and presence of NaCl. With 0.5 wt% T10 (aq) as eluent 0.25 wt% T500 is dissolved in the eluent and injected. The polymers are separated by the mechanism described above and can also be compared to fig.1a. The osmotic pressure difference between the eluent and the injected sample will provide a driving force for the water molecules to diffuse into the sample to gain osmotic equilibrium. To maintain a constant volume of the injected solution, other components have to diffuse out of the sample. The smallest components will have the highest rate of diffusion and leads to a peak of T10 which has been separated from T500 molecules due to osmosis and size exclusion. The retention time of T10 is the observed retention time for T10 also with water as eluent in the same column and under the same conditions, so a "complete" separation has taken place. With 50mM NaCl added to the eluent, the chromatogram is different as seen in fig.3. The components diffusing from the injected sample are mainly the salt ions. In a sample with several constituents the components with the fastest diffusion will take over the response to give osmotic equilibrium as water diffuses into the sample. As can be seen the T10 peak has almost disappeared.

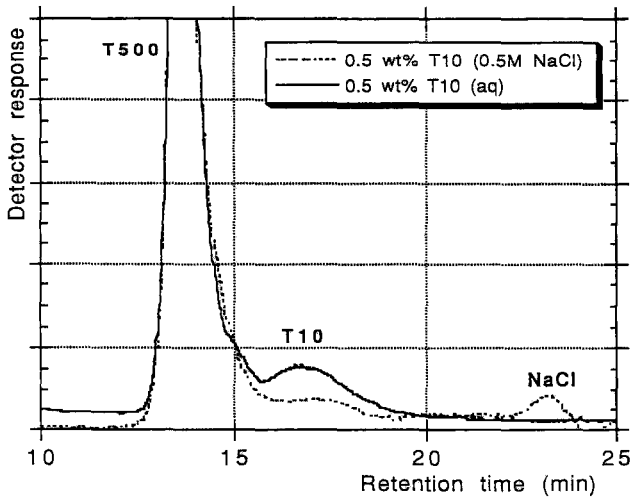

FIGURE 3. GPC-chromatograms, where the eluent is 0.5% T10(aq) (——) and 0.5% T10 in 50mM NaCl (·····). In both cases are 0.25% T500 dissolved in the eluents and injected into the column. The flow rate is 0.5 ml/min and an RI-detector is used.

Fig.4 compares the separation between two nonionic polymers and between a negatively charged polyelectrolyte and a nonionic polymer, both in the presence of salt. In chromatogram a) 0.6 wt% PEO4 in 10mM NaCl is the eluent and 0.25 wt% T500 is dissolved in the eluent and injected into the column. Separation of both PEO4 and NaCl from the larger dextran T500 is seen. The separation mechanism is the osmotic effect described above. NaCl does not dominate the diffusion as much as in fig.3, since the NaCl concentration is lower, 10mM compared to 50mM NaCl. This means that at a high enough salinity, the two polymers will not separate due to diffusion, only the salt will redistribute.

In chromatogram b) in fig.4 the sample is changed from the nonionic dextran, T500, to a charged polymer with the same molecular weight and at the same concentration, namely poly(styrene) sulfonate(Na-salt), PSS500. The eluent is the same, 0.6 wt% PEO4 in 10mM NaCl. Polyelectrolytes will give a larger osmotic effect due to the mobile counterions as is seen in the figure. The charged macromolecules will also influence the distribution of salt between injected sample and surrounding eluent, the Donnan effect. To obtain ion equilibrium across the "invisible membrane" between the injected sample and the eluent, an increased diffusion of NaCl from the sample must take place as is also seen in the figure. The reason for the higher retention time for the PEO4-peak in the polyelectrolyte sample is not understood.

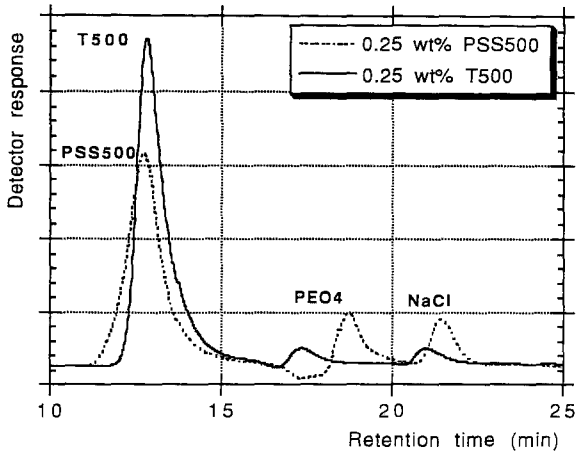

FIGURE 4. GPC-chromatograms where the eluent is 0.6% PEO4 in 10mM NaCl. Samples dissolved in the eluent and injected are 0.25% T500 (——) and 0.25% PSS500 (·····). The flow rate is 0.5 ml/min and an RI-detector is used.

In surfactant systems the consequences of the osmotic redistribution effects discussed above can be much larger. The eluent in fig.5 is 10mM of NaDDBS and the injected samples are a) 7mM NaDDBS and b) 20mM of NaDDBS which, as can be expected, gives one negative and one positive peak. The peaks at about 25 minutes are related to monomers and impurities like salt. If, however, the experiment is repeated after adding 10mM NaCl to the eluent and the injected samples, the result becomes quite different, see fig.6. In fig.6a the result is still a normal negative peak when 7mM NaDDBS is injected, but the injection of 20mM NaDDBS (fig.6b) produces a different and more complex result. The origin of the result in fig.6b can be understood by the chromatogram in fig.7. In fig.7 the eluent is 10mM NaDDBS (no salt) and the injected sample is 0.7mM NaCl dissolved in the eluent. As can be seen a strong negative peak is produced, at about the retention time for the micelles, together with the positive NaCl peak. The negative peak must be the effect of salt concentration on the micellar size. Addition of salt causes a growth in micellar size which will form micelles having a smaller retention time. As the larger micelles are chromatographically separated from the salt, they will start to shrink again and are probably spread out over the column leaving a negative peak behind. In fig. 6b variations in the salt concentration are generated by osmotic redistribution effects due to the higher surfactant concentration in the injected sample. The result can be quite complex as salt and surfactant are continuously redistributed as the different peaks propagate through the column. However, not all surfactants give these complex results since a requirement is that the micellar size is salt sensitive. For instance some ethoxylated sulfonates have been found to give

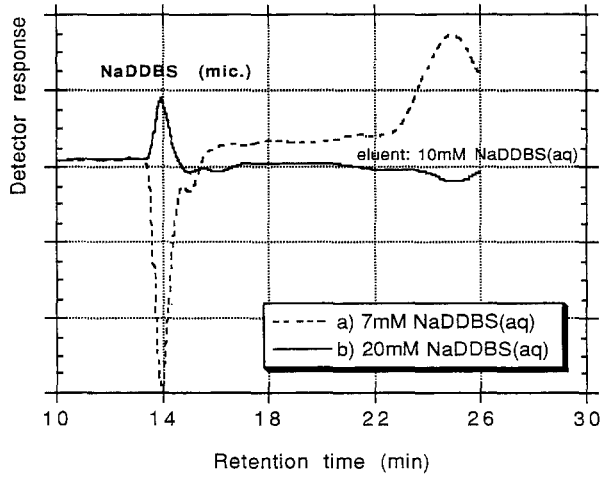

FIGURE 5. GPC-chromatograms where the eluent is 10mM NaDDBS(aq), in a) 7mM NaDDBS(aq) and in b) 20mM NaDDBS are injected. The flow rate is 0.5 ml/min and an RI-detector is used.

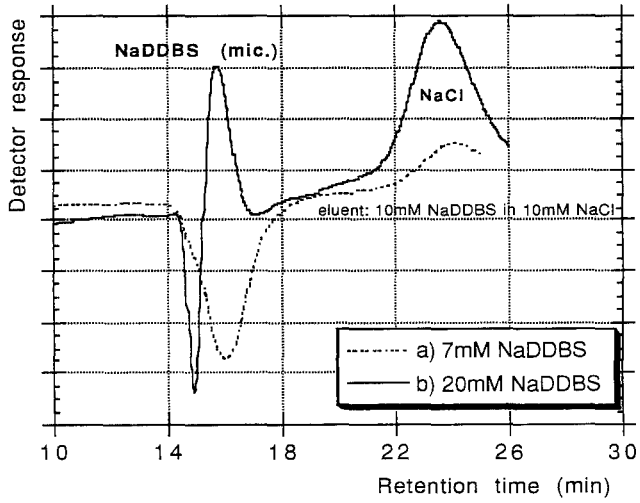

FIGURE 6. GPC-chromatograms where the eluent is 10mM NaDDBS in 10mM NaCl, in a) 7mM NaDDBS and in b) 20mM NaDDBS both in 10mM NaCl, are injected. The flow rate is 0.5 ml/min and an RI-detector is used.

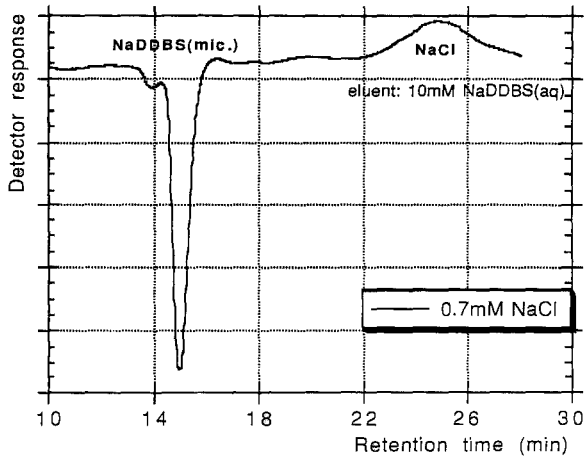

FIGURE 7. GPC-chromatogram where 10mM NaDDBS is the eluent. The injected sample is 0.7mM NaCl. The flow rate is 0.5 ml/min and an RI-detector is used.

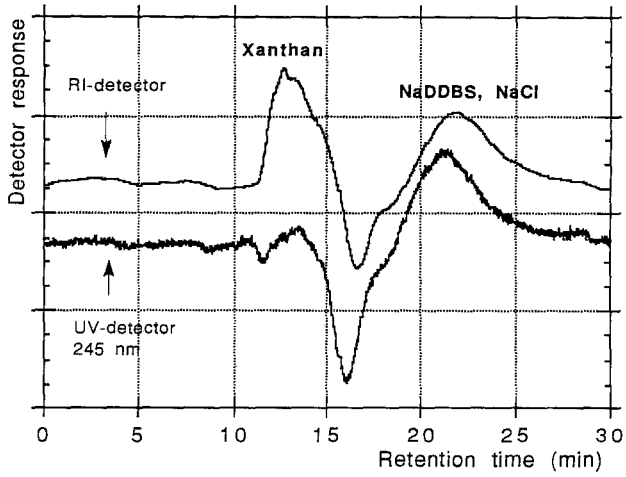

FIGURE 8. GPC-chromatograms where 10mM NaDDBS in 10mM NaCl is the eluent. 0.35% xanthan is dissolved in the eluent and injected. The flow rate is 0.5 ml/min and both an UV(245nm)- and an RI-detector are used.

"normal" chromatogram (data not shown) also with salt present in the eluent as higher or lower concentrations of surfactant are injected.

An example of polymer-surfactant chromatograms where the secondary separation mechanisms are large is shown in fig.8. The anionic biopolymer xanthan is dissolved in the eluent consisting of 10mM NaDDBS and 10mM NaCl. NaDDBS is an anionic surfactant, so there are repulsive interactions between the polymer and the surfactant micelles. Fig.8 shows chromatograms detected both with RI and UV(245nm). The xanthan peak is not detected by the UV-detector, but a redistribution of the NaDDBS micelles is clearly seen. Based on the results from figs.5-7, all effects; osmosis, Donnan effects and the salt effect on micellar size take part in these separations. Since secondary separation mechanisms are present also in nonionic systems electrostatic repulsions will not contribute to the total separation.

CONCLUSIONS

Using multicomponent solvents or eluents in a gel permeation chromatography column secondary separation mechanisms must be carefully considered. Osmotic pressure differences between the injected sample and the eluent will lead to separation of components even though the column is equilibrated with one of the components. The smallest components dominate the separation due to the highest diffusion rate. Charged macromolecules will give a lager osmotic effects due to the mobile counterions. Salt will redistribute due to Donnan equilibrium when polyelectrolytes are present. For surfactant solutions the salt redistribution may have large consequences, especially when the micellar size is very salt sensitive. The secondary separation mechanisms must be known to be able to understand the complex chromatograms obtained for polymer-surfactant systems having mutual repulsive interactions.

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DETERMINATION OF THE DEGREE OF POLYMERIZATION OF AGAR-TYPE POLYSACCHARIDES BY A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD

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ABSTRACT

A reversed-phase HPLC methods in combination with Diode Array Detection is described for separation of polysaccharides in acid hydrolysates from many different agar-type polysaccharides. Temperature is optimized at 30°C. The detection limit is about 5 pmol. Sugars show a linear response in a wide concentration range. This technique is able to resolve the different oligomers according to the respective degree of polymerization (DP) in a very rapid, easy, reliable and reproducible way.

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INTRODUCTION

The molecular weigth distribution is very important for characterization of natural and synthetic polymers in biochemistry, agriculture and the food industry. Given that the molecular weight of polysaccharides seem not be controlled during biosynthesis, a large range of components of different molecular weight will be formed (1).

Molecular weights and different structures of agar-type polysaccharides are specially important since the gel strength, gelling and melting temperatures are determinant properties for its use in biochemical techniques (immunodiffusion and diffusion; electroforesis of proteins, nucleic acids and polysaccharides; immunoelectrophoresis, electrofocusing, affinity chromatography, chromatofocusing, ...) and microbiological techniques (culture media) (2,3).

Several types of methods have been employed for determining molecular weights. Classical methods based on osmotic pressure, viscosity, light scattering and streaming biofringance measurements are not used appreciably at present. More recently new tecniques based in partition chromatography on cellulose or ion-exchange resins, separation of saccharides in the form of borate complexes on anion exchangers, gel filtration on cation exchangers or polyacrylamide gels and chromatography on silica with chemically bonded amino phases have been used (4), but only if the molecular weight of the polysaccharides are reasonably large can be effectively carried out (5,6).

The use of refractive index (IR) detectors (7,8) and UV detectors (4) are widely employed. In this study a reversed-phase HPLC in combination with Diode Array detection has been used for the separation and quantitative

determination of the different hydrolyzed oligomers forming agar polymers. The simplest and ideal methods of detection are those, that require none chemical reaction of the sugars, being detection of separated sugars without previous derivatization.

MATERIALS AND METHODS

Reagents

The oligosaccharides have been separated using water distilled and deionized water, filtered through GS Millipore membranes (0.22 μm pore size; Molshem, FRANCE). Samples were filtered through GV-Millex 013 Millipore membranes (0.22 μm pore size; Bedford, MA, USA).

Galactose, sucrose and maltooligosaccharides were purchased by Sigma. Agarose and Agar of *Gelidium* used as black control have been furnished by Hispanagar S.A. as aditional material for this research.

Apparatus

A 1090 HP Hewlett-Packard Liquid Chromatograph system (Waldbroon, Germany) equipped with a Diode Array Detector System, a Rheodyne loop injector of 20 μl and a Supelcosil LC-18 column, 15 cm x 4.6 mm (5 μm packing) and a W/guard (Supelguard LC-18, 2 cm x 4.6 mm, 5 μm packing; Supelco, Inc., Bellefonte, PA, USA) columns were used.for the chromatographic separation of the derivatives. A Hewlett-Packard System

consisting in a Workstation plus a Operation Software was employed to continuous on-line quantification of chromatographic peaks. UV wavelength for detection was of 190 ± 4 nm.

Biological samples

Gelidium sesquipedale (Clem.) Born et Thur., a red algae, has been the biological material for the extraction of the cell-wall polysaccharides. Algal treatment and cell-wall polysaccharides extraction was carried out as reported previously by Santos and Doty (9) modified by Torres et al. (10).

Sample preparation

Polysaccharides (0.015 g) were hydrolysed with hydrochloric acid (0.01 M, 1 ml) for 10 and 24 hours in a water bath (100°C). The resulting solution was keep to reach room temperature and then filtered through GV-Millex 0.13 Millipore membranes.

Chromatographic conditions were described by Vratny, P., et al. (4) with minor modifications, omitting the following addition of absolute ethanol to allow precipitation of polisaccharides. The flow rate was 1.2 ml/min. Analysis were carried out with 20 μl of sample.

Chromatographic conditions (Vratny, P., 1983):

Equipment for liquid chromatography manufactured by Laboratorní Prístroje (Prague, Rep. Czech) was used in the experiments. The instrument

consisted of a high-pressure HPP 4001 linear pump, an LCI-02 septum injection valve and an LCI-20 stop-flow valve with and RIDK 101 refractometric detector. A Supelcosil LC-18 column, 25 cm x 4.6 mm (5 μm packing) and a W/guard (Supelguard LC-18, 2 cm x 4.6 mm, 5 μm packing) columns were used.

To preparate the sample, add 1 g or starch to 10 ml of 0.2 M trifluoroacetic acid. Heat at 100 ^{o}C for one hour. Cool, then add 90 ml or absolute ethanol and store at -70 ^{o}C to allow precipitaded saccharides to settle. Decant the supernatant and remove all remaining traces of ethanol by evaporation under nitrogen (ethanol interferes with retention of the larger, DP5, oligomers). Redisolve the dried residue in 10 ml of deionized water and filter through a 0.45 μm filter. Store frozen samples until analysis. Glucose oligomers were obtain from corn syrup by precipitating them with ethanol as described above.

RESULTS

Oligosaccharides have been separated by a reversed-phase liquid chromatography method, in a very easy, rapid and reproducible manner, resolving the different compounds according to the respective DP (11,12). After injection of a polysaccharide-free sample there is disturbance of the detection baseline a which has been identified agreeing with Vratny as a peak of solvent. The monomeric compounds of galactose are detected from the second peak.

The separation of oligosaccharides has been achieved by optimizing the column temperature at 30°C, at this temperature the rate of interconversion between the α- and β-anomers of polysaccharides increase the presence of double peaks.

The standard calibration curve of galactose and sucrose units is showed in figure 1. Sugars showed a linear response in the concentration range 20 pmol-12000 nmol. The detection limit obtained by this method was about 5 pmol.

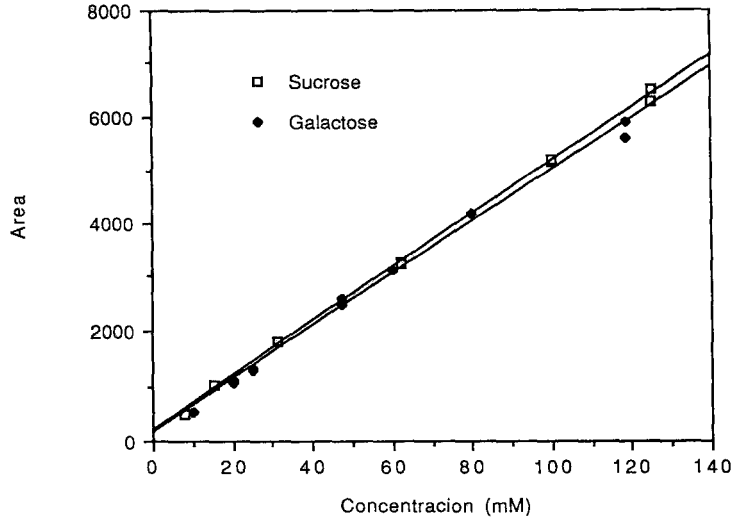

FIGURE 1: Standard calibration curve of galactose and sucrose units. Both of them are very similar, and showed a linear response in the concentration range 20 pmol-1200 nmol.

In table I appears the calculated concentration of degrees of polymerization corresponding to samples hydrolysed for 10 hours, from thallus, apical tips, unpurified agar from *Gelidium*, Bacto agar, agar and agarose from *Gelidium*. High concentrations of monomers and DP12 are observed, and couple monomers but no odd chain oligomers are detected.

Figure 2 shows a typical chromatogram obtained from maltooligosaccharides. The chromatographic retention times for the oligomers DP 4 to DP 9 were from 1.5 until 7 minutes.

Chemical hydrolysis for b-C1-C4 link is easier than a-C1-C3 (2), by this reason oligosaccharides are detected. In figure 3, appears the concentration at

TABLE 1

Concentration (mM) of Degrees of Polymerization Corresponding to Samples, after 10 Hours of Hydrolysis, from Agarosa from *Gelidium*, Agar, Bacto Agar, Tallus, Apical Tips and Unpurified Agar (Whole Plant). Retention Times Are Expresed + S.E.M., from at least 4 Different Assays.

DP	T.Ret.(min)	Agarose	Agar (Hispanagar)	Bacto Agar	Agar (Tallus)	Agar (Apical)	Agar (Whole plant)
1	1.45±0.03	988.77	1012.19	1100.78	1064.75	1061.27	1108.15
2	1.83±0.02	3.27	2.50	13.60	2.99	6.61	4.03
3	1.92±0.01						
3	2.09±0.12	13.60	10.00	30.91	10.00	6.89	32.09
4	2.28±0.04						
4	2.39±0.01	8.03	6.62	7.76	9.32	49.57	21.99
6	3.51±0.05						
6	3.83±0.05	2.90	8.50	2.89	2.03	2.88	60.73
8	5.81±0.20						
8	6.05±0.20	34.99	12.88	9.46	14.34	10.80	7.95
10	10.97±0.75						
10	12.57±0.18	–	1.33	–	0.70	–	–
12	12.98±0.30						
12	15.24±0.45	1430.45	1093.36	1005.61	1004.37	1452.91	1398.88

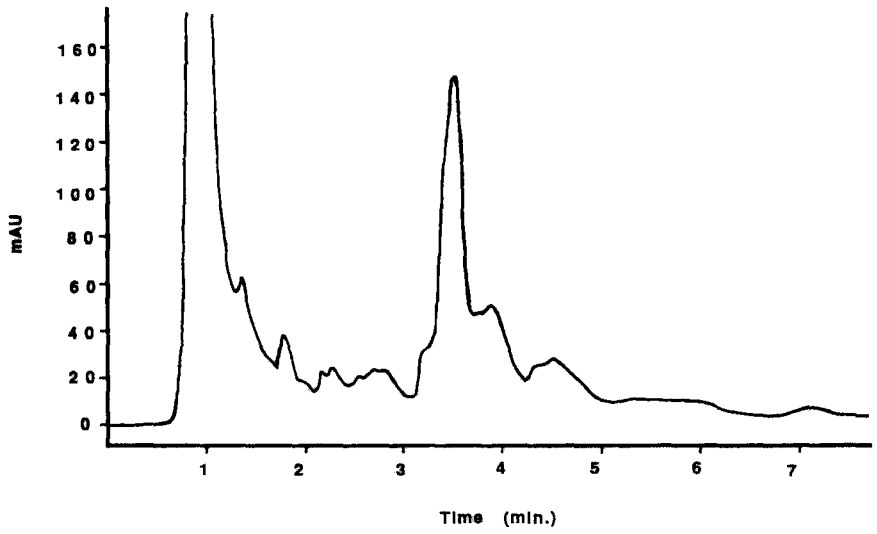

FIGURE 2: Chomatogram from maltooligosaccharides. We can see the typical double peaks for each respective degree of polymerization (DP). Retention times are expressed in table 1.

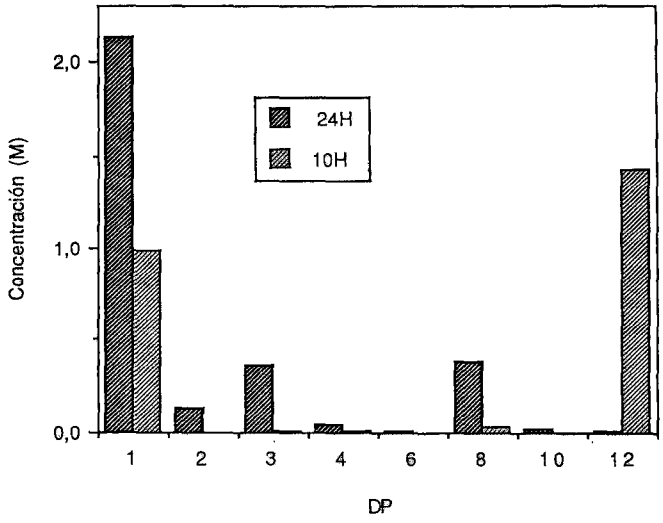

FIGURE 3: Concentration of the different DP after 10 and 24 hours of acid hydrolysis. Larger oligosaccharides are present at the minor hydrolysis time. Mainly oligosaccharides couples monomers are obtained in both of them, 10 and 24 hours.

different degree of polimerization after 10 and 24 hours of acidic hydrolysis. The appearance of that larger oligosaccharides is formed early in the analisis.

Each oligomer elution time has been calculated using five different samples. The variation coefficient (C.V.) was less than 5%. The sensitivity of the technique was similar than those reported by other authors (4).

DISCUSSION

The main effort was directed towards an exhaustive study of separation and quantification of agar by HPLC technique. Using the HPLC procedure described, we resolved DP1 to DP12 galactose oligomers without pre- or post-derivation. The used column provides several advantages, greater efficiency, faster separations and better resolution of the larger oligomers than other methods (11).

Agarose links are very resistant to enzymatic hydrolysis by bacteriane α- and β-agarases, so, acidic hydrolysis has been used to obtain the structural monomerics. To avoid interferences with retention times of oligomers larger than DP5 the latter addition of ethanol, was eliminated. UV detectors are very sensitive at low wavelengths (190 nm) and remain unaffected by temperature and pressure variations. In this method two additional important advantages are the use of a pressure-stable stationary phase, and a neutral and non-toxic cheap aqueous eluent.

In summary, our method allows the separation and the quantitative determination of the DP1-DP12 oligomers from galactose agar-type polysacharides. Thus, molecular size can be deduce and subsequent rheological properties could be established.

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NEW ORGANIC MONOSIZED MICROSPHERES FOR USE IN ENANTIOMER SEPARATIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Monosized nonporous resin particles were prepared in aqueous medium by polymerisation of urea with formaldehyde in the presence of

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soluble Fe(III) hydrolysis polymers. Measurements of the geometrical parameters showed that the material particle size distribution was in the range 2.5-3.5 μm (average particle diameter 2.99 μm with standard deviation 0.49), and had a sphericity index of 0.851. Particle pore-size was determined by gel permeation chromatography. The microspheres exhibited exceptional mechanical strength, chemical stability in the pH range between 1-13, and low tendency toward swelling in the solvents commonly used in liquid chromatography. The synthesis of a chiral urea-formaldehyde based resin for the enantiomer separation of underivatized phenylalanine by ligand exchange chromatography is reported.

INTRODUCTION

Silica-bonded packings are widely used in high performance liquid chromatography (HPLC), although these materials have certain disad_ vantages, which limit their use and shorten column lifetimes. These restrictions are mainly related to the Si-O-Si-C bonds, which are unstable in acidic and basic aqueous solvents, and to irreversible adsorption of basic solutes due to the residual surface silanol groups (1). Attempts to overcome such drawbacks were made by developing silica particles coated with a polymeric layer (2-4). These sorbents, which exploit a combination of mechanical rigidity and chemical stability, show low efficiency and loading capacity due to the difficulty of obtaining uniformly distributed layers inside the porous structure of the particles. Recent improvements in polymer technology have led to the production of highly cross-linked polymeric packings, that match rigidity and low swelling properties, and allow high eluent flow rates and possess excellent mass transfer properties (5-9). Procedures for the production of monodispersed polymer particles have been described in detail (10,11). Most of these stationary phases are formed from cross-linked polystyrene-

divinylbenzene. They can easily be functionalized by introducing them into network reactive groups (12), or using them without functionalization for reversed-phase chromatography. Other polymer packings currently available are the poly(acrylate)-based ones. Because of the presence in their matrix of labile ester linkages, polyacrylic sorbents can be useful over a restricted pH range; however, these materials exhibit higher chemical stability than silica gel. Polymeric sorbents have been designed to solve specific problems in liquid chromatography, i.e. the separation of underivatised amino acids, amines, biopolymers, carbohydrates and inorganic ions, as well in steric exclusion chromatography. Another field of great interest is the development of chemically stable chiral stationary phases (CSPs). Owing to the high cost of most of the chiral selectors, the potential of virtually indestructible resin-type CSPs appears to be enormous, even in view of their use for preparative scale separations. Therefore, as an alternative to silica gel packings, a number of CSPs made of synthetic and natural polymers are suitable for low pressure chromatography (13-18). However, very few chiral polymeric sorbents for HPLC have so far been designed (19-21), even though a number of chiral selectors, such as cyclodextrins, proteins, ion-pairing agents, etc. may exhibit high enantiodiscrimination when used in the presence of alkaline aqueous eluents (22).

Recently we reported the synthesis and chromatographic evaluation of a new kind of CSP consisting of non porous (NP) and rigid polyamide particles, obtained by copolymerization of urea (U), formaldehyde (F) and a chirally selective monomer, L-leucinamide (23). This packing showed excellent chemical stability and specific enantioselectivity toward a series

of carboxylic acid racemates, but low column efficiency, due to the poor size uniformity of the particles. In the present work we deals with an improved procedure for the production of U-F based CSPs. Firstly, NP microbeads with narrow particle size distribution were prepared by adding to the reaction mixture of water soluble Fe(III) hydrolysis polymers. The synthesis was then extended to the preparation of a chiral sorbent containing (+) L-2-amino-5-ureidopentanoic acid moieties bonded to the polyamide matrix. The resolution of underivatized phenylalanine by ligand-exchange chromatography is reported.

EXPERIMENTAL

Chemicals

$Fe(NO_3)_3 \cdot 9H_2O$, sodium bicarbonate, propylene glycol, urea, formaldehyde (40 % m/v) and copper (II) acetate were purchased from Carlo Erba (Milan, Italy). Other chemicals and solvents of reagent grade were obtained from Merck (Darmstadt, Germany). DL-, D- and L-phenylalanine, and (+) L-2-amino-5-ureidopentanoic acid were from Sigma (St. Louis, MO, USA). n-Alkanes, and tetrahydrofuran (THF) and methanol of HPLC grade were purchased from Fluka (Buchs, Switzerland). Water was filtered through Millipore (Bedford, MA, USA) type GS (0.22 μm) filter-disks.

Synthesis of monosized particles

Two spherical microparticulate materials were synthesised according to the following procedures.

Packing I. To 10.7 g of $Fe(NO_3)_3 \cdot 9H_2O$ (26.5 mmol), dissolved in 50 ml of bidistilled water, were added under stirring small aliquots (300 mg)

of sodium bicarbonate for a total of 5 g, the final pH of the red solution was 2.20. Then to the solution were added propylene glycol (150 ml), urea (13.5 g, 0.225 mol) and 40% m/v formaldehyde (40 ml, 1.06 mol). Viscosity measurements of the mixture at 25 °C gave δ = 1.072 g/cm^3 and η = 8.22 cP (dynamic viscosity). The solution was transferred to a round bottomed glass flask connected to a rotary flash evaporator, without applying vacuum, and allowed to react at room temperature overnight. The material was collected on a filter, washed with water, and then treated under stirring with 50 ml of 3 N HCl for 20 min, filtered and washed successively with water, water-methanol (1:1), methanol and acetone. After drying under vacuum at 40 °C, 5.30 g of resin were obtained. Elemental analysis gave : N = 30.83; C = 29.34; H = 5.48.

Packing II. Spherical particles containing a chiral monomer were prepared according to the procedure described above using 14.8 g (0.08 moles) of (+) L-2-amino-5-ureidopentanoic acid, 5.1 g (0.084 moles) of urea and 30 ml (0.8 moles) of 40% (v/m) formaldehyde. Elemental analysis gave : N = 16.17; C = 52.28; H = 6.88. Acid-base titration (24) showed that the resin contained 1.7 mequiv. of COOH groups per gram.

Column packing

Column packing was obtained by the high performance slurry technique, commonly used for silica-based sorbents. A suspension of resin (3.5 g in 25 ml methanol) was forced at 6000 p.s.i. through stainless-steel tubes (150 x 0.46 cm I.D.) using methanol as pressurising solvent.

Particle and pore size measurements of packing I

Particle geometric parameters were measured on a Model VIDS IV (Analytical Measuring Systems, Cambridge, UK) image analyser, equipped with a 286 Unibit IBM computer and a VIDS IV software, and

with a Model TK-870E camera. The analyser was connected to a Model MP 3502M (Swift & Son, London, UK) trinocular polarising microscope, with a 500 x magnification lens. Data obtained on 51 randomly selected beads were elaborated using STAT graphic software. A sphericity index value of 0.851 and an average diameter of 2.99 μm (with standard deviation of 0.49) were obtained.

Scanning electron micrographs (SEM) were performed on a Cambridge 100 scanning secondary electron microscope using the gold-sputtered procedure.

Pore-size properties were investigated by gel permeation chromatography (GPC) (25). The following procedure was used: the column was connected to the solvent delivery pump and to a refractive index (RI) detector (Water Associates, Model R401) and then conditioned with anhydrous tetrahydrofuran. Solute standards included n-alkanes (n-pentane, n-hexane, n-decane, n-dodecane and n-pentadecane), and a poly(styrene) standard with narrow molecular weight range of 20.000 (Aldrich). Duplicate injections of each aliphatic hydrocarbon gave retention volumes similar to V_0 obtained for poly(styrene) standard.

Chromatography

The liquid chromatographic system consisted of a Perkin Elmer (Norwalk, CT, USA) Model Series 400 solvent delivery pump equipped with a Rheodyne 7125 injection valve, and connected to a Varian (Walnut Creek, CA, USA) Model 2550 variable wavelength detector. Chromatographic data were acquired using a PE Nelson Model 1020 Plus PC-based integrator (Perkin Elmer) or recorded on a Shimadzu (Kyoto, Japan) Chromatopac CR3A integrator.

The mobile phase used for the enantiomer separations consisted of 0.05 M acetate buffer prepared from acetic acid solutions, the pH was adjusted to 8.5 with potassium hydroxide, and to the eluent a small amount of copper (II) acetate was added (0.1 mM). Before use the eluent was filtered through a Millipore MF filter (0.45 μm) and degassed with helium. After washing with water, the column was loaded with copper (II) ions by flushing with a 0.05 M copper (II) acetate aqueous solution.

Samples were prepared by dissolving suitable amounts of D-, L- and DL-phenylalanine in the eluent in order to obtain a concentration of approximately 5 mg/ml. Sample volumes of about 2-4 μl were injected for the experiments.

RESULTS AND DISCUSSION

Two main steps are known to occur in the polymerisation between formaldehyde and urea to form resins (26). The first is an addition reaction of formaldehyde to introduce the hydroxymethyl group and is illustrated in the equation below:

$$R\text{-}NH_2 + HCHO \longrightarrow R\text{-}NHCH_2OH$$

The second step involves the linking together of monomer units with the formation of linear polymer chains. This condensation reaction is catalysed by acids.

$$n\,NH\text{-}CH_2OH + n\,H_2NR \longrightarrow \longrightarrow (\text{-}HNCONHCH_2NHCONHCH_2\text{-})_{n/2} \qquad (1)$$

Acidic groups contained on the surface of inorganic polymer materials (for example silanols in silica gel) are known to favour reaction *(1)* with the formation of spherical shaped agglomerates coarceving the solid particles. According to this reaction, Iler (27) developed a method for the production of porous silica gel particles for HPLC, and, using a similar procedure, Stout *et al.* (28) reported the preparation of macro porous microspheres for SEC, the porogenic sol silica being subsequently removed from the agglomerates with hydrofluoric acid. In the present work we used water soluble Fe(III) hydrolysis polymers as the catalysing hydroxides.

Several metal ions are known to yield soluble polymers as intermediates in the precipitation of insoluble hydroxide (29). Hydrolysis polymers have been extensively studied by gel filtration chromatography and identified as polydispersed products with high molecular weight (30-33). Owing to the acidic character of such inorganic compounds, urea-formaldehyde polymers were expected to grow around the Fe(III) hydrolysis species, giving rise to monosized particles with high mass compactness. Data related to the geometric parameters of 51 randomly chosen particles (Packing I) were statistically processed and are reported as a frequency histogram in Figure 1. Scanning electron micrography (SEM) of the particles are shown in Figure 2. Figure 2b shows the microporous surface structure of beads at 24 K magnification.

Microporosity was confirmed by gel chromatography of a series of different molecular weight hydrocarbons and poly(styrene) standards. As expected, the compactness of linear polymer agglomerates gives the sorbent excellent mechanical strength. Figure 3 shows the back

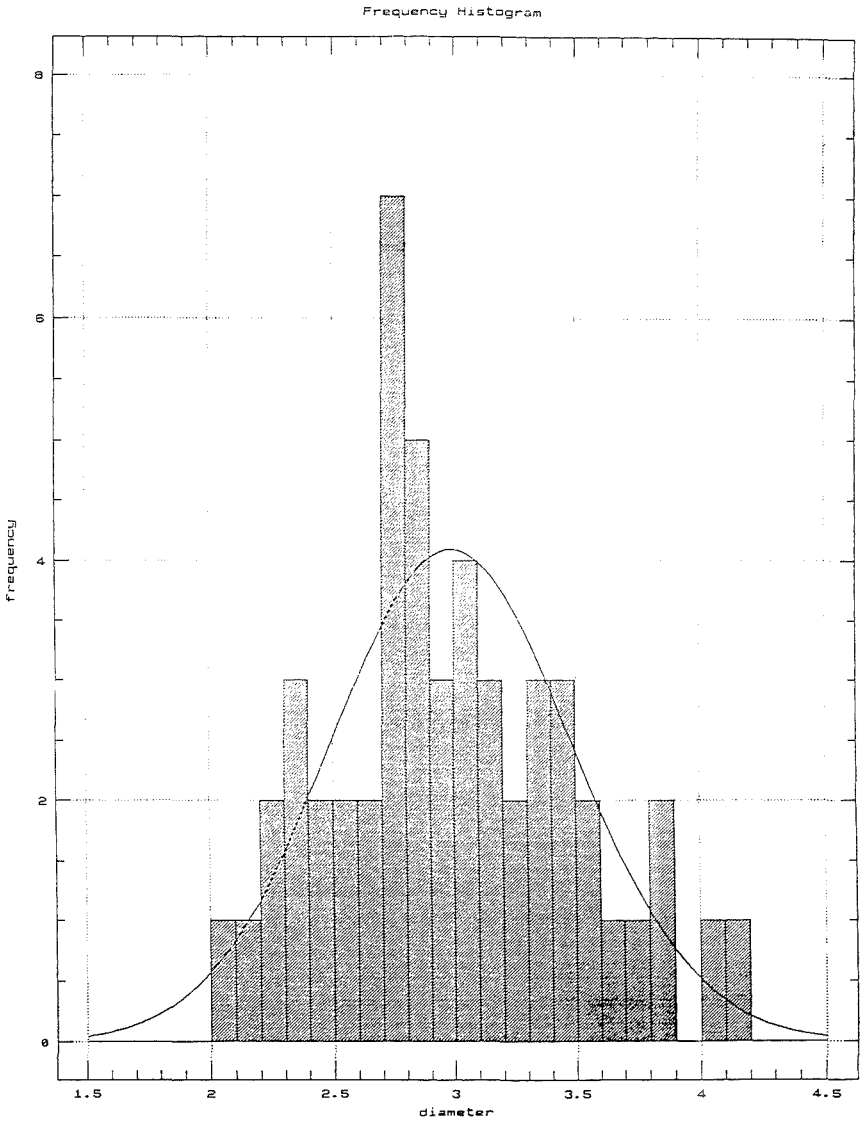


FIGURE 1: Particle size diameter distribution of the urea-formaldehyde resin represented as frequency histogram.

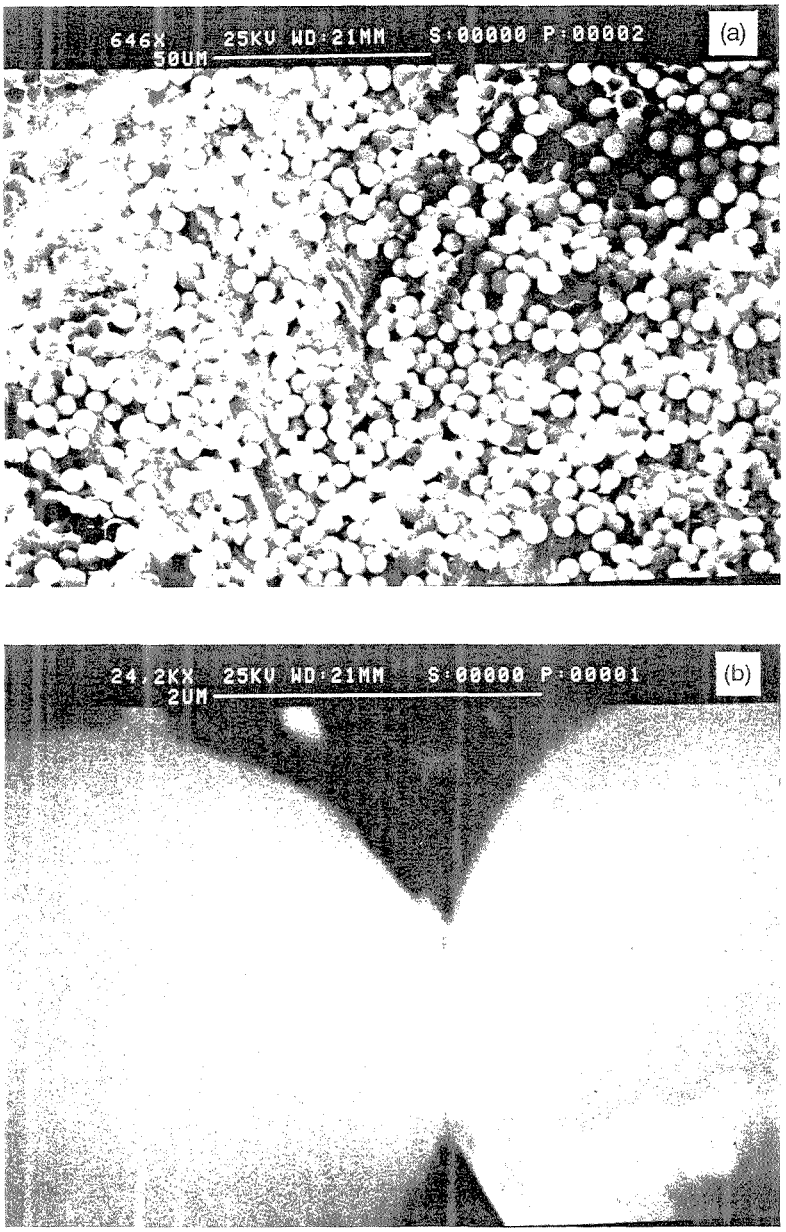


FIGURE 2: Scanning electron micrography (SEM) of the (a) spherical polyamide gel particles; (b) surface structure, performed at 24 K magnification.

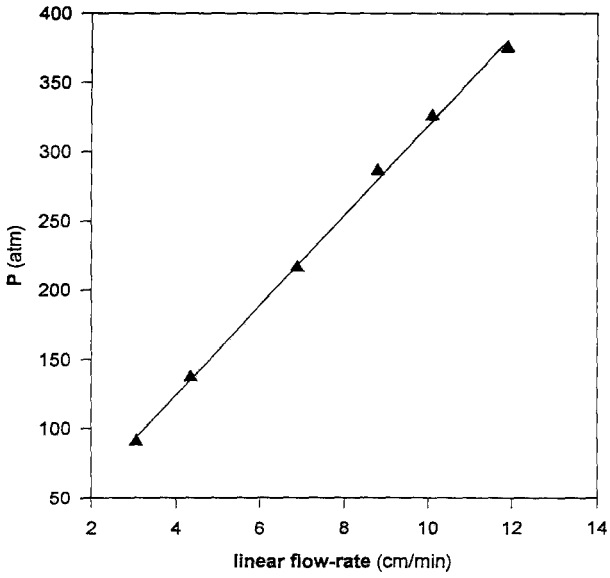


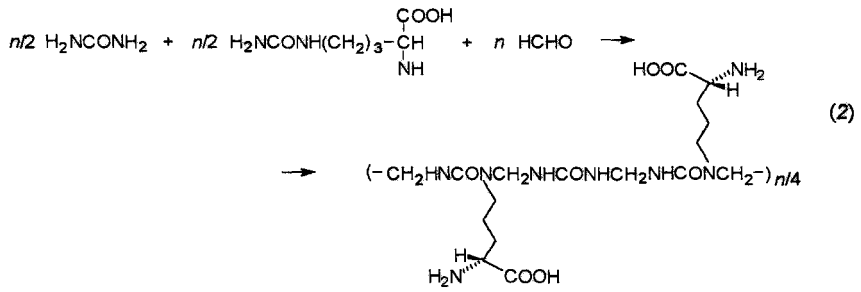
FIGURE 3: Pressure plot for a column packed with urea-formaldehyde resin *vs.* linear flow-rate. Eluent, water-methanol (1:1 v/v).

pressures of a packed column as a function of the linear velocity using a water-methanol mixture (1:1 v/v) as eluent. This plot is comparable to that found when using silica based packings.

Two procedures can be used for the functionalisation of the sorbent : a) grafting the superficial matrix by acylation and conversion of polyamide moieties to the corresponding iminium salts (34) or b) direct copolymerization of urea and formaldehyde in the presence of an urea-derivative selector. The second method appears of greater applicability and reproducibility of yield because it avoids a multi-step modification of the original structure and insures an uniform distribution of the bonded species. In both cases modified sorbents may exhibit selectivity only by

the superficial bonded ligands, the inner ones being masked from the gel's non porous morphology.

The copolymerization procedure was used to prepare a packing containing an amino acid derivative, (+) L 2-amino-5-ureidopentanoic acid as the chirally selective monomer (packing **II**). According to reaction (*1*) monomers are assumed to copolymerise as in the reaction below :



(*2*)

Elemental analysis and acid-basic titration measurements showed that only a part of the chiral selector reacts in accordance with equation (*2*), and is available for the enantiodiscriminative process. However, ligand coverage was far higher then that commonly obtained by grafting reactions on silica gel (35), and appears comparable to the high yield of surface modification of poly(styrene) beads (12).

Figure 4 shows the resolution of free DL-phenylalanine on packing **II**, and the enantiomer elution sequence, L > D. Owing to the lack of hydrophobic interaction on the polyamide matrix (data not reported), the complexation interaction between the enantiomer analytes and the bonded Cu(II)-ligand complexes seems to be the dominant one, and the chiral recognition similar to that proposed by Davankov to explain the

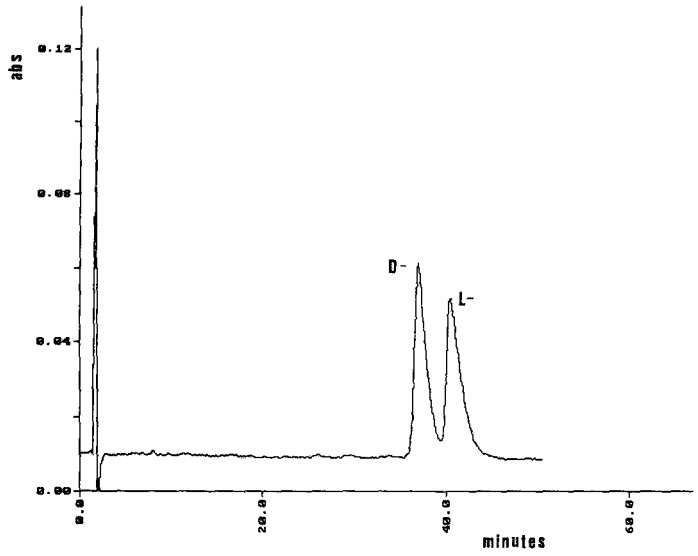


FIGURE 4: Enantioseparation of underivatized phenylalanine. Column (150 x 4,6 mm I.D.) packed with (+) L-2-amino-5-ureidopentanoic acid-urea-formaldehyde resin. Eluent, 0.05 M potassium and 0.1 mM copper (II) acetate buffer at pH 8.5; flow-rate, 0.8 ml/min; detection, UV at 254 nm; room temperature.

resolution of underivatized amino acids on grafted polyacrylamide-type resins (36). According to this model, the =C=O carbonyl group present in the selector moiety probably occupies the lower axial site of the Cu(II) coordination sphere, hindering the rotation of the D-amino acid and side chain, and, thus decreasing its retention in comparison with the L-enantiomer. Chromatographic data are summarised in Table I. A resolution (R_S) value of 1.32 appears reasonably high as in the separation of free amino acid enantiomers.

Concluding, urea and formaldehyde were found to copolymerize in monosized microspheres that exhibited excellent properties as packing

TABLE 1

Chromatographic data for the separation of underivatized phenylalanine enantiomers. Column, 150 x 0.46 cm I.D.; eluent, 0.05 M potassium and 0.1 mM copper (II) acetate buffer at pH 8.5; flow-rate, 0.8 ml/min; UV detector, 254 nm; room temperature.

k'_D	k'_L	α^a	N^b	$R_S{}^c$
20.1	22.1	1.1	3691	1.32

[a] $\alpha = k'_L / k'_D$; [b] $N = 5.545 (t_R / W_h)^2$, refereed for the second eluted peak; [c] R_S, calculated from the fundamental resolution equation.

for high-performance liquid chromatography. Because a large number of mono- and polyfunctional amides are commercially available or can easily be synthesised, several new stationary phases with a wide range of selectivity could be produced. Owing to the low cost of the starting compounds, the procedure described in the present work appears of considerable interest in the development of new CSPs to be used for preparative scale separations.

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UNUSUAL ANALYTE ADSORPTION EFFECTS ON INERT LC COMPONENTS

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Abstract

The use of injector loops made of the inert polymer PEEK™ was studied for the analysis of non-steroidal anti-inflammatory drugs (NSAID) using microbore LC with peak compression. Surprisingly it was found that the use of PEEK not only failed to prevent adsorption of analyte in the loop but, in this instance, actually exacerbated the problem. The effect of loop overfill volume, competing NSAID in the sample solution and percentage organic component in the mobile phase on this phenomenon was investigated. Comparison was also made between PEEK™ and other materials used for injector loops.

INTRODUCTION

There is increasing awareness that loss of analyte through adsorption in the LC injection system is a possible problem when the solvent used to dissolve the sample is weaker in elution strength than the LC mobile phase. (1-4) In particular

this is an issue in the use of low dispersion forms of LC, where often it is necessary to use a "weak" solvent to bring about peak compression (5).

Injection of samples in solutions of low eluting strength in comparison with the mobile phase may be carried out in order to induce on-column focusing or "peak compression" of the sample band on injection of the sample onto the analytical column. By use of this technique the sample band is concentrated on the top of the analytical column before subsequent elution by the mobile phase. In this way peak dispersion is reduced thus allowing the injection of larger sample volumes than would be the case with the injection of samples in mobile phase solution (6-8). Peak compression is of particular importance when using narrow-bore columns, since in its absence, very small injection volumes are necessary to avoid the problem of volume overload of the column.

In view of the adsorption problems experienced when using peak compression using sample injectors fitted with conventional Vespel™ valve rotor seals and stainless steel sample loops (5), it follows that when working in this area, efforts should be made to use inert, non-absorbing materials. In this respect the use of a TEFZEL™ (ETFE) valve rotor seal had already been adopted (5). It was also decided that the use of injector loops made from PEEK™ should be investigated for microbore LC of NSAID

MATERIALS

Apparatus

The chromatographic system consisted of a Shimadzu LC-10AD pump, SPD-6AV UV/visible detector fitted with an 8 µl flow cell and C-R5A integrator (all Dyson Instruments, Houghton-le-Spring, U.K.). Stainless steel connecting tubing was 0.007" x 9 cm pre-column and 0.007" x 9 cm post-column. A Rheodyne 7125 injection valve was used and fitted with a Tefzel™ (ETFE) rotor seal (Anachem, Luton, U.K.). External sample loops of 20 µl volume were used. The stainless steel and fused silica loops were cut to required lengths in - house. Fused silica tubing (uncoated) 0.25 mm i.d. x 0.4 mm o.d. was supplied by Chrompack (U.K.) Ltd., London, UK and the 20 µl PEEK™ loop was supplied by Whatman (Millipore (UK) Ltd.) Watford, U.K. Spherisorb ODS2 (5 µm), 12 cm x 2.1 mm

i.d. and Spherisorb ODS2 (5 μm), 12 cm x 4.6 mm i.d. stainless steel columns were supplied by Capital HPLC, Bathgate, U.K. and were used with a Spherisorb (10 μm), 10 cm x 4.6 mm i.d. silica pre-column in line before the injector. The column temperature was maintained at 25 °C with a water jacket and Tecam TE-7 Tempette pump/heater supplied by B.D.H., Poole, U.K.

Chemicals

Water was glass distilled and de-ionised (MilliQ purification system, Millipore (UK) Ltd. Watford, U.K.). Methanol was supplied by Rhone-Poulenc Ltd., Manchester, U.K. Nitric acid GPR, 69-72% v/v, Di-sodium hydrogen orthophosphate, AR grade and orthophosphoric acid (85%), GPR, were supplied by B.D.H., Poole, U.K. Indomethacin, flufenamic acid and naproxen were supplied by Sigma, Poole, U.K.

METHODS

Mobile phase and sample solution preparation

The mobile phase, methanol - 0.02 M phosphate buffer (pH 7.0) (58:42, v/v), was filtered and degassed in an ultra-sonic bath before use. The flow rate used throughout when using the 2.1 mm i.d. column was 0.378 ml min^{-1}, the flow rate used with the experiment conducted on a 4.6 mm column was 1.0 ml min^{-1}. Indomethacin and flufenamic acid solutions, 250 ng ml^{-1}, were prepared in 0.02 M phosphate buffer (pH 7.0), mobile phase and a range of other methanol-aqueous buffer solutions indicated later in the text. Various volumes of these solutions were injected into the chromatographic system described above and peak area data were obtained.

Investigation of the effect of increasing the loop fill volume on the resulting peak areas of indomethacin

A Rheodyne injection valve was fitted with a Tefzel™ rotor seal and 20 μl PEEK ™ loop. A series of 20 μl injections were made using increasing volumes of indomethacin solution, 250 ng ml^{-1} in aqueous phosphate buffer 0.02 M, (pH 7.0)

to flush through the loop and injector during loading prior to injection. At least two injections were made for each loop flush volume. This was followed by a similar series of injections of indomethacin solution 250 ng ml^{-1} in mobile phase. The same experiments were carried out with a 20 µl stainless steel loop, a 20 µl fused silica loop and a commercially obtained (Whatman) 20 µl PEEK™ loop. The stainless steel loop had previously been connected to a Rheodyne valve and flushed with 20% aqueous nitric acid for 20 minutes with a flow rate of 1.0 ml min^{-1}. The experiments using the PEEK™ loop were repeated using flufenamic acid instead of indomethacin. The effect of the presence of naproxen on the peak areas of indomethacin was investigated by passing increasing loop flush volumes of a combined solution of naproxen 500 ng ml^{-1} and indomethacin 250 ng ml^{-1} in aqueous phosphate buffer through the PEEK™ loop.

Further studies of indomethacin interaction with valve components

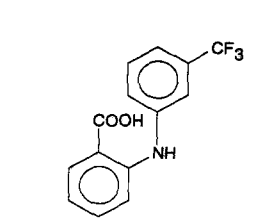
A 20 µl PEEK™ loop was fitted to another injection valve and increasing loop flush volumes of indomethacin 250 ng ml^{-1} in aqueous phosphate buffer passed through the injection valve and sample loop . The loop was then washed free of indomethacin solution by passing 100 µl of blank aqueous buffer through the loop. The loop was then fitted onto the original valve, and flushed with a further 100 µl of buffer before injection.

Loop flush volumes of 100 µl, 250 µl and 500 µl of indomethacin, 250 ng ml^{-1} in a range of methanol - aqueous buffer ratios from 0% to 58% methanol were passed through the 20 µl PEEK™ loop prior to injection. A similar experiment was also conducted with a 20 µl stainless steel loop but in this instance a 4.6 mm i.d. analytical column was used.

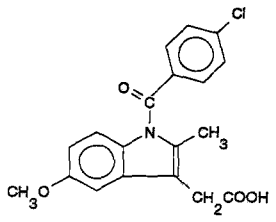
RESULTS AND DISCUSSION

It is common when operating valve injectors to flush a volume of sample solution through the valve in excess of the loop volume to ensure that the sample loop is completely filled. It is recommended that at least five times the loop volume is used for this purpose to give an RSD of 0.1% on replicate injections (9).

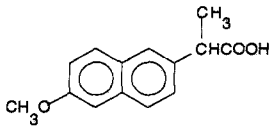
However if analyte adsorption was taking place in the injection system, then the amount of analyte swept onto the column by the mobile phase would increase



Flufenamic Acid



Indomethacin



Naproxen

FIGURE 1. Structures of NSAID.

with increasing loop flush volume. This has already been clearly illustrated (5). The purpose of this study was to attempt to minimise adsorption in the injector sample loop in microbore LC of NSAID (Figure 1) by substituting a PEEK™ loop for a stainless steel loop. However it was found (Figure 2) that the peak areas of indomethacin obtained on injection of solutions of indomethacin in aqueous phosphate buffer using the PEEK™ loop increased more rapidly with increasing loop flush volume than occurred when using a stainless steel injection loop. Furthermore, this increase in area did not reach an upper limit followed by a plateau as would be expected if available adsorption sites were being progressively filled. In fact, on increasing the loop flush volume a maximum peak area was reached whereafter the peak areas were reduced. This effect was also noted using a commercially obtained PEEK™ sample loop (Figure 3) believed to be from a different batch of PEEK™.

Using a stainless steel loop of the same volume and i.d., a 13% rise in area for a 100 µl loop flush volume was observed on changing the injection solution from mobile phase to aqueous buffer. As has been demonstrated (5), this rise may be attributed to adsorption in the valve and sample injection loop. The

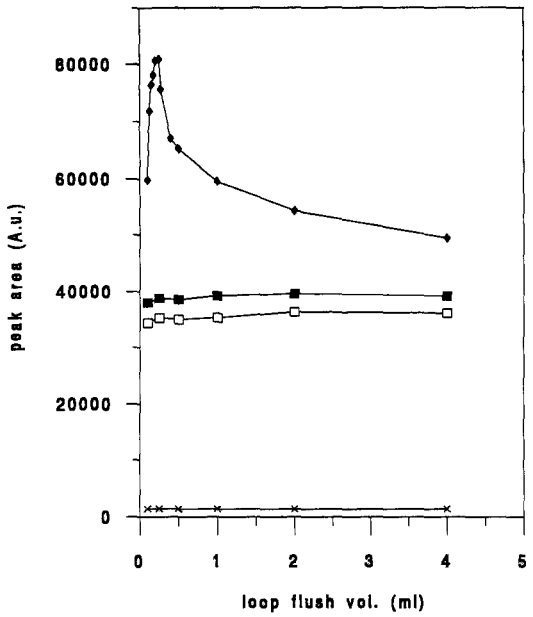


FIGURE 2. The effect on peak areas of indomethacin of increasing the loop flush volumes. ◆ injections of aqueous buffer solution with PEEK™ loop. ■ injections of aqueous buffer solution with stainless steel loop. □ injections of mobile phase solution with stainless steel loop. X injections with PEEK™ loop following blank aqueous buffer wash. Spherisorb ODS2 (5 μm), 12 cm x 2.1 mm i.d.column, mobile phase, methanol - 0.02 M phosphate buffer (pH 7.0) (58:42, v/v), flow rate 0.378 ml min^{-1}. Loop size 20 μl.

corresponding rise for the PEEK™ loop was +81% and this rose to a maximum of +137% when the loop flush volume was increased to 250 μl. The subsequent decrease in areas with higher loop flush volumes was unexpected. It is possible that this unusual behaviour may be due to some kind of aggregation phenomenon. There is a precedent that such a phenomenon may give rise to a sharp change in behaviour in that, for example, the formation of micelles does not take place until a critical concentration has been reached.

Subsequently an experiment was conducted whereby the PEEK™ loop was flushed with increasing quantities of indomethacin solution in aqueous buffer

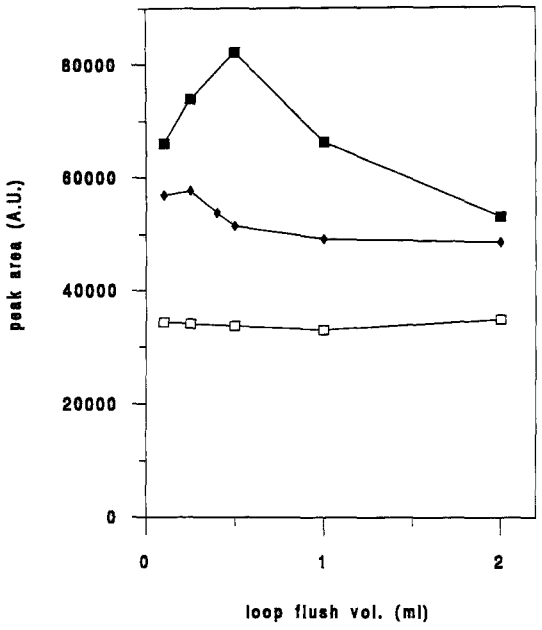


FIGURE. 3. The effect of the addition of naproxen on the peak areas of indomethacin with increasing loop flush volume. ■ injections of indomethacin solution in aqueous buffer, ◆ injections of indomethacin and naproxen solution in aqueous buffer, □ injections of indomethacin solution in mobile phase. A Whatman 20 µl PEEK™ sample loop was used, all other chromatographic conditions as Figure. 2.

when fitted to another injection valve. The solution was then flushed from the loop with a 100 µl blank aqueous buffer wash and then transferred back to the original valve, washed again with 100 µl of blank aqueous buffer and then an injection made. The results Figure 2 show that although some indomethacin had remained adsorbed in the loop after the blank aqueous buffer wash, the quantity was much reduced and did not increase as the loop flush volume was increased up to 250 µl as had been found previously when the loop was filled in a conventional manner.

The areas obtained on the injection of 100 µl flufenamic acid solution in aqueous buffer were 48% greater than a 100 µl injection in mobile phase when

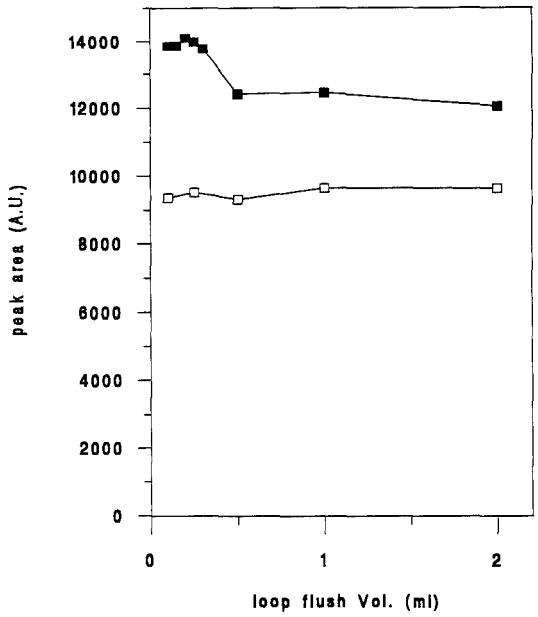


FIGURE 4. The effect on the peak areas of flufenamic acid of increasing loop flush volumes through PEEK™ loop. ■ injections of flufenamic acid solution in aqueous buffer, □ injections of flufenamic acid solution in mobile phase. Chromatographic conditions as Figure 2.

using the PEEK™ loop but the areas did not show the same percentage rise with increasing loop flush volume as had been noted for indomethacin (Figure 4). This shows that whatever the mechanism of this anomalous effect, flufenamic acid is not affected to the same degree as indomethacin. However the areas obtained using loop flush volumes of solutions in aqueous buffer of more than 500 μl were less than those obtained for 100 μl loop flush volumes as had been noted with indomethacin. Again this is not consistent with what would be expected from an adsorption process eventually reaching a saturation limit as had been noted in previous work using stainless steel loops (5). The lower absolute values for flufenamic acid peak areas compared to indomethacin are due to the lower molar absorptivity of flufenamic acid at 254 nm.

If an absorption or aggregation process was occurring in the injector then the presence of a similar solute may be expected to alter the observed effects due to either interaction with the analyte or competition with the analyte for adsorbing surfaces within the loop. This was investigated by adding naproxen to a solution of indomethacin in aqueous buffer. On injection of this solution it was found that the rise in peak areas of indomethacin with increasing loop flush volume was much less than when naproxen was absent. This suggested that either naproxen interacts with indomethacin preventing the complete interaction of the indomethacin with PEEK™ or that naproxen competitively interacts with PEEK ™ to prevent complete interaction of the PEEK™ with indomethacin. The indomethacin peak areas were still 72% greater for the buffer solution than the solution in mobile phase for 100 μl loop flush volumes but this may be dependant on the concentration of naproxen or other competitive species in the injection solution.

In the experiments described above NSAID injection solutions had been prepared in aqueous buffer i.e. the extreme conditions for obtaining peak compression. Further work was therefore carried out to determine whether similar behaviour took place in the presence of an organic modifier (methanol) in the injection solution, given that it would still be possible to obtain peak compression when using such methanol containing solutions. Varying the percentage of methanol in the injection solution followed by sample loading through the PEEK™ loop showed that even small reductions in the methanol concentration relative to that in the mobile phase produced a considerable rise in the observed indomethacin peak areas, indicating that adsorption was occurring (Figure 5). However, it was found that the unusual rise and fall in peak areas previously observed with increasing loop flush volumes did not occur for solutions of greater than 10% methanol concentration. This suggested that the interactions taking place in the aqueous buffer / PEEK™ system were probably hydrophobic in nature.

This study was completed by looking at other possible inert loop materials. It was found that, in part, the amount of sample adsorption which takes place within a sample loop depends on the condition of the internal surfaces of the loop. For example, a stainless steel loop which had been pre-flushed with 20% aqueous nitric acid gave negligible sample adsorption compared to that found when it was

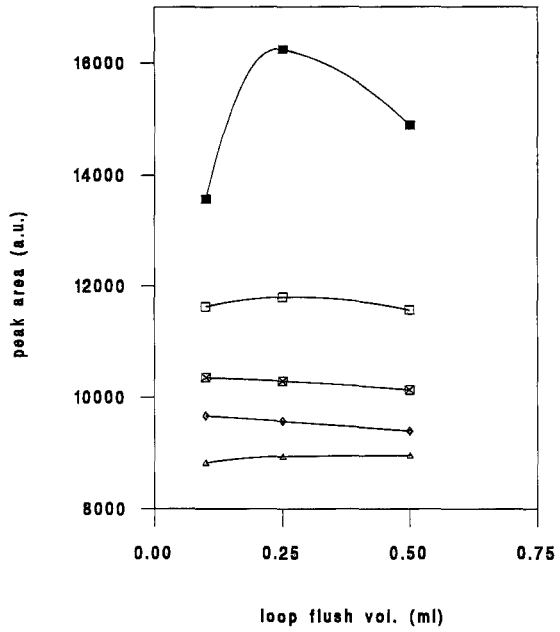


FIGURE 5. The effect on the peak areas of indomethacin on changing methanol concentration of the injection solvent and increasing loop flush volume through PEEK loop. ■ sample solvent, aqueous buffer, □ sample sovent, methanol-buffer, 10:90, ☒ sample solvent, methanol -buffer 20:80, ♦ sample solvent, methanol-buffer 30:70, Δ sample solvent, mobile phase. Chromatographic conditions as Figure 2.

untreated. A previously un-used fused silica loop also gave minimal adsorption of indomethacin. However, the degree of sample adsorption in both cases was not zero due to the contributing factors of the other internal valve surfaces. Also, although the percentage adsorption was reduced with the pre-treated stainless steel loops compared with that obtained with PEEK™, again adsorption occurred with only small reductions of the quantity of methanol present in the injection solvent (Figure 6). Furthermore the increase in peak area on increasing the loop flush volume from 100 μl to 500 μl became increasingly significant as the methanol concentration was reduced. This result highlighted the potential source

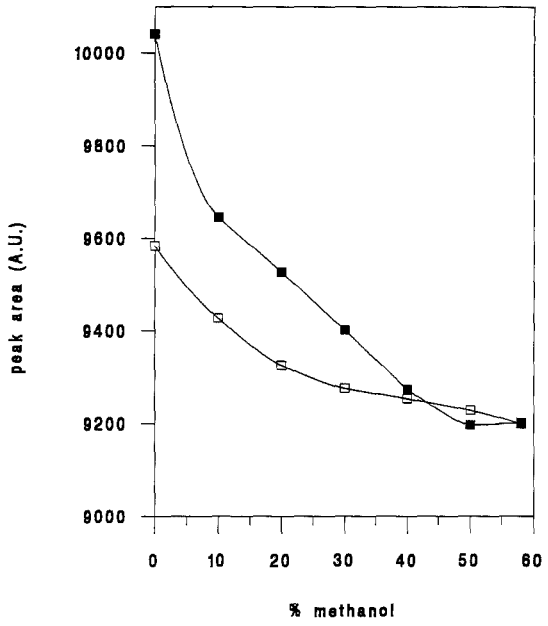


FIGURE 6. The effect on the peak areas of indomethacin on changing the methanol concentration of the injection solvent and increasing loop flush volume through a stainless steel loop. ■ 500 µl injections, □ 100 µl injections, Spherisorb ODS2 (5 µm), 12 cm x 4.6 mm i.d. column, mobile phase, methanol - 0.02 M phosphate buffer (pH 7.0) (58:42, v/v), 1.5 ml min^{-1}. Loop size 20 µl.

of bias and imprecision which could be introduced into an analytical procedure if loop flush volumes were not consistent between one injection and another and an adsorption process was occurring.

CONCLUSIONS

Relative success in avoiding the adsorption of NSAID when injecting samples of low solvent strength was obtained by using fused silica and acid washed stainless steel sample loops. However the main conclusion from this study was that although steps may be taken to minimise sample adsorption, it may occur when it

is least expected. In addition, minimal adsorption or absence of absorption may be related to factors such as (i) the nature of components present in the sample other than the analyte - this may change and therefore the degree of adsorption may change from sample to sample; (ii) the regularity of acid treatment of stainless steel loops - this is inconvenient, may not always be effective and may have a long term adverse effect on the accuracy of the loops and the condition of other components in the injector. It is therefore recommended that, since analyte adsorption is very difficult to avoid with certainty when using weakly eluting solvents for sample solutions, it is best to proceed as though adsorption will occur. Consequently, in our ongoing work in the area of microbore LC with peak compression, the commonly used loop overfill method is being avoided and a half loop fill procedure is being used.

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DETERMINATION OF GLYPHOSATE AND SOME RELATED COMPOUNDS BY ION-EXCHANGE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Anion exchange liquid chromatographic methods are described for the determination of N-phosphonomethyl-glycine (PMG), which is known also as glyphosate, its monomethyl-ester (PMG-Met), N,N-bis(phosphonomethyl)-glycine (bis-PMG), N-methyl, N-phosphonomethyl-glycine (PMG-NMet) and glycine (Gly). Five different anion exchange columns were tested for the separation of the above mentioned compounds. Effects of the eluent pH, ionic strength and organic solvent composition were studied. It was found that only some of the tested columns were applicable for the separation of the PMG and PMG-NMet critical pair, which may be due to the different structure, the ratio of the hydrophobic and hydrophilic surface area of the ion exchanger. Analytical performance of the methods was investigated. Some examples are also shown for the application of these described methods.

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INTRODUCTION

N-phosphomonethyl glycine is widely used in herbicide formulations (e.g. Roundup or Rodeo from Monsanto or Glialka from Alkaloida Ltd) as a weedkiller. Different analytical methods were applied for the determination of PMG in a wide range of matrices. In herbicide formulations or as technical samples, glyphosate can be analyzed by ion exchange chromatography due to its ionic and polar character (1,2) and ultraviolet and refractive index detectors were used. However, analysis of glyphosate in biological (3) or environmental samples (4) requires pre- or post-column derivatization and fluorescence detection for the more sensitive and specific determination. For this purpose fluorenylmethyl chloroformate (FMOC), orto-phtaldialdehyde (OPA) or phenylisothiocyanate (PITC) has been successfully applied. Metabolism of PMG in water and soil was followed by thin-layer chromatography and metabolites, mainly aminomethyl-phosphonic acid (AMPA) were analyzed by gas chromatography-mass spectroscopy after derivatization (5,6). The sample preparation was carried out by column chromatography on ion exchanger columns. For the analysis of technical glyphosate and bis-PMG, complexometric determinations were also developed (7,8). Solid phase analysis of PMG and its salts has been carried out by Fourier-Infrared spectroscopy (9,10). NMR spectroscopy has also been applied to the analysis of PMG (1).

In our present work anion exchange liquid chromatographic method was developed for the analysis of technical PMG and formulations and for its impurities. We also evaluated the data obtained using different anion exchanger stationary phases and eluents, which will be discussed later.

EXPERIMENTAL

Chemicals and liquid chromatographic instrumentation

All chemicals were of analytical or chromatographic grade. Acetonitrile was obtained from Romil Chemicals (Shepsed, United Kingdom). Water was purified using a Milli-Q instrument (Millipore, Milford, USA). Potassium dihydrogen ortophosphate and ortophosphoric acid were obtained from Reanal (Budapest, Hungary). PMG reference standard and technical samples, Bis-PMG, PMG-Met, PMG-NMet and Gly were obtained from Alkaloida Ltd., (Tiszavasvári, Hungary). The liquid chromatographic system consisted of the following instruments: a Waters 600E pump with 3000A system controller, a Rheodyne 7010 injection valve (equipped with 20 μl sample loop), and a Biotronik BT 3030 variable wavelength ultraviolet detector operated at 195 nm

wavelength. Data acquisition was carried out using a Waters Maxima 820 data station running on NEC APC IV. Eluent (eluent A) for the routine determination of Gly, PMG-Met and PMG contained 5(v/v)% acetonitrile in 3 mM potassium dihydrogenphosphate solution, pH 3.15. Eluent (eluent B) for the determination of bis-PMG contained 5 (v/v)% acetonitrile in 50 mM potassium dihydrogenphosphate solution pH 2.40. In both case, pH of the eluent was adjusted to the desired value using 1 M phosphoric acid solution using a Jenway 3020 pH meter (Jenway Ltd., Felsted, Dunway, Essex, United Kingdom). For these routine determination a Dionex Omnipac PAX-500, 250x 4mm ID column was used. Other columns used for comparison are listed in Table 1. together with their main characteristics.

Sample preparation

Approximately 15-20 mg of the glyphosate sample was weighed with analytical precision and was dissolved in 10 ml of the mobile phase and was homogenized. Reference solutions were prepared by weighing of 2-3 mg of the reference materials with analytical precision and were dissolved in the mobile phase and it was homogenized. These samples were stable at least for 96 hours from the preparation. Both reference and sample solutions were prepared with the freshly prepared mobile phase. During the preparation of the eluent, the transmittance of the solvent was regularly checked and pH adjustments were made with a precision of 0.1 pH unit.

Evaluation of the method

Linearity

Linearity was tested by injecting solutions containing the target compounds in different, known concentrations. Two or three replicate injections were performed. We found that peak response-solute concentration functions were linear for all compounds between the concentrations of about 100 μg/ml and 1500 μg/ml.

Reproducibility

The reproducibility of injection was tested by injecting the same reference solution containing the analytes at 100 μg/ml. RSD of the injection reproducibility was found to be 0.90 % (n=5). Reproducibility of retention times was also calculated and RSDs of the retention times were better than 5 % except bis-PMG, where the reproducibility of the retention times was ca 9%. Retention times of the different compounds are given in Table 2.

Table 1. Stationary phases and columns used in the experiments

Stationary phase	Column dimensions	Functionality	Manufacturer
Dionex PAX-500	250x4 mm ID	agglomerated type anion exchanger, polymer	Dionex, Sunnyvale, California, USA
Chrompack Ionosphere 5A	100x3 mm ID glass cartridge	anion exchanger on silica	Chrompack, Raritan USA
Waters IC Pak A HC	150x4 mm ID	quaternary amine anion exchanger on methacrylate polymer	Waters, Milford, USA
Merck Polysphere AA NA	120x4.6 mm ID		Merck, Darmstadt, Germany
Hamilton PRP X-100	150x4.6 mm ID	quaternary amine anion exchanger on polystyrene-divinylbenzene polymer	Hamilton, Bonaduz, Switzerland

Table 2. Retention times of the solutes

Solute	Eluent	Retention time, min
glycine	A	1.82
glyphosate-monomethylester	A	2.59
glyphosate	A	5.72
gliphosate	B	2.05
bis-phosphonomethyl glycine	B	5.38

Detection limit, limit of quantitation

Detection limits were determined by injecting reference solutions at different concentrations. Detection limit for Gly was found to be 5 μg/ml, for PMG and PMG-Met 10 μg/ml, and for bis-PMG 25 μg/ml.

Recovery

Recoveries were tested in the concentration range of 0.11-2.81 percent of the main component PMG by the addition of the impurities to the reference PMG solution. The solutions were analyzed and recoveries were calculated from the found amount of the compound in question and the added amount of the same compound. Values of recovery varied between 91 % and 122 %, the average was 106 % (n=10).

Analysis of technical PMG

Using the methods described above, glyphosate batches obtained from the Alkaloida Ltd, were analyzed. Chromatogram of the reference solution containing PMG, PMG-Met and Gly is shown on Fig. 1. Comparing the chromatogram of th reference solution to the chromatogram of technical PMG, we concluded that unknown impurities are present in the technical PMG in low concentration (Fig.2.). Fig. 3. shows the typical impurity profile of the technical glyphosate. In order to identify the unknown compounds in the samples, PMG-NMet and aminonomethyl phosphonic acid were included in the tests. Unfortunately, using the described system and conditions, aminomethyl phosphonic acid was not detectable even at greater concentration. PMG- NMet gave similar retention as PMG, so the separation and determination was not possible. This fact will be discussed in more detail later. Solution of On Fig 4 separation of PMG and bis-PMG using eluent B is demonstrated.

Attempts were made to analyze the bis-PMG content of PMG together with the other two impurities using gradient elution by changing the eluent pH or ionic strength. Unfortunately, the high baseline drift did not allow the sensitive detection of the analytes. Using more selective detection system, e.g. post column derivatization and fluorescence detection, gradient elution of the compounds either by pH or ionic strength gradient seems to be possible.

DISCUSSION

Comparison of different stationary phases for the separation of PMG and its derivatives

The analytical methods described in the previous part employed a Dionex PAX500 column, which is special, multifunctional stationary phase with anion exchanger groups and hydrophobic surface, which enables the separation of the analyte both in ion exchange and reversed phase mode. The packing material in this column is a so-called agglomerated type anion exchanger (11). To evaluate the effect of ionic strength and pH of the eluent, the separation was tested at different pH and buffer concentration. Fig. 5 shows the dependence of the logarithm of the capacity factors of the solutes on the reciprocal of the buffer concentration at constant pH of 3.15. This correlation was found to be linear except the highest and lowest buffer concentrations. Logarithm of the capacity factors showed linear dependence using the least square method on the logarithm of the buffer concentration. Slopes of these functions are equal to the ratio of the charge on the analyte and the counterion of the ion exchanger. These data together with the correlation coefficients are given in Table 3. However, using the initial chromatographic system,

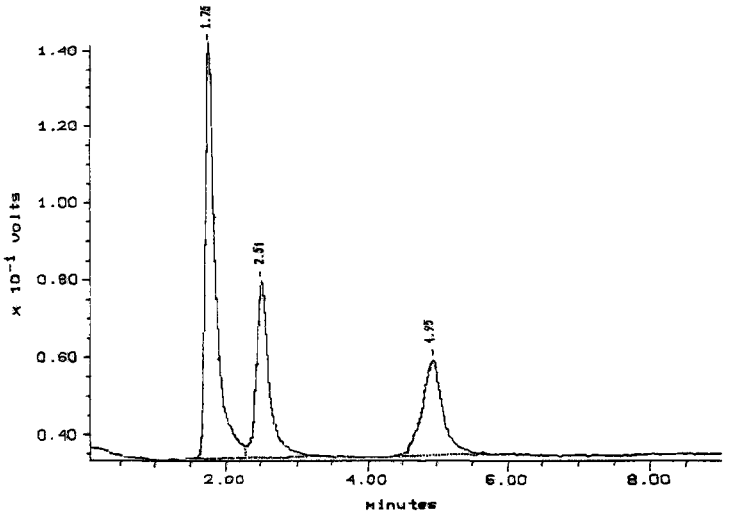


Fig. 1. Chromatogram of the reference solution
Gly (1.75 min), PMG-Met (2.51 min), PMG (4.95 min)

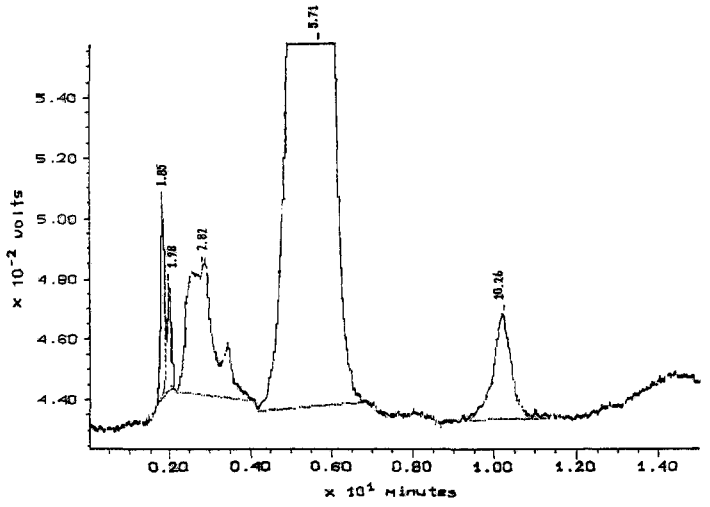


Fig. 2. Chromatogram of the technical PMG sample

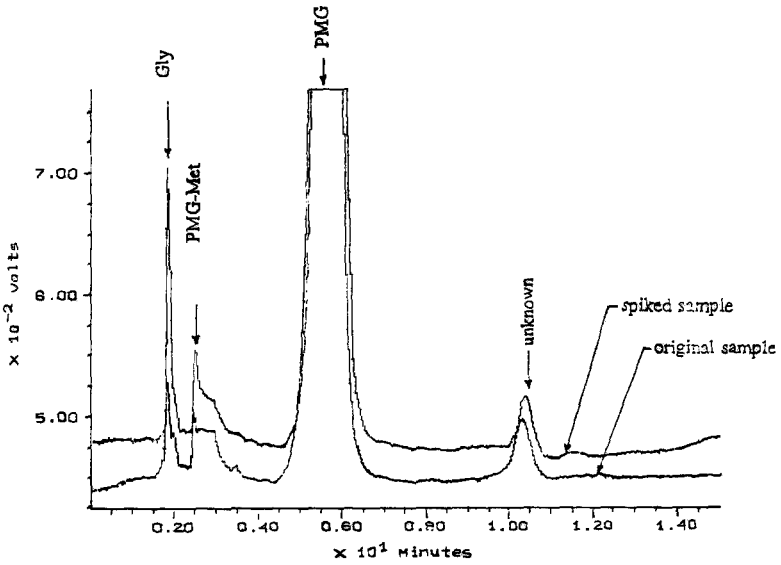


Fig. 3. Typical impurity profile of the technical PMG. Solutes are marked on the figure. Lower trace: original sample; upper trace: sample after spiking with Gly and PMG-Met

separation of PMG and PMG-NMet was not achieved. Thus, capacity factors were measured as a function of pH (Fig. 6b). We found that by using the Dionex column, separation of PMG and PMG-NMet is possible only at higher pH.

The retention of the solutes was not influenced significantly by varying the acetonitrile concentration in the eluent between 1 and 20 (v/v)%. However, the peak response decreased gradually as the acetonitrile content of the eluent was increased according to the poor solubility of the analytes in acetonitrile-water mixtures with higher acetonitrile content.

For the other columns, the dependence of the logarithm of the capacity factor on pH was also studied (Fig. 6a,c,d,e). The five column can be roughly divided into three groups: (1) Dionex PAX-500 and Chrompack Ionosphere A; (2) Waters IC-Pak A and Hamilton PRP X-100; (3) Merck Polysphere AA NA. Using the columns in the second group, separation of PMG and PMG-NMet is not possible in the pH and ionic strength range studied. These

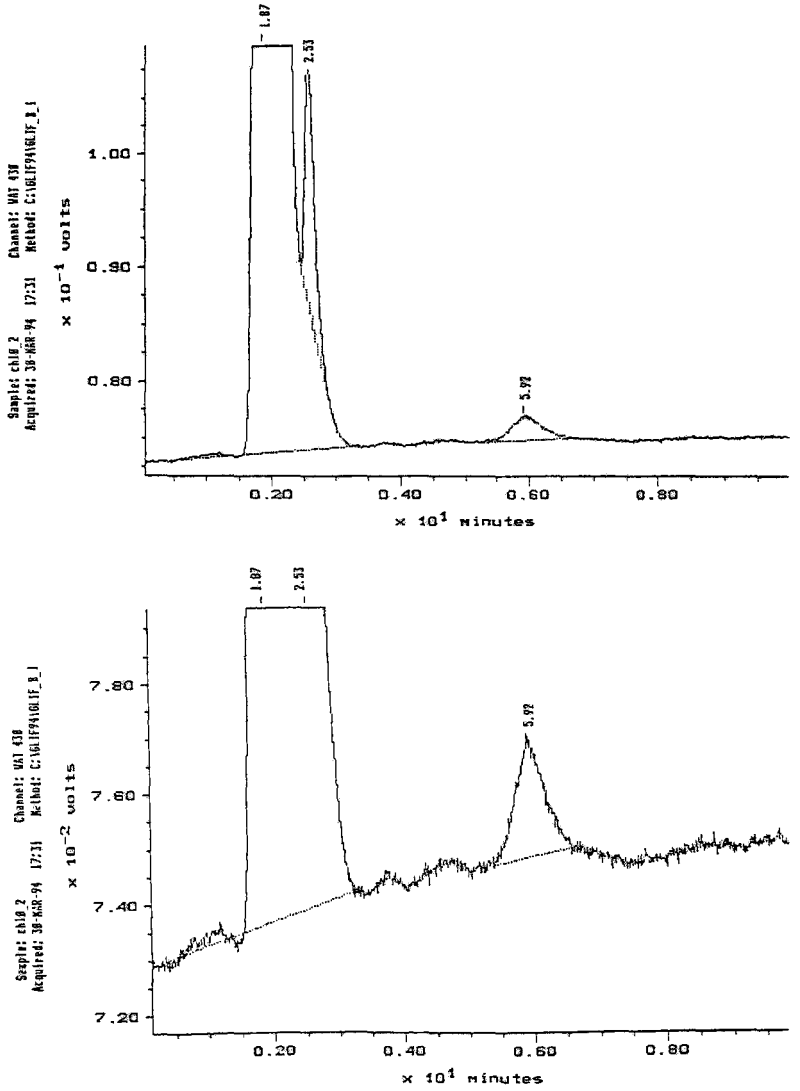


Fig. 4. Separation of PMG and bis-PMG in a technical PMG sample

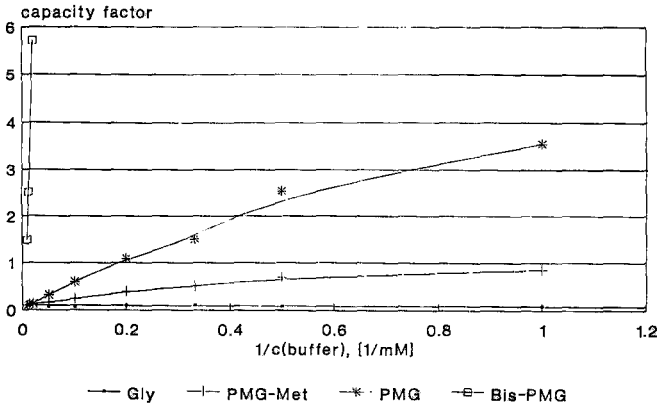


Fig. 5. Plot of the capacity factors for the different solutes versus the reciprocal of the potassium dihydrogenphosphate concentration in the eluent

Table 3. Slopes and regression coefficients for the linear regression of log k vs. logarithm of potassium-dihydrogenphosphate concentration (expressed in mM) in the eluent at pH 3.15

Compound	Slope	r	Concentration range
PMG-Met	-0,486	-0,987	1-100 mM
bis-PMG	-1,957	-0,999	10-100 mM
PMG	-0,8315	-0,997	1-100 mM

columns have quaternary alkylammonium groups bound to polymer surface (in case of IC-Pak A this polymer is polymethacrylate, in case of PRP X-100 the polymer skeleton is polystyrene-divinylbenzene copolymer). Columns in the first group are applicable for the separation of PMG and PMG-NMet. One of these columns, namely the Chrompack Ionosphere A is based on silica gel, where secondary interaction may occur between the silanol groups and the secondary and tertiary amino groups of PMG and PMG-NMet, respectively. In the case of the PAX-500 column, differentiation between PMG and PMG-NMet can not be explained easily. This column - as it was mentioned before - is of agglomerated type based on a highly crosslinked core and a functionalized latex layer

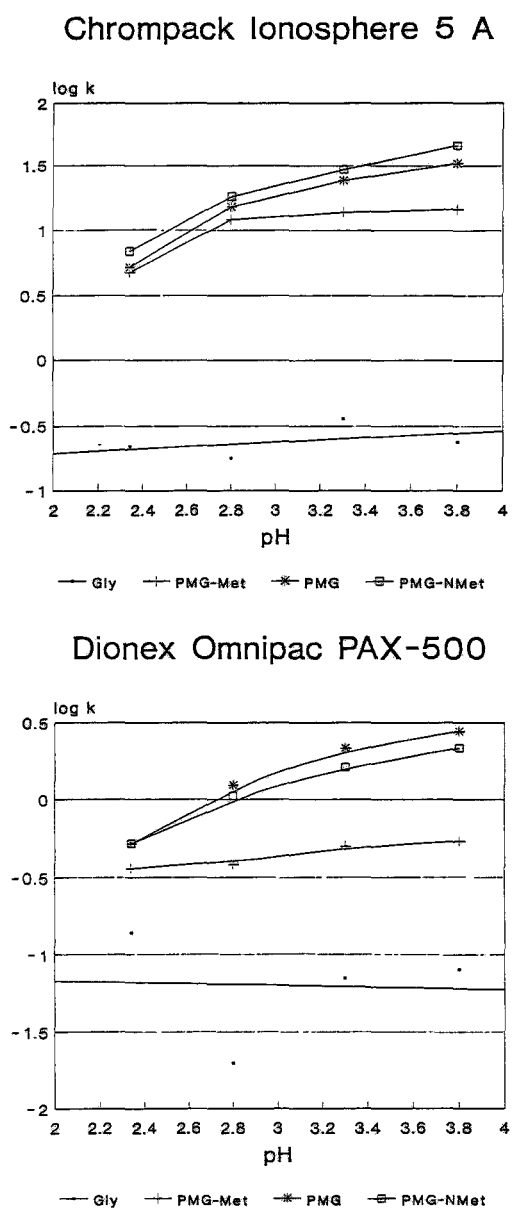
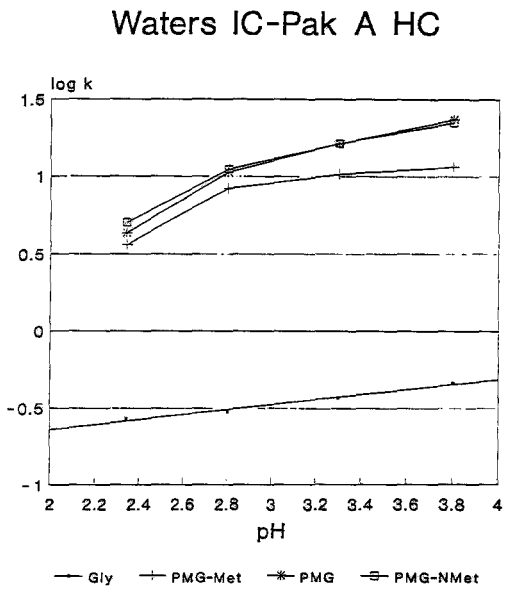


Fig. 6. Plot of the log k for PMG, Gly, PMG-Met and PMG-NMet versus the eluent pH on different anion exchanger columns



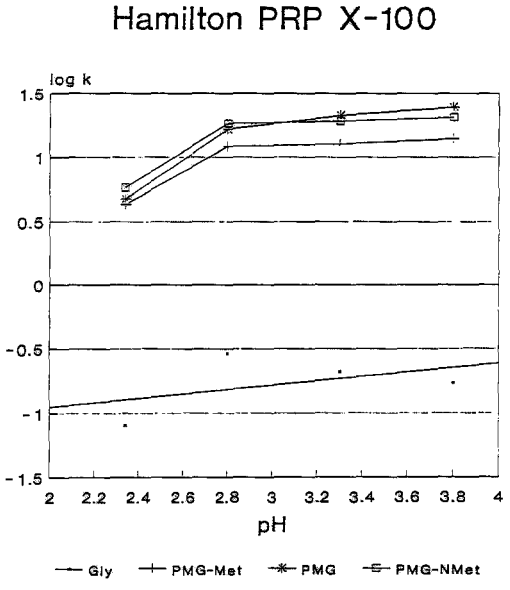


Fig. 6 (continued)

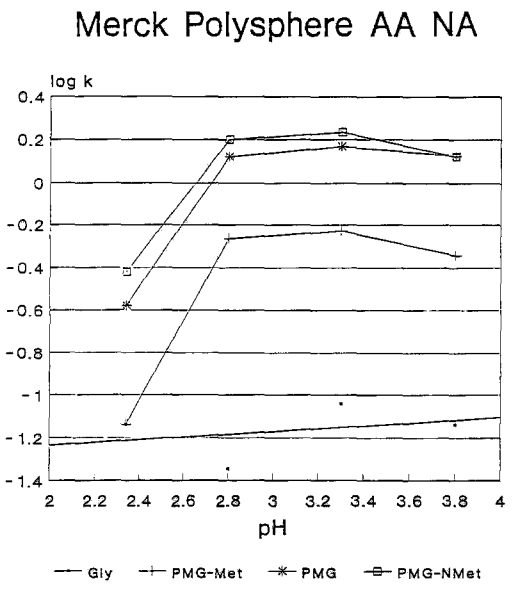


Fig. 6 (continued)

bound by electrostatic forces on the surface of the cores. The latex on the surface of the cores has quaternary ammonium groups but the alkyl groups attached to the quaternary ammonium contain hydroxy functions as well (11). Separation of PMG and PMG-NMet may be due to the residual sulphonated groups of the core which are not covered by the aminated latex, or, by a secondary interaction of the weakly acidic (pKa ca. 10) hydroxy functions on the latex-coated hydroxy groups by secondary equilibria. However, the experimental data show that separation of the two compounds depends on the pH in the pH range 2.2-3.0 but remains approximately constant between pH 3.0-3.6. In this pH range the ionization of strongly acidic sulphonic acid groups become complete and these dissociated groups can retain PMG and PMG-NMet by their different basicity. However, concentration of such uncovered groups must be low because the peak shape was not distorted due to secondary equilibria.

Polysphere AA NA exhibited unique retention of the tested solutes. The retention was relatively small compared to the other column but good efficiency and fair good separation of the analytes was provided. This column was originally offered for the analysis of amino

acids. Unfortunately, information are not available on the structure of functional group structure of this column except that this is an anion exchanger on polymer skeleton.
Summarizing, we can conclude that columns from the first group are the best choice for the detailed analysis of PMG including PMG-NMet. Silica based packing materials can be used in the pH range where this separation is made, and show very good mechanical stability over polymer based columns.

SUMMARY
Liquid chromatographic methods using anion exchanger columns were developed for the determination of the herbicide glyphosate and some related compounds. These methods were applied to the analysis of technical glyphosate batches. Anion exchanger columns obtained from different vendors were tested and compared. Further development of these methods has to be made mainly by improving the detection of the compounds and application of gradient-compatible detection methods.

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SIMULTANEOUS DETERMINATION OF EPHEDRINE AND 2-IMIDAZOLINES IN PHARMACEUTICAL FORMULATIONS BY REVERSED-PHASE HPLC

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ABSTRACT

A simple,rapid, and accurate method for the determination of naphazoline, oxymetazoline, xylometazoline, and ephedrine in rinological solutions using reversed-phase HPLC was developed. It involves the use of alumina coated with polybutadiene as the stationary phase, acetonitrile-aqueous 10^{-3} M NaOH (10:90) as the initial mobile phase with a linear gradient from 10 to 80% acetonitrile in 25 min, and detection at 224 nm. The only sample preparation is its dilution with water. Linearity and precision of the method have been assessed. The assay results obtained for various formulations were in agreement with the declared amounts.

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INTRODUCTION

Some 2-imidazoline derived drugs have a potent vasoconstrictor action. Among them naphazoline (I), oxymetazoline (II) and xylometazoline (III) display a rapid and prolonged effect in reducing swelling and congestion when applied to mucous membranes. They represent the active principle of many commercially available nasal solutions.

Also ephedrine (IV) salts have been used alone or in combination with other agents, especially 2-imidazolines, for the symptomatic relief of nasal congestion associated with the common cold, hay-fever, or rhinitis or sinusitis.

The determination of the above compounds has been essentially performed by gas liquid chromatography (1,2) or reversed-phase high performance liquid chromatography (RP-HPLC) (1, 3-5). The major problem associated with the analysis of basic drugs by RP-HPLC is peak tailing caused by ionic interactions between the analytes and free silanol groups of the packing material. Mobile phase additives (6) represent the first form of in situ column modifications to reduce peak asymmetry and to effect selectivity changes.

Alkylamines and alkylsulfonated ion-pairing reagents are the most common ones. Alkylamines act as silanol-masking agents, primarily by hydrogen bonds, thereby reducing ion-exchange and/or adsorption effects (7). The addition of counter ions and/or basic modifiers to the eluent may cause deterioration of the column packing and usually shortens column life. Therefore there is a growing interest in developing reversed phase sorbents from alkaline-stable support materials such as alumina.

A different approach to the analysis of basic drugs has been the use of base-deactivated columns (8) in which

free silanol groups are masked through various procedures. Kountourellis and Raptouli proposed the use of a basic alumina column and non-aqueous mobile phase containing very small amounts of ammonia for determining some 2-imidazolines in pharmaceutical preparations.

We describe here the simultaneous analysis of compounds I-IV by RP-HPLC performing the separation with an alkaline-stable stationary phase, namely Aluspher^{R} RP-select B. Validation data are presented for the determination of the drugs in commercial samples of rinological formulations.

EXPERIMENTAL

Chemicals

All reagents used were of analytical-reagent grade (Merck, Darmstadt, Germany) and were used as obtained. The pure pharmacologically active ingredients were naphazoline nitrate (I), ephedrine hydrochloride (IV) (Lepetit SpA, Milan, Italy), oxymetazoline hydrochloride (II) (Procter&Gamble, Milan, Italy), and xylometazoline hydrochloride (III) (Zyma SpA, Varese, Italy). Acetonitrile was of HPLC grade (J.T.Baker, Deventer, Holland). Water was deionized and doubly distilled from glass apparatus. All solvents and solutions for HPLC analysis were filtered through a nylon Milllipore filter (pore size 5 μm).

HPLC Equipment

Chromatography was performed on a Varian 9000 liquid chromatograph equipped with a 10 μL sample loop, a Varian Polychrom 9065 photodiode-array detector, serially connected with a 2550 Varian variable-wavelength detector and a personal computer IBM PS/2. The analytical column was a LiChroCART (125 mm x 4.0 mm I.D.) packed with 5 μm Aluspher^{R} RP-select B (Merck,Darmstadt, Germany).

HPLC Conditions

The operating conditions were as follows: initial mobile phase, acetonitrile-water containing 10^{-3} M sodium hydroxide (10:90, v/v), then a linear gradient up to 80% acetonitrile in 25 min;flow-rate, 1.2 mL min^{-1}; injection volume, 10 μL; column temperature 25 °C detection wavelength, 224 nm.

Calibration Standards

Stock solutions were prepared by dissolving 100 mg of compounds II,III, and IV and 50 mg of compound I in 100 mL of water. They were kept refrigerated at 4 °C. Working standard solutions were prepared by diluting aliquots of the stock solutions to give concentrations ranging from 10 to 800 μg mL^{-1} for (II), (III), and (IV), and from 2 to 160 μg mL^{-1} for (I). The calibration graphs were constructed by plotting the peak areas obtained at the wavelength of 224 nm versus the amounts (μg) injected.

Sample Preparation

An accurately measured volume (1-2 mL) of each commercial nasal solutions was transferred into a volumetric flask and diluted to volume with water. Usually the dilution factor was 10. After filtration through a nylon filter (pore size, 0.45 μm) the solution was injected into the liquid chromatograph.

RESULTS AND DISCUSSION

As mentioned in the introduction, the separation of organic bases on silica-based reversed phase materials is usually very problematic. This is due to the presence of active sites which complicate the retention mechanism and lead to peak tailing with polar solutes. Aluspher^{R} RP-

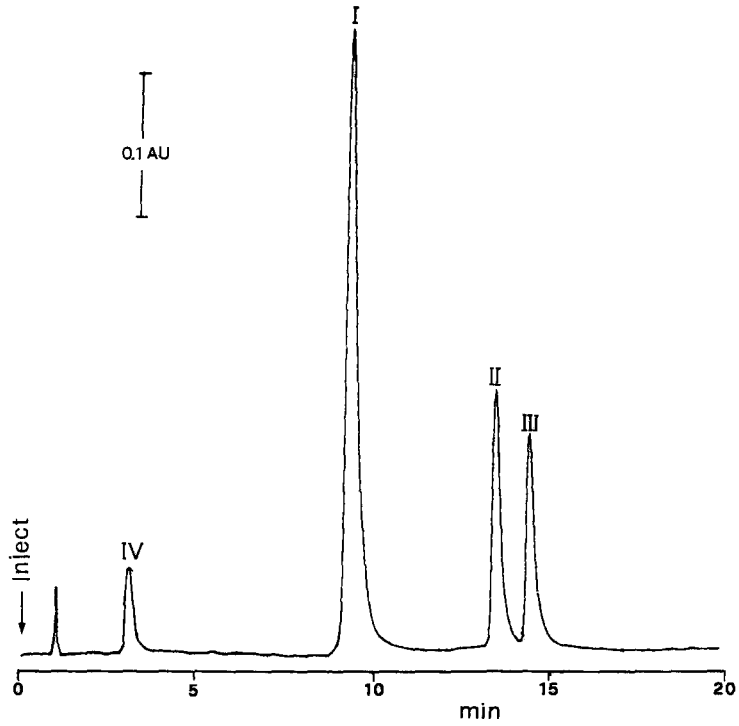

Figure 1 Typical chromatogram obtained at 224 nm for a standard solution containing 0.25 mg mL^{-1} of (II), (III), and (IV), and 0.125 mg mL^{-1} of (I).

select B is a new alkaline-stable stationary phase based on porous aluminium oxide coated with polybutadiene, which displays characteristic hydrophobic properties which are comparable to those shown by silica-based RP-phases (10).

Figure 1 shows the chromatogram of a standard solution of compounds I-IV, obtained with this stationary phase. As can be seen, a very good separation without peak tailing was achieved.

The detection wavelength of 224 nm was chosen since this value allowed the simultaneous analysis of the compounds investigated with a good sensitivity. In case the drugs are to be analyzed alone the following wavelengths can be selected to obtain the maximum sensitivity: 259 nm for ephedrine, 283 nm for naphazoline and oxymetazoline, and 224 nm for xylometazoline.

The photodiode-array detector allowed the estimation of the purity parameter format values (11). These values represent an absorbance-weighted mean wevelength of a spectrum and are very useful in the analysis of a pharmaceutical preparation both in confirming peak purity and peak identification, since these values provide an absorbance-weighed mean wavelength of a spectrum. The purity parameters were calculated for the compounds of interest over the range 220-367 nm and are reported on Table 1.

The capacity factors (reported in Table 1) were reproducible under the experimental conditions used, the coefficient of variation (CV) ranging from 1.0 to 1.5 for within-day and from 1.8 to 2.7% for between-day studies. The average analysis time was about 20 min.

The linearity was evaluated over the range of concentrations reported in the experimental section. The equations obtained by the least square regression fit are reported in Table 2.

The limits of detection, defined as the lowest concentration of each compound resulting in a signal-to-noise ratio of 3:1, are reported in Table 1.

The procedure was applied to the determination of active ingredients in six commercially available formulations. The results obtained are shown in Table 3. The quantities found were in conformity with the values claimed by the manufacturers.

TABLE 1
Analytical Parameters for Compounds I-IV[a]

Compound	Capacity factor	Purity parameter mean(nm) ± SD	Ideal detection wavelength (nm)	Detection limit at 224nm (ng injected)
I	8.3	223.82 ± 0.09	283	0.5
II	12.3	226.23 ± 0.11	283	1.0
III	13.3	223.58 ± 0.09	224	1.0
IV	2.0	221.59 ± 0.10	259	5.0

[a] Each value is the mean of five determinations

TABLE 2
Calibration Curves for Compounds I-IV: Linear Regression of the amount injected (x) versus the peak area (y); mean value ± standard deviation at 95% confidence interval (t=3.18; n=5)

Compound	Intercept	Slope	R^2
I	(-30.0 ± 0.6)E3	(95.4 ± 0.9)E4	0.999
II	(-5.2 ± 0.3)E3	(14.4 ± 0.2)E4	0.999
III	(-3.5 ± 0.1)E3	(12.4 ± 0.1)E4	0.999
IV	(-2.3 ± 0.2)E3	(2.11 ± 0.04)E4	0.998

TABLE 3
Analysis of Pharmaceutical Formulations*

Commercial preparation	Naphazoline Recovery(%)	CV	Oxymetazoline Recovery(%)	CV	Xylometazoline Recovery(%)	CV	Ephedrine Recovery(%)	CV
A					98.7	0.2		
B					92.2	0.3		
C					98.9	0.2		
D	99.1	0.2					96.7	0.1
E			99.8	0.3				
F	98.7	0.3						

*mean of five determinations.

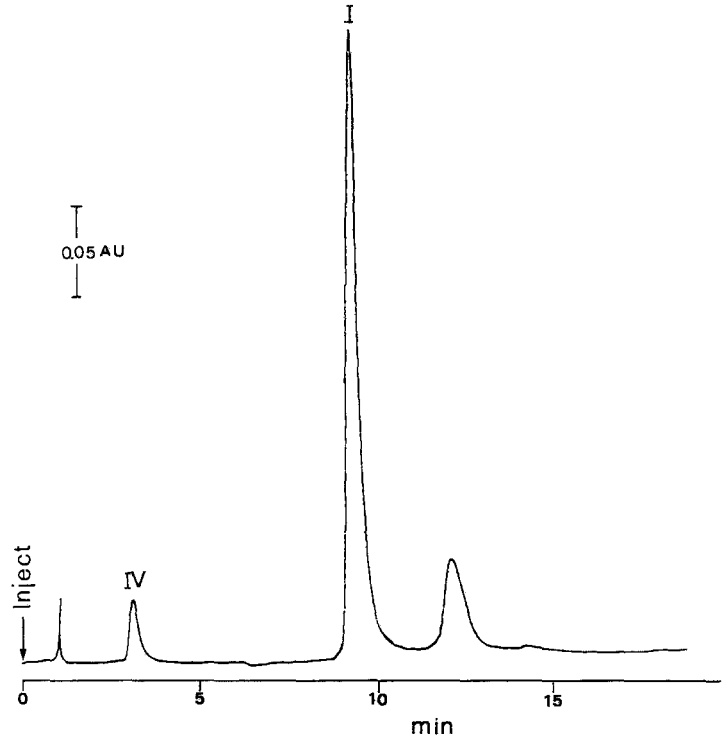

Figure 2 Chromatogram obtained after injecting the pharmaceutical formulation D, containing naphazoline nitrate and ephedrine hydrochloride as active ingredients.

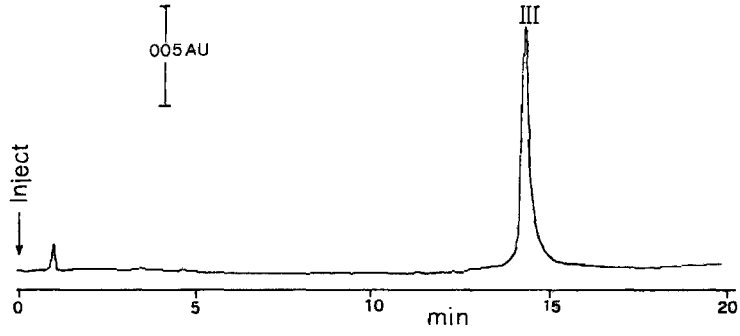

Figure 3 Chromatogram obtained after injecting the pharmaceutical formulation A, containing xylometazoline hydrochloride as active ingredient.

Figures 2 and 3 show the chromatograms obtained from the analysis of the commercial preparations D and A, respectively. No interference was observed from the excipients. In the case of sample D where both active compounds naphazoline and ephedrine are present, no problem was encountered with the possible interfering paraben, added as preservative to the preparation, since its peak eluted as the last one.

The analytical results obtained lead to the conclusion that the developed method is simple, rapid, and precise and therefore it can be successfully adopted for the routine analysis of I-IV in liquid pharmaceutical formulations.

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DETERMINATION OF NEMATICIDE ALDICARB AND ITS METABOLITES ALDICARB SULFOXIDE AND ALDICARB SULFONE IN SOILS AND POTATOES BY LIQUID CHROMATOGRAPHY WITH PHOTODIODE ARRAY DETECTION

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ABSTRACT

Solid phase extraction has been combined with High Perfomance Liquid Chromatography using a Photodiode Array Detector to isolate, determine, quantify and recover trace concentrations of aldicarb and its toxic metabolites, aldicarb sulfoxide and aldicarb sulfone, in soils and potatoes. A method is proposed which eliminates matrix interferences. The procedure involved extracting with acetonitrile or dichloromethane, initial separation and clean-up with a Sep-Pack Florisil or Silica cartridges. The extract can be successfully analyzed by HPLC-DAD and recoveries for pesticides were not influence by the use of this extraction method. The results have been applied to the systematic study of these compounds in growns of patatoes. The lowest detectable concentration for each pesticide is 40 μg kg-1 in soil and 15 μg kg-1 in potatoes.

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INTRODUCTION

Aldicarb, [2-methyl-2-(metylthio)propionaldehyde-O-(methylcarbamoyl)oxime;Temik®] is a systemic insecticide widely used for the protection of the potatoes cultures. The aldicarb (A) is degraded by oxidation to aldicarb sulfoxide (AX) and sulfone (AN) , although these two metabolites can undergo subsequent hydrolysis to their respective non toxic oximes (1). However, aldicarb, its sulfoxide and its sulfone are know to be the most toxic of these compounds. aldicarb sulfoxide is the most important compound, accounting for the high systemic activity and the long-term persistence of the insecticidal activity in soil (2, 3).

Previous methods for determining residues of aldicarb, aldicarb sulfoxide and aldicarb sulfone involved gas chromatography (4, 5). However, the increasing availability of liquid chromatographic methods is mainly due to the result of their sensibility for thermally labile and polar pesticides, which require derivatization prior to gas chromatographic analysis (6).

UV absorbance has been the most commonly used detection method in HPLC determination of N-methylcarbamate pesticides (7-9). UV detection is subject to interferences of other co-extractives taken from the sample and also to the lack of sensitivity for some compounds. The most serious drawbacks of UV detectors arise from matrix interferences and from working below 205 nm (10).

The development of diode-array detectors has been the most important advance in HPLC for the quantitative identification of compounds (11, 12). It provides an opportunity to explore all wavelengths in the UV and choose the monitoring wavelength which maximizes instrumental sensitivity. With the multi-signal capability of a diode-array detector, all the compounds can be analyzed at, or close to, their maximum wavelengths.

Most environmental sample extracts will require a preliminary clean-up procedure before determination by HPLC (13-15). The extent of clean-up required depends upon the type of sample being analyzed, the detection limit required and the detection technique used. Combining solid phase extraction with HPLC-DAD, the above-mentioned limitation can be overcome.

This paper describes a method for the high pressure liquid chromatographic using a Photodiode Array Detector in order to determine the quantity of aldicarb, aldicarb sulfoxide and aldicarb sulfone. A valid alternative method must also provide for elimination of possible interferences in soil and potatoes without increasing the analysis time.

MATERIALS

Instrumentation

A Hewlett-Packard (Palo Alto, CA., USA) model 1050 HPLC equipped with a HP 1040 M Diode Array detector has been utilized. A Hewlett-Packard data station has been used for data storage, comparison and mathematical manipulation of the acquired spectra.

The column was a cyanopropyl of 25 cm length and 4 mm ID., with a paking type Spherisorb of 5 μm (Tracer, Barcelona, Spain).

A centrifuge (Selecta, Barcelona, Spain) Model Centronic and a Selecta Model Vibromatic-384 have been used in the extraction procedure.

A Vac-Elut sample preconcentration system (Varian, Harbor City, CA., USA) for solid-phase extraction has been used in the purification procedure.

In the solid phase extraction two types of cartridges (Waters Associates, Milford, MA., USA) have been used. The Sep-Pack Florisil containing 500 mg of

sorbent to stage of extraction in soil and the Sep-Pack 50 mg of Silica for extraction in potatoes.

Chemicals and Solvents

The pesticide standards were supplide by the Laboratory of Dr. Ehrenstorfer GmbH (Angsburg, Germany) with a purity of 99.9%.

Standard stock solutions were obtained by the dissolution of 10 mg of aldicarb, its sulfoxide and sulfone in 100 ml of acetonitrile. These solutions were diluted to final working solution in acetonitrile-water (30:70).

HPLC-grade solvents were used. The acetonitrile (LiChrosolv, Merck, Darmstadt, Germany) used in the mobile phase has a transmitance of 50% at 195 nm and 80% at 200 nm. Mobile phase was desgassed with helium prior to use. Distilled water was obtained from a Millipore (Milford, MA., USA) Milli-Q water purification system.

All solvent and samples were filtered through 0.22 μm Millipore membrane filters before injection.

METHODS

Chromatographic Conditions

In preliminary studies we tested the chromatographic behaviour of aldicarb and its metabolites using as mobile phase solvents which generally are used in liquid chromatography with UV detection: water, methanol, acetonitrile or mixture of them. The best result for those three compounds is obtained when it is used a mixture acetonitrile-water. Figure 1 shows the influence of acetonitrile content in the mobile phase on the capacity ratio k´. With increasing acetonitrile content, the

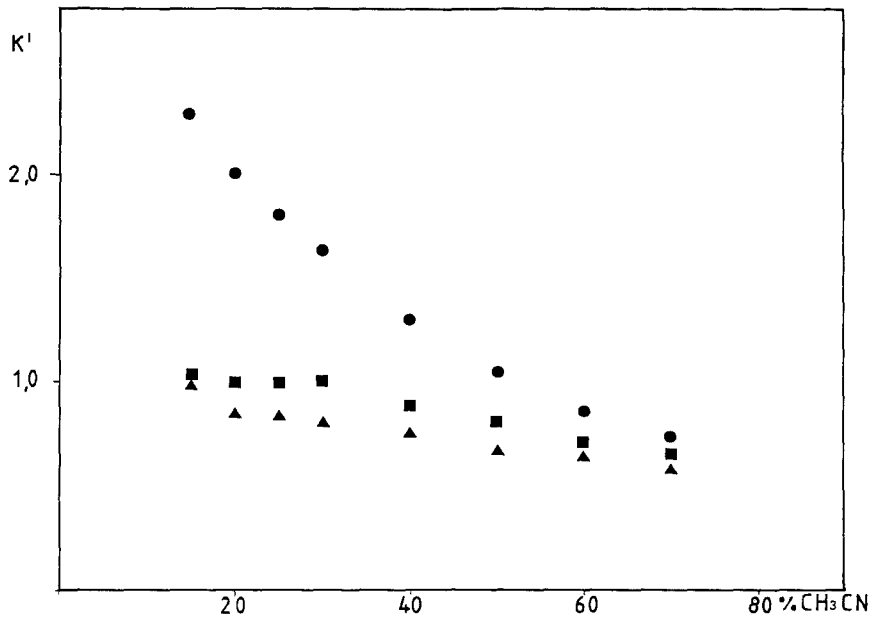

FIGURE 1. Influence of acetonitrile content in the mobile phase on the capacity ratios (k') of aldicarb (●), aldicarb sulfoxide (▲) and aldicarb sulfone (■). Flow rate: 1.2 ml min^{-1}, injection volumen: 50 µl, λ analytic: 202 nm

capacity ratio k'of aldicarb decreased, whereas those of aldicarb sulfoxide and aldicarb sulfone were influenced only slightly. As a result, the acetonitrile content was fixed at 30% V/V for experiments, and good separations were obtained in 4 minutes.

The separation of pesticides was carried out using isocratic elution at a flow rate of 1.2 ml/min with injection volume of 50 µl.

The UV absorbance of the elution profile of analytes was recorded at 202 nm for all measurements.

Extraction and Clean-up Procedures

The extraction and clean-up procedures are simple and can be adapted to plants and soils.

Extraction and Clean-up in Soil: A 25 g sample of homogeneized soil sieved to 2 mm was extracted with 25 ml of acetonitrile and shaken in a capped centrifuge tube for 30 min, centrifuged for 5 min at 3000 rpm (1000 g) and filtered by suction.

A Florisil cartridge was washed with 10 ml of acetonitrile for activation of the sorbent. The sample extract was passed through the cartridge by negative pressure of 7 in of Hg by station Vac-Elut.

3 ml was taken from the liquid which passes through the cartridge and contains aldicarb, aldicarb sulfoxide and aldicarb sulfone. The volume was made up to 10 ml with water and was injected into the HPLC system.

Extraction and Clean-up in Potatoes: A total of 20 g of the homogeneous sample of potatoes was extracted in a capped centrifuge tube with 10 g anhydrous sodium sulfate and 20 ml of dichloromethane shaken for 30 min. This mixture was centrifuged at 3000 rpm (1000 g) for 5 min and the supernatant collected over anhydrous sodium sulfate.

Silica cartridge was washed with 5 ml of anhydrous dichloromethane for activation of the sorbent. The extract was passed through the cartridge by negative presure of 7 in of Hg. The remaining solvent in the cartridge was evapored under a nitrogen purge at 5 psi for a period of 10 min.

The concentrated sample was eluted with 2 ml of acetonitrile. The extract was diluted with water at ratio 1:2 acetonitrile-water and injected into the HPLC.

RESULT AND DISCUSSION

The use of DAD detection has allowed the selection of the wavelength which determinate each pesticide in the analytical procedure in order to get improvement in sensitivity and selectivity. aldicarb and its metabolites exhibit strong UV absorbance characteristics at the shorter wavelength of 202 nm, in addition to secondary maximum at 248 nm for aldicarb and 236 nm aldicarb sulfoxide. In FIGURE 2 spectrum in optimal conditions is shown.

The linear dynamic range of the detector response was checked for the pesticides at a wavelength of 202 nm between 0.25 and 500 ng. A typical chromatogram of pesticide mixture under the best conditions is shown in Figure 3.

Linearity of response using area peak at 202 nm vs. quantity injected, was studied by sucessive injections of 50 µl of working solutions. The linear calibration corresponds to the equations:

$A_p = 1.747+1.838$ [A] $r = 0.9998$

$A_p = 0.504+1.641$ [AX] $r = 0.9999$

$A_p = 1.773+1.859$ [AN] $r = 0.9998$

where A_p is in mUA per second and concentration in ng.

The limits of detection are 0.06 ng of aldicarb, 0.05 ng of aldicarb sulfoxide and 0.1 ng of aldicarb sulfone for a relation signal/noise ratio S/N = 3.

Ten replicate injections of the standard solution containing 5 ng each to aldicarb, aldicarb sulfoxide and aldicarb sulfone were carried out to determine the precision of the method. The coefficients of variation were 1.72%, 2.45% and 2.92% for aldicarb, aldicarb sulfoxide and aldicarb sulfone respectively.

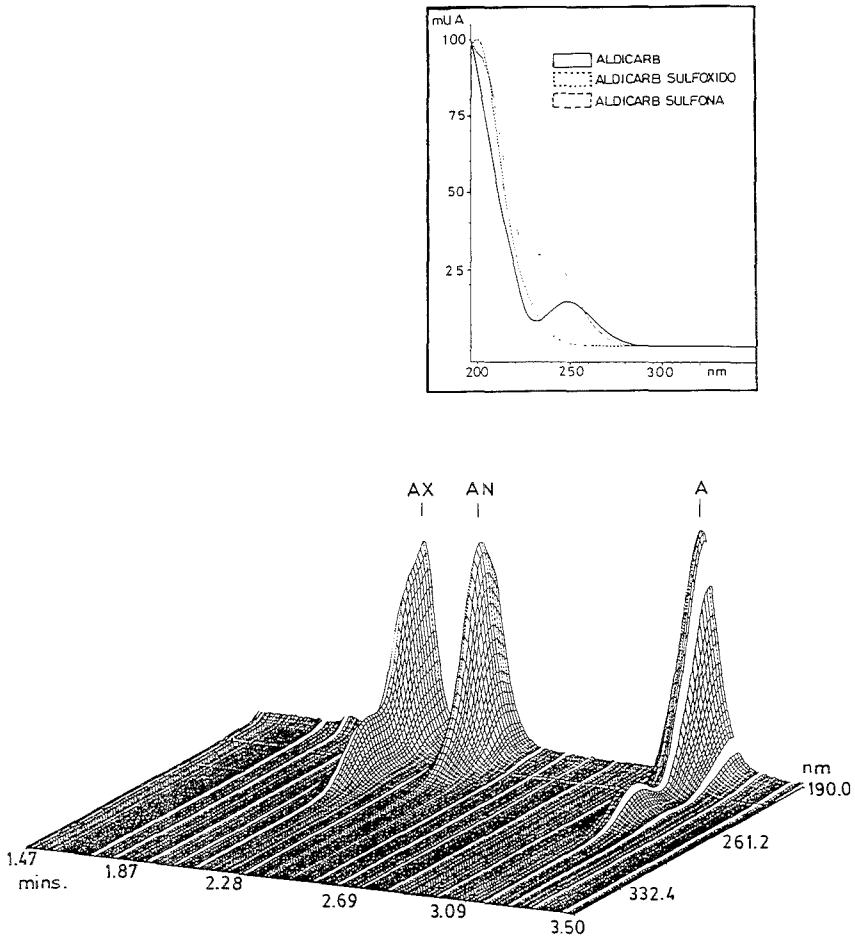

FIGURE 2. Evolution of the absorption spectra for the mixture of pesticides. Range λ: 190 - 350, Step: 2 nm, Threshold: 0.1 mUA, Total Spectra number: 1043, [A] = [AX] = [AN] = 500 ng injected
Other parameters in Figure1.

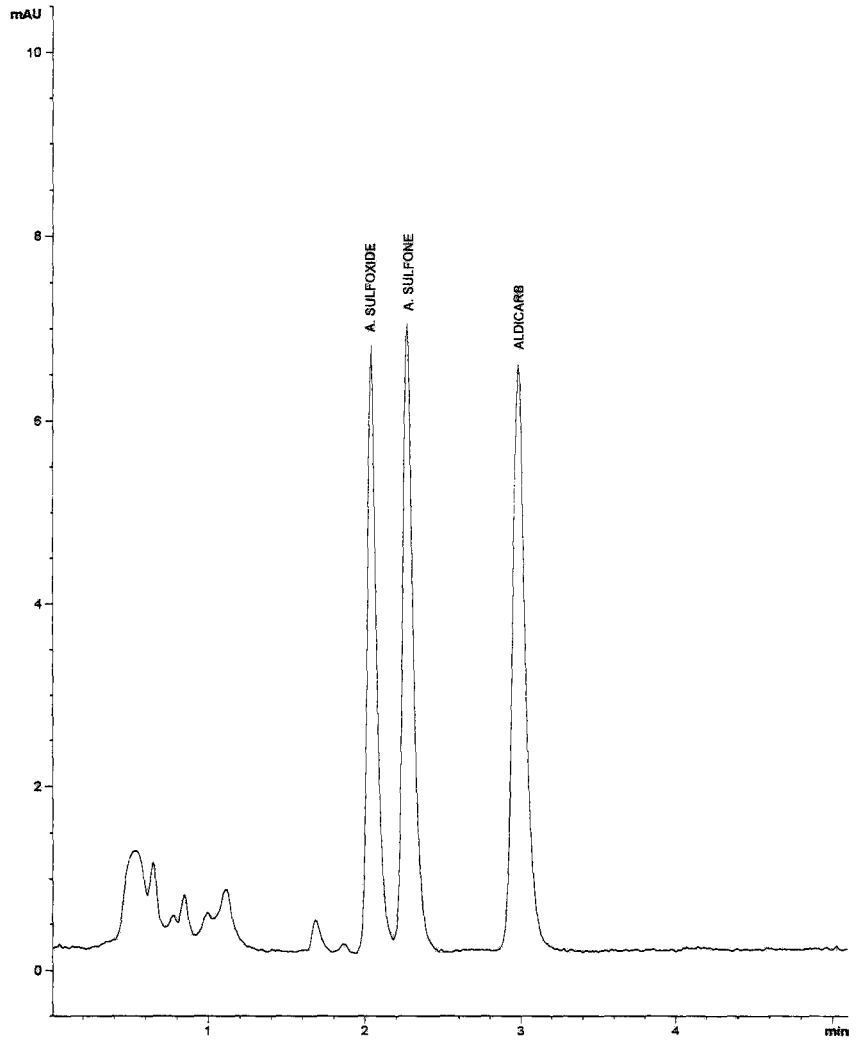

FIGURE 3. Liquid chromatogram for aldicarb and its metabolites.
Flow rate: 1.2 ml min^{-1}, injection volumen: 50 µl, λ analytic: 202 nm, [A] = [AX] = [AN] = 35 ng injected

The validity of the proposed method was proven by spiking with aldicarb and its metabolites in potato and soil samples over the range of 5 to 1000 μg kg^{-1} and subjecting the samples to the described analytical procedure. Average recoveries from fortified samples were 80-100% for aldicarb, aldicarb sulfoxide and aldicarb sulfone in soil and 60-100% for aldicarb and 80-100% for aldicarb sulfoxide and aldicarb sulfone in potatoes.

The proposed analytical method allows to reach the detection limits of 40 μg kg^{-1} in each pesticide in soil and 15 μg kg^{-1} in potatoes.

The proposed method is so quick and efficient that permits the systematic use to determine these compounds in soils and plants. It has been used to study the degradation of aldicarb in soils from Alava plain in the Basque Country (North of Spain).

The field treatments have taken place in grounds destined to be used for farming of sow and consume potatoes. The selection of grounds for this study was carried out taking account edaphologic criteria and that the area is representative as for surface, production and agricultural yields in the culture of potatoes.

In order to prove the possible change of behaviour that the metabolism can have for the plaguicide aldicarb depending on nature of the soils, were selected two grounds of diferent texture: clayey and sandy. The characteristics of soils selected for study can be observed in Table 1.

In these grounds, the Temik®, granule at 10% aldicarb, at rates of 20 kg/Ha was applied on April 1994. The samples of soil were collected in the course of the culture cycle of in periods of: 7, 15, 60 and 120 days from different depths: surface, 15 cm and 30 cm.

The samples of potatoes were taken randomly at the time of the harvest (October 1994).

TABLE 1

Characteristics of Soils Selected for Study

SOIL 1			
	SURFACE	**15 CM DEPTH**	**30 CM DEPTH**
TEXTURE	LOAM	LOAM-SAND	LOAM-SAND
HUMIDITY (%)	2.80	3.07	3.57
CONDUCTIVITY (μS/cm)	60.0	65.8	76.0
ORGANIC MATTER TOTAL (%)	1.27	1.40	1.30
pH (H_2O)	7.10	7.05	7.07
pH (KCl)	6.39	6.36	6.40

SOIL 2			
	SURFACE	**15 CM DEPTH**	**30 CM DEPTH**
TEXTURE	LOAM-CLAY	LOAM-CLAY	LOAM-CLAY
HUMIDITY (%)	3.72	3.90	4.01
CONDUCTIVITY (μS/cm)	100.6	108.8	94.7
ORGANIC MATTER TOTAL (%)	1.84	1.84	1.80
pH (H_2O)	7.91	7.82	7.85
pH (KCl)	7.35	7.27	7.36

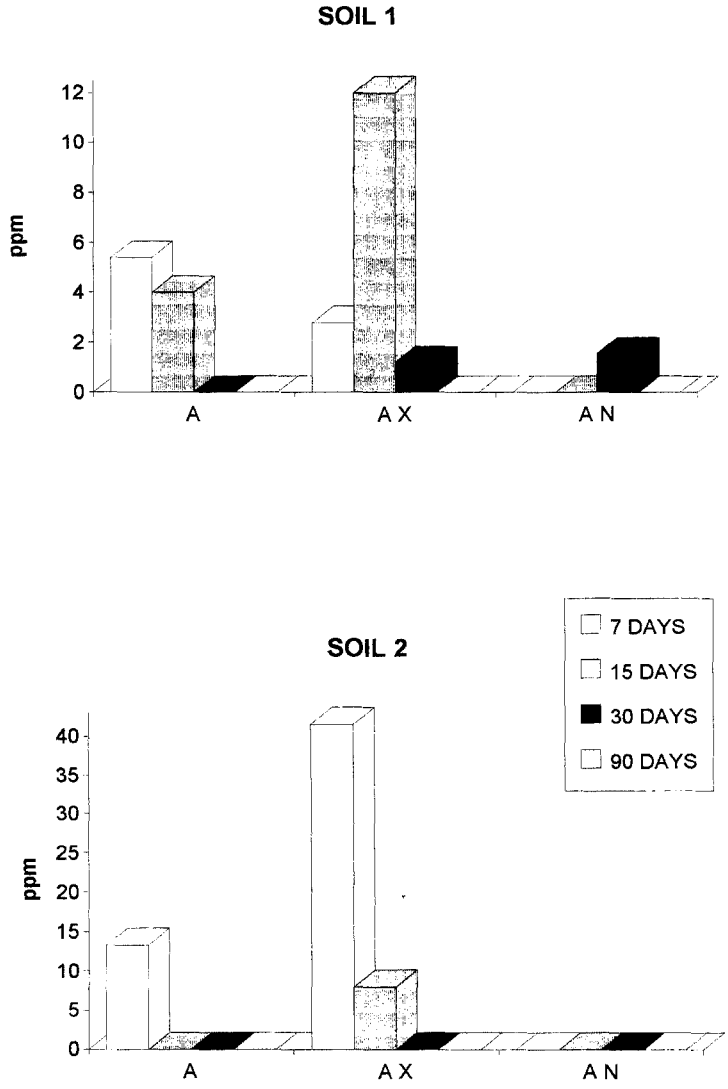

FIGURE 4. Persistence of nematicide aldicarb (A) and its metabolites aldicarb sulfoxide (AX) and aldicarb sulfone (AN) in the whole culture cycle in both selectionated soils.

With regard to the studies carried out with samples of soil one might consider that the maximum quantity of aldicarb has been observed during the first week after the treatment and at 15 cm depth.

The first product which is observed in the degradation process of the aldicarb in soils is the aldicarb sulfoxide, increasing its concentration progressively up to 15 days after the application; at this time, aldicarb sulfoxide and its respective aldicarb sulfone appear together.

120 days after the application were observed neither aldicarb nor its metabolites.

The Figure 4 shows the result obtained. It can be seen that the degradation of the aldicarb is favoured in those soils with clay-like texture (soil 2) compared to those of sandy texture or loamy (soil 1). This is so because the oxidation reaction is catalized by the components of clay.

The result obtained from potatoes samples allows us to conclude that none of the plaguicides are present in this vegetable at consumption moment for concentrations up to the detection limits.

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A VALIDATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PARACETAMOL AND ITS MAJOR METABOLITES IN URINE

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ABSTRACT

A reversed-phase high-performance liquid chromatographic (RP-HPLC) method employing UV-detection (248 nm), 5 μm Resolve C18 as stationary phase (in a 8 x 100 mm Waters Radial-Pak cartridge) and 0.1 M potassium phosphate monobasic/methanol/glacial acetic acid (95:4:1, v/v/v) as mobile phase was developed for the rapid and simultaneous determination of paracetamol and its major metabolites (its glucuronide and sulphate conjugates) in urine. The method requires only minimal sample preparation and chromatographic run time is only 6 min. For determination of paracetamol, the precision (inter-assay RSD ranged from 1.60-0.66% between 5 and

100 μg mL^{-1}) and limits of detection (2 ng mL^{-1}) were satisfactory, as were these parameters for determination of the major metabolites. In the course of studies on paracetamol bioavailability and metabolism, over 1500 samples have been assayed using this method. Herein we report its use for monitoring of the levels of paracetamol and its major metabolites in the urine of fifteen normal healthy volunteers given a single oral dose of 500 mg.

INTRODUCTION

Paracetamol or acetaminophen (N-acetyl-*p*-aminophenol) is a mild analgesic-antipyretic used in the treatment of somatic pain (e.g. myalgia, arthralgia, neuralgia), often where aspirin (acetylsalicylic acid) is contraindicated (for example, in cases where there is gastrointestinal intolerance, hyperuricaemia, gastritis, asthma or von Willebrand disease) or when the analgesic is to be administered in conjunction with other drugs, such as non-steroidal inflammatory agents or oral anticoagulants [1].

Following oral administration of a therapeutic dose of paracetamol, it is rapidly absorbed from the gastrointestinal tract. Peak plasma levels are reached within 30 to 120 min, and the therapeutic half life is about 3 h, the bulk (ca. 98%) of the drug being metabolized in the hepatic system, primarily to its glucuronide and sulphate conjugates [2-4]. The incidence of the first pass effect on drug bioavailability depends on the dose, dosage interval and route of administration [5-7].

Many analytical techniques have been used to determine paracetamol in biological fluids, among them colorimetry [8,9], gas-liquid chromatography [10-15],

thin layer chromatography [16], high performance liquid chromatography [17-22] and immuno-assay techniques [23,24]. Nonetheless, since these assays were largely developed for use in clinical toxicology, they are not necessarily suitable for pharmacokinetic studies, which require precise measurement of very low levels of paracetamol and its metabolites and often involve analysis of large numbers of samples in short periods [25-28].

In this work we aimed to develop a paracetamol assay suitable for use in studies of its bioavailability and metabolism. In order to minimize sample preparation, a highly specific technique was called for. The technique chosen was HPLC with UV detection, which as well as specificity offered high sensitivity, and the possibility of semi-automation. The chromatographic run time was minimized by employing a Waters Radial-Pak cartridge and optimizing chromatographic efficiency, selectivity and resolution. The assay developed allows simultaneous determination of paracetamol and its major metabolites (its glucuronide and sulphate conjugates) in urine. The limits of detection and precision of the assay are reported, and also a specimen set of data for paracetamol levels in the urine of fifteen normal healthy volunteers over a 12 h period.

EXPERIMENTAL

Materials

Paracetamol was obtained from C. Barcia (Barcelona, Spain). Paracetamol sulphate and paracetamol glucuronide

were synthetized according to the methods of Burkhardt [30] and Shibasaki [31], respectively, in the Pharmaceutical Chemistry Laboratory, Pharmacy Faculty, University of Santiago de Compostela (Spain). HPLC grade methanol and glacial acetic acid were obtained from E. Merck (Darmstadt, Germany). Analytical grade monobasic potassium phosphate was obtained from Probus (Barcelona, Spain). Water for HPLC was purified using the Milli Q Water Purification System (Millipore, Bedford, MA, USA).

Sample collection

Following administration of a 500 mg dose of paracetamol, urine samples were collected after set time intervals. Urine volume was recorded and a 20 mL aliquot was stored at -20°C (at this temperature paracetamol and its conjugates are stable in plasma and urine) [32]. A urine blank, i.e. urine free of paracetamol and its metabolites, was also procured.

Preparation of standards and test samples

Stock solutions of 1 mg mL^{-1} paracetamol, paracetamol glucuronide and paracetamol sulphate in deionized water were prepared. Aliquots of each solution were then diluted with the urine blank to give standards having analyte concentrations of 5, 10, 15, 20, 25, 50 and 100 $\mu g\ mL^{-1}$.

The test samples were prepared by diluting each thawed 20 mL urine sample (including the urine blank) with a suitable volume of deionized water and filtering

this solution through a 0.45 μm hydrophilic membrane (Millex HV filter unit, Millipore, Bedford, USA). A sample of the filtrate was transferred to an injection in preparation for chromatography.

Chromatography

The HPLC system comprised a Waters 510 pump, a Waters Intelligent Sample Processor 712 automatic injection module, and a Waters Guard-Pak precolumn and Radial-Pak cartridge (8 mm i.d. x 100 mm) - the latter housed in a Waters Radial Compression Module (RCM8x10) - both packed with 5 m particle-size Resolve C18. The pressure of the system was maintained at 1200 p.s.i. UV-detection was by a Waters Lambda Max 490 (set at 248 nm) and data handling by a Waters 745 B Data Module. The mobile phase was 0.1 M potassium phosphate monobasic/methanol/glacial acetic acid (95:4:1, v/v/v), degassed by sonication; the flow rate was 2.5 mL^{-1} min. In all cases, 20 μL of sample/standard was injected onto the column.

Calibration

Calibration curves for each analyte were constructed by least-squares regression of peak area (mean of three replicates) on analyte concentration in the standard solutions. The peak areas for the test samples were converted to concentrations (in μg mL^{-1}) by multiplying them by the reciprocal slope of the corresponding calibration curve.

Precision

For evaluation of the precision, the paracetamol and the paracetamol glucuronide and sulphate standards were assayed as described four times and the relative standard deviation (RSD) for each of the seven concentrations (5 to 100 $\mu g\ mL^{-1}$) was calculated.

Application

Fifteen normal healthy volunteers were given a single oral dose of 500 mg paracetamol. Urine samples were collected after 1, 2, 3, 4, 5, 6, 8, 10 and 12 h and processed and assayed (three replicates) as described.

RESULTS AND DISCUSSION

Table 1 shows the parameters for the calibration curves. All three were linear with correlation coefficients > 0.998 over the range of concentrations studied.

Typical chromatograms for a diluted, filtered urine sample and the correspondingly processed urine blank are shown in Figs. 1(a) and 1(b) respectively. Figure 1(a) corresponds to urine obtained from a subject 3 h after a single oral dose of 500 mg of paracetamol. The overall sample run time was 6 min, and the retention times for paracetamol glucuronide, paracetamol sulphate and unchanged paracetamol were 2.15, 3.48 and 4.47 min, respectively. Fig. 1(b), showing the chromatogram of the urine blank, confirms that there were no signals due to

TABLE 1

Parameters of the Calibration Curves for the Determination of Paracetamol and its Glucuronide and Sulphate Metabolites in Urine (using peak area counts).

	Glucuronide	Sulphate	Paracetamol
Slope	1539543	1602243	124298
Intercept	6343569	2310349	54499
RSD of slope (%)	1.67	3.29	1.10
Conc. range ($\mu g\ mL^{-1}$)	5-100	5-100	5-100
Number of standards	7	7	7
Correlation coeficient	0.9993	0.9987	0.9997

endogenous compounds at the retention times of the analytes. When endogenous compounds in the urine gave peaks close to these retention times, limits of detection were slightly affected. Notwithstanding, as little as 2 ng mL^{-1} of paracetamol and 5 ng mL^{-1} of its glucuronide and sulphate conjugates (in paracetamol equivalents) could be detected as a distinct peak, which is more than satisfactory since, even 12 h after therapeutic doses, urine concentrations of paracetamol are generally measured in terms of milligrams.

The precision is shown for paracetamol in Table 2. The relative standard deviation ranged from 1.60-0.66% between 5 and 100 $\mu g\ mL^{-1}$ paracetamol, indicating that precision was satisfactory in the concentration range studied. For paracetamol glucuronide and sulphate, the RSDs ranged from 0.4-1.5% and 0.7-2.4%, respectively, in the same concentration range.

The method described here has been successfully used to assay paracetamol in over 1,500 urine samples in the course of studies on paracetamol bioavailability and

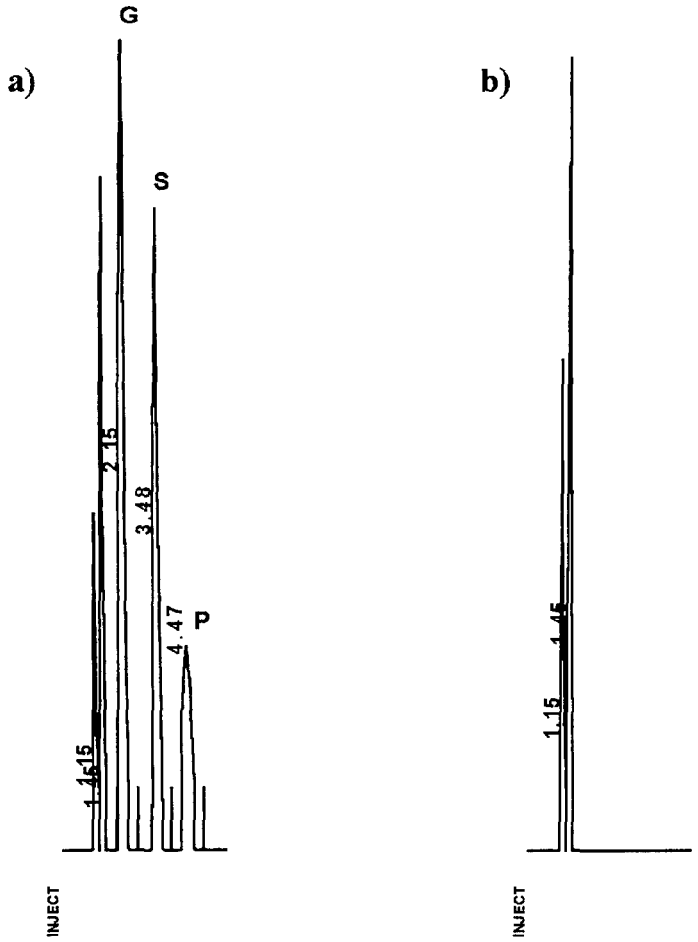

FIGURE 1. (a) Chromatogram of paracetamol (P) and its glucuronide (G) and sulphate (S) metabolites in urine. Concentrations P = 3.04 μg mL^{-1}; S = 27.72 μg mL^{-1}; G = 49.33 μg mL^{-1}. (b) Chromatogram of a processed urine blank.

TABLE 2

Precision (RSD) of the HPLC Assay for Paracetamol (5-100 $\mu g\ mL^{-1}$) in Urine (using peak area counts).

Paracetamol conc. ($\mu g\ mL^{-1}$)	Peak area counts. Mean of four runs ± SD	R.S.D (%)
5	640302±10250	1.60
10	1369182±19237	1.40
15	1916115±23723	1.23
20	2435508±32270	1.32
25	3133128±45283	1.44
50	6424224±54275	0.84
100	12430191±83242	0.66

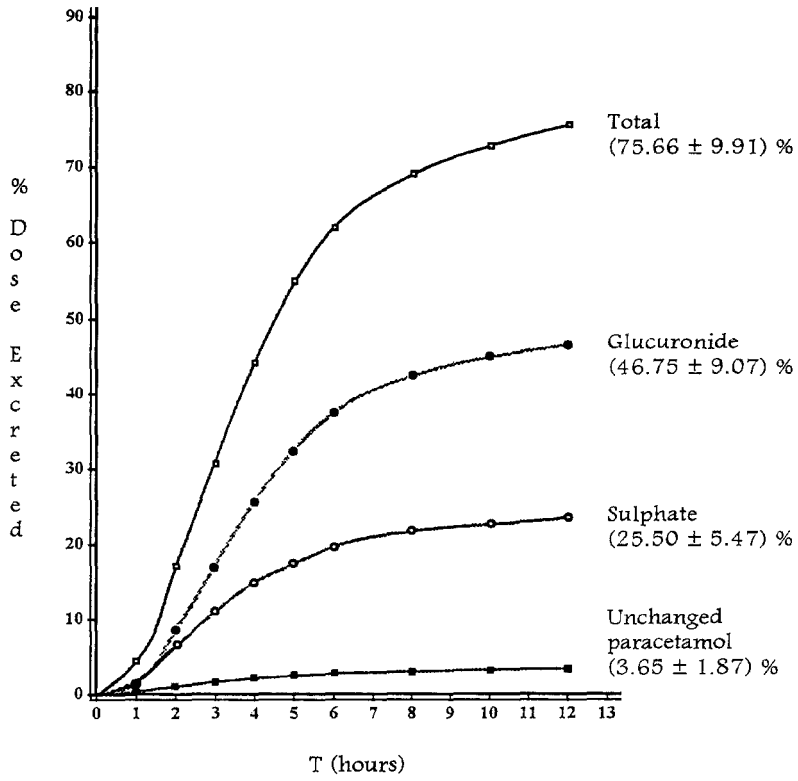

FIGURE 2. Cumulative curves of % dose excreted *vs* time for unchanged paracetamol, its glucuronide and sulphate conjugates, and the sum of these, after oral administration of a 500 mg dose to fifteen normal healthy volunteers.

metabolism [29]. Figure 2 shows the urinary profiles obtained for paracetamol and its glucuronide and sulphate conjugates in the short study described here. After 12 h, 3.65 ± 1.87% of the ingested paracetamol was excreted unchanged, and 25.50 ± 5.47% and 46.75 ± 9.07% was excreted as the sulphate and glucuronide conjugates, respectively (total, 75.66 ± 9.91%). These results are in keeping with those of HPLC assays for paracetamol employing conventional columns [17, 19, 21, 33].

CONCLUSIONS

An HPLC method was developed for the determination of paracetamol and its major metabolites, its glucuronide and sulphate conjugates, in urine. This method requires only minimal sample preparation and has a short chromatographic run time. The calibration curves are highly linear, and precision and limits of detection are satisfactory. This rapid method is especially suited to pharmacokinetic studies involving analysis of large numbers of samples, and has already been used to assay over 1500 samples in the course of studies on paracetamol bioavailability and metabolism.

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DETERMINATION OF DEGRADATION PRODUCTS OCCURRING IN ACIDIC SOLUTIONS OF A 21-AMINOSTEROID (TIRILAZAD) USING A GRADIENT HPLC METHOD

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ABSTRACT

A high performance liquid chromatographic (HPLC) method quantitating degradation products of a 21-aminosteroid (tirilazad mesylate) in acidic solutions is described. Mobile phase elution consists of a 58 minute gradient from 5 to 95% mobile phase A, where mobile phases A and B are (81:9:10:0 v/v) and (5.4:0.6:10:84 v/v) acetonitrile, tetrahydrofuran, 0.2 M ammonium formate/ formic acid buffer (pH 3.5) and water. A base deactivated octadecyl silane column thermostatted at 42°C, and fixed wavelength detection at 254 nm are used for this method. The assay is compatible with thermospray mass spectrometry, offering several advantages for the identification of impurities. Validation of the method, along with the use of mass spectrometry for peak identity and purity is discussed.

INTRODUCTION

Tirilazad mesylate (U-74,000F), a 21-amino substituted steroid [16-α-methyl- 17-α-hydrogenpregna-1,4,9(11)-triene-3,20-dione-21-[2,4-bis-(1-

pyrrolidinyl)-6-piperazinylpyrimidine] methane sulfonate], inhibits lipid peroxidation (1,2). Activity in experimental models of head, spinal injury (3,4), and hemorrhagic shock (5) were found. Because the solubility of tirilazad decreases with increasing pH, administration in-vivo uses acidic aqueous solutions (pH 3). Tirilazad degrades in solution and quantitative methods were needed to determine the amounts of these degradation products. Secondarily, the method was used to determine the amounts of process and degradation related impurities in the bulk tirilazad mesylate. In acidic solutions, tirilazad degrades from four reactions: hydrolysis of the piperazine-pyrimidine bond, oxidation of the C-20 ketone, dienone-phenol rearrangement of the steroid A ring and oxidation to produce the amine side-chain (6). Figure 1 shows the major degradation pathways.

Development of a HPLC method that is compatible with thermospray mass spectrometry generally entails the use of volatile buffers and offers several advantages. Information regarding peak identity and purity is obtained. Isolation of unknowns is easier as thermospray mass spectrometry compatible systems use volatile buffers that can be removed via freeze-drying. Compounds with low UV absorbance are detected (assuming sufficient ionization efficiency). Thermospray mass spectrometry compatible systems do have some disadvantages. Peak shape may be compromised because competing amines are not typically used because of their lack of volatility and their affect on sensitivity (compete for protons). In the absence of post-column mixing, mobile phase pH is usually restricted either to acidic pH's or to the use of unbuffered systems. To achieve ionization, acidic pH's are preferred for basic analytes. The buffers may compromise UV detectability as carboxylic acids absorb significantly below 230 nm.

None of the above restrictions significantly affected developing a method for tirilazad. The newer base-deactivated columns made possible the development of high efficiency separations for basic analytes without using competing amines. This report discusses the development of a thermospray mass spectrometry compatible method for degradation

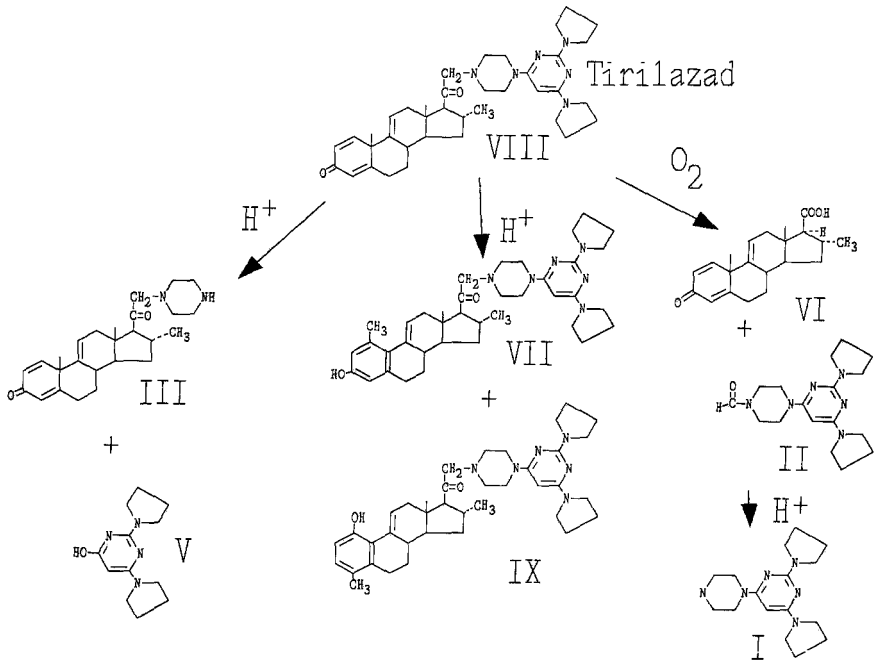

Figure 1. Degradation of Tirilazad mesylate (compounds are numbered to conform with Reference 6).

products in tirilazad, including improvements over the methods used in the identification of the degradation products (6).

MATERIALS AND METHODS

Reagents/Compounds

All solvents were HPLC grade (Burdick and Jackson, Muskegon, MI). Other reagents were analytical reagent grade. Tirilazad mesylate (**VIII**) and compounds **I**, **II**, **III**, **V** and **VI** were prepared in the research labs of The Upjohn Company (6).

Equipment

The gradient pump was a low-pressure, mixing system (Model 2249, LKB, Bromma, Sweden). A line-source detector at 254 nm (UV Monitor III, LDC, Rivera Beach, Fl) was used in the validation experiments. A photo-diode array HPLC detector (Model 990, Waters Assoc., Milford, MA) was used to obtain UV spectra of the degradation products and peak homogeneity of the tirilazad . The column was a 4.6 X 250 mm, 5 µm, octadecylsilane column (Inertsil®, GL Sciences, Tokyo, Japan). Other columns that were evaluated included: YMC-Basic® (YMC, Morris Plains, NJ, USA), Develosil® (DyChrom, Sunnyvale, CA, USA), Rexchrom® (Regis, Morton Grove, IL, USA) and Asahipak ODP-50®, an ODS modified polymeric column (Asahi Chem. Co., Kawasaki-ski, Japan).

An octadecylsilane TLC plate (RP-18, WF254s, EM Science, Cherry Hill, NJ, USA) was used with mobile phase A to evaluate the elution of all peaks from the solvent front. The plates were scanned at 254 nm (Camag, Muttenz, Switzerland) and developed using a starch-iodine mixture (dried at 50 °C for 30 minutes).

Mass spectra were acquired with either a quadrapole MS with thermospray interface (Model 4600, Finnigan, San Jose, CA) or a triple quadrapole system (Model 70, Finnigan, San Jose, CA). The instrument was operated in the discharge mode with a vaporizer temperature of 100 °C, a jet temperature of 250 °C and a repeller voltage of 80 V. To maintain uniform response, a cross-gradient was operated post-column thus maintaining a consistent solvent composition into the interface.

Methods

Column temperature was maintained at 42 ° C. A linear gradient began at 2 minutes and increased from 5% to 95% mobile phase A over a 58 minute period. Mobile phases A and B were (81:9:10:0) and (5.4:0.6:10:84) acetonitrile, tetrahydrofuran, 0.2 M ammonium formate/ formic acid buffer

(pH 3.5), and water. A flow rate of 1.0 ml/min was used. Blank peaks were reduced by passing the aqueous buffer solution through a octadecylsilane cartridge (Sep-pak, Water's Associates, Milford, MA).

Tirilazad mesylate solutions at 1.5 mg/ml were diluted 1:4 with a solution of methanol:water: ammonium formate buffer (used in mobile phase preparation) 50:45:5. A 10 µL injection volume was used.

RESULTS AND DISCUSSION

Method Development

HPLC systems for tirilazad and degradation products of tirilazad (Figure 1) were previously reported using isocratic reversed-phase (7,8) and gradient methods (6). Problems with these methods include insufficient retention of the more polar impurities, inadequate resolution of all known impurities from the tirilazad mesylate, and poor peak shape for tirilazad . Over the past few years, a large number of columns were claimed to give high efficiency for basic analytes. After comparing several of these columns for peak efficiency (Table 1), an Inertsil column was selected for further evaluation. Using the Inertsil column provided a highly efficient chromatographic system without using competing amines.

Using an acetonitrile gradient with pH controlled at 4.0 with ammonium formate as a starting point (6), modifications were made to improve both peak shape and resolution. Adding tetrahydrofuran to the mobile phase improved the separation of the more polar compounds, peaks **I**, **III** and **V**, and reduced peak tailing. The ratio of acetonitrile:tetrahydrofuran was optimized at a ratio of 9:1 and was maintained at that ratio throughout the gradient. The gradient slope, mobile phase pH, and column temperature were then optimized. The resulting chromatography is shown in Figure 2. Table 2 shows the relative retention times, response factors (254 mn), observed UV maximum and molecular ions from LC/MS.

Table 1
Columns Evaluated for use in Method (Parameters measured for tirilazad)

Column Description	Theoretical Plates	Retention Time (min)
YMC-Basic mixed alkyl chain length (4.6 x 150 mm)	3000	25
Develosil, C18 (4.6 x 250 mm)	3100	35
Asahipak ODP-50, C18 modified polymeric column (4.6 x 250 mm)	6500	23
Dupont C8 RX (4.6 x 150 mm)	1400	36
Inertsil C18 (4.6 x 250 mm)	14000	38

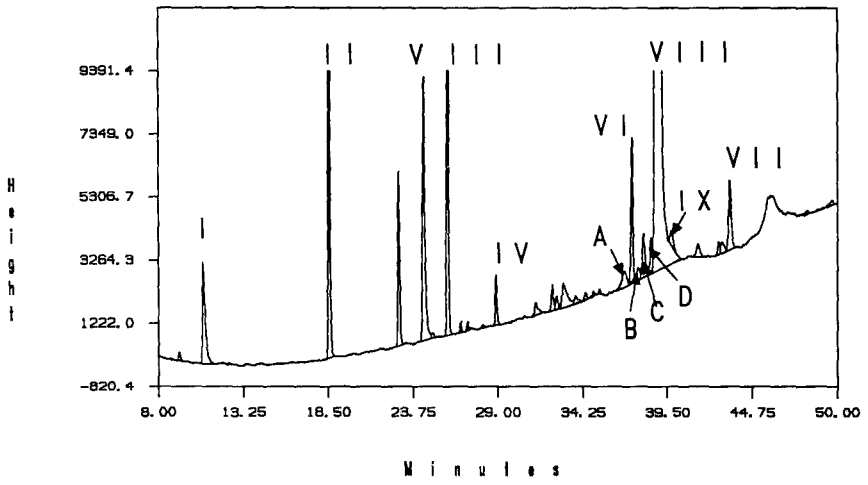

Figure 2. Chromatogram of Degraded Tirilazad mesylate (see Figure 1 for identity of peaks)

Table 2
Chromatographic and Mass Spectroscopic Data on Compounds
(compounds are numbered to conform with Reference 6.)

Compound	UV Absorption Maxima	Relative Response Factor (254 nm)	Relative Retention Time	Molecular Weight	LC/MS Ion (M+H)
I	245, 295	1.23	0.23	302.43	303
II	248, 296	1.76	0.44	330.40	331
III	242	0.48	0.63	408.6	409
IV	242	not known	0.73		339
V	238, 278	0.81	0.58	234.3	235
VI	244	0.73	0.92	326.43	327
VII	251, 293	1.0[b]	1.12	624.87	625
VIII (Tirilazad)	244, 295	1.0	1.0	624.87	625
IX	246, 294	1.0[b]	1.03	624.87	625

[b] Response factors were assumed to be 1.0.

Thermospray Mass Spectrometry

Using the newly developed method, thermospray mass spectrometry was performed on a sterile solution sample (1 month at 47°C, total impurities approximately 8%), an acid-degraded sterile solution (approximately 30%-40% total impurities), and a severely degraded bulk tirilazad mesylate sample (55 months at room temperature, approximately 20%-30% total impurities). Figure 3 shows the UV chromatogram and corresponding thermospray total ion current chromatogram for the degraded sterile solution.

Masses were obtained for significant unidentified impurities (Table 3). From this information, the unidentified impurity at r= 0.81 is the

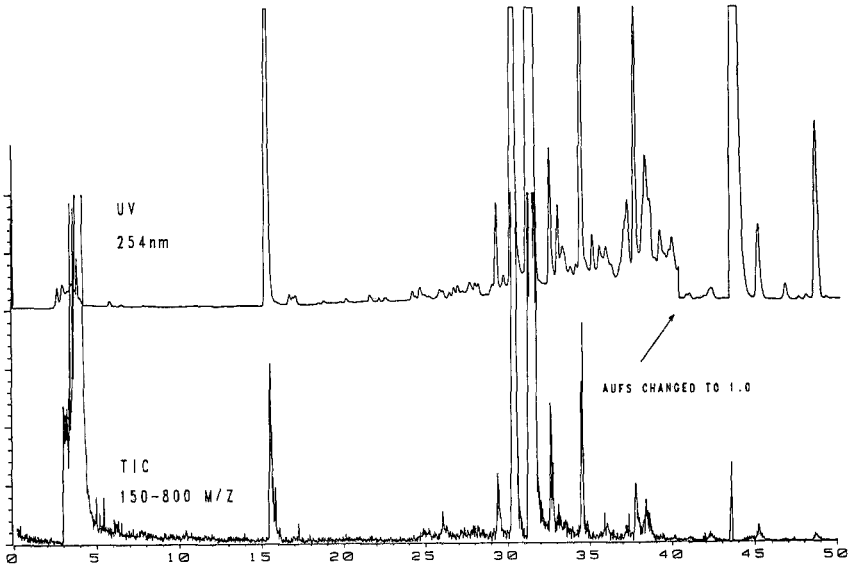

Figure 3. UV and Thermospray Total Ion Chromatograms of U-74,006F Aqueous Degradation Sample

Table 3
Mass Spectral Information for Unidentified Peaks

Relative retention	label	MH+	possible structural characteristics
0.81	IV	339	steroid
0.94	A	640	unknown
0.97	B	642	oxidation product
0.98	C	657	oxidation product
1.03	IX	626	tirilazad isomer

unknown steroid (M/Z = 339) previously discussed in reference 6. Based on the mass (M/Z=625) and the conditions in which the impurity is formed (low pH, high temperature) , the peak was identified as the *para* isomer of the dienone-phenol rearrangement product of tirilazad . Previous studies discussed the possible presence of this impurity, but it was never found (6). The resolution of this impurity is a significant improvement over previous work.

Mass spectrometry data also confirmed the identity and purity of the known degradation peaks (Table 2) and the absence of significant UV transparent peaks. Analysis of the edges of the tirilazad peak in all samples revealed that no extraneous ions were found. This confirms that no impurities with masses differing from the tirilazad were eluting on the front or tail of the tirilazad peak.

Validation

All known process and degradation related impurities were resolved from the tirilazad peak. As previously discussed, MS was used to confirm the absence of extraneous ions eluting under the tirilazad peak. TLC was used to evaluate whether all peaks elute in the chromatogram. No UV transparent peaks were found to be retained at the origin. UV scans of the plates at 254 nm confirmed that all peaks eluted from the solvent front in the bulk tirilazad mesylate sample. For the sterile solution samples, less than 0.2% of the degraded material remained at the origin for a sample that was degraded about 20%.

Chromatographic ruggedness was evaluated by slightly changing mobile phase pH, gradient slope and intercept and column temperature. Changes in pH over the range of 3.4 to 3.7 only cause minor changes in the resolution of the most significant impurities. Decreasing pH increases the resolution of peaks **V** and **III**. The effect of pH is more important for several minor peaks (**A-D**) that elute near tirilazad (see Figure 4). Peaks **A-D** were of little importance for assessing degradation but were important in

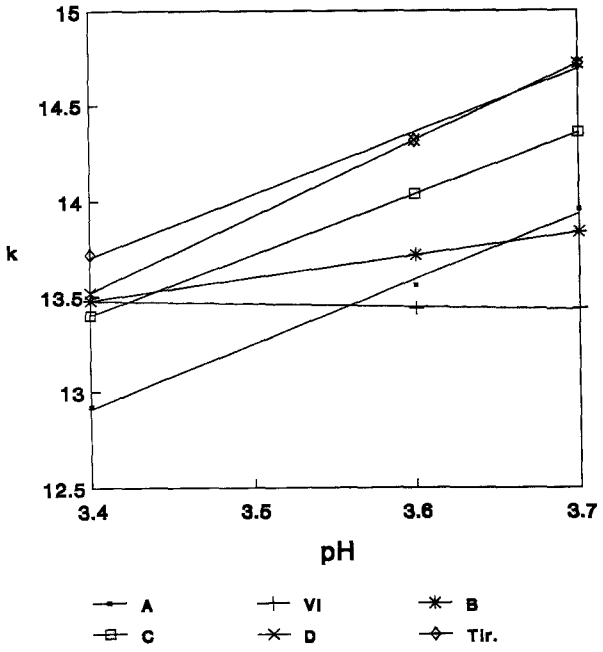

Figure 4. Effect of pH on Retention and Resolution (peaks eluting near tirilazad)

bulk tirilazad mesylate purity. The resolution of these impurities determined the final selection of mobile phase pH. Table 4 shows the effect of gradient slope, initial organic concentration, gradient delay, and temperature on the separation of the most critical peak pairs (tirilazad/**IX**) and (**III/V**). The resolution of tirilazad/**IX** varies from 2.6 to 3.1 over the parameter ranges studied. Only increases in gradient slope cause substantial decreases in resolution of peaks **V** and **III**. Other peak pairs were only moderately affected by these changes.

During the course of validation, some columns were found to cause a substantial increase in an unknown impurity at relative retention 0.97.

Table 4
Effects of Modifying Chromatographic Conditions on Resolution Factors (other variables held constant as specified in Experimental section)

Chromatographic Variable	Resolution Factor for Peak Pair	
	III/V	Tirilazad/IX
Gradient Slope (%/min change in A)		
1.11	10.6	3.1
1.38	7.7	2.9
1.64	5.7	2.8
Column Temperature (°C)		
38	5.1	2.6
40	5.3	2.8
42	5.4	2.8
Initial Percent A		
5	6.6	2.8
10	6.8	2.9
Gradient Delay (min)		
0	6.5	2.8
5	6.8	2.9

When only the column was switched, the same sample would show as much as a 300% increase in the impurity at 0.97 (total amount still less than 0.3%). Other experiments demonstrated that the increased degradation was not caused by the column frits or by instability in the mobile phase. The peak was concluded to be caused by on-column degradation.

Tirilazad peak area response was linear (R = 1.000) over the range of 0.0045 to 4.95 μg (amount injected). The residual relative standard

deviation from the regression was 0.45%. Linearity data provided response factors for peaks **I**, **II**, **III** and **V**. Correlation coefficients were >0.999 for all impurities. Repeated analysis of an impurity mix determined the precision for the quantitation of impurities. The relative standard deviations typically varied from 0.3% to 6% for impurities present at 5% to 0.2% (as a percentage of tirilazad plus other impurities). All impurities present at 0.1% or greater in samples stored under nominal conditions were resolved from other impurities.

Sample stability was evaluated over a 48 hr time-frame. The amount of the primary degradation impurity, **III**, increased over time, particularly in the bulk tirilazad mesylate sample. However, total impurity levels did not rise over the same time frame. A limit of 24 hours in a light protected environment ensures sample preparation stability.

For regulatory purposes a limit of quantitation of not more than 0.1% was needed. Root mean square (RMS) noise for seven blank injections was determined at six different 1 minute intervals. From these data, the worst case noise occurs after the onset of the gradient, near the elution of peak **I** (approximately 11 minutes). As compound **I** has the smallest peak height to peak area ratio, the worst case detection limits were calculated for this peak. Using a S/N ratio of 3, the detection limit was 0.007% (as a percentage of tirilazad). The quantitation limit, defined for a S/N ratio of 10, was 0.03%, meeting regulatory requirements.

Batch Analysis and Mass Balance Assessment

Several sterile solution lots stored at temperatures from 4 to 47 °C under various conditions were evaluated for impurities using the new method. The amounts of the degradation products in these lots ranged from 1.2 to 19% and are shown in Table 5. The average mass balance for these solutions was 99.2% with a relative standard deviation of 1.7% . The effect of heat, pH and oxygen on the formation of these degradation

Table 5
Degradation Product Data on Representative Samples (NQ = not quantitated, <0.05%; ND = not detected, approximately <0.02%)

Lot	Conditions	I	III	V	VI	VII	IX	Total Impurities
1	6 months, 8 °C	NQ	0.82	0.27	NQ	ND	ND	1.2
3	6 months, 25 °C	0.11	3.1	1.2	0.10	0.063	ND	4.9
4	3 months, 47 °C	1.4	9.9	4.2	0.17	0.62	0.19	19
6	9 months, 25 °C	0.64	4.5	1.9	NQ	0.074	ND	7.8
7	36 months, 4 °C	0.23	1.6	0.59	NQ	NQ	ND	3.3
8	6 months, 4 °C	0.11	0.75	0.24	NQ	NQ	NQ	1.9

products was previously discussed (6). The newly identified degradation product (**IX**, ***para*** isomer of dienone-phenol rearrangement) was only found in the sample stored at 47 °C.

CONCLUSIONS

A LC/MS compatible HPLC method for quantifying all significant degradation products in acidic solutions of tirilazad mesylate was developed. The success of this method was evidenced by a mass balance of 99.2%. Development of a method compatible with thermospray mass spectrometry proved advantageous in that molecular-ion information was obtained for nine compound, tirilazad peak homogeneity was evaluated and a significant unknown degradation impurity was identified. Use of an Inertsil column, designed for use with basic molecules, made the development of an efficient method easier as no competing amines were necessary to achieve satisfactory peak shape.

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EVALUATION OF ELECTROCHEMICAL AND FLUORESCENCE DETECTION IN LIQUID CHROMATOGRAPHY FOR THE ASSAY OF INDOMETHACIN IN AQUEOUS HUMOUR SAMPLES

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ABSTRACT

The performance of amperometric and fluorescence detection *vs* UV detection in high performance liquid chromatography (HPLC) have been evaluated for monitoring indomethacin at trace levels in rabbit aqueous humour. Amperometric detection at a potential of +1.20V (*vs* Ag/AgCl) did not present any advantage over UV detection. However, fluorescence detection after liquid-liquid extraction with ethyl acetate and pre-column hydrolysis yielded limits of detection (2 $\mu g\ l^{-1}$) and quantitation (5 $\mu g\ l^{-1}$), lowered by a factor of 2 by comparison with UV detection. Validation results for a fluorescence method in aqueous humour are presented concerning specificity, linearity, accuracy, precision, analytical recovery, limits of detection and quantitation.

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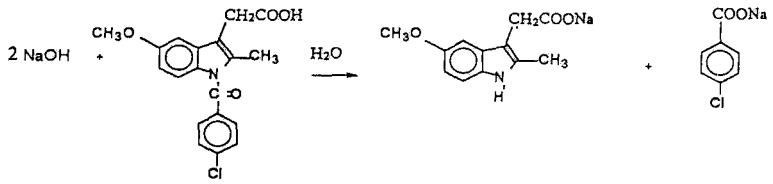
INTRODUCTION

The aim of this study was to develop sensitive and selective methods in HPLC to study the pharmacokinetics of indomethacin in rabbit aqueous humour (AH), following the administration of eye drops. The major problem in developing sensitive methods for this biological medium is related to the very small volumes of samples available (ca. 50-100-µl). The performance of electrochemical (EC) and fluorescence (FL) detection were investigated with the objective of improving the limit of quantitation (LOQ) of 10 $\mu g\ l^{-1}$ which can be attained in AH, using UV absorption detection (1).

BACKGROUND

Numerous HPLC methods have been proposed in the literature for the determination of indomethacin in biological samples; most of them deal with plasma, serum or urine (2-17), and some with AH (18-19). UV absorption is by far the most commonly used detection mode (1-11,19) with a LOQ which can be as low as 10-20 $\mu g\ l^{-1}$ in plasma and about 50-200 $\mu g\ l^{-1}$ in urine. In AH, the LOQ is not mentionned by ROVIRA (19), but a 10 $\mu g\ l^{-1}$ LOQ is feasible with ACN deproteinisation and UV detection at 266 nm (1). A few authors have used fluorimetric detection after pre (13, 15, 18) or post-column (12, 14, 16) hydrolysis of indomethacin into fluorescent dechlorobenzoylindomethacin (DBI) in plasma and urine (Figure 1). However, when DBI is present as a metabolite in these media, it is necessary to use a difference method with a pre-column reaction (13). In plasma, the best LOQ (and LOQ) for indomethacin is as low as 1 $\mu g\ l^{-1}$ (signal-to-noise ratio=2) (15). In urine, the lowest concentration used for calibration was about 250 $\mu g\ l^{-1}$ (12, 13). In aqueous humour, the LOD is 50 $\mu g\ l^{-1}$ with fluorescence detection after pre-column derivatization (18). The absence of DBI as metabolite in this medium has been mentionned by this author. Electrochemical detection (coulometric detection) has also been used by KAZEMIFARD for indomethacin assay in plasma (17). A LOD of



Indomethacin DBI 4-chlorobenzoic acid, sodium salt

Figure 1. Structure of indomethacin and its hydrolysis products.

20 μg l^{-1} was obtained which was claimed to be at least five-fold lower than UV detection at 254 nm. Since electrochemical and fluorescence detection were shown in a few papers (15, 17) to be significantly more sensitive than UV for the HPLC analysis of indomethacin, both these detection modes were investigated for the routine determination of indomethacin in aqueous humour.

MATERIALS AND METHODS

CHEMICALS

Indomethacin was received as a gift from Chauvin (Montpellier, France).

Methanol from Carlo Erba, Milano, Italy and acetonitrile from Merck, Darmstadt, Germany were of HPLC grade. MilliQ water was used throughout. All other chemicals were of analytical grade.

SOLUTIONS

Stock solutions of indomethacin (500 mg l^{-1} in methanol) for FL and EC detection were prepared and stored up to one month at 4°C. They were suitably diluted in a methanol-water mixture (65:35, v/v) for EC detection and in water for FL detection. These dilutions were stored at 4°C and used within 7 days.

ELECTROCHEMICAL DETECTION

A Hewlett Packard (Walbronn, Germany) Model 1050 isocratic pump and a Rheodyne injector Model 7125 fitted with a 20 µl-loop were used. A UV detector (Hewlett Packard Model 1050) was placed in series with an amperometric detector (Shimadzu L-ECD-6A, Touzart et Matignon, Vitry-sur Seine, France) which was equipped with a glassy carbon working electrode (WE) and a Ag/AgCl reference electrode (RE). The UV detector was set at 266 nm and the detection potential of the WE was set at 1.20V *vs.* Ag/AgCl, unless otherwise stated. This potential was selected from the hydrodynamic voltammogram obtained by successive injections of a standard solution of indomethacin (5 mg l^{-1}) at variable potentials of the WE (from +0.30 V to +1.20 V, *vs.* Ag/ AgCl). Injection was carried out using a Unicam (Cambridge, UK) Model PU 4247 autosampler . The signals were recorded on a dual channel Unicam Model PU 4880 integrator. Separation was carried out on a 125 x 4 mm i. d. Lichrospher (Merck, Darmstadt, Germany) 100 C18 column (5 µm). A disposable guard column (Merck, 4 x 4 mm, i. d.) packed with the same material was used to protect the analytical column. The guard column was replaced after 100 injections. The eluent was a methanol-50 mM sodium phosphate buffer pH 3 (65:35, v/v) mixture, at a flow rate of 1 ml min^{-1}.

FLUORESCENCE DETECTION

Preliminary studies were carried out to follow the formation of DBI together with disappearance of indomethacin in an alkaline medium. For this purpose, a Merck LMC System UV detector set at 266 nm (maximal absorbance wavelength of indomethacin in the mobile phase) was placed in series with a Shimadzu RF-551 fluorescence detector with excitation and emission wavelengths set at 305 and 380 nm, respectively. A Gilson (Villiers-le Bel, France) Model 307 pump fitted with a Rheodyne valve containing a 20-µl loop was used to deliver the mobile phase. The mobile

and stationary phases were the same as those used for EC detection. Signals were recorded on a Shimadzu C-4A dual channel integrator. Off-line derivatization and manual injection were used at this stage. For further experiments and method validation automated derivatization and injection with a Gilson Model 401C dilutor coupled to a Gilson Model 232 XL autosampler with a variable capacity loop were used. The extraction and derivatization procedures were carried out in 800-µl polypropylene minivials. The sample rack for derivatization was themostatted at 40°C ± 0.5°C. The pump and integrator were the same as those described for the preliminary studies.

EXTRACTION PROCEDURE FOR ELECTROCHEMICAL DETECTION

The blank AH matrix used to assess the specificity of EC detection *vs* endogeneous components was treated as follows : an aliquot (200-µl) of drug-free pooled rabbit AH was mixed with an equal volume of acetonitrile. The mixture was vortexed for 15 sec, then centrifuged at 2000 rpm for 10 min. A 20-µl aliquot of supernatant was injected onto the column.

EXTRACTION PROCEDURE FOR FLUORESCENCE DETECTION

To a 50-µl aliquot of AH, 10-µl of 0.1 M acetate buffer pH 5 and 400-µl of ethyl acetate were added. The mixture was vortexed for 2 min, then centrifuged at 2000 rpm for 10 min. The lower aqueous phase was removed by aspiration and discarded. The organic phase was evaporated at 30°C under a stream of nitrogen. The walls of the microvial were washed four times with 50-µl aliquots of ethyl acetate and the combined washings were evaporated again under the same conditions. The microvials were placed in the thermostatted rack of the autosampler for reaction and injection. A 50-µl aliquot of a 50 mM phosphate buffer pH

11.6 was automatically added to each vial and mixed by aspiration with the diluter; the solution was subjected to hydrolysis for 1 hr at 40°C and then 15-µl of a mixture 50 mM phosphate buffer pH 4-ACN (90:10, v/v) was added and mixed. A 50-µl aliquot of this solution was injected onto the chromatographic system.The separation was carried out with a mobile phase 25 mM phosphate buffer pH 6.6-acetonitrile (90:10, v/v) at a flow rate of 1 ml min^{-1}. Peak areas were used for quantitation.

Different criteria for method validation were evaluated. The absence of DBI as metabolite was established by applying the method to four different samples of AH withdrawn 45 min and 2 hr after administration of eye drops to four rabbits. The specificity of the method *vs* the endogeneous components of the matrix was assessed using ten independent sources of AH. Other criteria were assessed on pooled AH. The linearity was assessed on a same day from three calibration curves, each one prepared from matrices spiked between 5 and 50 µg l^{-1} (n= 6 concentrations) of indomethacin. Intra-day precision and accuracy were tested by applying the procedure twelve times on matrices spiked at three concentration levels (5, 20 and 50 µg l^{-1}). Inter-day precision and accuracy were tested on five days from a pool of matrices spiked at the same three concentrations. One aliquot at each concentration was assayed on each of five days with reference to a standard curve daily prepared in the matrix. Analytical recovery was calculated by comparing the peak areas from extracted spiked matrices and non-extracted drug dissolved in the reconstitution solvent at six concentrations (from 5 to 50 µg l^{-1}) of indomethacin on each of five days. Stability studies were carried out on a pool of AH spiked at 5 and 20 µg l^{-1} indomethacin under different conditions: at ambient temperature for 3 hr and 24 hr, after 3 freeze-thaw cycles at -18°C. The stability of the dry residue stored after extraction for 24 hr at -18°C was also tested to cover experimental delays. For each conditions, 6 determinations were carried out by comparison with a calibration curve prepared for each experiment. The results of the assays were compared to those obtained from samples freshly prepared.

RESULTS AND DISCUSSION

ELECTROCHEMICAL DETECTION

Figure 2 shows the plot of the hydrodynamic voltammogram for indomethacin. This compound is oxidised from a potential of +0.70 V (*vs.* Ag/AgCl) with a diffusion plateau observed from a +1.15 V potential. This potential value is higher than that of 0.95 V reported by KAZEMIFARD et al. (17) with a coulometric detector. This difference can be explained by the fact that the coulometric detector has a Pd/H_2 RE which has a higher potential than that of the amperometric detector we used. Figure 3 shows chromatograms recorded at 266 nm (UV) and +1.20V (EC) from drug-free AH and of a standard solution of indomethacin at 50 $\mu g\ l^{-1}$. The LOD in aqueous solutions for EC and UV detection are respectively 20 $\mu g\ l^{-1}$ and 50 $\mu g\ l^{-1}$. The repeatability of successive injections (n=8) of a 5 mg l^{-1} aqueous solution of indomethacin was 0.41% and 1.89% for UV and EC detection, respectively. The lower repeatability in EC detection is related to a progressive decrease in the response which is probably due to adsorption problems at the electrode. This observation together with the low gain in sensitivity shows that EC detection cannot be used routinely for the assay of indomethacin.

FLUORESCENCE DETECTION

PRELIMINARY EXPERIMENTS

Optimisation of the hydrolysis pH and reaction time

Since the hydrolysis of indomethacin is a base catalysed reaction (20-23) a buffer pH 11.6 was selected in our study. A phosphate buffer

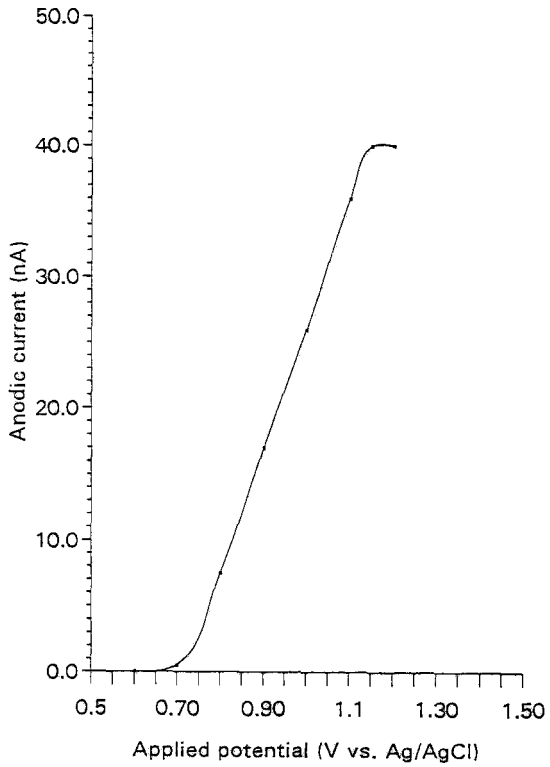


Figure 2. Hydrodynamic voltammogram for indomethacin with amperometric detection. For experimental conditions, see text.

was preferred to a sodium hydroxide solution (15) as it gave us less fluorescent interferences on the chromatogram. The hydrolysis kinetics of indomethacin in a phosphate buffer pH 11.6 (24) was confirmed using both UV and fluorescence detection. Figure 4 shows the formation of the non fluorescent p-chlorobenzoic acid and the fluorescent derivative DBI (13, 20, 25).

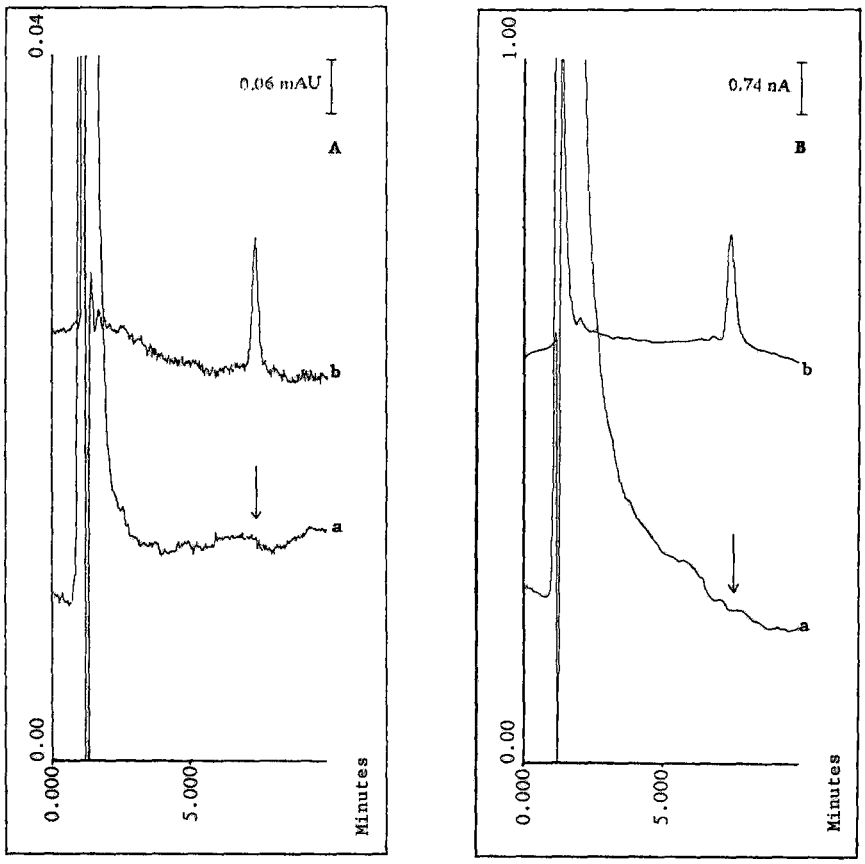


Figure 3. Comparison of UV (A) and electrochemical (B) responses for indomethacin:
(a) blank aqueous humour.
(b) aqueous standard solution (50 $\mu g\ l^{-1}$).
For experimental conditions, see text.

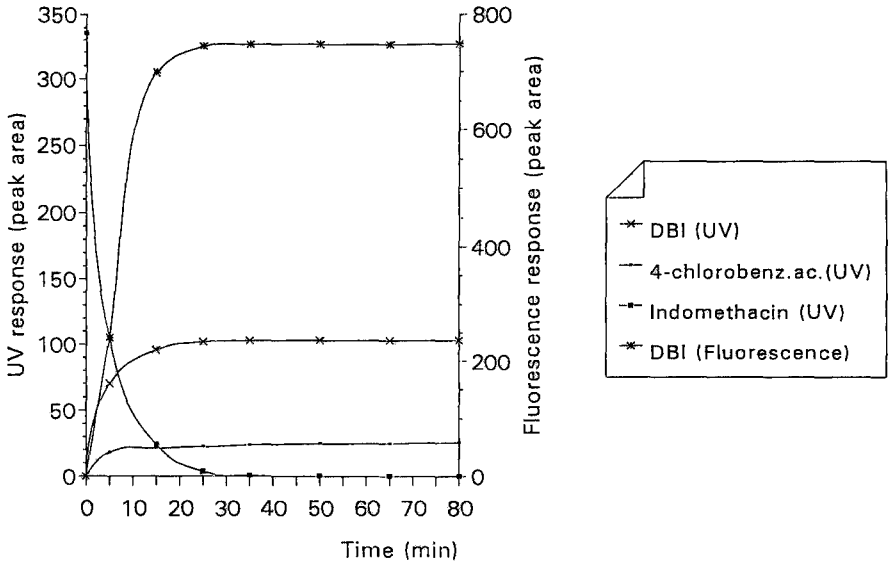


Figure 4. Hydrolysis kinetics of indomethacin followed by UV and fluorescence detection : UV response refers to the left axis (indomethacin, DBI and p-chlorobenzoic acid); fluorescence response (DBI) refers to the right axis. For experimental conditions, see text.

Optimisation of the mobile phase composition

The mobile phase composition was optimized with respect to pH and organic solvent. The influence of pH on the fluorescence response was studied off-line with a spectrofluorimeter, over a pH range compatible with the silica-based C18 column (from pH 3 to 7), keeping a proportion of methanol (65%) identical to that of the mobile phase previously used for chromatography. Increasing the pH resulted in a progressive increase in the fluorescence. A pH of 6.6 gave an increase by 300 % by comparison to a pH of 3. The replacement of methanol by acetonitrile did not produced any significant change in the fluorescence intensity. However, acetonitrile was preferred to methanol for

chromatography as it gave a higher efficiency. A 25 mM phosphate buffer pH 6.6-acetonitrile (90:10, v/v) was finally selected as mobile phase, which gave a typical retention time of 5.15 min (Figure 5) on three different batches of stationary phases.

Optimisation of the extraction procedure

Different solvents proposed in the literature for indomethacin extraction from plasma were used (2,5,9,10,13,15,18). Ethyl acetate with an acetate buffer pH 5 (13,18) was selected as it gave a "clean" blank AH chromatogram and a satisfactory recovery.

Search of DBI as metabolite

Samples withdrawn at different time intervals after administration of indomethacin eye drops to rabbits did not reveal the presence of DBI as metabolite under the operating conditions used. This confirms the observation of SANDERS et al. (18) and previous results of TLC using ^{14}C labelled indomethacin (1).

METHOD VALIDATION

Specificity

Ten individual sources of AH did not give any significant interference at the retention time of indomethacin (Figure 5).

Linearity

The relashionship between the peak areas and concentration of indomethacin was linear between 5 and 50 $\mu g\ l^{-1}$ with a determination coefficient (r^2) of 0.9904.

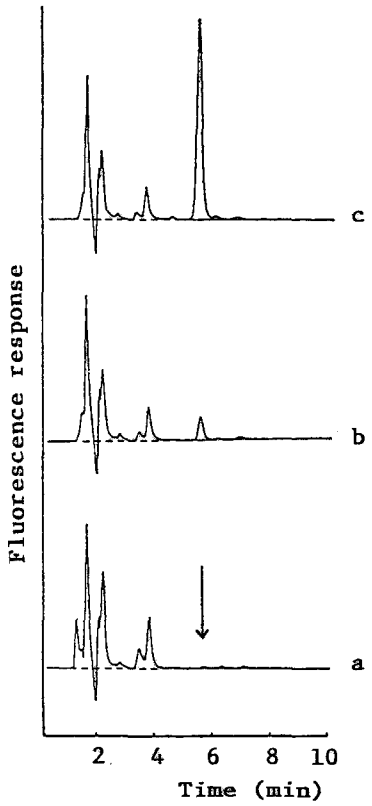


Figure 5. Typical chromatograms using HPLC with fluorescence detection.
(a) blank aqueous humour.
(b) aqueous humour spiked at the LOQ (5 $\mu g\ l^{-1}$).
(c) aqueous humour spiked at 50 $\mu g\ l^{-1}$.

The linear regression equation was :
area = 7943 (± 414) * conc. ($\mu g\ l^{-1}$) - 12507 (± 12561), with the confidence intervals calculated at p=0.05.

The accuracy was evaluated at each concentration by calculating the relative error (%) between the mean calculated value and the actual value. The results are given in Table 1. A concentration of 5 $\mu g\ l^{-1}$ with

TABLE 1. Evaluation of accuracy from linearity data.

Concentration ($\mu g\ l^{-1}$)	Mean bias (%)*
5	18.72
10	-5.53
20	-3.21
30	-0.66
40	-4.91
50	3.83

* n= 3 linearity graphs

TABLE 2. Intra-day and inter-day precision and accuracy

	Intra-day precision and accuracy*		Inter-day precision and accuracy**	
Concentration (µg l-1)	RSD (%)	Bias (%)	RSD (%)	Bias (%)
5	8.05	-1.40	15.15	-1.40
20	7.25	-9.95	5.27	7.25
50	4.89	-2.14	12.03	0.02

* 12 assays at each concentration, on a same day
** 1 assay at each concentration on each of 5 days

a relative error lower than 20% complies with the guidelines for accuracy of the Washington Conference (26) with respect to the LOQ. This concentration was further tested for both accuracy and precision using samples spiked at this level, independently from the calibration curve and from runs performed on different days.

Precision and accuracy

The results of intra-day (12 determinations per day) and inter-day (1 assay on each of 5 days) precision and accuracy at three concentration levels are given in Table 2. A two-way ANOVA showed that there was no day influence on the inter-day results.

TABLE 3. Analytical recovery of indomethacin from aqueous humour (n=5 days).

Concentration (µg l-1)	Mean Recovery (%)	RSD (%)
5	65.16	9.32
10	75.14	10.97
20	67.63	2.35
30	67.56	12.05
40	72.48	3.92
50	71.24	4.98
Mean	69.87	5.31

LOQ and LOD

Based on accuracy and precision results (lower than 20 %), a concentration of 5 µg l^{-1} can be considered as the LOQ (26) in AH. The LOD was determined to be 2 µg l^{-1} (S/N =3) and confirmed by analyzing ten different matrices spiked at this concentration.

Analytical recovery

Table 3 gives the results of recoveries over the concentration range studied. The mean recovery is approximately 70% with a RSD of 5.31%.

Stability of indomethacin in AH

The confidence intervals calculated on the results showed that the results were not affected by storage at 24 hr at ambient temperature or three freeze-thaw cycles. Moreover, dry extracts can be kept at 18°C for 12 hr should this be necessary.

CONCLUSION

The present study showed that ECD coupled with HPLC does not present any advantage over UV detection for indomethacin monitoring

in AH. However, liquid-liquid extraction of indomethacin from this matrix followed by automated pre-column hydrolysis and fluorescence detection yields a LOD and a LOQ lowered by a factor of two compared with to the UV detection method presently in use (1). Validation results with fluorescence detection showed that the proposed procedure complies with the validation guidelines for biological assays (26). It is well suited to the determination of indomethacin in AH, as the fluorescent derivative formed (DBI) is not present as metabolite in this matrix. However, this detection mode should be used only when a high sensitivity is required, as it is more time consuming and difficult to implement when compared with the HPLC-UV technique.

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HPLC ASSAY OF THEOPHYLLINE AND ZIDOVUDINE IN RAT SERUM

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ABSTRACT

A simple, sensitive, and reproducible HPLC method has been developed for the determination of theophylline and zidovudine (AZT) employing reverse phase high performance liquid chromatography with UV detection at 270 nm. The separation was performed on a Novapak C_{18}, 5 μm (3.9 x 150 mm) column. Acetonitrile (7.5%) in 0.2% acetic acid was used as the mobile phase and the run time was 8 min. Each drug was used as an internal standard for the other. The mean retention times of theophylline and zidovudine were 3.4 and 6.0 min, respectively. Linear response ($r > 0.998$) was observed over the range of 0.02 - 10 μg/ml for both drugs. There was no significant difference ($p < 0.05$) between inter- and intra-day studies for theophylline and zidovudine. The mean relative standard deviations (RSD%) of the results of within-day precision and accuracy of the two drugs were < 7%. The applicability of the assay was demonstrated in determining each drug concentrations in different groups of rats.

INTRODUCTION

Theophylline is widely employed as a bronchodilatant for the treatment of chronic obstructive airways diseases despite its low therapeutic index.[1-4]

Zidovudine (azidothymidine; AZT) is one of three approved drugs that are effective for treatment of neurological insult produced by HIV infection.[5-8] However, AZT remains the first-line agent in the treatment of patients with human immunodeficiency virus (HIV) infection.[9] It delays disease progression to acquired immunodeficiency syndrome (AIDS) and AIDS-related complex (ARC) disease.[10]

Several HPLC methods have been reported for theophylline and AZT.[11-18] However, these often consume more organic solvents, have longer run time, or use protein precipitation that resulted in many interfering peaks when tested in our laboratory. Some assays utilized solid-phase extraction which is time consuming and may lead to a lower drug recovery. Others have benefited of expensive detectors or equipment which are not on hand. The present study describes a sensitive, specific and rapid sample preparation assay for determining theophylline and zidovudine in rat serum with no interference from their metabolites. The assay was applied successfully to two different pilot studies for theophylline and AZT in three different groups of rats.

MATERIALS

Chemicals

Zidovudine (Lot number 8510567-177-W) was donated courtesy of M. Maguire, Burroughs Wellcome, Research Triangle Park, NC. Theophylline (Lot number 108F-0352) was purchased from Sigma Chemical Company, ST. Louis, MO. All other reagents and chemicals were analytical grade, and used as received.

Stock solutions of theophylline and AZT, 50 μg/ml, were prepared in methanol and stored in 15 ml amber glass vials at -4°C until used. Each drug was used as the assay internal standard (IS) for the other. Weekly dilutions were made in HPLC quality water to give AZT or theophylline concentrations of 0.02-10 μg/ml in rat serum, and a constant concentration of 0.5 μg/ml of the IS.

Instruments

Waters HPLC system was equipped with a Water 484 variable UV absorbance detector (set at 270 nm), and a Waters 717 autosampler. Waters 501 solvent delivery system was used to operate the gradient flow through a Novapak C18 column (3.9 x 150 mm) packed with 5 μm spherical particles. Flow rate was monitored by Waters automated gradient controller. The initial flow rate was 0.8 ml/min for 4 min and it was increased to 1.8 ml/min within 1 min. After 7 min flow rate was reduced to its initial value. Sample run time was 8 min. Chromatograms were recorded on a Waters 746 Data Module integrator chart. The HPLC system was operated at ambient temperature. Acetonitrile (7.5%) in

0.2% acetic acid solution was used as the mobile phase. Degassing was achieved by filtration through 0.22 μm filter. The injection volume was 50 μL.

METHODS

Drug Analysis

Two hundred microliters of blank rat serum was spiked with one of the two drugs and 10 μL of its IS (500 ng/ml) in 10 ml screw-capped test tube fitted with polyteflon-lined cap. Fifty microliter of isoamyl alcohol was added and the tube was vortexed for 30 seconds. The solution was mixed with 2 ml of chloroform, vortexed at high speed for 1 min, and centrifuged at 1000 rpm for 5 min. The aqueous layer was aspirated to a waste and the organic layer was transferred to a clean tube. The tube containing the organic layer was placed in a water bath (50°C) and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 100 μL of mobile phase prior to injection into the chromatograph for analysis.

To assess the accuracy and precision of the within-day assay, six extraction of serum sample of AZT or theophylline, at the following concentrations 0.05, 0.5 and 5 μg/ml, were performed on a single day. The reproducibility of the assay (within-day and between-day) was evaluated by comparing the linear regression analysis of three standard plots obtained from spiked rat serum samples at three different days over a two month period for each drug. The recovery of each drug was assessed by extracting plasma specimens spiked to contain each drug concentrations from 0.02 to 10 mg/ml. The peak area ratio (AZT/theophylline or theophylline/AZT) was then compared with the peak area ratio for aqueous standards containing equivalent amounts of the drug and IS without extraction.

Animals

Eighteen male Sprague- Dawely rats (262 - 314 g) were used in the pilot studies of both drugs. For AZT, 12 rats were divided into 2 groups (6 rats /group) and each group was housed in one cage. One group was used as a control and the other group was subjected to acute liver damage which was induced by a single oral dose of CCl_4 solution in paraffin oil. AZT (3 mg/kg) was IP injected after 24 hr of CCl_4 applications to both groups. Theophylline was given to 6 rats as 4 mg/kg IP doses. Food and water were available *at Libitum* at all times during the experiment. Rats were lightly anesthetized with ether only during blood sampling. Blood samples were collected from the orbital venous plexus 30 min, 1, and 2 hr after each drug administrations. Therefore, each data point is the mean of 6 replicates and only three blood samples were collected from each rat per day to avoid any damage to the eye. Serum samples were separated by centrifugation at 6,000 rpm for 15 min and stored at -4°C till assayed as described above.

Statistical Analysis[19]

All results are expressed as mean ± SD. The relative standard deviation RSD% was calculated for all values. The *t*-test was used to examine the concentration difference at each day, and one-way analysis of variance (ANOVA) was employed to evaluate the reproducibility of the assay. The level of confidence was 95%.

RESULTS and DISCUSSION

Figure 1 shows representative chromatograms of extracted drug-free serum (1A), serum samples taken from rats after IP administration of 3 mg/kg AZT (1B), and serum samples withdrawn from rats after IP administration of 4 mg/kg theophylline (1C). A comparison of Figure 1A with 1B and 1C indicates that theophylline and AZT peaks are free from matrix interference. Using the chromatographic conditions described, AZT and theophylline were well resolved with mean retention times of 6.0 and 3.4 min, respectively.

Least-squares regression calibration curves were found to be linear at serum concentrations between 0.02 to 10 μg/ml for AZT and theophylline. The mean

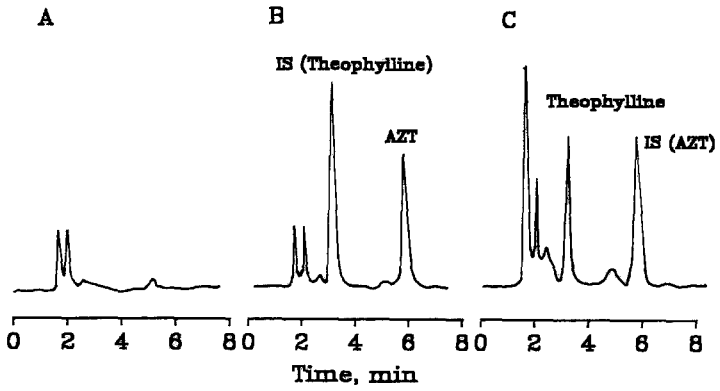

FIGURE 1

Chromatograms of extracted drug-free serum (A), serum samples (spiked with 0.5μg/ml of theophylline) taken from rat after IP administration of 3 mg/kg AZT (B), and serum samples (spiked with 0.5μg/ml of AZT) withdrawn from rat after IP administration of 4 mg/kg theophylline (C).

linear regression equations of the peak area ratios (Y) *vs.* drug concentrations (X) of AZT and theophylline were typically of the form Y = 0.858 X - 0.014 and Y = 1.55 X - 0.038, respectively. The mean correlation coefficients, r, were generally > 0.998. The detection limit of the assay, based on extraction of 0.2 ml of serum, was 20 ng/ml at a signal to noise ratio of >3.

Within-day precision and accuracy of the method were determined from replicate analysis (n=6) of 3 spiked serum test standards at concentrations within the linear range of the assay for each drug (Table 1). The mean percentage recovery of 0.02 to 10 μg/ml (n = 6) of theophylline and AZT were 97 ± 5% (RSD% = 5.3) and 94 ± 3.5% (RSD% = 3.7), respectively. Table 1 shows the recoveries of 3 different concentration of the tested drugs. Extraction efficacy was found not to significantly vary among different concentrations of AZT and theophylline.

The reproducibility of the assay was evaluated by comparing the linear regressions of three standard plots prepared at three different days over a three month period for each drug. The results of this evaluation are summarized in Table 2. The mean correlation coefficient was > 0.998 for both drugs with RSD% of the slopes of the three lines were 8 % and 6% for AZT and theophylline, respectively. Analysis of variance (Table 3) of the data indicated no significant

TABLE 1

Within-day Precision and Accuracy of Theophylline and Zidovudine Recoveries From Spiked Rat Serum Samples

Spiked Concentration (μg/ml)	Mean Measured Concentration (μg/ml), n = 6	Standard Deviation	Recovery %	Relative Standard Deviation %
Theophylline				
0.05	0.051	0.005	102	10.0
0.50	0.490	0.030	98	6.0
5.00	4.850	0.230	97	4.7
Zidovudine				
0.05	0.046	0.0037	92.0	8.0
0.50	0.458	0.0230	91.6	5.0
5.00	4.750	0.3100	95.0	6.5

TABLE 2
Reproducibility of Data from Standard Plots In Rat serum
n = 6

Standard Plot[a]	Slope[b]	Intercept[b]	Correlation Coefficient[b]
Theophylline			
1	1.64	- 0.056	0.999
2	1.57	- 0.034	0.999
3	1.45	-0.023	0.999
Zidovudine			
1	0.918	-0.0046	0.999
2	0.870	-0.0020	0.998
3	0.785	-0.0350	0.999

a Obtained from assays on three different days.
b The mean of six determinations.

TABLE 3

One-Way ANOVA for the Reproducibility of the Assay
(Within-Day and Between-Day)

Source of Variation	Sum of Squares	Degree of Freedom	Mean Square	F value
Theophylline:				
Total	0.9065	17		
Between	0.0748	2	0.0371	0.27
Within	0.8317	15	0.1386	
Zidovudine:				
Total	0.0466	17		
Between	0.0146	2	0.0073	1.37
Within	0.0320	15	0.0053	

F(95%) tabulated = 3.68, No significant difference at $p<0.05$

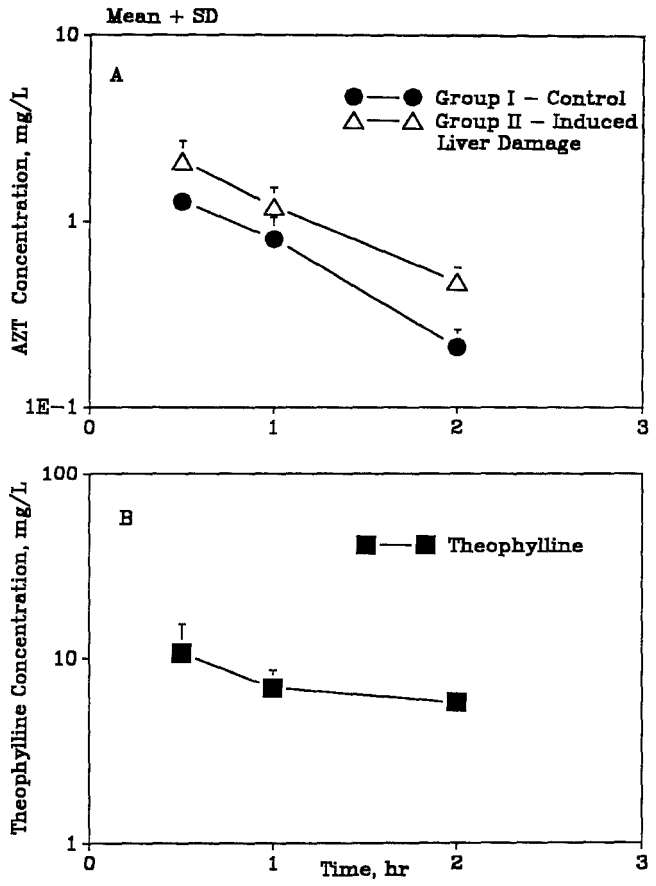

FIGURE 2

A plot of serum drug concentration - time profiles after IP administrations of AZT (3 mg/kg) given to 2 groups of rats (A); and theophylline (4 mg/kg) given to a group of rats (B).

difference in the slopes, within-day and between-day, of the calibration curves of the two drugs. The results confirmed the reproducibility of the assay method.

Figure 2A depicts a typical plot of AZT concentrations after IP administrations (3 mg/kg) to two groups of rats. Group I was the control and group II was with induced acute liver damage. While Figure 2B represents theophylline concentration time profile after 4 mg/kg IP dose to 6 rats. Figure 2 demonstrates the usefulness of the method for analysis of theophylline and AZT in serum. Re-analysis of samples several weeks after the initial analysis showed no loss in both drugs.

CONCLUSION

The HPLC method described herein has sufficient sensitivity to determine the pharmacokinetics of theophylline and AZT following a single IP dose. The assay is simple, accurate, and reproducible, and has been employed on a routine basis over the last two years for the analysis of samples of both drugs in different studies.

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EVALUATION OF FOUR METHODS FOR CATECHOLAMINE ANALYSIS IN PLASMA OBTAINED FROM SUBJECTS DURING INCREMENTAL CYCLE ERGOMETRY

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ABSTRACT

Plasma was collected from a subject during incremental cycle ergometry in order to determine the anaerobic threshold. Norepinephrine and epinephrine were extracted using alumina, strong-cation exchange/alumina, or weak-cation exchange, and then separated using reversed-phase ion-pair chromatography. Amperometric detection was used following chromatography for all three types of extractions (Methods A, B and C), while coulometric detection was only used following chromatography and alumina extraction (Method D). The upper limit of linearity was the lowest for Method C. The extraction for Method B took about twice the amount of time as the other extractions. The standard curve for Methods A, B and D was linear to 5000 ng/L. The lowest limit of quantification and relative standard deviation was obtained using Method D. In summary, Method D was the best method for this purpose.

INTRODUCTION

The inflection in blood lactate concentration that occurs during increased work may be caused by muscle anaerobisis, fiber recruitment or hormonal control

via catecholamines (1). Therefore, changes in plasma catecholamine concentrations are quantitated as part of the process of determining their influence on the lactate threshold during graded exercise. Initially, plasma catecholamine concentrations are low because the subject is at rest. By the time the subject reaches volitional exhaustion, plasma catecholamine concentrations are significantly above normal concentrations.

Plasma catecholamine concentration can be determined by several methods as described in three excellent reviews (2 3 -4). "However, it is now very clear from the ever-growing volume of publications on the subject, that high performance liquid chromatography with amperometric or fluorometric detection is emerging as a pre-eminent tool for metabolic fingerprinting of these important biomolecules (2)." Besides amperometric detection, coulometric detection can be used (3). Sample pretreatment is required to remove endogenous interferences (2,3). Catecholamines have been extracted using alumina (5 ,6), weak-cation exchange (7), strong-cation exchange followed by alumina (8), boric acid gel (9) and organic solvents (10 ,11).

Our review of the literature revealed that none of the HPLC methods with electrochemical detection (ECD) meet all of our criteria for quantitating norepinephrine and epinephrine concentrations in plasma of subjects during incremental cycle ergometry (2-11). For this purpose, the limit of quantitation must be low enough to allow for the determination of basil norepinephrine and epinephrine levels. This is especially difficult for epinephrine because basil levels are frequently less than 100 ng/L (10,11). The method must be accurate and have a relative standard deviation of less than 5% for norepinephrine and epinephrine. Accurate and precise concentrations are required to determine the onset of the anaerobic threshold. The range of linearity must be large, because the concentration of plasma norepinephrine and epinephrine in these subjects increases above basil values by a factor of about 10 to 20. This is especially

important for norepinephrine because the level of norepinephrine can be as high as 5000 ng/L. Finally, the method must be practical because there are typically about 15 samples per subject. The most common shortcomings of previously reported methods was the limit of detection that was too high for epinephrine and upper limit of linearity for norepinephrine was not high enough. To the authors knowledge, this is the first direct comparison of these methods and should provide researchers or clinicians new to this field with a valuable comparison of electrochemical methods for catecholamine analysis.

Therefore, the purpose of this research was to ascertain which of the four methods (Figure 1) described here, if any, meet all of the criteria, as described in the preceding paragraph, for determining the concentration of plasma norepinephrine and epinephrine in subjects who have exercised to volitional exhausting during incremental cycle ergometry. In all cases, separations were done using a reversed-phase ion-pair HPLC. Three types of extractions were used with amperometric detection: **1.** aluminum oxide (Method A), **2.** strong-cation in series with aluminum oxide (Method B) and **3**. weak-cation (Method C). The fourth method was an aluminum oxide extraction followed by coulometric detection (Method D). We did not evaluate boric acid gel because this gel is relatively expensive when compared to aluminum oxide. We did not evaluate liquid-liquid extraction, because this extraction is limited due to relatively poor selectivity of this extraction compared to alumina, although the recovery is higher (10).

EXPERIMENTAL

Materials

Reduced glutathione (96%), sodium 1-octanesulfonate (98%), 3,4-dihydroxybenzylamine hydrobromide (98%), and 70% (w/v) perchloric acid

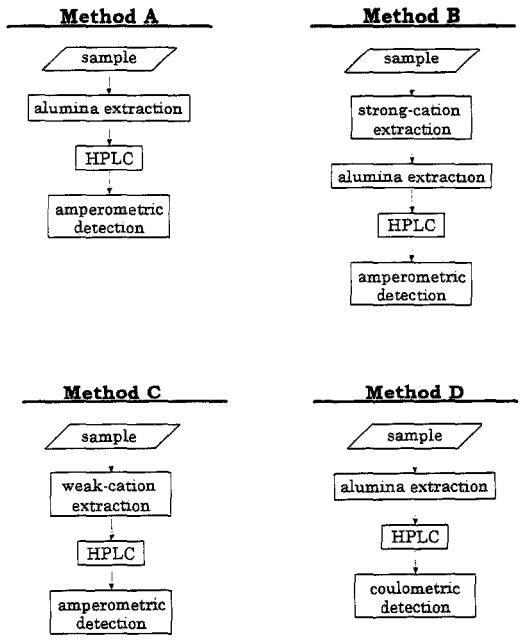

FIGURE 1. Methods Evaluated for the Quantification of Plasma Catecholamine Concentrations During Incremental Cycle Ergometry.

(99.999%) were purchased from Aldrich Chemical Company (Milwaukee, WI). HPLC grade monobasic potassium phosphate, water, methanol, and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA) as well as Gelman 0.20-μm x 45-mm Nylaflo membranes and 13- x 75-mm Vacutainer blood collection tube containing 0.048 mL of a 15% tripotasssium ethylenediaminetetreacetic acid. Ultrapure grade tris-(hydroxymethyl)aminomethane (TRIS) was supplied by Research Organics (Cleveland, OH). Ethylene glycol-bis(β-aminoethylether) N,N,N'-tetraacetic acid (97%, EGTA) was obtained from Sigma Chemical Company (St. Louis, MO). Microfilters, RC58 membranes, a octadecyldimethylsilyl, 3-μm, 3.2-x100-mm cartridge column and holder, acid

washed aluminum oxide, norepinephrine bitartrate, epinephrine bitartrate, and an electrode polishing kit were obtained from Bioanalytical Systems (W. Lafayette, IN). An octadecyldimethylsilyl, 3-μm, 4-x100-mm glass-lined column was obtained from Scientific Glass Engineering (Austin, TX). Strong-cation and weak-cation exchange solid-phase extraction columns, each containing 100 mg of sorbent, and a solid-phase extraction manifold were acquired from Supelco, Inc. (Bellefonte, PA). All other chemicals were reagent grade.

Methods

Sample collection. An indwelling catheter was placed in a vein of the subject's antecubital fossa the morning after an overnight fast. A mixture containing 2.5 g of glutathione, 3.0 g of EGTA, and 33.5 mL of distilled water was prepared in a 50-mL Erlenmeyer flask with a ground-glass stopper. The mixture was continuously stirred during use. During exercise to volitional exhaustion on a cycle ergometer, 3 mL of whole blood was collected during the last 30 s of each 2-min work stage and immediately after maximal exercise for analysis. The blood was transferred to a 13-x100-mm tube containing 100 USP units of lithium heparin. A 70-μL aliquot of glutathione and EGTA mixture was added to the whole blood and gently rocked. The blood was centrifuged at 1000g for 5 min. The plasma was stored in 1.5-mL polyethylene Eppendorf micro test tubes at -70°C.

Pooled Plasma Samples. Pooled plasma A was prepared from one subject at rest. Blood was collected in six 13- x 75-mm Vacutainer tubes via venipuncture of a vein in the subject's antecubital fossa. The Vacutainer tubes were centrifuged at 1000 g for 10 min and the resulting plasma was pooled. Aliquots (1.2 mL) of pooled plasma were stored in Eppendorf tubes at -70°C. Pooled plasma B was prepared by pooling all the plasma obtained while exercising a subject to volitional exhaustion. Pooled plasma C was prepared similarly but a different subject was used.

Aluminum Oxide Extraction. This extraction was performed as previously described (6) with the following exceptions: **1.** A pH 7.4 phosphate buffer was prepared by dissolving 0.472 g of potassium monobasic phosphate, 1.72 g of sodium dibasic phosphate, 0.42 g of sodium bicarbonate, 1.46 g of sodium chloride, 0.95 g of sodium metabisulfite, 0.31 g of reduced glutathione, and 0.38 g of EGTA in 150 mL of HPLC grade water. The pH was adjusted to a pH of 7.4 with 1 M HCl or 1 M NaOH as required. The solution was transferred to a 200-mL volumetric flask. The flask was filled to the mark with HPLC grade water. **2.** A combined stock solution containing 1 mg/L of norepinephrine and 1 mg/L epinephrine in 0.1 M perchloric acid was prepared from the 100 mg/L solution of norepinephrine and 100 mg/L solution of epinephrine (6) using 1.0-mL disposable plastic pipets and a 100-mL volumetric flask. This solution was stored at 4°C. **3.** Polypropylene centrifugation tubes (15-mL) were used for the dilutions of the norepinephrine and epinephrine solution containing 1 mg/L of each catecholamine, and for the extractions. **4.** The combined stock solution was diluted using disposable plastic pipets and 0.1 M perchloric acid. Standards were prepared from the resulting dilutions of combined stock solution by adding 10-μL aliquots (using a micropipet with disposable plastic tips) of each dilution to 1.0 mL of the pH 7.4 phosphate buffer. Standards were prepared before each use. **5.** All plasma samples were centrifuged for 4 min in a Beckman Microfuge E. **6.** The extraction was performed by rocking for eight minutes. **7.** The aluminum oxide was washed three times with 5-mL of vacuum degassed HPLC grade water.

Strong-Cation/Aluminum Oxide Extraction. This extraction was done as described elsewhere (8) with minor modifications: **1.** Standards were prepared in accordance with the procedure described under *Aluminum Oxide Extraction.* **2.** The samples or standards were extracted under a vacuum of 400 Pa (3 mm of Hg) at a rate of ~100 μL every 5 seconds. **3.** The catecholamines were eluted into 15-mL polypropylene centrifugation tubes under a vacuum of 400 Pa at a rate of ~100 μL every 5 seconds. **4.** An aluminum oxide extraction was then performed

as described under *Aluminum Oxide Extraction* except 2 mL of TRIS buffer was added to the eluate followed by 50 mg of aluminum oxide.

Weak-Cation Extraction. Slight alterations were made to Supelco's extraction procedure (7): **1.** Standards were prepared in accordance with the procedure described under *Aluminum Oxide Extraction.* **2.** An aliquot of sample (0.75 mL) was diluted with an equal volume of HPLC grade water in a 1.5-mL polyethylene Eppendorf micro test tube. The internal standard was then added. **3.** The samples or standards were extracted under a vacuum of 400 Pa (3 mm of Hg) at a rate of ~100 μL every 15-20 seconds. **4.** The catecholamines were eluted into a 1.5-mL polyethylene Eppendorf micro test tube.

Amperometric Detection. Chromatographic conditions were established as recommended by Bioanalytical Systems (6) using the 3.2-x100-mm column. The Bioanalytical Systems LC4A detector was set to 1 nA/V x 1 (B) and +0.65V. The mobile phase flow was set to 1.2 mL/min. The mobile phase was conditioned by recirculation as required. The glassy carbon electrode was polished in accordance with the manufacturer's procedure. The mobile phase was continuously sparged with helium. The electrodes were connected in the dual series mode.

Coulometric Detection. The mobile phase was a 50 mM, pH 2.6 phosphate buffer containing 2.0 mM sodium 1-octanesulfonate, 60 mL/L of methanol, 40 mL/L of acetonitrile and 0.25 mM Na_2EDTA. The mobile phase was pumped through the 4-x100-mm column at a flow of 0.8 mL/min. The potentials of the ESA 5100A coulometric detector with a 5011 analytical cell and a 5021 conditioning cell were set as follows: conditioning cell = +0.35V, detector 1 = +0.05V, and detector 2 = -0.30V.

Method Evaluation. The method evaluation protocol of reference 12 was followed. Recovery was determined by adding 500 pg of norepinephrine and 500 pg of epinephrine to 1.0 mL of a pooled plasma sample. The slope (m), intercept

(b), and correlation coefficient (r) were determined using a linear regression. The limit of detection was determined as described elsewhere (12). The limit of quantification was determined by calculating the mean of phosphate buffer blanks and adding 10 times the standard deviation of the mean (13). The pooled relative standard deviation (13) for norepinephrine concentration determined using Method A was calculated from the relative standard deviation for pooled plasma-A-I, pooled plasma-A-II and the standards(Table 1) (14). The calculation was similar for determining the pooled relative standard deviation for epinephrine except the pooled plasma-A-I and A-II values were not available, because the values were below the limit of quantification. For Method B, the pooled relative standard deviation was calculated using pooled plasma-B and pooled plasma-C (Table 1). For Method C, only the 1000 ng/L standard was used so a pooled relative standard deviation was not calculated. For Method D, the pooled relative standard deviation was determined using the pooled plasma-A and the standards (Table 1).

RESULTS AND DISCUSSION

Method A.

The standard curve was linear to 5000 ng/L for both norepinephrine ($r = 0.996$) and epinephrine ($r = 0.999$). The average concentration of the 100, 500, or 1000 ng/L norepinephrine and epinephrine STD-I (Table 1) was not statistically different from the expected value of 100, 500, and 1000 ng/L, respectively, at the 0.01 significance level (15). The same was true for all three STD-II's (Table 1). The average concentration of norepinephrine in pooled plasma-A (A-I of Table 1) using one standard curve was not statistically different from the concentration of norepinephrine found in a second extraction of pooled plasma-A (A-II of Table 1) using a different standard curve at the 0.01 significance level (15). The limit of

TABLE 1.

Results for Methods A, B, C AND D[1].

	Sample	Nor[2] ± RSD[4] (n)	Epi[3] ± RSD[4] (n)
Method A[1]	100 ng/L STD-I[5]	100 ± 5.0 (5)	108 ± 7.4 (5)
	100 ng/L STD-II[5]	103 ± 7.9 (2)	90 ± 9.0 (2)
	Average[6]	102 ± 1.9 (2)	99 ± 12.9 (2)
	500 ng/L STD-I[5]	539 ± 5.4 (5)	498 ± 13.0 (5)
	500 ng/L STD-II[5]	530 ± 1.5 (2)	462 ± 1.5 (2)
	Average[6]	534 ± 1.3 (2)	480 ± 5.3 (2)
	1000 ng/L STD-I[5]	1047 ± 6.7 (6)	1040 ± 5.6 (6)
	1000 ng/L STD-II[5]	1033 ± 3.1 (2)	954 ± 5.3 (2)
	Average[6]	1040 ± 1.0 (2)	997 ± 6.1 (2)
	pooled plasma-A-I[7]	292 ± 2.8 (4)	< LOQ[8] (4)
	pooled plasma-A-II[7]	286 ± 3.2 (4)	< LOQ[8] (4)
Method B[1]	pooled plasma-B[7]	647 ± 5.4 (4)	255 ± 8.1 (4)
	pooled plasma-C[7]	397 ± 4.6 (4)	134 ± 5.7 (4)
Method C[1]	1000 ng/L STD[5]	987 ± 9.6 (3)	887 ± 8.5 (3)
Method D[1]	25 ng/L STD[5]	25 ± 6.2 (6)	25 ± 5.0 (5)
	100 ng/L STD[5]	106 ± 3.0 (4)	96 ± 3.2 (6)
	500 ng/L STD[5]	498 ± 1.6 (6)	494 ± 2.1 (6)
	1000 ng/L STD[5]	943 ± 3.1 (5)	988 ± 4.6 (6)
	pooled plasma-A[7]	253 ± 8.8 (4)	36 ± 7.9 (4)

[1]c.f. Figure 1, [2]norepinephrine concentration (ng/L), [3]epinephrine concentration (ng/L), [4]relative standard deviation, [5]standard prepared as described in the section on methods, [6]Average of STD-I and STD-II, [7]pooled plasma prepared as described in the section on methods, and [8]limit of quantification.

TABLE 2.

Comparison of the Methods for Norepinephrine Quantification.

	LOD[2]	LOQ[3]	Linearity[4]	RSD[5] (n)
Method A[1]	14	29	5000	5.1 (24)
Method B[1]	6	11	5000	5.0 (8)
Method C[1]	ND[6]	ND[6]	1000	9.6 (3)
Method D[1]	6	8	5000	3.8 (21)

[1]c.f. Figure 1, [2]limit of detection, [3]limit of quantification, [4]upper limit of linearity, [5]relative standard deviation, and [6]not determined for this method.

detection was 14 ng/L (n=4) and the limit of quantification was 29 ng/L (n=4) for norepinephrine (Table 2). The limit of detection was 30 ng/L (n=4) and the limit of quantification was 43 ng/L (n=4) for epinephrine (Table 3). The pooled relative standard deviation was 5.1% (n=24) for norepinephrine concentrations (Table 2) and 9.0% (n=16) for epinephrine concentrations (Table 3). The recovery relative to dopamine is 98 ± 6% for norepinephrine (n=3) and 96 ± 4% (n=4) for epinephrine. We found that chromatographic conditions had to be optimized in order to prevent an endogenous compound from coeluting with norepinephrine and a different endogenous compoumd from coeluting with epinephrine (Figure 2).

Method B.

This standard curve was linear to 5000 ng/L for both norepinephrine (r = 0.995) and epinephrine (r = 0.999). The limit of detection was 6 ng/L (n=2) and the limit of quantification is 11 ng/L (n=2) for norepinephrine (Table 2). The limit of detection was 10 ng/L (n=2) and the limit of quantification is 21 ng/L (n=2) for epinephrine (Table 3) (13). Further evaluation was not done because we felt the method was too time consuming. A relatively large broad peak eluted before norepinephrine (Figure 3).

TABLE 3.

Comparison of the Methods for Epinephrine Quantification.

	LOD[2]	LOQ[3]	Linearity[4]	RSD[5] (n)
Method A[1]	30	43	5000	9.0 (16)
Method B[1]	10	21	5000	7.0 (8)
Method C[1]	ND[6]	ND[6]	1000	8.5 (3)
Method D[1]	5	7	5000	3.9 (21)

[1]c.f. Figure 1, [2]limit of detection, [3]limit of quantification, [4]upper limit of linearity, [5]relative standard deviation, and [6]not determined for this method.

Method C.

This standard curve is linear to about 1000 ng/L for norepinephrine (r = 0.999) and 1000 ng/L for epinephrine (r = 0.999). Typical chromatograms are shown in Figure 3. Further evaluation was not done because the method did not meet our criteria for the upper limit of linearity for norepinephrine. Compared to Method A and B, Method C did not provide a clean extraction as demonstrated by the noisy baseline (Figure 4).

Method D.

The standard curve was linear to 5000 ng/L for norepinephrine (r = 0.999) and epinephrine (r = 0.999). The average concentration of the 100, 500, or 1000 ng/L norepinephrine and epinephrine standard (Table 1) was not statistically different from the expected value at the 0.01 significance level. The limit of detection was 6 ng/L (n=5) and the limit of quantification is 8 ng/L (n=5) for norepinephrine (Table 2). The limit of detection was 5 ng/L (n=5) and the limit of quantification was 7 ng/L (n=5) for epinephrine (Table 3). The pooled relative standard deviation was 3.8% (n=21) for norepinephrine concentrations (Table 2)

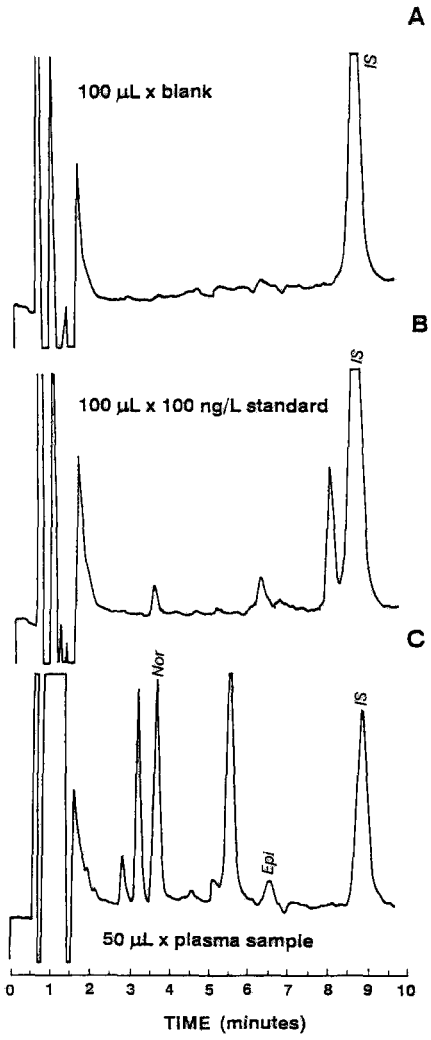

FIGURE 2. Method A Chromatograms: Samples were extracted using alumina and following HPLC separation the compounds were detected amperometrically as described in the methods section. Phosphate buffer blank with internal standard (IS) (A). Standard containing 100 ng/L of norepinephrine (Nor) and epinephrine (Epi) (B). Extracted plasma sample taken from a subject after 20 minutes of exercise on the ergometer (C).

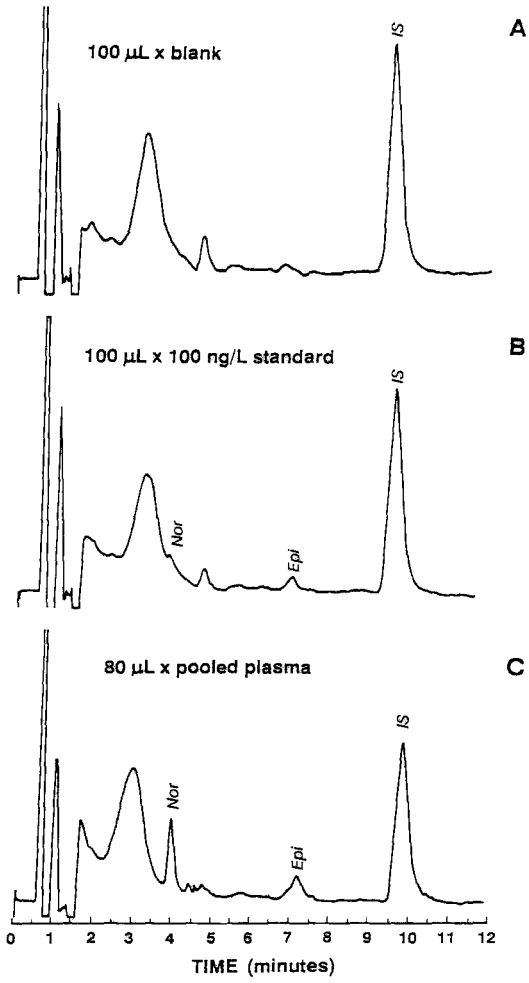

FIGURE 3. Method B Chromatograms: Samples were extracted using strong-cation exchange and then alumina as described in the methods section. Following HPLC separation the compounds were detected amperometrically. Phosphate buffer blank with internal standard (IS) (A). Standard containing 100 ng/L of norepinephrine (Nor) and epinephrine (Epi) (B). Extracted pooled plasma-B (Table 1) (C).

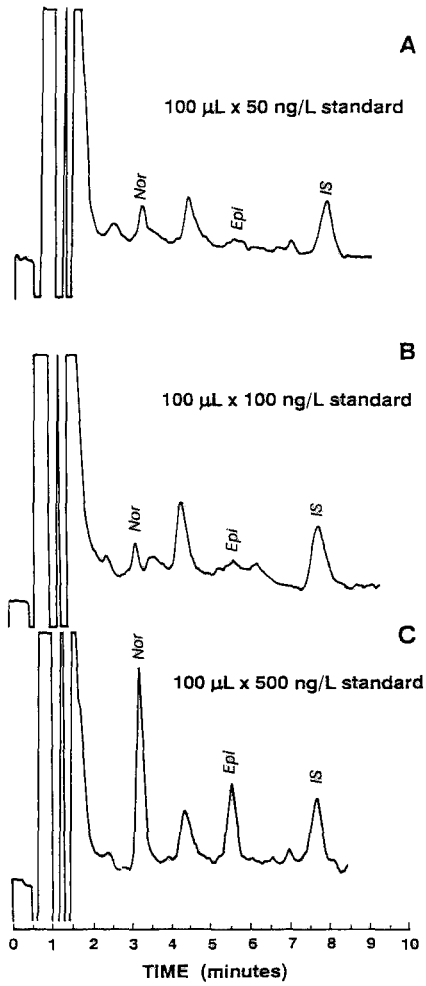

FIGURE 4. Method C Chromatograms: Samples were extracted using weak-cation exchange and following HPLC separation the compounds were detected amperometrically as described in the methods section. Standard containing 50 ng/L of norepinephrine (Nor) and epinephrine (Epi) (A). Standard containing 100 ng/L of norepinephrine (Nor) and epinephrine (Epi) (B). Standard containing 500 ng/L of norepinephrine (Nor) and epinephrine (Epi) (C).

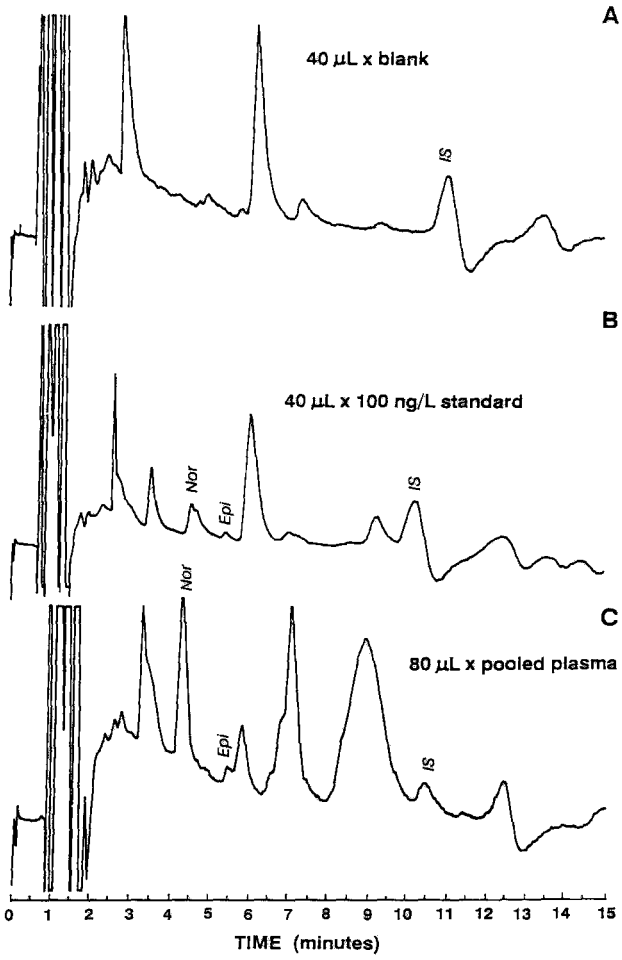

FIGURE 5. Method D Chromatograms: Samples were extracted using alumina and following HPLC separation the compounds were detected coulometrically as described in the methods section. Phosphate buffer blank at twice the gain with internal standard (IS) (A). Standard containing 100 ng/L of norepinephrine (Nor) and epinephrine (Epi) (B). Extracted pooled plasma-A (Table 1) (C).

and 3.9% (n=23) for epinephrine concentrations (Table 3). The recovery for the aluminum oxide extraction was the same as for Method A. The chromatographic conditions were carefully optimized to prevent compounds from coeluting with norepinephrine and epinephrine (Figure 5).

Summary.

The aluminum oxide extraction required about 1 hour for six samples; however, the combined strong-cation exchange and aluminum oxide extraction required 2 hours to complete for the same number of samples. Given amperometric detection, the advantage to doing the strong-cation/aluminum oxide extraction was that the limit of quantification is lower for norepinephrine and epinephrine and the peak that elutes just before epinephrine was greatly minimized. However, Method B was not further evaluated, because it was too time consuming. Method C was not completely evaluated, because the standard curve was not linear over the required range.

Method D was the best of the four methods described here (Table 2 and 3) for determining plasma norepinephrine and epinephrine concentrations in exercise research samples as well as clinical samples. The aluminum oxide extraction was completed in the shortest amount of time. The limit of quantification for norepinephrine and epinephrine was the lowest. The pooled relative standard deviation was the lowest for norepinephrine and epinephrine. The standard curve was linear over the required range.

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CHIRAL SEPARATION OF ACEBUTOLOL BY DERIVATIZATION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY#

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ABSTRACT

A simple and specific high performance liquid chromatographic (HPLC) method is described for the determination of R-(+)- and S-(-)acebutolols.

The method is based on the derivatization of the hydroxyl and amino functions of acebutolol with N-benzyloxycarbonyl-L-phenyl-alanine and acetic anhydride, respectively. The resulting derivatives were separated on a reversed phase C-18 column and monitored with a UV detector, leading to a base line resolution of the diastereomeric derivatives of S-(-) and R-(+) acebutolols.

#This work was presented in part at the annual meeting of the Pharmaceutical Society of the R.O.C., Taipei, Dec. 17th, 1994.

INTRODUCTION

It has been reported [1-4] that enantiomers of certain β–adrenergic blockers have quite different biological activities and pharmacokinetic profiles. Therefore, the stereospecific analysis of β–adrenergic blockers has recently received widespread attention [2,5-6].

Acebutolol is one of β–adrenergic blockers that are frequently used for the treatment of the diseases related to hypertension and ventricular arrhythmia [7]. Acebutolol is a chiral compound but its racemic form is used as a drug for clinical treatment. Several chiral reagents including (S)-(-)-N-trifluoroacetyl-L-prolyl chloride (TPC) [8], (R)-(+)-1-phenyl-ethyl isocyanate [9], (S)-(-)-1-phenylethyl isocyanate [6] and (S)-(+)-1-(1-naphthyl) ethyl isocyanate [10] have been used for the enantiomeric analysis of acebutolol as diastereomers, but TPC is unstable and easily racemized in storage [8]. The isocyanate reagents are moisture sensitive and must be handled with care.

In this study, a modification method based on our previous report [11] using commercially available N-benzyloxycarbonyl-L-phenylalanine (N-CBZ-L-Phe) and acetic anhydride was developed for the chiral separation of racemic acebutolols. Derivatization of acebutolol with N-CBZ-

L-Phe and acetic anhydride was conducted in mild and simple conditions. The resulting derivatives from racemic acebutolol can be easily separated as the diastereomers on a reversed phase C-18 column and monitored with a UV detector.

METHODS

Chemicals and Solutions

N-Acetyl-O-(N-benzyloxycarbonyl)-L-phenylalanyl acebutolol (ACB-D) was synthesized in our laboratory, and its structure was confirmed by MS, NMR and elemental analysis. Acebutolol was simply prepared by neutralization of acebutolol hydrochloride (May & Baker, Dagenham, U.k.) with sodium bicarbonate solution and structurally identified by MS, NMR and elemental analysis. 9-Acetylanthracene (Aldrich, Milwaukee, WI, U.S.A.), N-benzyloxycarbonyl-L-phenylalanine (N-CBZ-L-Phe) (Sigma, St. Louis, MO, U.S.A.), 4-N, N-dimethylaminopyrine (DMAP) (Tokyo Kasei, Tokyo, Japan), acetic anhydride (Ac_2O), toluene, dicyclohexylcarbodiimide (DCC) and silica gel 60 for column chromatography (Merck, Darmstadt, Germany), dichloromethane (without ethanol as stabilizer), methanol, acetonitrile and tetrahydrofuran

(THF) of HPLC grade (Fisher, Springfield, NJ, U.S.A.), ethyl acetate, n-hexane and acetone (Lab-Scan, Dublin, Ireland) were used without further purificiation. All other chemicals were of analytical reagent grade.

Solutions of 9-acetylanthrancene (internal standard, I.S.), acebutolol, DMAP, DCC, N-CBZ-L-Phe and acetic anhydride at various concentrations were prepared by dissolving the respective compound in dichloromethane. Deionized and distilled water was used to prepare related aqueous solutions.

Liquid Chromatography

Isocratic HPLC conditions consisting of a Waters Millipore 501 LC pump, a U6K injector, a C-18 reversed phase column (Waters Millipore, 150 X 3.9 mm I.D., 4 μm) with a disposable Nova-Pak C-18 precolumn (10 μm; bed volume < 100 μL) and a Waters 486 tunable absorbance detector were applied. The mobile phase of methanol/water (60/40, v/v), degassed with vacuum filter through a 0.45-μm filter, was used at a flow rate of 1.3 mL/min and the column eluate was monitored at 254 nm.

Mass Spectrometry

Mass spectrum was obtained on a VG Biotech Quattro 5022 mass spectrometer with fast atom bombardment (FAB).

Derivatization Procedure

A 0.5-mL volume of acebutolol solution was added to a 10-mL glass-stoppered test tube pre-cooled in an ice bath, containing 0.1 mL of N-CBZ-L-Phe solution (55 mM), 0.1 mL of DMAP solution (14 mM) and 0.1 mL of the I.S. solution (95 nM). Then 0.1 mL of DCC solution (0.24 M) was added and the reaction mixture was shaken mechanically at 0 ° C for 0.5 h shaker. At the end of the reaction, 0.1 mL of acetic anhydride (1.06 M) was added and the resulting reaction mixture was shaken mechanically at 30 ° C for 15 minutes in a thermostated shaker. After the reaction, 1.0 mL of methanol was added and mixed. The resulting solution was used for HPLC analysis.

RESULTS AND DISCUSSION

In order to optimize the derivatization conditions for acebutolol, several parameters such as reaction solvent, the concentrations of DMAP and N-CBZ-L-Phe, the amounts of DCC and acetic anhydride, and reaction time were investigated. The amount of acebutolol used for study was 0.34 μmol (in 0.5 mL of dichloromethane solution), unless otherwise indicated. The effects of the parameters on the derivatization

of acebutolol were evaluated by the peak-area ratios of the derivative to the I.S.

Effect of Reaction Solvent

The effects of various organic solvent (excluding alcohols) on the derivatization of acebutolol were studied according to the Derivatization Procedure. The solvents tested included acetone, acetonitrile, dichloromethane, ethyl acetate, THF and toluene. Dichloromethane was found to be the best solvent for the derivatization.

Effect of N-CBZ-L-Phe

The concentration of N-CBZ-L-Phe solution (0.1 mL) required for the derivatization of acebutolol to a plateau formation of the derivative is around 36 mM as shown in Figure 1, but a higher concentration of N-CBZ-L-Phe solution at 55 mM was selected for the derivatization, equivalent to a molar ratio (N-CBZ-L-Phe to acebutolol) of 16.6.

Effect of DMAP

The effect of the DMAP solution (0.1 mL) at various concentrations on the formation of the acebutolol derivative is shown in Figure 2. The results indicate that the concentration range of DMAP is suitable

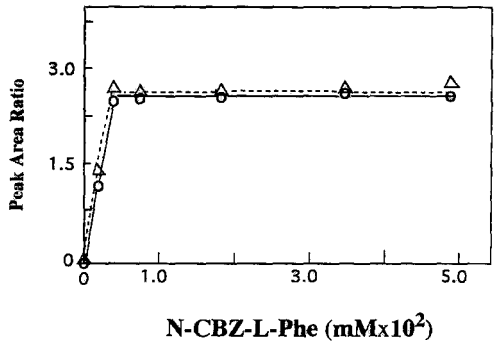

FIGURE 1. Effect of N-CBZ-L-Phe concentration on the formation of acebutolol derivatives. o : S-acebutolol derivative; Δ : R-acebutolol derivative.

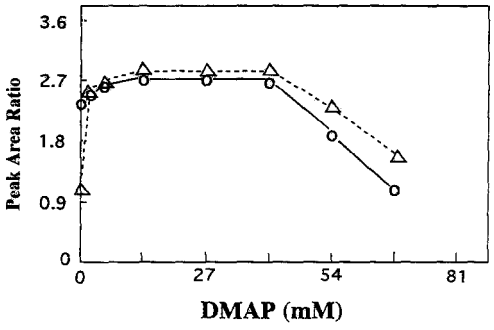

FIGURE 2. Effect of DMAP concentration on the formation of acebutolol derivatives. o : S-acebutolol derivative; Δ : R-acebutolol derivative.

over 6.85- 40.5 mM, and DMAP solution at the concentration of 14 mM was used for derivatization.

Effect of DCC

The effect of DCC solution (0.1 mL) at varied concentrations up to 0.5 M on the formation of the acebutolol derivative was studied. As shown in Figure 3, the optimal concentration of DCC for the derivatization of acebutolol is above 0.087 M and DCC at 0.24 M was used for derivatization.

Effect of Acetic Anhydride

The concentration of Ac_2O solution (0.1 mL) required for the derivatization of acebutolol to a plateau formation of the derivative is around 0.04 M as shown in Figure 4, but a higher concentration of this simple reagent at 1.06 M was used for the derivatization.

Effect of Reaction Time

The reaction time required for the esterification of acebutolol with N-CBZ-L-Phe to give a constant formation of the acebutolol ester is about 10 minutes at 0 ° C as shown in Figure 5; and that for the acetylation of the acebutolol ester to give a constant formation of the final

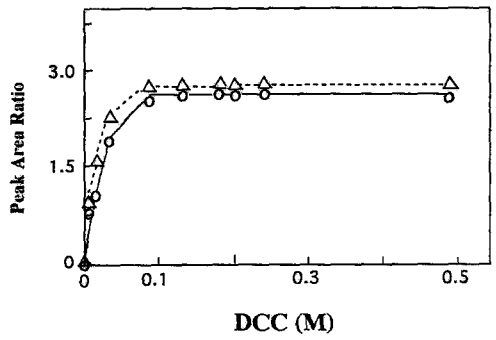

FIGURE 3. Effect of DCC concentration on the formation of acebutolol derivatives. o : S-acebutolol derivative; Δ : R-acebutolol derivative.

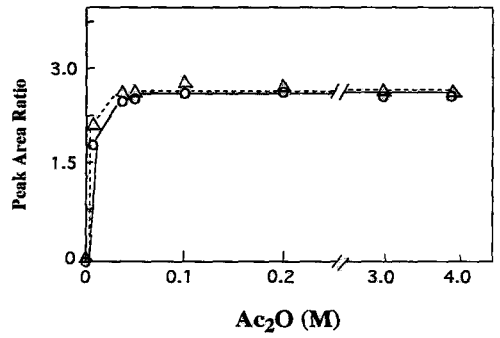

FIGURE 4. Effect of Ac_2O concentration on the formation of acebutolol derivatives. o : S-acebutolol derivative; Δ : R-acebutolol derivative.

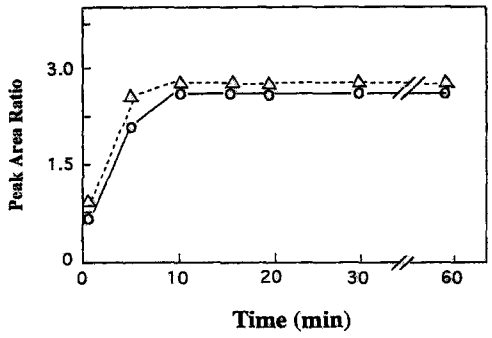

FIGURE 5. Effect of reaction time on esterification of acebutolols.
o : S-acebutolol derivative; Δ : R-acebutolol derivative.

derivative is about 10 minutes at 30 ° C as shown in Figure 6. The reaction times for esterification and acetylation were set 30 min and 15 min, respectively.

Based on the optimum derivatization conditions obtained above, a derivatization procedure was formulated under the METHODS section. Derivatization of acebutolol only with N-CBZ-L-Phe resulted in an ester derivative which was unstable, reflecting on gradually decreasing the peak-area ratios of the derivative to the I.S. after derivatization, due probably to the intramolecular degradation as postulated in Figure 7. Therefore, derivatization of acebutolol successively with esterification and acetylation is essential. The resulting derivative is stable up to 4 days tested as shown in Figure 8.

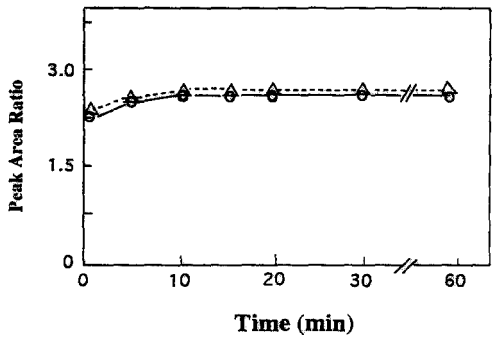

FIGURE 6. Effect of reaction time on acetylation of acebutolol esters. o : S-acebutolol derivative; Δ : R-acebutolol derivative.

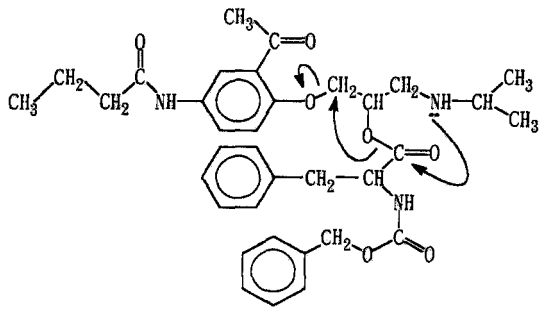

FIGURE 7. Assumed intramolecular degradation of O-(N-benzyloxy-carbonyl)-L-phenylalanyl acebutolol.

Analytical Calibration and Typical Chromatograms

To evaluate the quantitative applicability of the method, six different concentrations of acebutolol over the range 0.01 - 0.32 μmol, (each in 0.5 mL of dichloromethane solution) were determined to construct a calibration graph. The results indicate good linearity for the determina-

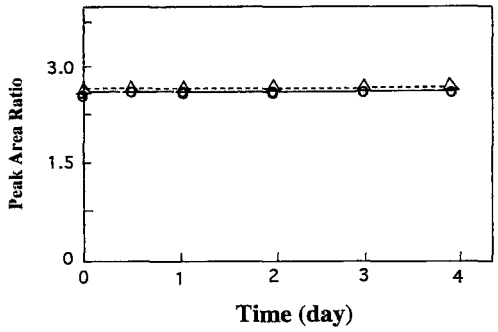

FIGURE 8. Stability of acebutolol derivatives after derivatization.
o : S-acebutolol derivative; Δ : R-acebutolol derivative.

tion of acebutolol over the range studied; linear regression equations, $y = 9.5497\chi - 0.0031$ (for S-acebutolol) and $y = 9.7451\chi - 0.0016$ (for R-acebutolol) were obtained with correlation coefficients of 0.9997 and 0.9998, respectively; where y is the peak-area ratios of the acebutolol derivative to the I.S., and χ is the amount of acebutolol in μmol. The derivatization yield of acebutolol is nearly quantitative (> 94%) as presented in Table 1, based on the calculation of the peak-area ratios of acebutolol tested to the I.S. in comparison with that of the acebutolol derivatives (ACB-D) synthesized. The observed C.V. for the intra-assay (n=6) at two levels of acebutolol is below 5% as shown in Table 2.

A typical liquid chromatogram is presented in Figure 9, illustrating a good resolution of the acebutolol derivatives (Resolution >1.5) and they

TABLE 1

Derivatization Yield of Acebutolol

Acebutolol tested(μmol)	The derivative found(μmol)*	Yield(%)
S 0.16	0.15 ± 0.009	94
R 0.16	0.16 ± 0.006	102
S 0.32	0.32 ± 0.004	99
R 0.32	0.34 ± 0.004	106
S 0.64	0.65 ± 0.011	102
R 0.64	0.69 ± 0.013	108

* Mean ± S. D. of three replicate analyses.

TABLE 2

Precision for the Determination of Acebutolol

Acebutolol tested(μmol)	The derivative found(μmol)*	C.V. %
S 0.06	0.06 ± 0.003	4.8
R 0.06	0.06 ± 0.001	2.1
S 0.44	0.45 ± 0.004	1.0
R 0.44	0.44 ± 0.008	1.8

* Mean ± S. D. of three replicate analyses.

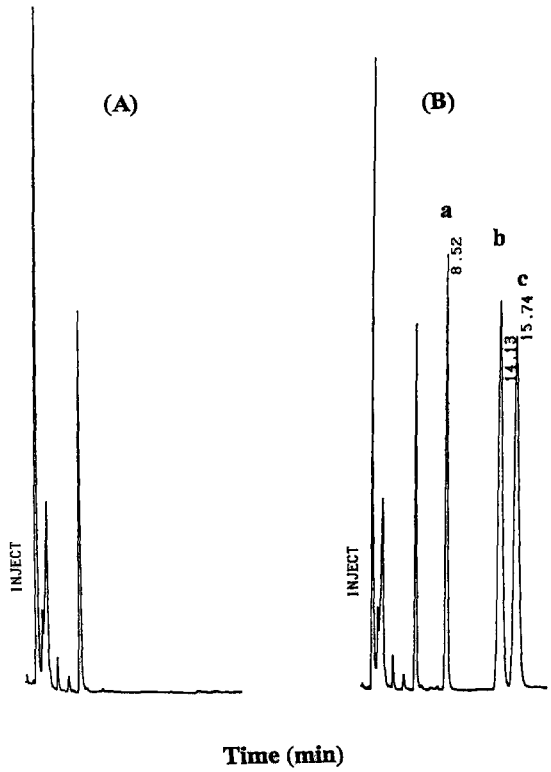

FIGURE 9. HPLC chromatograms of (A) reagent blank (B)derivatives of enantiomeric acebutolol. Peaks: a, internal standard; b, S-acebutolol derivative; c, R-acebutolol derivative. HPLC conditions: column, Nova-Pak C18(15cm x 3.9 mm I.D.); mobile phase, 60%(v/v) methanol in water; flow rate 1.3 mL/min; detector, UV 254 nm, 0.02AUFS.

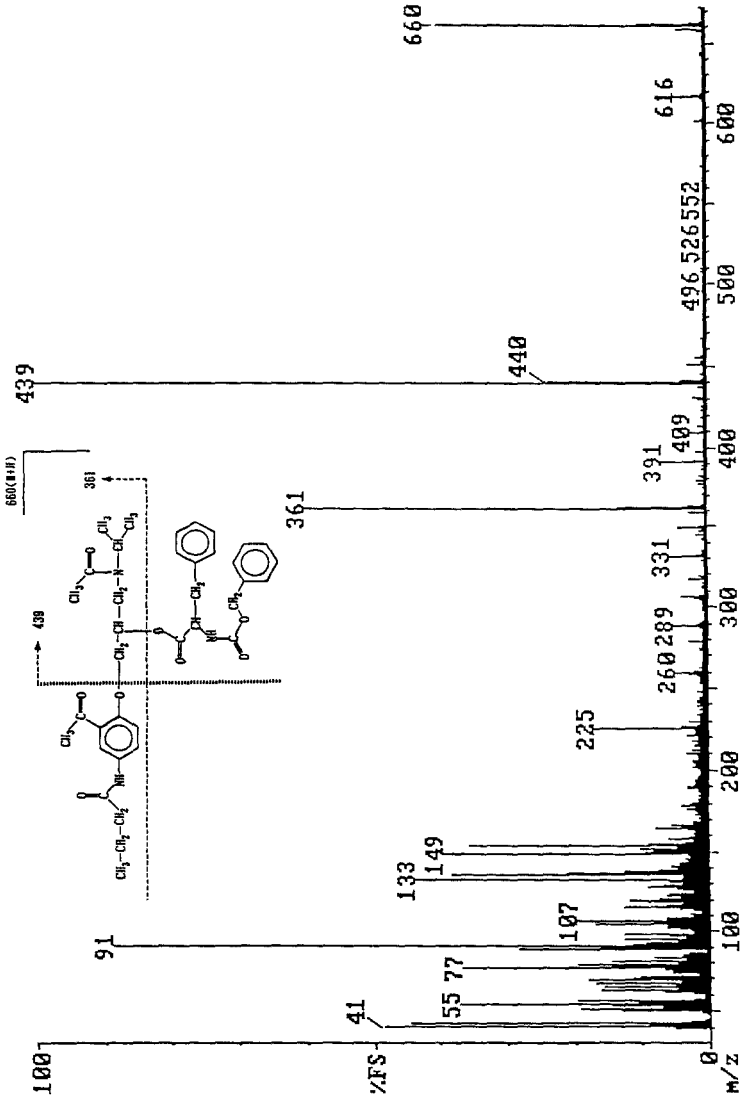

FIGURE 10. FAB-MS spectrum of acebutolol derivative. See text for conditions.

are eluated in reasonable time (16 min). The structure of the acebutolol derivative isolated was determined by FAB-MS (Figure 10) with nitrobenzyl alcohol as a matrix, giving a pseudomolecular ion at m/z=660 (M +H) and a diagnostic peak at m/z=361, equivalent to the fragment of a substituted acetamide (CH_3CONRR'). This indicates that the amino nitrogen of acebutolol is acetylated. Therefore, the derivative is tentatively assigned as N-acetyl-O-(N-benzyloxycarbonyl)-L-phenylalanyl acebutolol (ACB-D).

In conclusion, a simple and specific HPLC method was developed for the chiral analysis of acebutolol, based on the derivatization of acebutolol with N-CBZ-L-Phe and acetic anhydride in mild conditions. Further application of the method to the chiral analysis of related β-blocker alcohols will be very attractive.

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NORMAL-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD WITH DANSYLATION FOR THE ASSAY OF PIPERAZINE CITRATE IN DOSAGE FORMS

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ABSTRACT

The assay of the anthelmintic agent piperazine citrate in dosage forms has been accomplished by normal-phase HPLC after converting the drug to a UV-absorbing dansyl (DNS) derivative. For this purpose, an aliquot of an aqueous extract or dilution from tablets, powders, effervescent granules or syrups was first mixed with a solution of 1-benzylpiperazine, the internal standard, and next with solutions of DNS-chloride and sodium carbonate. Sonication of the reaction mixture led to the formation of the DNS-derivatives, with bis-DNS-piperazine separating as a crystalline product. After extraction into chloroform, and dilution of the extract with mobile phase, the DNS derivatives were separated on a cyanopropyl column with hexane-isopropanol (85:15) as the mobile phase. At a flow rate of 1.5 mL/min and a detection wavelength of 335 nm, DNS-1-benzylpiperazine and bis-DNS piperazine eluted at 4.0 and 8.5 min, respectively. Detector responses were linearly related to on-column concentrations of piperazine citrate ranging from 8-50 μg. The recovery of analyte from synthetic formulations simulating the various dosage forms was in all cases > 99.0% (range 99.7-102.0%). Assay results by the proposed method agreed closely with those obtained by the gravimetric method of USP 23.

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INTRODUCTION

Piperazine, a cyclic ethylenediamine, is an anthelmintic agent used to treat human and animal infestations by roundworms and pinworms (1). Following oral administration, piperazine and its salts interact with susceptible intestinal worms to cause reversible muscle paralysis, presumably by blocking the response of muscles to acetylcholine (2,3). The paralyzed worm is eventually expelled in the stools by the normal peristaltic activity of the intestine (2).

Piperazine citrate is the salt recognized as official in the United States (4); however, the hexahydrate form as well as the adipate, tartrate and phosphate salts are also considered to be therapeutically important in foreign markets (5,6).

Piperazine and its salts have been analyzed by a variety of analytical methods that have included gravimetry (4,7), UV-Vis spectrophotometry (8-12), IR spectrophotometry (7,13), NMR spectroscopy (14), turbidimetry (15), fluorometry (16), polarography (17,18), titrimetry (4-6,19-21), and gas-chromatography (22,23). In the USP (4), the drug substance is assayed by a nonaqueous titration while a tedious gravimetric approach is required for the dosage forms. More recently, piperazine has been assayed in dosage forms by HPLC methods, either on a cationic ion-exchange column with refractometric detection (24), or by the reversed-phase (RP)mode with photometric detection in the visible range after derivatization with 4-chloro-7-nitro-benzofurazane (NBD)-chloride (25).

The purpose of this report is to describe a HPLC assay method for piperazine citrate which utilizes precolumn derivatization with DNS-chloride at room temperature, followed by normal-phase (NP)-HPLC with photometric detection in the UV range. This method was found suitable for the assay of the title drug in commercial tablets, powders, effervescent granules and syrups.

EXPERIMENTAL

Samples and Materials

The piperazine citrate was a USP reference standard (U.S. Pharmacopeial Convention, Inc.). The dansyl chloride (DNS-Cl), anhydrous sodium sulfate (Sigma Chemical Co.), and 1-benzylpiperazine (Aldrich Chemical Co.) were used as received. Tablets (275.75 mg), syrups (1.25 g/5 mL, 650 mg/5 mL, or 550 mg/5 mL), effervescent granules (0.2 g/5.71 g) and powders(1.65 g/envelope) were obtained from domestic and foreign commercial sources. The acetone, isopropanol and hexane were of HPLC grade (J.T. Baker); the anhydrous sodium carbonate was of analytical reagent grade (Fisher).

Reagents

a. DNS-Cl solution - Prepared by dissolving DNS-Cl in HPLC grade acetone and filtering. This solution, containing 5mg/mL, was stored in an amber glass bottle and in the refrigerator.

b. Basic solution - Prepared by dissolving 550 mg of anhydrous sodium carbonate in 300 mL of water, adding 300 mL of HPLC grade acetone, and mixing. This solution was stored in an amber glass bottle at room temperature.

c. Internal standard solution - Prepared by dissolving 1-benzylpiperazine in acetone-water (1+1) to a concentration of 4 mg/mL.

Sample Preparations

a. Piperazine citrate standard preparation - An accurately weighed quantity of piperazine citrate (about 200 mg) was transferred to a 100 mL volumetric flask, dissolved in about 50 mL of water, brought to volume with water, and mixed.

b. Tablet preparation - A group of 20 piperazine citrate tablets were weighed and reduced to a fine powder. A portion of powder, equivalent to about 200 mg of piperazine citrate, was transferred to a 100 mL volumetric flask, mixed with about 50 mL of water, and sonicated for about 15 min. After bringing to volume with water and mixing, the solution was filtered through a 0.45 μm membrane filter with the aid of pressure.

c. Syrups - A 5.0 mL volume of syrup was transferred to a 100 mL volumetric flask, diluted to volume with water, and mixed. An aliquot of the solution, containing about 200 mg of piperazine citrate (15.0, 30.0 or 40.0 mL aliquots for syrups containing 1.25 g/5 mL, 650 mg/5 mL, or 550 mg/5 mL, respectively), was transferred to a 100 mL volumetric flask, diluted to volume with water, and mixed.

d. Effervescent granules and powders - An accurately weighed quantity of granules or powder, equivalent to about 200 mg of piperazine citrate, was transferred to a 100 mL volumetric flask, and dissolved in about 50 mL of water (added slowly and with gentle swirling). After sonication for 10 min, the solution was brought to volume with water, mixed, and filtered through a 0.45 μm membrane filter with the aid of pressure.

e. Synthetic piperazine tablet preparation - To a 100 mL volumetric flask, an accurately weighed quantity of piperazine citrate (about 200 mg) and a starch-lactose (1+1) mixture (about 100 mg) was added. After the addition of 50 mL of water, the mixture was sonicated for 15 min, diluted to volume with water, and mixed. A portion of the solution was filtered through a 0.45 μm membrane filter with the aid of pressure.

f. Synthetic piperazine citrate syrup preparation - An accurately weighed quantity of piperazine citrate (about 100, 130 or 250 mg) was transferred to a 100 mL volumetric flask, mixed with 1 mL of simple syrup, diluted to volume with water, and mixed.

g. Synthetic effervescent granules preparation - An accurately quantity of piperazine citrate (about 200 mg), together with sodium bicarbonate (2.46 g), tartaric acid (1.94 g), and citric acid (0.63 g) were transferred to a 100 mL volumetric flask, dissolved in about 50 mL of water (added slowly with gentle swirling), and sonicated for about 10 min. The solution was diluted to volume with water, mixed, and filtered through a 0.45 μm membrane filter with the aid of pressure.

Dansylation Method

To a 125 mL glass-stoppered Erlenmeyer flask, 3.0 mL of sample preparation, 2.0 mL of internal standard solution, 10 mL of DNS-Cl, and 10 mL of basic solution, were added in succession and mixed with gentle swirling. The flask was loosely stoppered, placed in an ultrasonic bath, sonicated for 10 min, and allowed to stand in the dark for at least 30 min. The reaction mixture was diluted with water (20 mL), mixed with chloroform (20 mL), and vigorously shaken for about 1 min. After allowing the phases to separate, the chloroform layer was carefully removed by aspiration with a pipet, and passed through a layer of anhydrous sodium sulfate, held on a funnel fitted with a glass wool pledget, into an amber glass vial. A 2.0 mL volume of the filtrate was transferred to a 5 mL volumetric flask, diluted to volume with mobile phase, and a portion of the solution was immediately injected into the liquid chromatograph.

HPLC Method

a. Apparatus - Consisting of a Series 10 liquid chromatograph, LC 90 UV spectrophotometric detector (Perkin-Elmer Corporation), and ChromJet electronic integrator (Spectra-Physics). Samples were introduced through a Model 7125 injection valve fitted with a 20 μL sample loop (Rheodyne).

b. Chromatographic conditions - Separations were performed on a 25 cm x 4.6 mm i.d., CN5 SG cyanopropyl, 5 μm, column (Burdick & Jackson). Samples were eluted with a mobile phase composed of hexane-isopropanol (85:15). The flow rate was 1.5 mL/min, and the detection wavelength was 335 nm.

Calculations

The quantity of piperazine citrate in the sample preparation was calculated from one of the following equations:

$$\text{mg/tablet} = (R_{sp}/R_{st}) \times C \times (W_1/S) \times 100$$
$$\text{mg/g effervescent granules} = (R_{sp}/R_{st}) \times C \times (W_2/S) \times 100$$
$$\text{mg/envelope} = (R_{sp}/R_{st}) \times C \times W_3/S) \times 100$$
$$\text{mg/5 mL syrup} = (R_{sp}/R_{st}) \times C \times 100F$$

where R_{sp} and R_{st} = peak responses of sample/internal standard in the sample preparation and standard/internal standard in the standard preparation, respectively; C = the amount of piperazine citrate in the standard preparation, mg/mL; W_1 = the average tablet weight, mg; S = the amount of sample taken for the analysis, mg; W_2 = 5,710 mg; W_3 = the weight of the contents of one envelope, mg; and F = 100/15 for 1.25 g/5 mL syrup, 100/30 for 625 mg/5 mL syrup, and 100/40 for 550 mg/5mL syrup.

To convert the value in piperazine citrate to that in piperazine hexahydrate, multiply the value by 0.8968 (obtained from [mol. wt. piperazine hexahydrate/mol. wt. piperazine] x [3 mol. wt. piperazine/mol. wt. piperazine citrate]). The reverse conversion can be accomplished by dividing by the same factor.

RESULTS AND DISCUSSION

DNS-Cl is an electrophilic reagent that has been widely used for the precolumn derivatization of a number of drugs of therapeutic interest (26). The formation of the DNS-derivatives is usually carried out with an excess of DNS-Cl in an aprotic solvent and under alkaline conditions, by leaving the reaction mixture to stand for several hours at room temperature or for shorter periods at elevated (30-50°C) temperatures (27). In this manner, primary and secondary amines with little or no UV absorbing properties are converted to stable sulfonamides that are analyzable by HPLC with either photometric or fluorometric detection (26,27). In the present study, formation of bis-DNS-piperazine occurred over several hours upon standing, and in less than 5 minutes upon sonication of the reaction mixture. In both instances, the derivative separated as a light yellow crystalline product, m.p. 258-260°C, whose mass spectrum exhibited abundant peaks at m/e 84,155, 171, 318, and 552, and a molecular ion peak corresponding to a molecular weight of 552.14.

A sample preparation amenable to direct NP-HPLC analysis was obtained by extracting the aqueous reaction mixture with chloroform, drying the chloroform extract with anhydrous sodium sulfate and diluting the extract with the mobile phase. Alternatively one could use a RP-HPLC approach, but this course of action will complicate the analytical procedure because it will require to evaporate the chloroform extract to

Table 1

Retention times relative to DNS-piperazine of DNS-derivatives of N-containing heterocyclic compounds of potential utility as an internal standard

DNS-derivative	Column[a]			
	A[b]	B[b]	C[b]	D[c]
Piperazine	1.00	1.00	1.00	1.00
2-Methylpiperazine	0.95	0.94	0.89	0.93
1-Benzylpiperazine	0.50	0.36	0.31	0.48[d]
1-Phenylpiperazine	0.46	0.41	0.45	0.47[d]
1-(2-Aminoethyl)piperazine	2.41	2.05	-e	2.94
Piperidine	0.42	0.32	0.36	0.41[d]
4-Benzylpiperidine	0.41	0.36	0.40	0.41[d]
4-Hydroxypiperidine	0.95	0.54	0.74	1.79

[a]Column identification: A= CN5 SG cyanopropyl (Burdick & Jackson); B = Microsorb-MV CN (Rainin); C = μBondapak CN (Waters); D = Econosphere CN (Alltech).
[b]Solvent: hexane-isopropanol (85:15), 1.5 mL/min.
[c]Solvent: hexane-isopropanol (97.5:2.5), 1.2 mL/min.
[d]Only partially resolved from the solvent front.
[e]Derivative was retained on the column.

dryness, and to reconstitute the residue in a suitable solvent. In comparison to other procedures for the precolumn derivatization of piperazine citrate (25), the use of DNS-Cl offers advantages such as the possibility of conducting the reaction at ambient temperature, of omitting the addition of an amine to the mobile phase, and of monitoring the elution within the UV spectral range.

Several hydroxy- phenyl- and methyl-piperazine and piperidine compounds were evaluated for their suitability as an internal standard. Table 1 lists the retention times of the corresponding DNS-derivatives on various brands of cyano columns relative to that of piperazine. On the basis of its ready reaction with DNS-Cl to form a DNS-derivative that was quantitatively coextracted with that of piperazine, and chromatographically well resolved from the peak of bis-DNS-piperazine (R >2.0), 1-benzylpiperazine was judged as appropriate. However, at the completion of this study, it was verified that homopiperazine could serve as an attractive alternative to 1-benzylpiperazine by virtue of

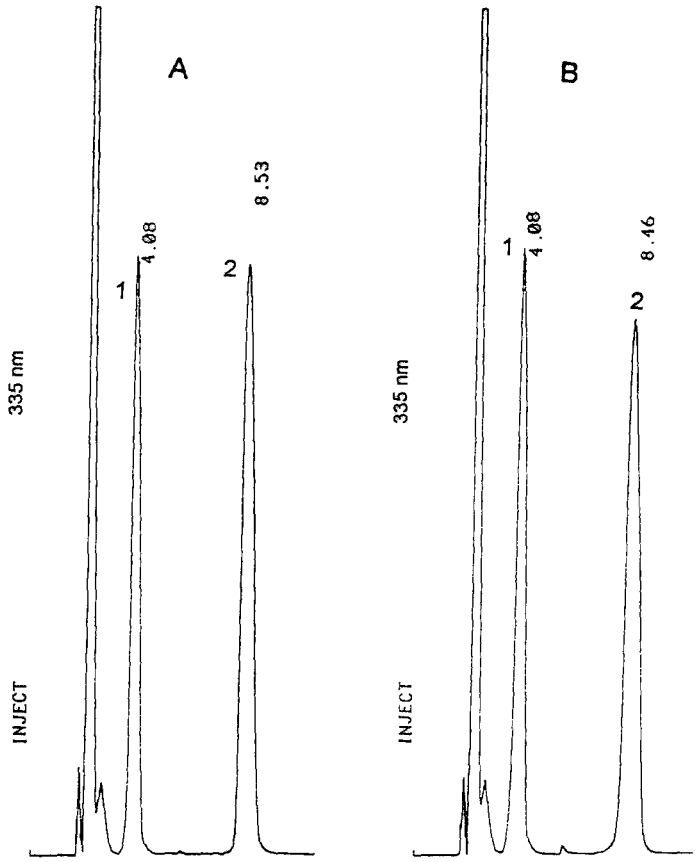

Figure 1. Typical high-performance liquid chromatograms of 1, DNS-1-benzylpiperazine and 2, bis-DNS-piperazine in (A) a standard preparation and (B) a commercial syrup. Flow rate was 1.5 mL/min.

its more convenient (ca. 6.5 min) retention time. In addition, it was verified that the elution behavior of certain DNS-derivatives varied from column to column, notably on the μBondapak cyano and Econosphere CN. For instance, the μBondapak CN column was the least retentive of all the columns tested, with some DNS-derivatives eluting with the solvent front even when the concentration of isopropanol in the mobile phase was less

Table 2

Results of recovery of piperazine citrate from spiked synthetic dosage forms by proposed HPLC method

Synthetic formulation	Piperazine citrate found, % of added			
	Run 1	Run 2	Mean	SD
Tablet, 200 mg	99.5	99.8	99.7	0.15
Syrup, 250 mg/mL	99.7	100.7	100.2	0.71
Syrup, 130 mg/mL	99.9	99.5	99.7	0.28
Syrup, 100 mg/mL	101.9	99.7	100.8	1.55
Granules, 0.2 g/5.71 g	102.7	102.9	102.8	0.14

than 5 parts per 100. In contrast, the Econosphere CN column retained certain DNS-derivatives (e.g. DNS- 1-(2-aminoethyl)piperazine) under the recommended experimental conditions. Typical separations of the DNS derivatives of the analyte and internal standard are presented in Figure 1.

A linear relationship between detector responses (ratio of peak heights or areas of bis-DNS-drug to DNS-internal standard) and on column concentrations of piperazine citrate was observed over the concentration range of 8-50 μg (line equation, $y = 0.86x - 0.026$; $r = 0.999$), with the curve passing through the origin. Based on these results, assays were routinely conducted using a sample preparation that contained about 2 mg/mL of analyte. The reproducibility of the method was assessed on the basis of peak areas (RSD = 1.92%) and peak heights (RSD = 0.58%) for six consecutive injections of a standard mixture of bis-DNS-piperazine and DNS-1-benzylpiperazine. The accuracy of the proposed method was assessed by spiking synthetic formulations, prepared to simulate tablets, syrups and effervescent granules, with known amounts of piperazine citrate and subjecting the samples to the assay procedure described for commercial dosage forms. As presented in Table 2, the mean recovery ($n = 2$) of piperazine citrate by the proposed HPLC method was in all cases better than 99.5% (range 99.7-102.0%) of added. No recovery study was conducted on a synthetic powder dosage form owing to the fact that the formulation ingredients were not disclosed by the manufacturer.

Table 3

Results of the assay of piperazine citrate in commercial dosage forms by proposed DNS-HPLC method and USP 23 gravimetric method

Lot No.	Piperazine citrate found, % of declared					
	HPLC			Gravimetry		
	Run 1	Run 2	Mean	Run 1	Run 2	Mean
	Tablets, 275.5 mg/tablet[a]					
1	100.1	101.3	100.7	99.2	98.0	98.6
	Effervescent granules, 0.2 g/5.71 g[b,c]					
1	103.0	103.7	103.4	104.2	104.9	104.5
2	103.7	103.8	103.8	104.8	104.7	104.8
	Powder, 1.65 g/envelope[d]					
1	99.9	101.3	100.6	99.5	98.8	98.7
	Syrup A, 1.25 g/5 mL[b]					
1	85.9	87.2	86.6	85.4	85.5	85.5
2	85.9	86.9	86.4	85.5	85.3	85.6
3	87.4	87.6	87.5	85.8	85.8	85.8
	Syrup B, 625 mg/5 mL[b]					
1	93.3	91.8	92.6	90.9	91.7	91.8
	Syrup C, 550 mg/5 mL[c]					
1	85.2	84.0	84.6	83.3	82.2	82.8
2	84.0	85.1	84.5	83.4	82.9	83.2
3	83.1	84.3	83.7	82.7	83.3	83.0

[a]Manufactured in U.S.A.
[b]Manufactured in the Philippines.
[c]Declared as piperazine hexahydrate.
[d]Manufactured in Peru.

Assay values for commercial dosage forms of piperazine citrate by the proposed HPLC method are summarized in Table 3. These values agreed closely with those obtained by the gravimetric method of USP 23, which is based on the precipitation of piperazine as the picrate salt. Intermethod assay differences based on the labeled amount were about 2.1% for tablets, 0.7-1.9% for syrups, 1.9% for powders, and 1.1% for effervescent granules. Except for two brands of syrup (syrups A and C), all of the samples conformed to the compendial requirements for labeled amounts. No interferences were noted from either excipients of tablets and effervescent granules or from ingredients of syrups.

In summary, the analysis of piperazine citrate in commercial solid and liquid dosage forms can be accomplished in a simple, specific and accurate manner by the NP-HPLC method presented here. This method will yield assay results that are comparable to those obtained by the slower and more cumbersome gravimetric method of USP 23.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 18(16), 3359-3363 (1995)

THE BOOK CORNER

ANALYSIS OF ADDICTIVE AND MISUSED DRUGS, edited by John A. Adamovics, Marcel Dekker, Inc., New York, New York, 1995, viii + 671 pp. , $ 195.00; ISBN: 0-82 47-9230-6.

This book is a welcome addition in the field of the analysis of drugs abuse with the testing for banned substances in athletes. The book examines both the chromatographic and non chromatographic methods available to identify, analyze, screen for these controlled-use drugs.

The book consists of two parts. The first part consists of ten chapters written by fifteen international contributors who are experts in the field. Chapter 1 discusses the use of enzyme immunoassays in the analysis of drugs of abuse. This is followed by a chapter describing the development of biosensors for the direct, sensivitive, and selective assay of misused drugs. The third chapter describes a paper chromatographic technique extensively used to screen for drugs in biological matrices. The next chapter is devoted to the uses of reversed--phase high-performance liquid chromatography in the analysis of drugs of forensic interest, which is followed by a chapter on the often overlooked use of underivatized silica gel with polar solvents. Chapter 6 assesses the applicability of the relatively new and exciting technique of capillary electrophoresis for the separation of illicit drugs. Chapter 7 discusses a simple and sensitive identification system for the detection of a broad spectrum of drugs using thin-layer chromatography for screening and gas chromatography/mass spectrometry for confirmation. For analysts overwhelmed by a large number of samples, Chapter 8 presents the most current robotic technology. The next chapter presents a comprehensive sports drug testing program. Finally, Chapter 10 is devoted to the approaches to solving forensic problems in developing countries with limited resources.

The second part of the book consists of an appendix printed in 236 pages. This appendix provides a comprehensive alphabetic listing of over 400 drugs identified by either the U. S. Drug Enforcement Adminstration or the International Olympic Committee. This appendix is tabulated to include the drug name, sample matrix, handling procedure, testing procedure, mode of detection and references which render it a valuable quick reference source for forensic analysis.

I congratulate the Editor for including this appendix, as it represents a compendium for all testing laboratories that analyze for abused and misused drugs.

The book contains over 1700 bibliographic citations up to 1993, with over 1100 references present in the appendix alone.

This book is highly recommended to own as it presents the state-of-the-art in this field and can be used as a practical daily reference for analytical, clinical, forensic, pharmaceutical chemists, pharmacologists, toxicologists, graduate students among other disciplines who are involved with abused and misused drug analysis. This book is also a must for analysts involved in drugs of abuse testing and athletic drug testing.

HPLC: A PRACTICAL USER'S GUIDE, by Marvin C. McMaster, VCH Publishers, Weinheim, Germany, 1994, xii + 211 pp., DM 98.00; ISBN: 1-56081-636-8

This book is very useful manual for all scientists and technicians whose work involves the use of HPLC, either routinely, or in research applications. It focuses on setting up and running an HPLC system efficiently, cleaning and trouble shooting column and system problems, means for obtaining separations and maximizing information from a system.

The book consists of three parts. Part I discusses the advantages and disadvantages of HPLC and basics in selecting and running an HPLC system. Part II, entitled HPLC optimization, and consists of six chapters as follows:

- Separation Models
- Column Preparation
- Column aging, diagnosis and healing
- Modification of Partition Chromatography
- "Non Partition" Chromatography
- Hardware Specifics
- Trouble Shooting and Optimization

This part represents the basic core of information the chromatographers should be aquainted with.

Part III discusses HPLC utilization and consists of five chapters. A chapter is dedicated to preparative chromatography and another to sample preparation and methods development. The other three chapters deal with automation, interfacing to computers and data acquisition devices, HPLC/mass spectrometry and with application logic. The author includes the following five appendices at the end of the book: (a) personal separation guide to point out starting points for chromatographic separations and trends in usage of columns, mobile phases and detectors, (b) a glossary of HPLC terms, (c) HPLC trouble shooting quick reference. This appendix is helpful to tackle quick problems which may arise; (d) laboratory experiments. This appendix consists of three experiments designed to familirize the student with several HPLC techniques used in HPLC systems such a as system start-up and column calibration, sample preparation and method development and column and solvent switching; (e) selected reference list to give a beginner starting point for literature in the field.

The book is recommended for all undergraduate and graduate students using HPLC as a tool for separation and analysis. Also, analytical chemists in pharmaceutical, biotechnology, environmental industries and other related fields as well as academic professionals who will find this book of high practical value.

HPLC DETECTION: NEWER METHODS, edited by G. Patonay, VCH Verlagsgesellschaft, Weinheim, Germany, 1993; xii + 236 pages, ISBN: 3-527-78219X; DM 158 + £ 65.00

This book provides an extremely valuable survey for the most powerful and less conventional detection methods used in HPLC. With the advancement

of several analytical methods, new detection methods have become available which have more advantages than the conventional detection methods, thus offering chromatographers new horizons in separation analysis techniques. Long-lived luminescence, fourier transform infrared spectroscopy (FTIR), HPLC-mass spectroscopy are among the topics discussed. The book consists of 9 chapters written by several contributors who are experts in the field of HPLC. Each chapter concludes with a list of references up to 1991, and most of the chapters contain good illustrations and figures.

In Chapter 1 measurement concepts are presented for detection in micro-HPLC separations using lasers. In Chapter 2, the advantages of using long-lived luminescence detection methods are discussed to illustrate its applications with trace concentrations. The utility of chemiluminescence in HPLC detection is presented in Chapter 3, Chapter 4 discusses near infrared semiconductor laser fluorescence, one of the latest emerging detection methods of ultra trace concentrations. The somewhat more conventional electrochemical detection method is discussed in Chapter 5, however, with a special emphasis on less conventional applications. Chapter 6 discusses powerful photothermal detection methods. The last three chapters focus on detection methods that are providing information about the structure and identification of the analyte molecule by the detector.

The titles of these chapters are:

- HPLC detection using fourier transform infrared spectroscopy
- HPLC detection using mass spectrometry
- HPLC chromatography proton nuclear magnetic resonance on-line coupling

These methods could offer enough information to identify the solute(s), thus, improving the utility and application of HPLC. The authors of these chapters offered an overview for those modern detection techniques and updated status to the developments required to improve the potential of these techniques.

This book is an excellent contribution to HPLC modality and is essential to be in any library in academia, industry, government and hospitals. It is also recommended for concerned chromatographers to have their own copy.

Reviewed by:

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JOURNAL OF LIQUID CHROMATOGRAPHY, 18(16), 3365 (1995)

ANNOUNCEMENT

BASIC PRINCIPLES OF HPLC
and
HPLC SYSTEM TROUBLESHOOTING

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The courses, which are offered for presentation at corporate laboratories, are aimed at chemists and technicians who work with HPLC. They cover HPLC fundamentals and method development, as well as systematic diagnosis and solution of HPLC module and system problems.

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- Introduction to HPLC Theory
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- Mobile Phase Selection & Optimization
- Ion-Pairing Principles
- Gradient Elution Techniques
- Calibration & Quantitation
- Logical HPLC Troubleshooting

The instructor for the courses, Dr. Jack Cazes, is Editor-in-Chief of the Journal of Liquid Chromatography, of Instrumentation Science & Technology journal, and of the Chromatographic Science Book Series. He has been intimately involved with liquid chromatography for more than 30 years; he pioneered the development of modern HPLC technology. Dr. Cazes was also Professor-in-Charge of the ACS short course and the ACS audio course on Gel Permeation Chromatography for many years.

Details of these in-house courses may be obtained from Dr. Jack Cazes, Post Office Box 2180, Cherry Hill, NJ 08034-0162, USA; Tel: (609) 424-3505; FAX: (609) 751-8724.

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(16), 3367-3372 (1995)

LIQUID CHROMATOGRAPHY CALENDAR

1995

OCTOBER 1: Ion Chromatography & Capillary Electrophoresis Short Course, Dallas, Texas. Contact: R. Cassidy via Email (Richard.Cassidy@chem.utas.edu.au) or Paul Haddad via Email (Paul.Haddad@chem.utas.edu.au).

OCTOBER 1 - 5: International Ion Chromatography Symposium (IICS'95), The Grand Kempinski, Dallas, Texas. Contact: IICS'95, Century International, Inc., P. O. Box 493, 25 Lee Road, Medfield, MA 02052, USA; (508) 359-8777; FAX: (508) 359-8778.

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 23 - 25: Bio-West'95 & Expo, San Jose, California. Contact: Bioconferences International, Inc., 4405 East-West Highway #501, Bethesda, MD 20814-4536, USA. Tel: (301) 652-3072; FAX: (301) 652-4951.

OCTOBER 23 - 25: 6th Annual Frederick Conference on Capillary Electrophoresis, Hood College, Frederick, Maryland. Contact: Margaret L. Fanning, Conference Coordinator, PRI, NCI-FCRDC, P. O. Box B, Frederick, MD 21702-1201

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 2: Anachem Symposium, Dearborn, Michigan. Contact: Prof. C. Evans, University of Michigan, Chem Dept, 4807 Chemistry Bldg, Ann Arbor, MI 48109-1055, USA.

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

NOVEMBER 12 - 17: AIChE Annual Meeting, Fontainbleu Hotel, Miami Beach, Florida. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

NOVEMBER 14 - 16: Kemia'95: Finnish Chemical Congress, Helsinki Fair Center, Helsinki, Finland. Contact: The Associatioon of Finnish Chemical Societies, Hietaniemenkatu 2, FIN-00100 Helsinki, Finland.

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 5 - 7: PrepTech'96, Sheraton Meadowlands Hotel, East Rutherford, New Jersey. Contact: Dr. R. Stevenson, 3338 Carlyle Terrace, Lafayette, CA 94549, USA.

FEBRUARY 25 - 29: AIChE Spring National Meeting, Sheraton Hotel, New Orleans, Louisiana. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

MARCH 3 - 8: 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.

MARCH 31 - APRIL 4: 7th International Symposium on Supercritical Fluid Chromatography and Extraction, Indianapolis, Indiana. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

MARCH 31 - APRIL 4: 7th International Symposium on Supercritical Fluid Chromatography & Extraction, Indianpolis, Indiana. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

APRIL 17 - 19: VIIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Université de Nice, Fracnce. Contact: Prof. W. R. G. Baeyens, University of Ghent Pharmaceutical Institute, Lab or Drug Quality Control, Harelbekestraat 72, Ghent, Belgium. Email: willy.baeyens@rug.ac.be.

MAY 7 - 9: VIIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Monte Carlo, Monaco. Contact: Prof. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Harelbekestraat 72, B-9000 Ghent, Belgium.

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.

JULY 14 - 18: 5th World Congress of Chemical Engineering, Marriott Hotel, San Diego, California. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 9 - 14: 31st Intersociety Energy Conversion Engineering Conference (co-sponsored with IEEE), Omni Shoreham Hotel, Washington, DC. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 17 - 20: 31st National Heat Transfer Conference, Westin Galleria, Houston, Texas. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

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

SEPTEMBER 1 - 6: 11th Symposium on Quantitative Structure-Activity Relationships: Computer-Assisted Lead Finding and Optimization," Lausanne, Switzerland. Contact: Dr. Han van de Waterbeemd, F. Hoffmann-La Roche Ltd., Dept PRPC 65/314, CH-4002 Basle, Switzerland.

SEPTEMBER 9 - 12: Saftey in Ammonia Plants & Related Facilities, Westin at Copley Place, Boston, Massachusetts. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, 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.

NOVEMBER 10 - 15: AIChE Annual Meeting, Palmer House, Chicago, Illinois. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, 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.

APRIL 14 - 19: Genes and Gen Families in Medical, Agricultural and Biological Research: 9th International Congress on Isozymes, sponsored by the Southwest Foundation for Biomedical Research, Hilton Palacio del Rio, San Antonio, Texas. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, 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 scientists in every issue of the journal. To be listed in the Liquid Chromatography Calendar, we will need to know:

a) Name of the meeting or symposium,

b) Sponsoring organization,

c) When and where it will be held, and

d) Whom to contact for additional details.

Incomplete information will not be published. You are invited to send announcements to **Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P.O. Box 2180, Cherry Hill, NJ 08034-0162, USA.**

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Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

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