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ANALYSIS OF COLLOIDS. VI*. SEMICONDUCTOR COLLOIDS OF HIGH MONODISPERSITY BY PREPARATIVE SIZE EXCLUSION CHROMATOGRAPHY

CHRISTIAN-HERBERT FISCHER
Hahn-Meitner-Institut
Abt. CK (Kleinteilchenforschung)
Glienicker Straße 100
D-14109 Berlin, Germany

ABSTRACT

The recently described method of size exclusion chromatography (SEC) of very small colloidal semiconductor particles was transferred from the analytical scale to the preparative one. The particular difficulties dealing just with SEC and the special situation when working with inorganic colloids are described. The influence of flow rate, sample volume, sample concentration and fraction volume were studied using CdS-sols with particle diameters ranging from 2nm to 8nm. Up to 200mg samples were separated in less than one hour. The fractions were generally characterized by analytical SEC and in two instances by transmission electron microscopy showing excellent consistency and standard deviations down to 11%.

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* The last papers of this series were:
V: Ch. -H. Fischer, M. Giersig, T. Siebrands, J. Chromatography in press
IV: T.Siebrands, M.Giersig, P.Mulvaney, Ch.-H.Fischer, Langmuir 9,2297 (1993)
III: Ch. - H. Fischer, M. Giersig, Langmuir, 1992, 1475

INTRODUCTION

Very small semiconductor and metal particles of less than 20nm diameter to as small as 1.3nm are a relatively new and rapidly growing field of colloidal chemistry [1]. Particles of this small size are interesting because of phenomena such as a quantum size effect of absorption spectra and fluorescence spectra, possible application for solar energy conversion and electronic devices. It has been shown that size exclusion chromatography (SEC) is a fast and powerful tool for rapid size determination not only for organic polymers but also for these inorganic, so called Q-particles and especially in combination with a diode array detector for the elucidation of their basic phenomena [2]. Monodisperse colloids are of particular interest to physicochemists, because special properties can be directly correlated with a certain particle size. So it was the scope of this work to scale-up the conditions of analytical SEC, i.e. to optimize the chromatographic parameters such as polydispersity, separation time (flow rate), and sample amount (sample concentration and volume, fraction volume). Unfortunately all these parameters are connected in such a way, that improving one leads to a deterioration of another. Fig.1 demonstrates the special situation in preparative SEC of a sample with a broad size distribution. In all other chromatographic methods two or more peaks corresponding to different chemical species are separated. So the column can be overloaded easily as long as the two peaks are more or less baseline separated and only with beginning overlap the product purity decreases. However the problem in the SEC is completely different, whenever a size distribution should be separated. Then the whole peak has to be considered as a superposition of elemental chromatograms each relating to the species of one size. Thus any increase of sample amount leads immediately to a bigger overlap of the elemental chromatograms and therefore to a higher polydispersity of the product collected in the fractions. Therefore with preparative SEC it is not only a question of how much substance is needed but also of how monodisperse should it be. The performance of the separation is a necessary compromise between monodispersity and yield. Though numerous papers dealing with SEC of polymers exist [3],

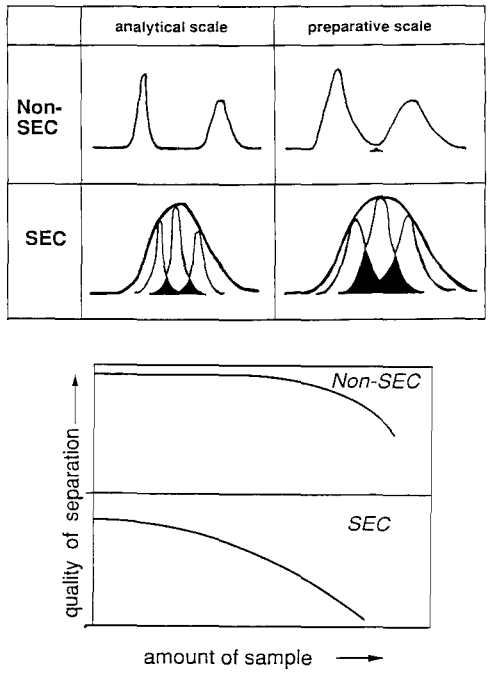

FIGURE 1. The effect of overloading in chromatography: Schematic comparison of non-SEC-methods and SEC with broad distributions. The overlapping areas of the peaks are marked in black. The SEC peak has to be considered as an overlay of numerous elemental chromatograms of one particular size. The graph on the bottom describes in principle the quality of the separation (size distribution or product purity, respectively) as a function of the amount of sample.

a rather special situation in the case of inorganic solid crystalline particles was found. For the actual investigations cadmium sulfide sols were used, because this material is one of the best known semiconducting materials. One general difficulty was the relative instability of the colloids. Despite the presence of polyphosphate as a stabilizer against coagulation in both the sample and the eluent the particle size changed with time. So all comparative experiments, such as optimization experiments had to be carried out over a short time frame in order to exclude sample changes.

MATERIALS

Cadmium perchlorate (Alfa), sodium polyphosphate (Riedel-de Haën), hydrogen sulfide (AGA) were of highest available purity and were used without further purification. Water was purified by a Milli-Q system (Millipore).

METHODS

HPLC

The equipment consisted of a Merck-Hitachi pump, type L 6000, and a Merck Hitachi L4200 UV/vis detector with a 2mm cell for the preparative work and a 5mm cell for the analytical, operating at 250nm. A set of two 125mm long Knauer columns were used, the first packed with Nucleosil 500 C4 (5μm) and the second Nucleosil 1000 C4 (5μm) from Macherey and Nagel. The mobile phase was an aqueous solution of $1x10^{-3}$ M cadmium perchlorate and $6x10^{-3}$ M sodium polyphosphate (referring to the formula $NaPO_3$) . For preparative separations the column diameter was 32mm with a flow rate of 4.5mL/min, except within flow rate optimization experiments where the sample loop volume varied from 200μL to 2000μL. Fractions were taken using an automatic fraction collector (Isco, model 328). The fraction volume was 450μL, where no other value is given. For analytical runs columns of 4mm diameter were used with a sample volume of 20μL. Concentrated original samples were diluted down to 1mM. Data collection and SEC calculations were carried out using a Bruker Chromstar system.

Preparation of colloidal cadmium sulfide

Hydrogen sulfide gas or aqueous sodium hydrogen sulfide solution was injected through a septum into an aqueous cadmium perchlorate / sodium polyphosphate solution, through which nitrogen had been bubbled for ten minutes. After shaking, the solution could be used.

RESULTS AND DISCUSSION

In the following the effects of flow rate, sample volume, sample concentration and fraction size on the separation of colloidal cadmium sulfide are discussed . The characterization of the fractionation was carried out by reinjecting fractions in the analytical column set (using the same stationary phase, the same column length, but with a diameter of only 1/8 of the preparative columns). The results were compared by the half width of the analytical peaks and/or by the diameter-polydispersity D_d determined by the chromstar computer program (as defined below). Fig.2 exemplified this general procedure. It shows the analytical chromatograms of the diluted original sample and of the fractions. The much smaller peak widths of the fraction chromatograms compared with that of the original sample were evident as well as the shift of the peak maxima with increasing fraction number. Interestingly the analytical chromatogram of the raw sol almost completely enveloped those of the fractions, when it was plotted in an adjusted size .

1. Effect of Flow Rate

In the experiment of Fig.3 the sample consisted of a cadmium sulfide sol prepared from a 5mM cadmium perchlorate and 30mM polyphosphate solution (based on the formula $NaPO_3$) by the addition of 1mM sodium hydrogensulfide. 200µl were injected three times on the preparative column the flow rate being varied from 2.25mL/min over 4.5mL/min to 9.0mL/min. 0.9mL fractions were collected and some of them re-injected in the analytical setup. In Fig.3 (top) for each run the analytical elution times of these preparative fractions were plotted over the corresponding preparative elution volumes. The slower the flow rate the higher was the initial slope and the earlier and more pronounced deflected the straight line towards saturation, i.e. the smallest particles eluted delayed in the preparative mode. They had the highest diffusion rate and were able to re-diffuse back into the same pore, if a rapid stream did not push them forward. Two reasons could be responsible for the decreasing slope with

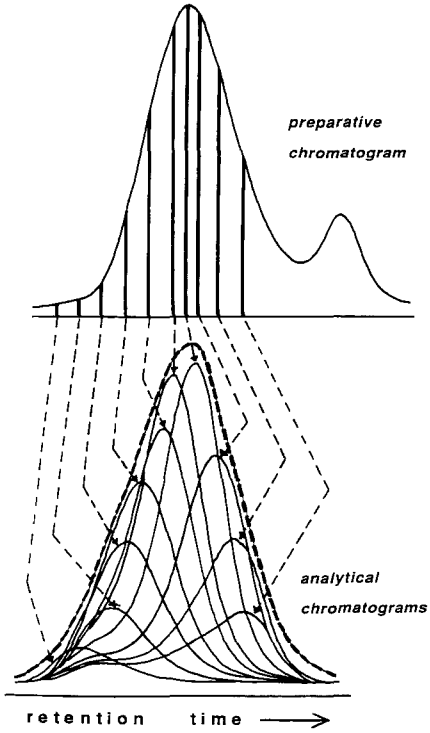

FIGURE 2. Characterisation of a preparative fractionation of a cadmium sulfide sol. Preparative chromatogram (**top**). The small peak is due to polyphosphate. Ten fractions (marked in black) were collected and re-injected in the analytical SEC (**bottom**), dashed line: diluted original sol, peak height adapted). Both time scales are different. Chromatographic conditions are given in the experimental part.

increasing flow rate: Firstly the residence time of the particles inside the pores was independent of the flow rate. But during this constant period the differing volumes of eluent, which passed through the interstitial volume, were dependent on the flow rate. Secondly, because of high flow rate fewer particles found the way into the pores, the other part is purged forward by the rapid stream. This also was obvious from the peak broadening (vide infra). The highest flow rate showed a linear relation

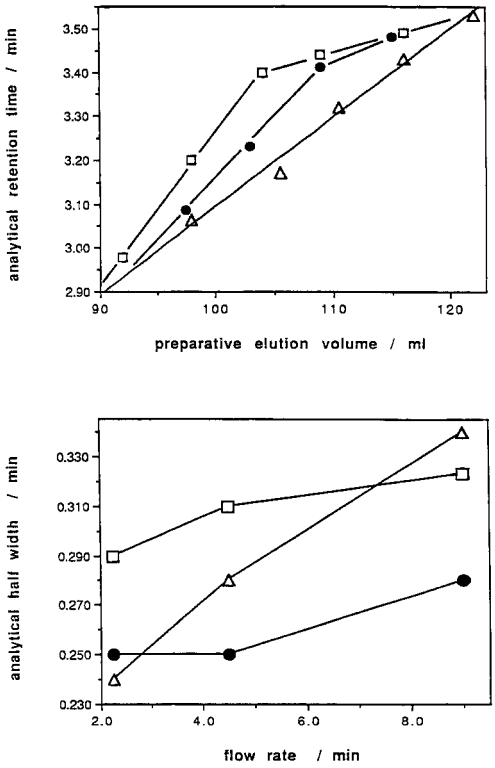

FIGURE 3. Effect of flow rates on the separation of a cadmium sulfide sol (5mM $Cd(ClO_4)_2$,30mM polyphosphate, 1mM H_2S).
Top: Analytical retention time of re-injected fractions as a function of their preparative elution volume (□ 2.25 mL/min, ● 4.5 mL/min, Δ 9mL/min)
Bottom: Half width of the analytical peak as a function of flow rate:
● fraction in the preparative peak maximum, Δ fraction at the frontal deflection point, □ fraction of the tail deflection point.

over the whole range with the smallest slope. Therefore one could be inclined to choose this condition. However, first it was necessary to characterize the quality of the separation. For this purpose three fractions of each run were analyzed: the fractions in the maximum of the preparative peak and in the two points of inflection. In Fig.3 (bottom) their analytical half widths were plotted over the flow rate. In the flow range of this experiment the peaks became broader with increasing flow rate, since there was not sufficient time for all the particles to diffuse into the accessable pore volume. Generally the peaks of the main fractions were the narrowest, while those of the smaller particles the broadest. Two exceptions were noted and both could be explained in terms of diffusion: Firstly, at the lowest flow rate the peak width of the main fraction had not decreased further, because the diffusion is dependent not only on the diffusion coefficient, but also on the concentration and the main fraction was more concentrated than the side fractions. Therefore the rate of *back* diffusion into the pores became relevant compared to the forward flow. Secondly, at the highest flow rate the peak of large particles became broader than that of small ones. The slow diffusion hindered the large particles to a higher extent to reach the pores thus causing peak broadening.
Normally the initial sol has its mean size in the desired range. Therefore the optimization of the main fraction suffices. With regard to the time consumption the medium flow rate of 4.5mL/min was used for further work.

2. Effect of Sample Volume

To study the influence of the sample volume a 10mM cadmium sulfide sol was used prepared from a 10mM cadmium perchlorate / 60mM polyphosphate solution by adding 1mM hydrogensulfide and then evaporation of the water to one tenth of the original volume. This sol was injected on to the preparative column several times using sample loops of 200µL, 500µL, 1000µL and 2000µL, respectively (constant flow rate of 4.5mL/min; 0.9mL fractions). From the normalized chromatograms (Fig.4, left part) it could be seen, that with increasing sample volume the

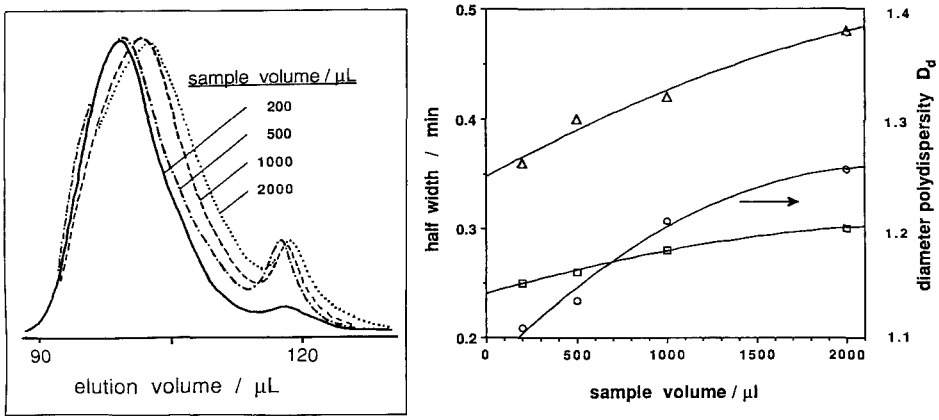

FIGURE 4. Effect of sample volume on the separation of a cadmium sulfide sol (10mM $Cd(ClO_4)_2$, 60mM polyphosphate, 1mM H_2S).
Left part: Preparative chromatograms. Loop sizes 200, 500, 1000 and 2000 µl.
Right part: Diameter polydispersity (O), half width of preparative peak (Δ) and analytical one (□) of the peak fraction as a function of the sample volume. (The preparative width was divided by ten, in order to compare all three parameters in one plot !)

peak width and the retention volume increased. The latter was shifted by 4.5mL in the case of the 2000µL loop compared to the 200µL one. This shift was much bigger than the theoretical half sample volume. Enhanced absorption was most probably the reason for the retardation. Though the sample concentration was the same in all cases, a larger sample volume was less diluted than a small one during the passage through the column. Especially the higher electrolyte concentration led the absorption increase (vide infra).

The widths of the analytical peaks of the main fractions and those of the preparative ones divided by 10 (in order to visualize both in one figure) were plotted over the sample volume in Fig.4, right part. Both values changed proportionally. For a more expressive characterization we used the *diameter* polydispersity D_d, defined in equation 1, where d_w is the weight average diameter of the particles in the sol and d_n the number

average diameter, respectively, according to equations 2 and 3. n_i is the number of particles with the diameter d_i and the weight w_i. An ideal monodisperse sol would have $D_d = 1$. These expressions are very similar to the related molar weights M_w and M_n and their polydispersity D, used in polymer chemistry. However, for colloid chemists the size is a more useful value, because crystalline particles do not swell and have a measurable, constant size, independent of the solvent used, which is not the case for the coils of organic polymers. When a commercial SEC-program is fed with diameters instead of molecular weights as calibration data, d_w , d_n and D_d are automatically calculated. Fig. 4, right part, shows that the diameter polydispersities of the main fractions lay between 1.11 for the 200µL loop and 1.26 for the 2000µL loop. The peak widths reflect the diameter polydispersities. However, the latter describes the broadness of the size distribution more quantitatively, because the same peak width has a different quantitative meaning depending on the elution volume because of the logarithmic relation in SEC.

$$D_d = \frac{d_w}{d_n} \qquad (1)$$

$$d_w = \frac{\sum n_i d_i w_i}{\sum n_i w_i} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3} \qquad (2)$$

$$d_n = \frac{\sum n_i d_i}{\sum n_i} \qquad (3)$$

3. Effect of Sample Concentration

For the studies of the concentration influence a colloidal cadmium sulfide stock solution was prepared from a 20mM cadmium perchlorate/

120mM polyphosphate solution by the addition of 2mM hydrogensulfide. 0.5mM and 1mM CdS sols were obtained by dilution with water and a 20mM CdS sol by evaporation of the solvent. Dilution of this concentrated sample led to 10mM, 5mM and 2.5mM sols. As checked by analytical SEC the evaporation process was not accompanied by particle growth. 500μL of all samples were injected into the preparative column (flow rate 4.5mL/min; 0.9 mL fractions). Some of these preparative chromatograms are shown normalized in Fig.5 (top). With increasing concentration the peaks became broader. That absorption played an important role revealed the inspection of the polyphosphate peak at about 29min, which could be used as an internal standard. Its size relatively to the CdS-peak increased tremendously with increasing sample concentration, though the relative concentrations were the same in all samples. This indicated the loss of particles by adsorption on the surface of the stationary phase. Following analytical SEC experiments investigating the peak area as a function of concentration also showed such a mass loss in the concentrated solutions compared to the diluted samples, when water was used for the dilution. On the other hand a linear relation between area and concentration was obtained, when the dilution was carried out with a solution of the same electrolyte composition as the concentrated solution. It is well known in colloid chemistry that high concentrations of electrolytes destabilise colloids by reducing the repelling charges on the surface of the colloidal particles. These charges do not only prevent against particle combination, but also against the adsorption of the colloidal material on any other surface carrying the same charge. The samples in question contained still electrolytes from the preparation: cadmium perchlorate, perchloric acid and polyphosphate. This was the reason for the concentration effect. The preparative peak widths and the diameter polydispersities of the main fractions were plotted as a function of CdS concentration in Fig.5 (bottom). Both parameters reflect the worse separation with increasing sample concentration. As in the case of organic polymers the slope is steeper for lower concentrations than for higher ones [4].

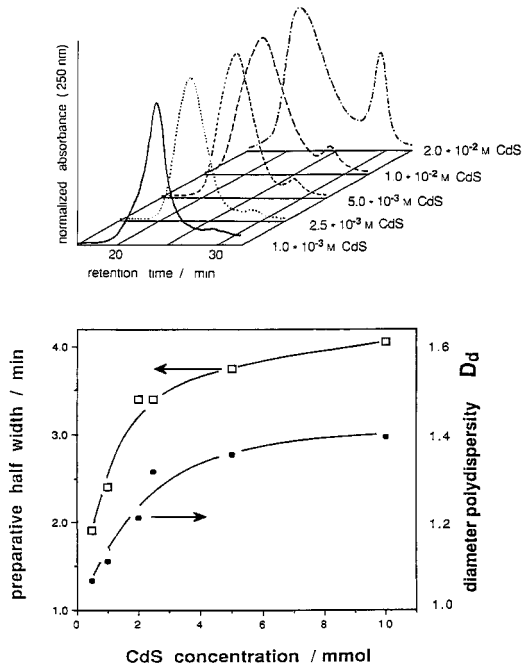

FIGURE 5. Effect of sample concentration on the separation of 500µl cadmium sulfide sol from $[Cd(ClO_4)_2]$: [polyphosphate] : $[H_2S]$ = 10 : 60 : 1 (details in the text).
Top: Normalized preparative chromatograms from 1mM, 2.5mM, 5mM, 10mM and 20mM CdS sols.
Bottom: Preparative half width and radius polydispersity of the main fraction as a function of CdS concentration.

4. Optimization of the Yield: Sample Concentration versus Sample Volume

In order to compare both, the options of increasing amounts of sample, either higher concentration, or larger sample volume, a cadmium sulfide sol prepared from 10mM cadmium perchlorate / 60mM polyphosphate / 10mM hydrogensulfide, was separated three times: 500µL non-diluted, 1000µL diluted 1:1 by water and 2000µL diluted 1:3, respectively. So

every time the same amount was injected and equal peak areas in the resulting chromatograms could be expected. Surprisingly, the peak areas were reproducibly different (Fig.6, left part) and decreased from 100% (2000µL) to 84% (1000µL) and 58% (500µL). Since the detector operated in a range, where Lambert-Beer`s-law was valid, it was concluded that increase of the sample concentration led to less CdS being eluted from the column. An irreversible adsorption effect took place on the stationary phase which increased with increasing concentration. This was in accordance with the observation that precolumns had a yellow surface, when they were used for a while. The reasons for the enhanced adsorption were discussed in the previous paragraph. Fig.6, right part, shows comprehensively as a function of sample volume and sample concentration: The relative peak areas of CdS normalized on the most diluted sample, the fraction of lost CdS calculated from the relative peak area under the assumption that in the most diluted sample all CdS eluted and the diameter polydispersity of the particles. The main fractions of all three runs had the same radius polydispersity of 1.2 compared to 1.5 in the case of the original sample. This behaviour was different from normal organic polymers, which gave worse polydispersity for higher concentration [5]. The constant polydispersity can also be explained in terms of irreversible or long time adsorption of colloidal particles on the stationary phase, increasing with the concentration. Therefore the material loss in the case of higher concentrations led to lower effective concentrations and consequently to smaller differences between the sample concentrations. Obviously the effect of these remaining differences balanced just the opposit effect of sample volume. In conclusion, a larger sample volume with correspondingly lower concentration is recommended for a high yield with low polydispersity. For organic polymers the same conditions are preferred, however, there it is based on changes in polydispersity [5], whereas irreversible adsorption and yield do not play any important role.

5. Effect of Fraction Volume

200µl of a cadmium sulfide sol (from cadmium perchlorate / polyphosphate/ sodium hydrogensulfide, 10mM / 60mM / 10mM) with

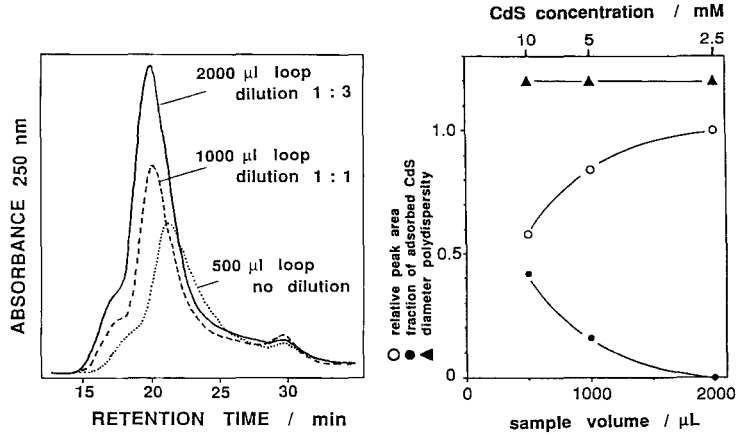

FIGURE 6. Comparison of the combined effects of sample concentration and sample volume on the separation of colloidal cadmium sulfide. The original sol was prepared from 10mM $Cd(ClO_4)_2$, 60mM polyphosphate, 10mM H_2S. The sample volumes were 500µl, 1000µl and 2000µl, but the latter two were diluted 1:1 and 1:3, respectively with water.
Left part: Preparative chromatograms of the three concentration / volume combinations
Right part: Relative peak area (non-diluted = 1), fraction of adsorbed CdS (calculated from the relative peak areas under the assumption that the most dilute sample has no loss by adsorption) and diameter polydispersity of the main fraction as a function of sample volume and concentration.

d_w = 6.9nm and D_r = 1.45 was separated, the fraction size being 0.9mL (0.2min). Analytical SEC was carried out from the collected material in the preparative peak maximum. For studying the effect of fraction volume it was expanded stepwise from 0.9mL to 8.1mL. This was achieved in one run by successive addition of the next four and the previous four fractions to the central fraction no.19 in the following sequence: 20, 18, 21, 17, 22, 16, 23, 15. After every addition an analytical run was carried out. The polydispersity increased exponentially from 1.197 to 1.272. A dramatic broadening started only when the collected fraction volume was more than 15 times the injected sample volume (Fig.7).

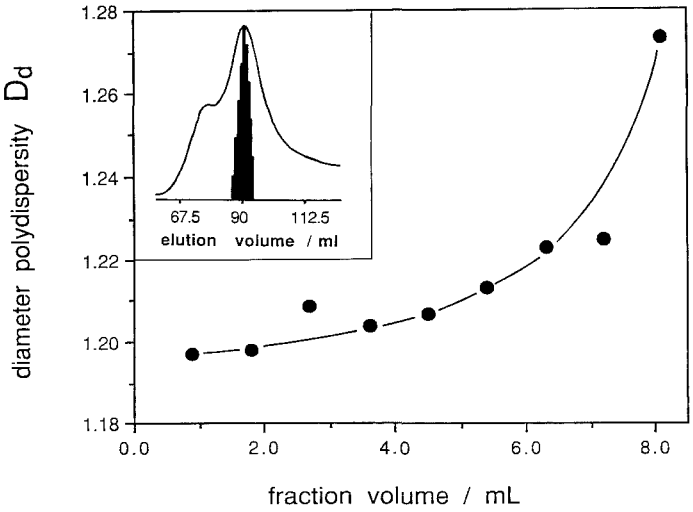

FIGURE 7. Effect of fraction volume on the separation of 200µl cadmium sulfide sol (10mM $Cd(ClO_4)_2$, 60mM polyphosphate, 10mM H_2S). Diameter polydispersity of the main fraction as a function of the fraction size. The inset shows the preparative chromatogram and the size of the fractions. Experimental details are explained in the text.

Consequently the fraction volume is the least critical point, when high yield with high monodispersities are considered.

6. Comparison of the Results by Transmission Electron Microscopy (TEM)

In order to check the efficiency of the SEC separation as well as the reliability of the SEC size analysis by a second independent method, two cadmium sulfide sols of very small and of medium size were separated (200µL sample volume). The first was prepared by mixing 5mM sodium hydrogensulfide and 5mM cadmium perchlorate/ 30mM sodium polyphosphate solution (volume /volume 4 : 1) and the latter from cadmium perchlorate/sodium polyphosphate solution and

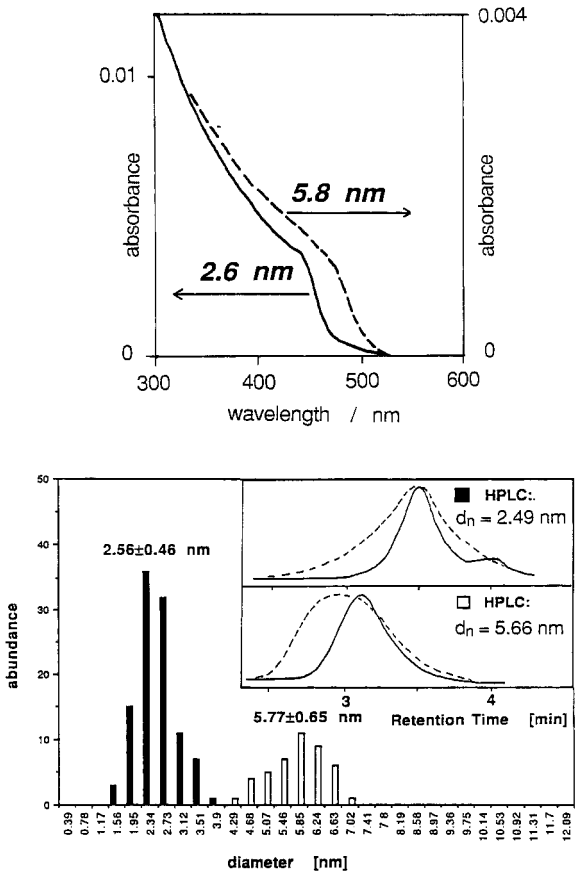

FIGURE 8. Bottom: Size distribution of two cadmium sulfide sols after preparative HPLC fractionation obtained by transmission electron microscopy (smaller particles: 5mM $Cd(ClO_4)_2$ /30mM polyphosphate and 5mM NaHS mixed 4 : 1 (v/v); bigger particles: 10mM $Cd(ClO_4)_2$, 60mM polyphosphate, 10mM H_2S). The inset shows the normalised analytical chromatograms of the diluted original samples and of the fraction analysed by TEM.
Top: UV/vis spectra of the two fractions from preparative HPLC. The shift of the onset of absorption is due to the size quantization effect. The steepness of the step indicates the narrow size distribution.

TABLE 1
Comparison of the SEC and TEM Results from the Experiment of Figure 8

sample	d_n [nm]		d_w [nm]		$D_d = d_w / d_n$	
	HPLC	ELMI	HPLC	ELMI	HPLC	ELMI
original	2.32		3.91		1.69	
1 fraction	2.49	2.56	2.82	2.81	1.13	1.10
	σ = 0.46 (18 %)					
original	6.05		9.09		1.50	
2 fraction	5.66	5.77	6.73	5.97	1.18	1.04
	σ = 0.65 (11 %)					

hydrogensulfide (10mM/60mM/10mM). One fraction of both separations was examined by transmission electron microscopy and also by SEC. Fig.8 (bottom) shows the histograms of the size distribution from TEM and in the insert the normalized chromatograms of the fractions in comparison to that of the original samples. On the top a typical phenomenon of nm-semiconductor particles is demonstrated: The size quantization effect (Q-effect) of the absorption spectra, i.e. the onset of absorption shifts with decreasing particle size towards shorter wavelengths [6]. It is due to the increasing energy gap between the valence band and the conductivity band. The very steep slope indicates the high monodispersity of the two fractions. Table 1 summarizes the quantitative results of this experiment. In both cases the monodispersity of the fractions increased significantly. The diameter polydispersity decreased from 1.7 or 1.5 to 1.1 or 1.04, respectively. Both fractions have size distributions with standard deviations of 11% and 18%, which is very narrow for cadmium sulfide colloids. The data of TEM and SEC were in excellent agreement.

CONCLUSION

The results have established the method of preparative SEC as a powerful tool for colloidal chemistry of nm-particles. The separation was carried out in less than one hour. With columns of 32mm internal diameter up to 200mg of sample was injected. Generally, for high yields larger sample volumes should be applied rather than higher concentrations. When larger amounts have to be separated one can work either in cycle, which is easily automated, or with larger columns, which is faster but also more expensive. In contrast to SEC, gel electrophoresis, which has also been used for the separation of CdS sols [7], needs time consuming preparation of the electrophoresis gel, the separation itself requires six hours and the column is destroyed on collection of the fractions. The analytical SEC on the other hand has been demonstrated to be a very reliable method, which is ideal just for routine size determinations because of its very short run time of less than five minutes.

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ADAPTATION OF A CAROTENOID PROCEDURE TO ANALYZE CAROTENOIDS, RETINOL, AND ALPHA-TOCOPHEROL SIMULTANEOUSLY

CHERNG-ZEE CHUANG, GENEVIEVE TROSCLAIR, AND ALFREDO LOPEZ-S
Department of Medicine
Section of Nutrition
School of Medicine
Louisiana State University Medical Center
New Orleans, Louisiana 70112

ABSTRACT

An isocratic procedure for carotenoids (Nelis and De Leenheer, 1983) was extended by monitoring at an extra wavelength (280 nm) and use of tocopheryl acetate as internal standard to include retinol (vitamin A) and α-tocopherol (vitamin E) with carotenoid analysis in a single chromatographic run. Peaks were validated by retention times, spiked test and signal ratio (313 nm/280 nm). Within-run CVs (n=10) were 4.7% and 2.9% and between-run CVs (n=9) were 7.3% and 5.1% for vitamins A and E respectively. Linearities were up to at least 1.8 ug/mL (vitamin A) and 46.5 ug/mL (vitamin E). Vitamins A and E of twenty serum samples were analyzed by this procedure (I) and a reference procedure (II) and their correlations were 0.8484 and 0.9919 respectively. Linear regressions were I=II(0.806)+0.225 ug/mL (vitamin A) and I=II(0.974)+0.52 ug/mL (vitamin E). Recoveries were 118% and 111% for vitamins A and E respectively. This isocratic procedure can analyze at least five carotenoids and vitamins A and E in serum simultaneously. It is simple and economic in time and reagents. Chromatographic run is within 20 minutes. Only a double channel absorbance detector is needed, without the use of programmable absorbance detector, diode-array detector or gradient elution. It will be a useful tool to study the role of carotenoids, vitamins A and E in human chronic diseases such as coronary heart disease and cancer.

INTRODUCTION

Carotenoids, vitamins A and E have been postulated to play an important role in human chronic diseases such as cancer and coronary heart disease (1-3). Simultaneous high-performance liquid chromatographic (HPLC) analyses for these vitamins usually need gradient elution (4-6), a programmable UV detector or an expensive photodiode array detector (5-9). Alternatively, carotenoids and vitamins A/E are analyzed by different mobile phase systems (10). Nelis and De Leeheer's procedure (11) that can analyze at least 5 individual carotenoids simultaneously in the serum is currently one of the best for carotenoid analysis.

Here, we extended Nelis and De Leeheer's procedure by monitoring at an extra wavelength of 280 nm, and use of α-tocopheryl acetate as internal standard (IS) to include retinol and α-tocopherol with carotenoid analysis in a single chromatographic run.

MATERIALS AND METHODS

A) Reagents/Standards

Retinol, α-tocopherol and α-tocopheryl acetate (IS) were obtained from Sigma Chemical Co. (St. Louis, MO) and prepared in absolute ethanol. The actual concentration of each standard was determined spectrophotometrically using absorptivity values (1%, 1cm): retinol=1780 at 325 nm, α-tocopherol=75.8 at 292 nm, and α-tocopheryl acetate= 43.6 at 285 nm (12). Hexane and components of mobile phase (described below) were of HPLC grade.

B) HPLC system

The HPLC system (Waters) included an U6K injector, an M6000A solvent delivery system, an M400 absorbance detector (436/280 nm

for sample analysis and 313/280 nm for peak identification), an 820 Maxima work station, a Nova-Pak C-18 column (250x3.9 mm) and a Nova-pak guard column. Carotenoids were detected at 436 nm and vitamins A and E at 280 nm, which is the best filter for our system to detect both retinol and α-tocopherol. Mobile phase is acetonitrile: methylene chloride: methanol (7:2:1), degassed by vacuum for 30 minutes and pumping at 1.0 mL/min flow rate. All analyses were performed in ambient temperature.

C) Sample preparation

Serum (150 uL) was deproteinized with 150 uLs of ethanol containing echinenone and α-tocopheryl acetate as internal standards. After vortexing for 10-20 seconds, 400 uLs of hexane were added. The mixture was vortexed again for 30-40 seconds to extract retinol, tocopherols and carotenoids and centrifuged at 3000 rpm for 10 minutes to separate the phase. Upper layer (hexane) was aspirated to another tube, dried under nitrogen and reconstituted with 100 uL mobile phase. Finally, 80 uLs were injected to HPLC for analysis.

C) Qualitative analysis

Six serum samples were randomly selected and prepared without adding tocopheryl acetate internal standard to check any endogenous interfering peaks which might coelute with tocopheryl acetate. Peak identification was validated with retention time, spiked test and 313/280 nm ratio response.

D) Quantitative analysis

Within-run precision was evaluated by analyzing a randomly chosen serum sample 10 times and between-run precision was calculated from values obtained from a serum sample (chosen for

quality control) which was included in each batch analysis. Recovery was studied by adding both vitamins A and E to a serum sample and the amount obtained from the experiment (difference of spiked and unspiked samples) was compared to the actual amount added. Three calibration standards containing vitamins A (0.22-1.8 ug/mL) and E (5.8-46.5 ug/mL) were analyzed to evaluate linearity. Serum samples collected from twenty patients undergoing angiographic examinations were analyzed with this procedure (I) and Bieri's procedure (II, reference 12) for vitamins A and E in the comparison study. Linear regression and correlation coefficient were analyzed by Statistix software version 4.0 (Analytical Software, St. Paul, MN).

RESULTS AND DISCUSSION

A) Qualitative analysis

Since α-tocopheryl acetate is a widely used IS for the analyses of retinol (9), tocopherols (8,9,12) and carotenoids (9), it is chosen in this study. All six samples without adding α-tocopheryl acetate (IS) were not found to have any apparent peaks at retention time of α-tocopheryl acetate, validating its use as an IS. Both retention time and spiked test confirmed the identity of retinol A and α-tocopherol peaks. Signal ratios (peak height) at 313 nm/280 nm for vitamins A/E peaks from standard and serum samples are shown in Table 1. Close agreement of signal ratio between standard and samples for vitamins A (2.71 vs 2.66) and E (0.0947 vs 0.0862) further validate the peak identity.

B) Quantitative analysis

The coefficients of variation (CV) of within-run precision (n=10) for both vitamins were below 5% and those of between-run precision (n=9) were below 8% (Table 2). These are comparable to

Table 1. Signal ratios at 313nm/280nm of vitamins A/E peaks from standard and serum samples

	A (std)	A (serum)	E (std)	E (serum)
N	2	6	2	6
Mean	2.71	2.66	0.0947	0.0862
Minimum	2.63	2.55	0.0942	0.0803
Maximum	2.79	2.81	0.0952	0.0909

A (std): signal ratio for vitamin A peak from standard.
A (serum): signal ratio for vitamin A peak from serum sample.
E (std): signal ratio for vitamin E peak from standard.
E (serum): signal ratio for vitamin E peak from serum sample.

Table 2. Within-run and between-run precision

Precision	N	Mean, ug/mL	Standard deviation, ug/mL	Coefficient of variation, %
Vitamin A				
Within-run	10	0.573	0.0268	4.7
Between-run	9	0.618	0.0452	7.3
Vitamin E				
Within-run	10	6.21	0.178	2.9
Between-run	9	11.7	0.596	5.1

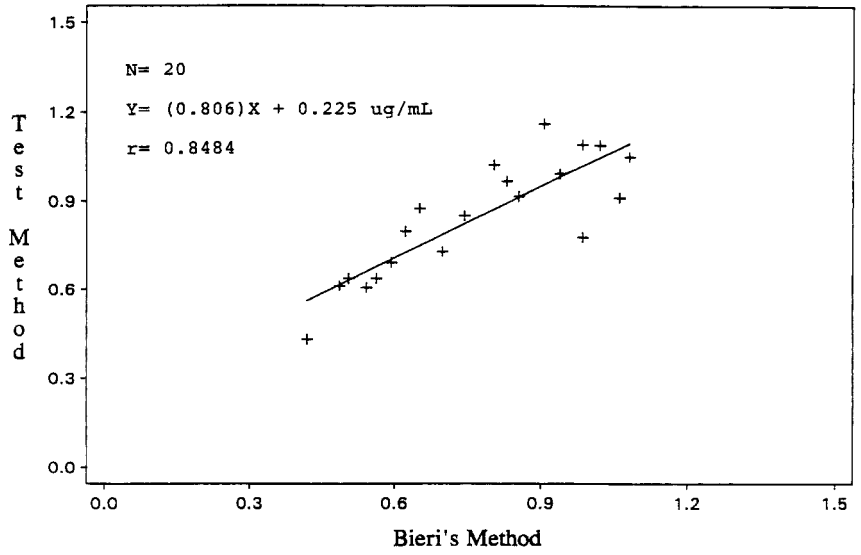

Figure 1. Comparison study of vitamin A (n=20).

other simultaneous procedures. Recoveries were 118% and 111% for vitamins A and E respectively. Analytical linearities were up to at least 1.8 ug/mL (vitamin A) and 46.5 ug/mL (vitamin E).

Comparison studies are shown in Figures 1 and 2 and chromatograms monitored at both 436 nm and 280 nm are shown in Figure 3. Retinol, α-tocopherol and α-tocopheryl acetate peaks are resolved and the run is within 10 minutes for vitamins A/E and 20 minutes for carotenoids, which is similar to other isocratic procedures (5,8) using similar column length. But the run time is shorter than gradient procedures (5,6) and total analysis time is more economic than running carotenoids and vitamins A/E separately (10). A minor peak between α-tocopherol and α-tocopheryl acetate was found in about 20% (8 out of 40) of samples and its retention time is very sensitive to variation in the components of the

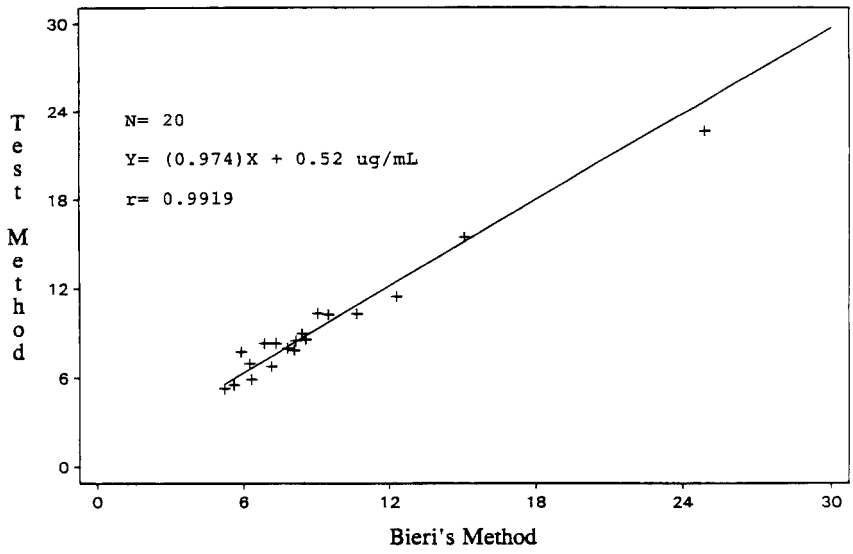

Figure 2. Comparison study of vitamin E (n=20).

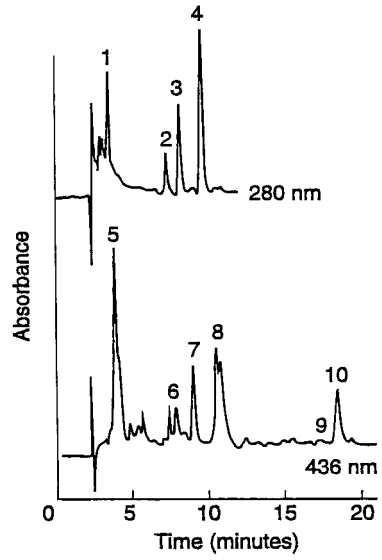

Figure 3. Chromatogram of a typical serum sample. Peak identification: 1=retinol, 2=Γ-tocopherol, 3=α-tocopherol, 4=α-tocopheryl acetate, 5=lutein and zeaxanthin, 6=cryptoxanthin, 7=echinenone, 8=lycopene, and 9=α-carotene, 10=ß-carotene.

mobile phase. Based on retention times of peaks of standard and samples, gamma-tocopherol was also separated by this system. Most simultaneous procedures (5-7,9) used a programmable UV/VIS detector or a photodiode array detector monitoring at three wavelengths of maximal absorbance for retinol, α-tocopherol and carotenoids to obtain their maximal sensitivity. One wavelength (280 or 292 nm) can be used to detect both retinol and α-tocopherol at the compromise of maximal sensitivity (4,8). This procedure only requires a double channel absorbance detector (280 nm and 436 nm) without the use of programmable UV/VIS detector, expensive diode-array detector or gradient elution.

Therefore, an isocratic carotenoid procedure was successfully extended to a procedure that can analyze carotenoids and vitamins A and E in a single run simultaneously. It is simple and more economic in total analysis time, reagents and equipment than many procedures commonly used at the present time. This simultaneous procedure will facilitate the study of roles of carotenoids, vitamins A and E in human research.

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DETERMINATION OF PAHs IN PARTICULATE AIR BY MICELLAR LIQUID CHROMATOGRAPHY

M. N. KAYALI, S. RUBIO-BARROSO*, AND L. M. POLO-DIEZ
Department of Analytical Chemistry
Faculty of Chemistry
Complutense University of Madrid
28040 Madrid, Spain

ABSTRACT

An acetonitrile / 0.20M SDS mobile phase was used to determine PAHs by HPLC with fluorimetric detection.

Because the peak area is greater the method is more sensitive than using an acetonitrile/water mobile phase. The method was applied to determine PAHs in particulate air samples and the results are in good agreement with those found by GC.

INTRODUCTION

The carcinogenic power of polyaromatic hydrocarbons (PAHs) and their importance in environmental studies is well accepted[1-6].

* Author to whom the correspondence should be addressed.

Due to the low concentration of PAHs in environmental samples and their complexity, the analytical techniques used most often for their determination are gas chromatography (GC) and high performance liquid chromatography (HPLC) with fluorimetric detection[7-10]; sensitivity has been increased by using a program of selected excitation and emission wavelength pairs[11], but the need to increase this sensitivity justifies further study.

Moreover, the sensitivity of the fluorimetric determination of PAHs has been increased using micellar solutions of anionic surfactants[12]. We have found no references to the behavior of PAHs in micellar liquid chromatography (MLC). However, information about other compounds is available[13,14]. To decrease the large retention times found using aqueous micellar phases the presence of small percentages (3%) of low molecular weight modifiers, such as alcohols, has been recommended for anthracene and other compounds containing one aromatic ring[15,16].

In this paper we present the results of separating 13 PAHs by HPLC using a sodium dodecyl sulfate (SDS) micellar solution as mobile phase. The possibility of using surfactants to decrease the percentage of organic eluents such as acetonitrile in the mobile phase is also evaluated.

EXPERIMENTAL

Apparatus and material

A HPLC system equipped with a high pressure gradient Milton Roy CM 4000 pump, a Rheodyne 7125 samples injector

with a 20μl loop, a Waters 420 fluorimetric detector with 254 and 375 nm (long-pass) excitation and emission filters, respectively and a Milton Roy CI 4100 integrator were used. The column was a C_{18} Nucleosil 5μm particulate size (150 x 4.6 mm, Phenomenex) thermostated in a P-Selecta Precisterms bath. An MCV high volume sampler equipped with a 15cm diameter Watman GF/A glass fibre filter was installed for collecting particulate air samples. A P-Selecta Ultrasons bath was used to extract samples and Heidoph W 2000 vacuum rotary evaporator to concentrate them. A P-Selecta Meditronic centrifuge capable of 4800 rpm (3700g) was used; Lida nylon membrane filters with 0.45μm pore size were used to filter the sample extracts and the eluents used to prepare the mobile phase.

Reagents

Standard stock methanolic solutions of different PAHs from Sigma at concentrations within the range 10^{-3}-10^{-4}M were prepared by weighing and dissolving the solid products in methanol (Carlo Erba). More dilute solutions were prepared by dilution with methanol.

An aqueous micellar solution of sodium dodecyl sulfate (SDS) ($C_{12}H_{15}NaSO_4$, FW=288.38 from Fluka) was prepared by weighing and dissolving the solid product in 1.0 l of water to give a final concentration of 0.20M; SDS critical micellar concentration (cmc)=8.1x10^{-3}M. More dilute solutions were prepared by dilution with water.

HPLC purity acetonitrile and methanol were from Carlo Erba. Water was obtained from a Milli-Q system (Millipore). All chemicals were of analytical reagent grade. Before use, all eluents were degassed and filtered under vacuum.

Procedure

1. Calibration graphs

They were prepared using the standard methanolic solutions of PAHs in the range 0.002-85.6 ng/μl. The injected volume was 20 μl. The PAHs were separated and quantified under the following conditions: the acetonitrile/0.20M SDS gradient specified in Table 1 was employed with a flow rate of 0.65 ml/min at 22°C; for fluorimetric detection the 254 and 375 nm (long-pass) excitation and emission filters were used; the areas of the peaks were used for quantification of PAHs. During the chromatographic analysis He was used to degas the mobile phase. The column was conditioned by applying the gradient specified in Table 2.

2. Air sample collection, extraction and clean-up

The sampler was placed in the open air in Madrid city center where 105,000 cars pass every day. The samples were taken for 24 hours each time at a flow-rate of 30 m^3/h in winter 1993.

The filters were treated twice with 50 ml of methylene chloride in the ultrasonic bath for 20 minutes and then

TABLE 1

Gradient Program

Time (min)	A (%)	B (%)
0.0	55	45
2.0	55	45
30.0	85	15
38.0	85	15
40.0	55	45

A: acetonitrile; B: 0.20M SDS or Milli-Q water

TABLE 2

Conditioning of the Nucleosil C_{18} Column

Time(min)	A(%)	B(%)	C(%)	Flow-rate(ml/min)
0.0	0	1	99	1
60.0	0	100	0	1
90.0	0	100	0	1
90.1	0	100	0	0.1
690.0	0	100	0	0.1
691.0	55	45	0	1
730.0	55	45	0	1

A: acetonitrile; B: 0.20M SDS; C: Milli-Q water; temperature 22°C

centrifugated at 4000 rpm. The extracts were collected and the solvent was evaporated in the vacuum rotary evaporator and finally the volume was reduced to 0.5 ml with a N_2 stream. Clean-up was carried out in a glass column (25 cm long and 1 cm internal diameter) containing 8g of silica gel, previously treated with methylene chloride, activated at 130°C for 24 hours and deactivated with 5% water. Elution was carried out first with 25 ml of n-hexane and then with 40 ml of n-hexane: methylene chloride (4:1). This second fraction was concentrated in a rotary vacuum evaporator and the solvent was eliminated under a N_2 stream. The residue was dissolved in 10 ml of methanol and the above calibration procedure was applied.

RESULTS AND DISCUSSION

Preliminary studies

As mentioned in the introduction, small amounts of alcohols such as propanol and butanol have been used as modifiers to decrease the retention time of anthracene[15,16].

According to our results, using 0.05-0.20M SDS mobile phases containing 3% of n-propanol, the naphthalene capacity factors were above 30, which are useless for practical purposes. Increasing the percentages of n-butanol to 20% decreased the naphthalene capacity factor to 21, which is still not practical.

However, the presence of acetonitrile in the micellar mobile phase drastically decreased the capacity factors. Compared with the results obtained using the acetonitrile/water mobile phase, both lower acetonitrile percentages and lower flow-rates were necessary to get the same retention factors when acetonitrile/SDS mobile phases were used.

The effect of methanol is similar to that of acetonitrile; however as the baseline drift found in the gradient technique is significantly higher, acetonitrile was preferred.

Chromatographic parameter optimization

a.- Conditioning of the column

The reproducibility of the capacity factors and resolution was clearly influenced by the previous chromatographic run. Thus, the column was conditioned using a 0.20M SDS mobile phase for 30 minutes, followed by acetonitrile/0.20M SDS: 55/45 (v/v) for 40 minutes at a flow-rate of 1 ml/min. The equilibrium was taken to have been reached when the retention time for naphthalene remained constant in the ±0.20 s range. In these conditions the behavior of the column was quite reproducible for about 200 runs. For practical reasons the column was conditioned over night once a month (see Table 2). This treatment seems to saturate the column with SDS retained by apolar interactions.

b.- Effect of SDS concentration in the mobile phase

Figure 1 shows the influence of SDS concentration on the PAH capacity factors. Retention factors decrease significantly with increasing SDS concentration. The capacity factor for dibenzo(ah)anthracene, which is most strongly retained in the column, decreases from 25 when using a mobile phase without SDS, to 18 when the mobile phase was 0.20M in SDS. Likewise, the retention times fell from 37 to 27 minutes.

As Figure 2 shows, lower acetonitrile/SDS flow-rates were necessary to achieve the same analysis time found when an acetonitrile/water mobile phase was used. It should be noted that the noise increases slightly in SDS although the resolution is good down the base line; on the other hand, the peak areas also increase significantly. The increase of sensitivity through an increase of the peak areas should be attributed to the sensitization of the fluorimetric detection in SDS mobile phase. This sensitization could not be explained by the change of flow rate because the opposite effect should be expected. In these experimental conditions SDS behaves as a modifier, decreasing the retention of PAHs with increasing concentration. According to the literature this behavior is typical of systems using micellar mobile phases[17]. The acetonitrile/SDS gradient was optimized in search of a compromise between resolution and analysis time. The recommended gradient is shown in Table 1. Lower acetonitrile concentrations considerably increase the

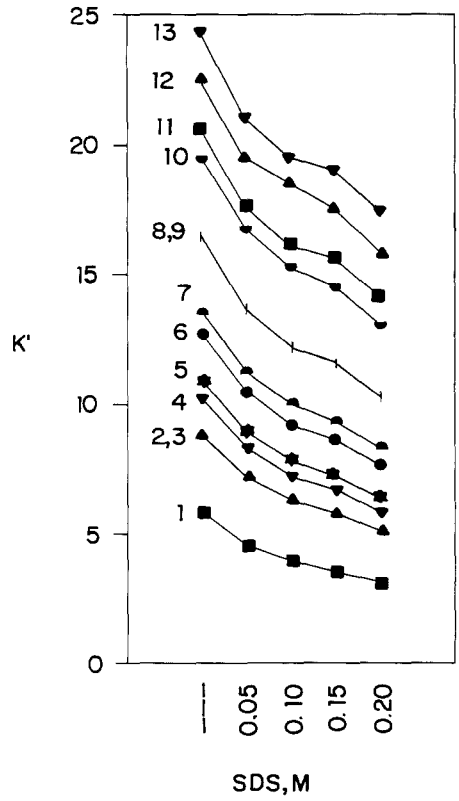

FIGURE 1. Effect of SDS concentration on the capacity factors of PAHs. The PAHs identified are indicated in Table 3.

capacity factors. On the other hand, the elution order of PAHs did not change with respect to that obtained with an acetonitrile/water mobile phase.

The use of methanol as a modifier on the SDS/acetonitrile mobile phase in percentages of 5-20% decreased the resolution of the following pairs: benzo(e)pyrene-benzo(a)pyrene and benzo(ghi)perylene-

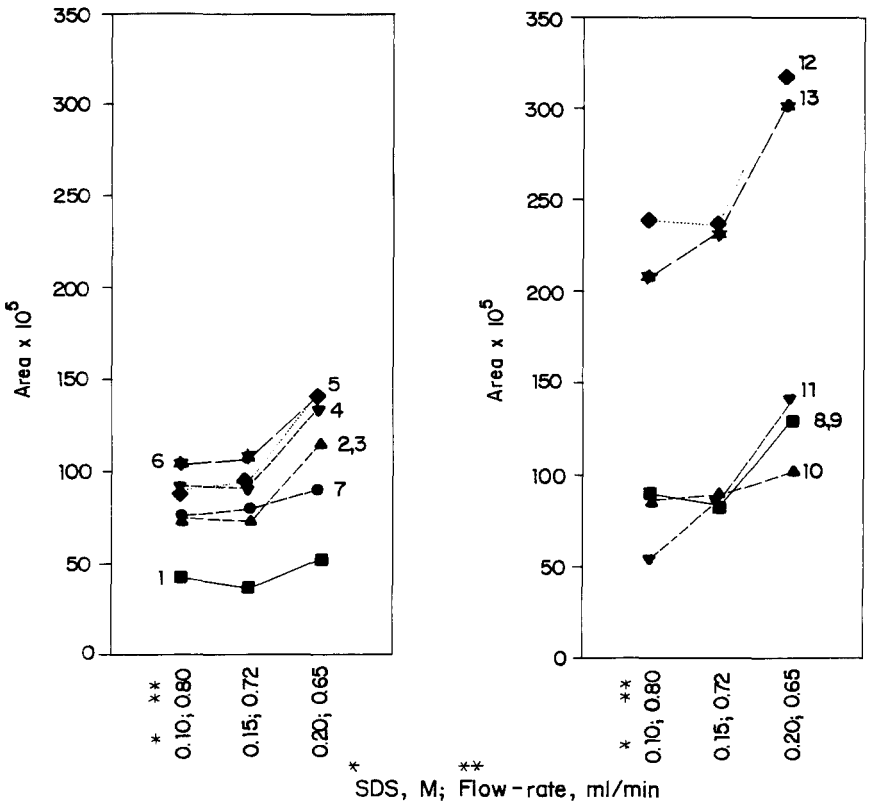

FIGURE 2. Effect of the SDS concentration and flow-rate on the chromatography peak area. The PAHs identified are indicated in Table 3.

dibenzo(ah)anthracene. Similar effects were found with n-propanol; percentages above 5-10% decreased resolution of the phenanthrene-anthracene and benzo(e)pyrene-benzo(a)pyrene pairs.

TABLE 3

Analytical Characteristics

Nº	PAH	x(ng/µl)	RSD,%		m_1/m_2*	DL(ng/µl)	
			1	2		1	2
1	Naphthalene	26	3.1	2.6	1.2	5.86	10.2
2	Fluorene	5.8	2.3	6.9	1.1	1.74	2.08
3	Acenaphthene	6.5	5.0	0.3	1.3	1.38	2.11
4	Phenanthrene	0.12	1.2	0.8	1.9	0.022	0.033
5	Anthracene	0.01	0.6	1.5	1.9	0.002	0.003
6	Fluoranthene	0.16	4.1	1.2	1.7	0.031	0.047
7	Pyrene	0.06	6.0	3.3	1.1	0.016	0.035
8	Chrysene	0.14	4.6	9.5	1.4	0.024	0.042
9	Benzo(a)anthracene	0.06	7.4	3.8	1.4	0.009	0.015
10	Benzo(e)pyrene	0.15	5.1	5.2	1.2	0.022	0.049
11	Benzo(a)pyrene	0.03	4.2	5.0	1.2	0.005	0.010
12	Benzo(ghi)perylene	0.25	1.3	3.8	1.4	0.019	0.039
13	Dibenzo(ah)anthracene	0.42	0.1	5.4	1.2	0.033	0.069

1: mobile phase acetonitrile/0.20M SDS; Flow-rate 0.65 ml/min.
2: mobile phase acetonitrile/ Milli-Q water; Flow-rate 1 ml/min.
* ratios of calibration graph slopes

c.- Effect of the flow-rate

As indicated above, a flow-rate of 0.65 ml/min gave an analysis time of 37 minutes, which is the same as is found for an acetonitrile/water mobile phase at 1 ml/min flow-rate. Flow-rates above 1 ml/min decreased the analysis time to 27 minutes and decreased resolution of the phenanthrene-

anthracene and benzo(e)pyrene-benzo(a)pyrene pairs. This means that the percentages of acetonitrile in the mobile phase can be decreased by exchanging to an SDS aqueous micellar phase, which decreases the waste of acetonitrile, with inherent advantages in cost and environmental protection.

The simultaneous effect of flow-rate and SDS concentration is shown in Figure 2, which shows that the peak areas increase quite significantly.

d.- Effect of temperature

According to the literature the viscosity of micellar mobile phases makes it necessary to work at above room temperature to avoid high pressures[16,18]. In this case there is no need to increase the working temperature because the pressure drop for the gradient specified in Table 1 is about 1600 psis.

Analytical characteristics

As shown in Table 3, the peak areas in acetonitrile/0.20M SDS mobile phase give higher slope ratios for the calibration graphs than in acetonitrile/water for the same analysis time, the flow rate being lower in micellar phase. As indicated in the table, the precision of the areas is about the same in both mobile phases. However detection limits are reduced in micellar phase, in inverse proportion

TABLE 4

Linearity of Calibration Graphs and Retention Times

PAH	Highest limit tested* (ng/μl)	t_r(min)** 1	2	RSD(%)*** 1	2
Naphthalene	59	9.24	9.85	0.13	0.28
Fluorene	10	13.68	14.01	0.72	0.20
Acenaphthene	17	13.83	14.24	0.32	0.22
Phenanthrene	0.27	15.13	16.27	0.21	0.42
Anthracene	0.03	16.18	17.27	0.19	0.34
Fluoranthene	0.39	18.54	19.88	0.19	0.41
Pyrene	0.20	20.20	21.02	0.31	0.25
Chrysene	0.34	23.40	24.92	0.18	0.26
Benzo(a)anthracene	0.12	23.60	25.18	0.12	0.32
Benzo(e)pyrene	0.28	29.03	29.76	0.38	0.31
Benzo(a)pyrene	0.06	31.04	31.28	0.87	0.22
Benzo(ghi)perylene	0.53	33.86	34.07	0.28	0.23
Dibenzo(ah)anthracene	0.94	36.81	36.86	0.30	0.29

1: acetonitrile/ 0.20M SDS mobile phase; Flow-rate 0.65 ml/min.
2: acetonitrile/ Milli-Q water mobile phase; Flow-rate 1 ml/min.
* for both mobile phases
** t_r = retention time
*** average of 4 determinations

to the increase area ratios. Noteworthy are the cases of pyrene, benzo(e)pyrene and benzo(a)pyrene, whose detection limits are clearly lower in micellar solution.

The precision of the retention times is also similar in both mobile phases.

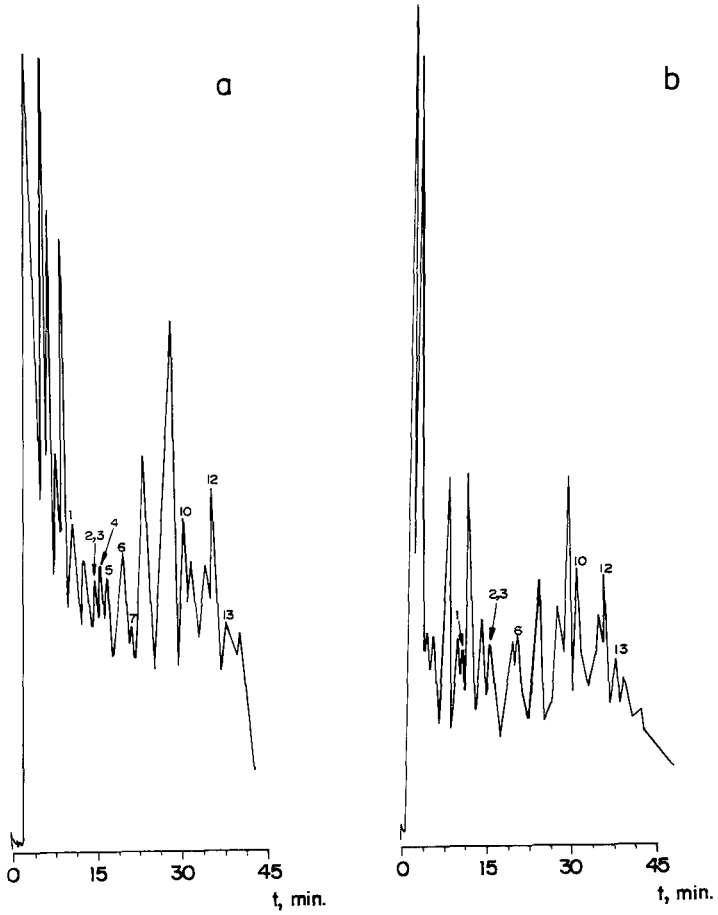

FIGURE 3. Chromatograms of a particulate air sample
a: acetonitrile/0.20M SDS mobile phase; Flow-rate= 0.65 ml/min.
b: acetonitrile/Milli-Q water mobile phase; Flow-rate= 1 ml/min.
The PAHs identified are indicated in Table 3.

TABLE 5

Determination of PAHs in Particulate Air Samples.

PAH	Concentration of PAHs (ng/m^3 air)* Sample 1		2		3	
	MLC**	GC***	MLC**	GC***	MLC**	GC***
Phenanthrene	1.0	1.3	3.3	6.0	1.5	1.8
Anthracene	0.4	0.2	0.7	1.2	0.3	0.3
Fluoranthene	2.1	11.3	1.5	12.7	0.5	6.1
Pyrene	5.2	11.9	3.5	16.5	---	7.9
Chrysene		6.5		13.3		8.2
	16		11.5		9.9	
Benzo(a)anthracene		5.0		7.3		6.9
Benzo(e)pyrene	19	2.0	15.8	6.3	14.7	2.1
Benzo(a)pyrene	3.9	3.9	7.8	7.6	3.2	6.0
Benzo(ghi)perylene	0.6	---	1.0	0.8	0.9	0.9
Dibenzo(ah)anthracene	4.8	2.8	3.3	3.5	9.3	9.9

* samples were collected in the city of Madrid in winter 1993
** n=3; average of 3 determinations of the same sample. Relative standard deviation (RSD) in the range 1 - 10%
*** Gas chromatography[19]

The linearity of the calibration graphs extends up to the higher concentration tested (Table 4) also in both mobile phases, the correlation coefficients being higher than 0.99.

The selectivity of the method was evaluated by comparing chromatograms obtained from the same particulate air sample. As shown in Figure 3, it is possible to detect more PAHs e.g. phenanthrene, anthracene and pyrene using a micellar mobile phase due to its higher sensitivity.

Determination of PAHs in urban particulate air

The MLC method was applied to determine PAHs in three particulate air samples from the city of Madrid. Results are shown in Table 5, which includes the results obtained by gas chromatography, applying the EPA method TO-13[19]. The results obtained by both methods agree well for phenanthrene, anthracene, benzo(a)pyrene, benzo(ghi)perilene and dibenzo(ah)anthracene. On the other hand, naphthalene, fluorene and acenaphthene are not detected by either of the two methods. The differences observed may be attributed to a different resolution of the two methods; for example chrysene and benzo(a)anthracene overlap in the HPLC method.

CONCLUSIONS

The use of the acetonitrile/SDS mobile phase to determine PAHs by HPLC with fluorimetric detection gives a higher sensitivity than that obtained with an acetonitrile/water mobile phase, due to the increase of the area ratios; moreover, lower flow-rates are necessary to achieve the same analysis time. This decreases the waste of acetonitrile and consequently, the cost and pollution. SDS modifies the surface of the stationary phase which then behaves as a modifier.

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ADVANTAGES OF TLC AS A PILOT TECHNIQUE FOR HPLC

JAN K. RÓŻYŁO, MAŁGORZATA JANICKA, AND RYSZARD SIEMBIDA
Faculty of Chemistry
Maria Curie-Skłodowska University
20-031 Lublin, Poland

ABSTRACT

In the paper two ways of using thin-layer chromatographic data for mobile phase optimization in column chromatography have been presented. The first method is connected with experimental correlations between thin-layer and column retention parameters. This moderately simple method needs to determine the correction parameter to transfer experimental thin-layer data to liquid column technique. In the paper the effects of mobile phase kind and cocentration, as well as chromatographed solute character on this parameter have been presented. The other method is based on thermodynamic description of adsorption systems with mixed mobile phases, i.e., adsorbent + binary solvent+ solute systems. This treatment is very precise, but it needs a lot of experimental thin-layer data.

In the paper the possibility of using TLC experimental data for the choice of optimal mobile phase kind and composition in two-component solvents and some organic model solutes has been presented.

INTRODUCTION

The problem of using thin-layer chromatography as a pilot technique for liquid column method is connected with two issues of general meaning. At first, it is necessary to solve problems of thin-layer effects, i.e., mobile phase demixing, preadsorption, the presence of gas phase or gradient of phase ratio and the others (1,2),

not only to obtain the recurrent experimental data, but for their proper interpretation.

The other problem is to find or choose the best method for transfering TLC experimental data to liquid column technique.

The most popular way of using TLC as a pilot technique for LCC is based on simple experimental correlations between thin-layer and column retention parameters. Different versions of this method have been with details described by Kaiser and Rieder (3), Siouffi and co-workers (4), Soczewiński (5,6), Gołkiewicz (7), Różyło and co-workers (8-10), Jost (11) and Hara (12).

In present paper the correlations between thin-layer and liquid column retention parameters have been described using Kaiser and Rieder relationship (3):

$$k'_{TLC} = c\, k'_{LCC} \qquad (1)$$

where k'_{TLC} is solute capacity factor obtained by TLC and k'_{LCC} by LCC measurements, and c is a constant, or the following modification of this relationship:

$$k'_{LCC} = \frac{1}{c}\, k'_{TLC} \qquad (2)$$

This relationship (1 or 2) is rather simple, but in practical use it requires evaluation of the value of c (or 1/c) parameter. As it was realized this parameter depends on mobile phase kind and composition as well as on chromatographed solute character, though from Kaiser and Rieder (3) works appears that it can be evaluated experimentally for standard solute in given chromatographic system.

The other method for using TLC as a pilot technique for LCC is based on thermodynamic description of adsorbent + binary solvent + solute systems. The detail description of this method has been presented in our previous papers (13-18).

In this treatment theoretical solute capacity factors k' in mixed mobile phase can be calculated by the means of the following equation:

$$\log k'_{12} = x_1^l \log k'_1 + x_2^l \log k'_2 + (x_1^s - x_1^l)(\log \frac{k'_1}{k'_2} + A_{s(12)}) \qquad (3)$$

TABLE I

Solute Capacity Factors obtained in hexane - carbon tetrachloride mobile phase.

k'_{TLC} - experimental k' values obtained by TLC;

k'_{LCC} - experimental k' values obtained by LCC;

$k'_{(th)}$ - theoretical k' values calculated according to eq.(3).

1/c correction factor from eq.(1)

Solutes	x_1^1=0.3				x_1^1=0.5			
	k'_{TLC}	$k'_{(th)}$	k'_{LCC}	1/c	k'_{TLC}	$k'_{(th)}$	k'_{LCC}	1/c
Naphtalene	1.32	1.30	1.05	0.80	1.16	0.94	0.83	0.72
Anthracene	2.00	1.97	1.58	0.79	1.62	1.60	1.23	0.76
Pyrene	1.95	2.20	1.78	0.91	1.86	1.70	1.38	0.74
Fluoranthen	2.19	2.73	2.45	0.90	1.74	2.01	1.82	1.05
2,3-dichloro-phenol	4.79	12.60	12.30	1.12	11.48	10.01	9.33	0.81
2,4-dichloro-phenol	15.14	16.62	16.60	1.10	11.75	12.39	11.75	1.00
2,5-dichloro-phenol	12.30	11.22	11.48	0.93	9.12	8.40	8.51	0.93
2,6-dichloro-phenol	6.46	10.19	10.00	1.55	5.07	7.61	7.41	1.46

where k'_1, k'_2 and k'_{12} are capacity factors of given solute in solvents 1 and 2, and in their mixture 1+2, respectivelly; x_1^1 and x_2^1 are molar fractions of solvents 1 and 2 in bulk phase; x_1^s is a molar fraction of solvent 1 in surface phase; $A_{s(12)}$ is a parameter describing molecular interactions existing in bulk phase.

The method of x_1^s and $A_{s(12)}$ parameters calculation from thin-layer experimental data are presented in papers (13-18). It is obvious that theoretical predictions of k' values according to equation (3) needs a lot of of TLC experimental data, i.e., not only k'_1 and k'_2 values, but a number of k'_{12} capacity factors in the mixed mobile phase 1+2, for the estimation of x_1^s and $A_{s(12)}$ parameters.

TABLE II.
Solute Capacity Factors obtained in hexane - benzene mobile phase.
k'_{TLC} - experimental k' values obtained by TLC;
k'_{LCC} - experimental k' values obtained by LCC;
$k'_{(th)}$ - theoretical k' values calculated according to eq. (3).
1/c correction factor from eq. (1)

Solutes	x_1^1=0.1				x_1^1=0.3			
	k'_{TLC}	$k'_{(th)}$	k'_{LCC}	1/c	k'_{TLC}	$k'_{(th)}$	k'_{LCC}	1/c
Naphtalene	1.34	0.78	0.78	0.58	0.95	0.38	0.35	0.37
Anthracene	1.82	1.09	1.29	0.71	1.05	0.37	0.48	0.46
Pyrene	1.70	1.24	1.34	0.79	1.12	0.44	0.54	0.48
Fluoranthene	2.04	1.33	1.58	0.77	1.20	0.38	0.60	0.50
2,3-dichloro-phenol	14.79	11.00	10.97	0.74	8.51	4.37	4.79	0.56
2,4-dichloro-phenol	15.14	12.06	12.59	0.93	8.13	5.00	5.62	0.69
2,5-dichloro-phenol	12.02	9.52	8.71	0.72	6.76	4.10	4.17	0.62
2,6-dichloro-phenol	6.31	7.15	6.92	1.10	3.89	2.70	2.95	0.76

In the paper these two ways of using thin-layer chromatography for the choice of optimal mobile phase in liquid column chromatography in systems with two-component mobile phases and some organic model substances have been presented.

MATERIALS AND METHODS

All experimental data presented in the paper have been obtained using adsorption liquid chromatography. Two techniques of this analytical method have been used, i.e., column chromatography and

TABLE III.
Solute Capacity Factors obtained in hexane - etylene chloride mobile phase.

k'_{TLC} - experimental k' values obtained by TLC;
k'_{LCC} - experimental k' values obtained by LCC;
$k'_{(th)}$ - theoretical k' values calculated according to eq. (3).
1/c correction factor from eq. (1)

Solutes	x_1^1=0.3				x_1^1=0.5			
	k'_{TLC}	$k'_{(th)}$	k'_{LCC}	1/c	k'_{TLC}	$k'_{(th)}$	k'_{LCC}	1/c
Naphtalene	1.20	0.68	0.58	0.48	0.89	0.30	0.27	0.30
Anthracene	1.35	0.92	0.89	0.66	0.81	0.32	0.25	0.31
Pyrene	1.41	0.88	0.79	0.56	0.95	0.44	0.32	0.34
Fluoranthene	1.78	1.10	1.00	0.56	1.17	0.38	0.35	0.30
2,3-dichloro-phenol	8.91	5.60	5.62	0.63	4.07	1.89	2.00	0.49
2,4-dichloro-phenol	9.55	6.18	6.31	0.66	4.27	2.00	2.34	0.55
2,5-dichloro-phenol	7.76	4.92	4.79	0.62	3.89	1.74	1.78	0.46
2,6-dichloro-phenol	4.37	4.10	4.17	0.95	2.45	1.32	1.41	0.58

Techma Robot Type 302 liquid chromatograph (Warsaw, Poland) equipped with UV detector (Labmim, Hungary). The column was fitted with Lichrosorb Si-60, d_p= 5μm (Merck). Mobile phase flow rate was v = 1 ml/min.

In thin-layer measurements sandwich chambers and precoated (Merck) silica gel (Si-60, d_p = 5μm) plates have been used.
Thin-layer chromatograms were visualized by Scanning Densitometer Cs-9000 (Shimadzu).
All substances and solutes used in TLC and LCC measurements were identical.
For detailed information see previous papers (14-18).

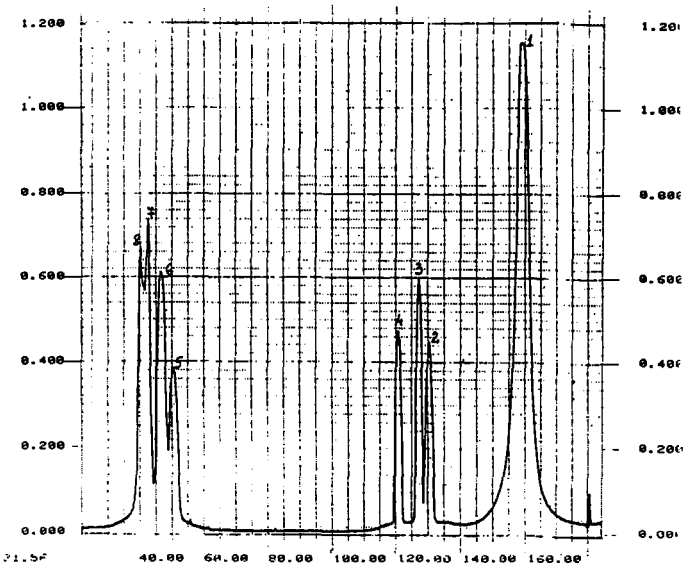

Fig. 1
Chromatogram obtained by TLC technique (Scanning Densitometer Cs-9000, Shimadzu, λ = 287 nm).
Mobile phase: hexane - carbon tetrachloride, $x_1^1 = 0.3$
solutes: (1)naphthalene, (2) anthracene, (3) pyrene, (4) fluoranthene, (5) 2,6-dichlorophenol, (6) 2,5-dichlorophenol, (7) 2,3-dichlorophenol, (8) 2,4-dichlorophenol

RESULTS AND DISCUSSION

Using TLC measurements experimental capacity factors k' of studied substances for all mobile phases have been obtained. These values are presented in Tables I-III as k'_{TLC} parameters. Experimental capacity factors of the same substances and mobile phases obtained by column chromatography are presented in Tables I-III as k'_{LCC} values.
Thin-layer chromatograms are presented on Figs 1-5, for chosen mobile phase systems.

For comparing experimental capacity factors corresponding to both techniques, 1/c correction factors have been calculated according to Kaiser and Rieder equation (1).

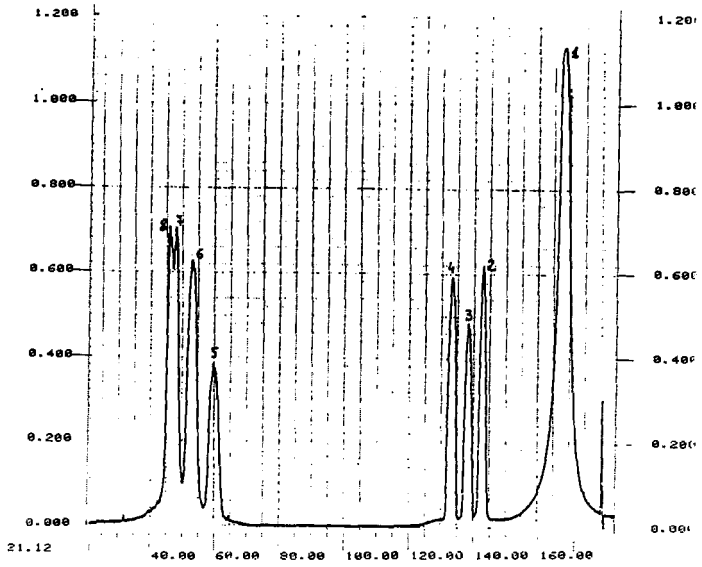

Fig.2
Chromatogram obtained by TLC technique (Scanning Densitometer Cs-9000, Shimadzu, λ = 287 nm).
Mobile phase: hexane - carbon tetrachloride, $x_1^1 = 0.5$
solutes: (1)naphthalene, (2) anthracene, (3) fluoranthene, (4) pyrene, (5) 2,6-dichlorophenol, (6) 2,5-dichlorophenol, (7) 2,3-dichlorophenol, (8) 2,4-dichlorophenol

These parameters are listed in Tables I-III for all studied substances and mobile phases.

Analysing these 1/c parameters it is obvious that they depend not only on chromatographed substances but on mobile phase properties as well.

But, for given two-component mobile phase one can notice that:

- 1/c values change with mobile phase composition;
- 1/c values are very similar to each other for the given group of chromatographed substances, i.e., for aromatic hydrocarbons or chlorophenols, especially in more active mobile phases hexane-benzene and hexane-ethylene chloride.

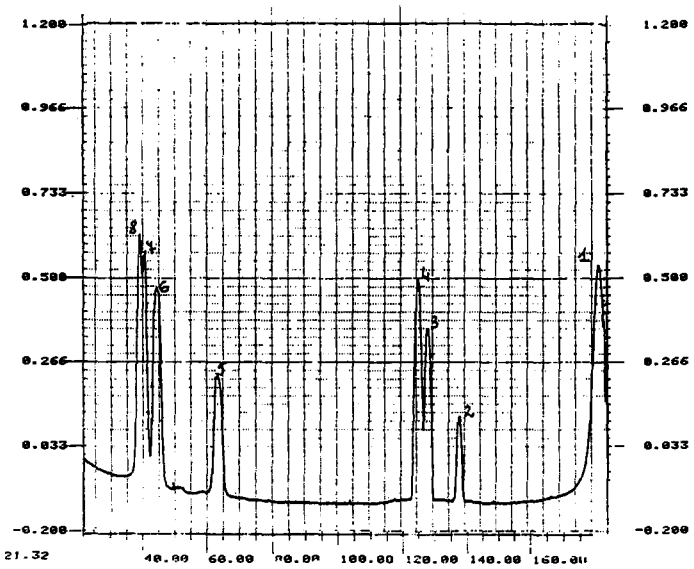

Fig. 3
Chromatogram obtained by TLC technique (Scanning Densitometer Cs-9000, Shimadzu, λ = 287 nm).
Mobile phase: hexane - benzene, $x^1_1 = 0.1$
solutes: (1)naphthalene, (2) pyrene, (3) anthracene, (4) fluoranthene, (5) 2,6-dichlorophenol, (6) 2,5-dichlorophenol, (7) 2,3-dichlorophenol, (8) 2,4-dichlorophenol

In the paper theoretical capacity factors $k'_{(th)}$ of studied substances (Table I-III) have been calculated using equation (3) resulting from thermodynamic description of adsorption chromatographic system.

These $k'_{(th)}$ values are capacity factors of studied substances in liquid column technique evaluated on the bases of experimental thin-layer data.

From the data presented in Tables I-III it seems that there is a very good agreement between theoretical and experimental k' values for all studied substances and mobile phases.

In the paper two ways of the choice of optimal mobile phase in LCC technique from TLC experimental data are presented.

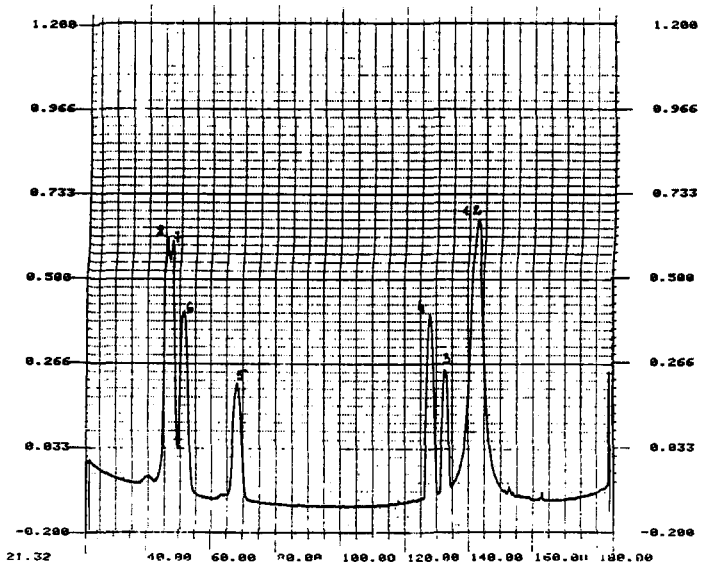

Fig. 4
Chromatogram obtained by TLC technique (Scanning Densitometer Cs-9000, Shimadzu, λ = 287 nm).
Mobile phase: hexane - benzene, $x_1^1 = 0.3$
solutes: (1) naphthalene, (2) anthracene, (3) pyrene, (4) fluoranthene, (5) 2,6-dichlorophenol, (6) 2,5-dichlorophenol, (7) 2,3-dichlorophenol, (8) 2,4-dichlorophenol

At first, for this purpose there have been used the correlations between experimental thin-layer and column retention parameters, i.e., between k'_{TLC} and k'_{LCC} values.
This method is rather simple, but for precise prediction of k'_{LCC} values it requires evaluation of 1/c correction factors. Though from the study appears that these 1/c values depend not only on the mobile phase kind but on its composition as well, they are characteristic for some groups of studied substances.

The other method is based on thermodynamic description of chromatographic systems. This method is very precise, but it needs much more experimental TLC information than the first one.

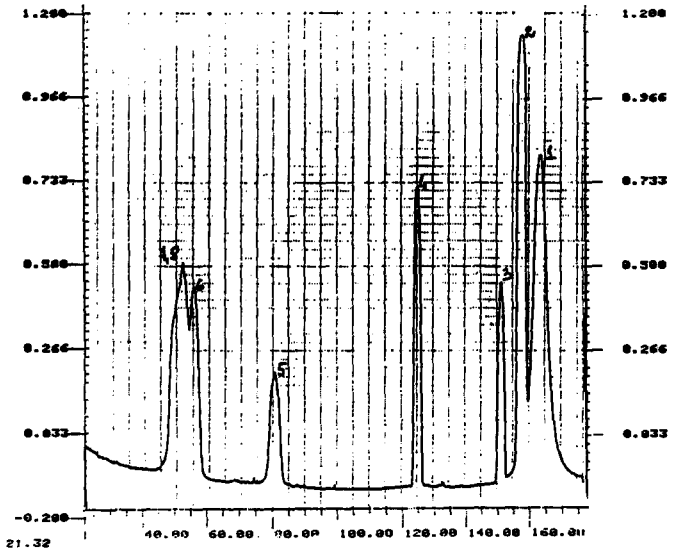

Fig. 5
Chromatogram obtained by TLC technique (Scanning Densitometer Cs-9000, Shimadzu, λ = 287 nm).
Mobile phase: hexane - ethylene chloride, $x_1^1 = 0.1$
solutes: (1)naphthalene, (2) anthracene, (3) pyrene, (4) fluoranthene, (5) 2,6-dichlorophenol, (6) 2,5-dichlorophenol, (7) 2,3-dichlorophenol, (8) 2,4-dichlorophenol

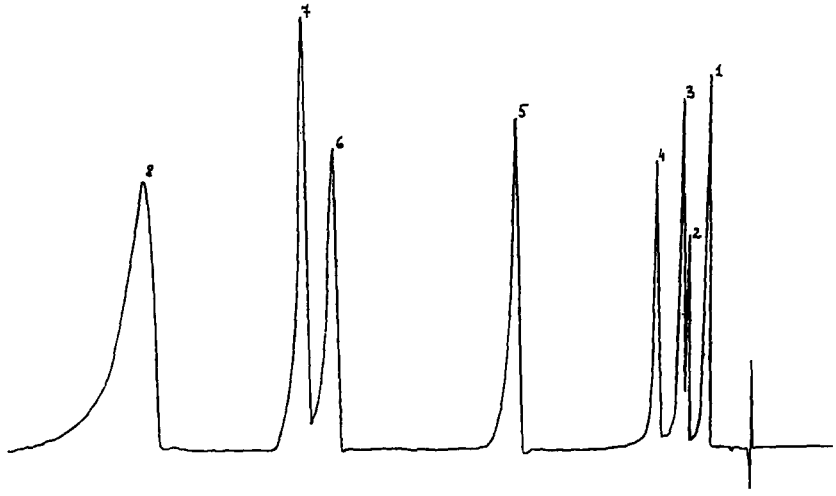

Fig. 6
Chromatogram obtained by LCC technique.
Mobile phase: hexane - carbon tetrachloride, $x_1^1 = 0.3$
solutes: (1)naphthalene, (2) anthracene, (3) pyrene, (4) fluoranthene, (5) 2,6-dichlorophenol, (6) 2,5-dichlorophenol, (7) 2,3-dichlorophenol, (8) 2,4-dichlorophenol

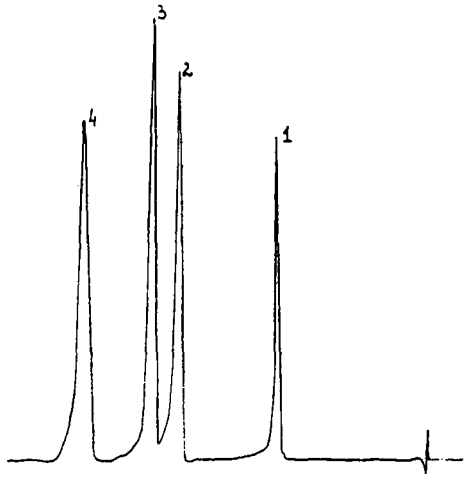

Fig.7
Chromatogram obtained by LCC technique.
Mobile phase: hexane - carbon tetrachloride, $x_1^1 = 0.5$
solutes: (1) 2,6-dichlorophenol, (2) 2,5-dichlorophenol,
(3) 2,3-dichlorophenol, (4) 2,4-dichlorophenol

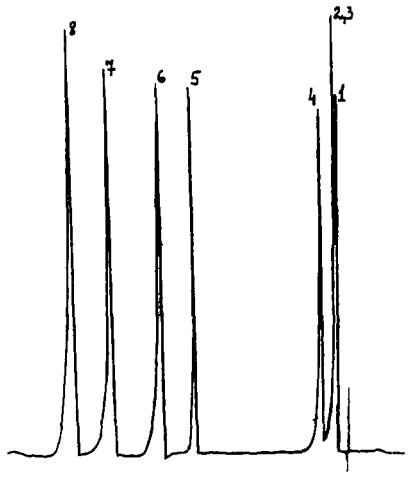

Fig.8
Chromatogram obtained by LCC technique.
Mobile phase: hexane - benzene, $x_1^1 = 0.1$
solutes: (1) naphthalene, (2) anthrecene, (3) pyrene, (4) fluoranthene,
(5) 2,6-dichlorophenol, (6) 2,5-dichlorophenol, (7) 2,3-dichlorophenol,
(8) 2,4-dichlorophenol

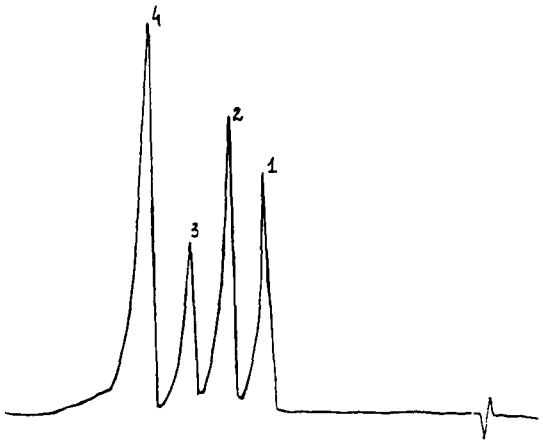

Fig. 9

Chromatogram obtained by LCC technique.
Mobile phase: hexane - ethylene chloride, $x_1^1 = 0.1$
solutes: (1) 2,6-dichlorophenol, (2) 2,5-dichlorophenol, (4) 2,3-dichlorophenol, (5) 2,4-dichlorophenol

From all experimental thin-layer measurements that have been performed in the study, four different mobile phases have been chosen as the most appropriate for liquid column measurements. Liquid column chromatograms obtained in these chosen systems are presented on Figs 6-9.

From LCC experimental data it appears that the best resolution for all studied substances has been obtained in hexane - carbon tetrachloride ($x_1^1 = 0.3$) as mobile phase. This mobile phase seems to be the most comfortable for the separation of aromatic hydrocarbons as well.

For the best separation of the group of chlorophenols it is much better to use hexane - benzene ($x_1^1 = 0.1$) as mobile phase.

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EFFECT OF THE MOBILE AND STATIONARY PHASES ON RP-HPLC RETENTION AND SELECTIVITY OF FLAVONOID COMPOUNDS

M. C. PIETROGRANDE[1] AND Y. D. KAHIE[2]
[1]*Chemistry Department*
Via L. Borsari, 46
I-44100 Ferrara, Italy
[2]*Analytical Chemistry*
Somali National University
P.O. Box 1081
Mogadishu, Somalia

ABSTRACT

The retention behaviour of an extended set of flavonoid compounds on phenyl and cyano reversed-phase HPLC columns was studied and compared to behaviour on octadecyl column. The selectivity properties of methanol, acetonitrile and tetrahydrofuran as organic modifiers on each stationary phase are reported. Both the stationary and the mobile phases appeared to significantly change HPLC system selectivity. Specific stationary phase selectivity effects proved more pronounced with methanol: in particular the phenyl phase showed a greater selective retention for unsaturated flavonoids while octadecyl proved more selective for glycoside compounds.

INTRODUCTION

Flavonoid compounds occupy a prominent position among natural phenols, particularly due to their conspicuous presence in green plants as well as to their importance in the flavour and nutritional quality of foodstuffs. Since Reversed-Phase HPLC plays a central role in the separation of complex flavonoid mixtures, many systematic collections and investigations of

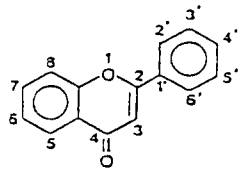
retention data have been published to date (1-3). The influence of the chromatographic variables (i. e., solvent, composition and type of stationary phase) in determining retention has been widely examined for an extended set of flavonoid compounds (4-8). Different solvents have exhibited significant selectivity features on octadecyl column; therefore an extended study of solvent effects on different stationary phases may lead to additional insights in retention selectivity.
In the present paper, the specific selectivity effects of organic modifiers (tetrahydrofuran and acetonitrile) combined with different stationary phases (phenyl and cyano) is exploited and related to solute molecular structure. This study will be useful in setting up a multichoice chromatographic separation set and in selecting uncorrelated retention systems for optimum analytical separations.

MATERIALS AND METHODS

The chromatographic measurements were made according to experimental procedure previously reported (7, 8). The retention data, log k,' were evaluated with a Waters 600 multi-solvent system, equipped with Rheodyne injection valve (20µl sample loop), and a Waters 990 photodiode-array detector, coupled with an APC III personal computer (NEC). Two reversed phase columns were used: 30 cm x 3.9 mm, 10 µm Ph µBondapak Phenyl column (Waters) and 30 cm x 3.9 mm, 10 µm CN µBondapak Cyano column (Waters). On these columns log k' values were measured at a minimum of six different mobile phase compositions (expressed as ϕ %, i. e., organic solvent % proportions). Acetonitrile and tetrahydrofuran used as solvent were of HPLC grade (Carlo Erba, Milan, Italy) and water was purified by a Norganic System (Millipore, Bedford, MA, USA). The aqueous phase was buffered at pH2-3 in 80mM acetic acid/8mM disodium hydrogenphosphate, analytical reagent grade (Carlo Erba, Milan, Italy).
The standards studied were from Sarsyntex (Merignac, France) and used as received. Standards (in methanol) had a concentration in the 10-100 ppm range. The set of flavonoid compounds selected, whose molecular structures are reported in Figure 1, represented the following classes: flavones, flavonols, flavanones and glycosides.

RESULTS AND DISCUSSION

The relationship between retention (log k') and mobile phase composition (ϕ) was calculated from the experimental data: the dependence of log k' on ϕ is linear for methanol and tetrahydrofuran, while it is parabolic for acetonitrile. These organic modifiers displayed the same behaviour as previously observed on the C-18 column (7). The best fit gave a correlation greater than 0.99 for all the systems examined: the correlation coefficients of the calculated equations are reported in Tables I-IV. The intercept value A (log k' value extrapolated to pure water), as a measure of the stationary phase retentivity independent of the organic modifier type, is a



No	Compound	3	5	7	2'	3'	4'
1	**Acacetin**		OH	OH			OMe
2	**Apigenin**		OH	OH			OH
3	**Apigenin 7-O-glucoside**		OH	O-Glu			OH
4	**Apiin**		OH	2Ap-Glu			OH
5	**Chrysin**		OH	OH		OH	OH
6	**Chryseriol**		OH	OH		OMe	OH
7	**Eriodictyol**		OH	OH			
8	**Galangin**	OH	OH	OH			
9	**Luteolin**		OH	OH		OH	OH
10	**Luteolin 7-O-glucoside**		OH	O-Glu		OH	OH
11	**Morin**	OH	OH	OH	OH		OH
12	**Naringenin**		OH	OH			OH
13	**Quercetin**	OH	OH	OH		OH	OH
14	**Quercetrin**	O-Rhm	OH	OH		OH	OH
15	**Rutin**	O-Rut	OH	OH		OH	OH
16	**Flavanone**						
17	**Flavone**						
18	**Flavanol**	OH					

Figure 1: Molecular structure of the flavonoid compounds studied.

TABLE I.
log k' vs. ϕ Parabolic Fitting according to the Equation:
$\log k' = A + B\,\phi + C\,\phi^2$
Organic Modifier: Acetonitrile; Column: Phenyl.
R = correlation coefficient; σ_{xy} = standard error of the regression.

	Compound	A	B	C	R	σ_{xy}
1	Acacetin	3.18±0.04	-8.24±0.20	5.00±0.22	1.00	0.01
2	Apigenin	2.86±0.11	-9.48±0.61	7.50±0.81	1.00	0.01
3	Apigenin 7-O-glucoside	2.55±0.02	-15.33±0.19	17.59±0.34	1.00	0.01
4	Apiin	2.39±0.04	-14.38±0.36	15.99±0.65	1.00	0.01
5	Chrysin	2.86±0.04	-7.16±0.20	4.00±0.22	1.00	0.01
6	Chrysoeriol	2.88±0.20	-9.40±1.14	7.36±1.50	0.99	0.01
7	Eriodictyol	2.58±0.08	-7.90±0.47	6.30+0.66	1.00	0.02
8	Galangin	2.97±0.02	-7.45±0.10	4.25±0.11	1.00	0.01
9	Luteolin	2.76±0.17	-10.47±1.03	9.37±1.45	0.99	0.03
10	Luteolin 7-O-glucoside	2.76±0.15	-15.47±1.16	18.46±2.07	1.00	0.01
11	Morin	2.68±0.01	-11.69±0.11	12.33±0.19	1.00	0.01
12	Naringenin	2.58±0.18	-7.93±1.05	5.79±1.39	0.99	0.01
13	Quercetin	2.44±0.11	-8.78±0.67	7.34±0.94	1.00	0.02
14	Quercitrin	2.76±0.03	-14.55±0.25	17.29±0.45	1.00	0.01
15	Rutin	2.55±0.14	-15.56±1.08	19.75±1.93	0.99	0.03
16	Flavanone	3.17±0.11	-7.12±0.85	3.75±0.56	1.00	0.01
17	Flavone	2.89±0.04	-7.31±0.20	4.50±0.22	1.00	0.01
18	Flavanol	3.04±0.06	-7.33±0.30	4.25±0.33	1.00	0.01

TABLE II.
log k' vs. ϕ Linear Fitting according to the Equation:
$\log k' = A + B\phi$
Organic Modifier: Tetrahydrofuran; Column: Phenyl.
R = correlation coefficient; σ_{xy}= standard error of the regression.

	Compound	A	B	R	σ_{xy}
1	Acacetin	2.51±0.20	-4.67+0.46	0.99	0.09
2	Apigenin	2.54±0.17	-4.97±0.41	0.99	0.10
3	Apigenin 7-O-glucoside	2.10±0.16	-5.52±0.54	0.99	0.08
4	Apiin	2.09±0.20	-5.90±0.67	0.99	0.10
5	Chrysin	2.45±0.18	-4.50±0.40	0.99	0.08
6	Chrysoeriol	2.15±0.16	-4.23±0.35	0.99	0.07
7	Eriodictyol	2.25±0.13	-4.13±0.29	0.99	0.06
8	Galangin	2.86±0.17	-5.06±0.38	0.99	0.07
9	Luteolin	2.34±0.15	-4.63±0.35	0.99	0.09
10	Luteolin 7-O-glucoside	2.12±0.20	-5.24±0.68	0.99	0.10
11	Morin	2.29±0.13	-4.60±0.30	0.99	0.08
12	Naringenin	2.41±0.14	-4.47±0.31	0.99	0.06
13	Quercetin	2.42±0.17	-4.60±0.3	0.99	0.07
14	Quercitrin	2.18±0.15	-5.22±0.50	0.99	0.07
15	Rutin	1.90±0.17	-4.63±0.56	0.99	0.08
16	Flavanone	2.26±0.13	-4.10±0.29	0.99	0.05
17	Flavone	1.90±0.13	-3.73±0.31	0.99	0.08
18	Flavanol	2.27±0.16	-4.07±0.36	0.99	0.07

TABLE III.
log k' vs. ϕ Parabolic Fitting according to the Equation:
$\log k' = A + B\phi + C\phi^2$
Organic Modifier: Acetonitrile; Column: Cyano.
R = correlation coefficient; σ_{xy} = standard error of the regression.

	Compound	A	B	C	R	σ_{xy}
1	Acacetin	2.22±0.11	-6.99±0.71	4.75±1.01	1.00	0.02
2	Apigenin	1.97±0.05	-7.33±0.41	5.76±0.66	1.00	0.02
3	Apigenin 7-O-glucoside	1.50±0.02	-7.53±0.22	5.73±0.54	1.00	0.01
4	Apiin	1.55±0.05	-8.14±0.57	5.91±1.40	1.00	0.01
5	Chrysin	2.08±0.06	-6.67±0.39	4.75±0.56	1.00	0.01
6	Chrysoeriol	2.04±0.05	-7.60±0.39	6.04±0.60	1.00	0.02
7	Eriodictyol	1.40±0.05	-5.92±0.47	5.38±0.91	1.00	0.01
8	Galangin	1.92±0.02	-5.54±0.28	3.25±0.11	1.00	0.01
9	Luteolin	1.83±0.09	-7.53±0.69	6.37±1.02	1.00	0.02
10	Luteolin 7-O-glucoside	1.53±0.10	-9.06±1.11	8.64±2.07	0.99	0.03
11	Morin	1.61±0.02	-7.41±0.21	7.20±0.41	1.00	0.01
12	Naringenin	1.47±0.05	-5.07±0.46	3.63±0.91	1.00	0.02
13	Quercetin	1.85±0.03	-8.16 ±0.33	8.17±0.66	1.00	0.01
14	Quercitrin	1.91±0.03	-8.79 ±1.06	9.09±1.58	0.99	0.02
15	Rutin	1.57±0.10	-14.57±1.08	21.81±1.97	0.99	0.03
16	Flavanone	1.83±0.04	-5.72±0.90	4.49±1.56	0.99	0.03
17	Flavone	1.83±0.04	-5.71±0.24	3.75±0.36	1.00	0.01
18	Flavonol	1.93±0.05	-5.67±0.31	3.50±0.43	1.00	0.01

TABLE IV.
log k' vs. ϕ Linear Fitting according to the Equation:
$\log k' = A + B\,\phi$
Organic Modifier: Tetrahydrofuran; Column: Cyano.
R = correlation coefficient; σ_{xy} = standard error of the regression.

	Compound	A	B	R	σ_{xy}
1	Acacetin	1.60±0.08	-4.49±0.21	0.99	0.02
2	Apigenin	1.61±0.07	-3.27±0.28	0.99	0.03
3	Apigenin 7-O-glucoside	1.24±0.05	-3.26±0.15	0.99	0.02
4	Apiin	1.19±0.04	-3.31±0.27	0.99	0.02
5	Chrysin	1.62±0.06	-4.58±0.15	0.99	0.01
6	Chrysoeriol	1.77±0.09	-4.10±0.19	0.99	0.02
7	Eriodictyol	1.61±0.06	-3.20±0.19	0.99	0.02
8	Galangin	1.76±0.07	-4.73±0.28	0.99	0.03
9	Luteolin	1.56±0.05	-3.59±0.15	0.99	0.01
10	Luteolin 7-O-glucoside	1.20±0.04	-3.27±0.18	0.99	0.01
11	Morin	1.53±0.04	-3.08±0.20	0.99	0.02
12	Naringenin	1.67±0.05	-3.22±0.17	0.99	0.02
13	Quercetin	1.55±0.04	-3.28±0.16	0.99	0.01
14	Quercitrin	1.31±0.03	-3.32±0.12	0.99	0.01
15	Rutin	1.17±0.03	-3.14±0.16	0.99	0.02
16	Flavanone	1.59±0.04	-3.97±0.19	0.99	0.02
17	Flavone	1.34±0.03	-4.00±0.11	0.99	0.01
18	Flavanol	1.52±0.03	-3.95±0.16	0.99	0.01

suitable parameter for comparing the different chromatographic systems. The experimental data obtained were related to those, previously reported, with methanol (MeOH) as solvent on the various stationary phases (8) and on the octadecyl (C-18) column with the different solvents (7). As stationary phase polarity increases and approaches that of the mobile phase, the stationary phase retentivity for each solvent decreases in the following order:

$$C\text{-}18 > Ph > CN.$$

The solvent strength S was computed in the interval between log k' = 1 and log k' = 0 and calculated as:

$$S = \Delta \log k' / \Delta\phi$$

The S values calculated for the different retention systems are reported in Table V. This parameter is very important for both isocratic and gradient elution to chose the best possible chromatographic conditions to optimize analytical elution (5, 7). The following order, as expected for solvent strength in RP-LC:

$$ACN > THF > MeOH$$

is not very clear for these data. A peculiar behaviour is shown by glycoside and aglycone classes. On the phenyl phase glycosides generally exhibit higher S values than do aglycones and, of the three solvents, the effect is most marked for acetonitrile. On the CN phase, methanol and tetrahydrofuran show the same solvent strength for both glycosides and aglycones, whereas when acetonitrile is the organic modifier glycosides show greater S values than do aglycones (see Table V). The same behaviour was previously observed for the C-18 phase. These results confirm the hypothesis previously assumed (8): specific interactions take place between the glycoside moiety and the CN group when acetonitrile is the bulk mobile phase, whereas there is no specific effect when CN is bonded to the stationary phase. On the basis of this information, the linear solvent strength (LSS) gradient theory developed by Snyder (9, 10) was useful in determining optimum gradient elution conditions for the separation of complex flavonoid mixtures.

The general pattern of the structure-retention relationship of these columns was studied by determining group contributions to retention (Δ log k') for various substituents in the benzopyran ring (i. e. the difference between retention of one molecule containing a particular substituent and that of a molecule which does not contain that group). Only experimental log k' data, roughly in the 0 - 1 range, were employed. Table VI reports mean Δ log k' values calculated from 4-5 different mobile phase compositions with their standard deviations. On the phenyl phase, a slight dependence of Δ log k' on ϕ is often observed in tetrahydrofuran and acetonitrile: more markedly in the case of glycosides. Similar behaviour had previously been observed on the C-18 phase (7). On the cyano phase, it can be seen that Δ log k' values are almost independent of mobile phase composition: the least retentive CN phase appears to be the least sensitive to changes in solute structure and solvent composition. Specific solute-stationary phase interactions on the different columns can be revealed by relating Δ log k' values for different column pairs. The resulting Δ log k' - Δ log k' correlations show good statistical coefficients (eqs. 1-9, Table VII).

TABLE V.
Mean Solvent Strength S of Methanol (MeOH), Acetonitrile (ACN) and Tetrahydrofuran (THF) on Phenyl and Cyano Columns.

	Compound	Phenyl column			Cyano column		
		MeOH	**ACN**	**THF**	**MeOH**	**ACN**	**THF**
1	Acacetin	4.8	3.3	4.3	4.8	3.7	4.0
2	Apigenin	5.0	3.8	4.7	3.7	4.2	3.9
3	Apigenin 7-O-glucoside	5.3	7.1	5.5	3.2	5.5	3.5
4	Apiin	5.3	7.1	6.6	3.4	6.2	3.7
5	Chrysin	4.5	3.5	4.2	4.0	3.6	3.8
6	Chryoeriol	5.0	3.7	4.5	4.0	4.2	4.0
7	Eriodictyol	4.3	3.7	4.2	2.4	3.6	2.9
8	Galangin	4.5	3.6	4.2	4.0	3.6	3.9
9	Luteolin	5.0	4.3	5.0	3.3	4.3	3.8
10	Luteolin 7-O-glucoside	5.3	8.3	5.9	3.2	6.2	3.8
11	Morin	5.0	4.7	5.0	2.9	4.5	3.1
12	Naringenin	4.3	3.7	4.3	2.9	3.3	3.1
13	Quercetin	5.0	4.0	4.7	3.2	4.3	3.3
14	Quercitrin	5.3	7.1	5.0	3.0	5.9	3.4
15	Rutin	5.3	7.3	4.3	3.0	8.3	3.9
16	Flavanone	4.2	3.5	3.7	3.3	2.7	3.1
17	Flavone	4.2	4.2	4.2	3.6	3.3	3.4
18	Flavanol	4.0	3.2	3.6	3.7	3.2	3.5
	Common mean and S. D.	4.8 ±0.5	4.7 ±1.7	4.7 ±0.8	3.4 ±0.6	4.5 ±1.4	3.6 ±0.4
	Aglycone mean and S. D.	4.6 ±0.4	3.7 ±0.4	4.3 ±0.4	3.6 ±0.6	3.7 ±0.5	3.5 ±0.4
	Glycoside mean and S. D.	5.3 ±0.0	7.3 ±0.5	5.7 ±0.6	3.2 ±0.2	6.4 ±1.1	3.7 ±0.2

TABLE VI.
Substituent Group Contributions to Retention (Δ log k')
with the Different Columns (Phenyl and Cyano) and Solvents (ACN and THF).
Data are reported as mean Δ log k' values with their standard deviations.

Group contribution	compounds	Phenyl ACN (0.2-0.4)*	Phenyl THF (0.3-0.5)*	Cyano ACN (0.1-0.4)*	Cyano THF (0.1-0.4)*
3-OH	1) 13-9	0.02±0.01	0.12±0.04	-0.02±0.02	0.08±0.01
	2) 18-17	0.08±0.03	0.23±0.07	0.08±0.02	0.19±0.02
	3) 8-5	0.02±0.02	0.16±0.06	0.03±0.03	0.11±0.01
3'-OH	4) 9-2	-0.20±0.05	-0.08±0.04	-0.13±0.03	-0.06±0.01
	5) 7-12	-0.22±0.04	-0.08±0.04	-0.15±0.02	-0.05±0.01
	6) 10-3	-0.21±0.04	-0.07±0.05	-0.14±0.03	-0.04±0.01
4'OH	7) 2-5	-0.35±0.05	-0.11±0.03	-0.20±0.01	-0.27±0.08
4'-OCH_3	8) 1-5	0.04±0.03	-0.02±0.02	0.03±0.04	-0.01±0.01
2, 3 unsaturation	9) 16-17	0.20±0.06	0.36±0.08	0.15±0.04	0.26±0.01
	10) 12-2	0.03±0.03	0.10±0.02	-0.04±0.08	0.07±0.01
	11) 7-9	0.03±0.01	0.08±0.02	-0.03±0.02	0.08±0.01
3-glycoside (rhamnose)	12) 14-13	-0.46±0.04	-0.53±0.11	-0.38±0.05	-0.33±0.01
3-glycoside (rutinose)	13) 15-13	-0.81±0.03	-0.80±0.18	-0.58±0.10	-0.44±0.02
7-glycoside (glucose)	14) 3-2	-0.65±0.06	-0.60±0.12	-0.48±0.05	-0.37±0.01
	15) 10-9	-0.66±0.03	-0.56±0.13	-0.47±0.09	-0.36±0.01
7-glycoside (apiosylglucose)	16) 4-2	-0.78±0.16	-0.74±0.12	-0.55±0.08	-0.43±0.01

* Mobile phase composition range.

TABLE VII.
Statistic Coefficients of Correlations between Mean Δ log k' Data Obtained for Different Stationary Phase and Modifier Pairs:
$\Delta \log k'_1 = A + B \Delta \log k'_2$.
R = correlation coefficient; $\sigma_{y.x}$ = standard error of the regression.
Data for methanol taken from Refs. 7 and 8.

Stationary phase pairs (1, 2)	Modifier	A	B	R	$\sigma_{y.x}$	Eq.
Phenyl-C-18	MeOH	-0.05±0.02	0.61±0.07	0.926	0.07	(1)
	ACN	-0.01±0.01	0.91±0.03	0.991	0.05	(2)
	THF	-0.03±0.02	0.95±0.05	0.982	0.06	(3)
Cyano-C-18	MeOH	-0.01±0.04	0.67±0.09	0.881	0.10	(4)
	ACN	-0.01±0.01	0.64±0.03	0.988	0.04	(5)
	THF	-0.01±0.01	0.62±0.03	0.984	0.04	(6)
Cyano-Phenyl	MeOH	0.02±0.04	0.99±0.16	0.854	0.11	(7)
	ACN	-0.01±0.01	0.71±0.02	0.992	0.03	(8)
	THF	0.01±0.02	0.62±0.05	0.954	0.07	(9)
Modifier pair (1, 2)	Column	A	B	R	$\sigma_{y.x}$	Eq.
ACN - MeOH	C-18	0.05±0.07	1.13±0.19	0.840	0.20	(10)
	Ph	0.12±0.06	1.71±0.21	0.906	0.15	(11)
	CN	0.02±0.02	1.04±0.13	0.907	0.10	(12)
THF - MeOH	C-18	0.13±0.08	1.06±0.23	0.781	0.24	(13)
	Ph	0.18±0.08	1.65±0.30	0.823	0.21	(14)
	CN	0.08±0.05	0.93±0.17	0.821	0.14	(15)
THF - ACN	C-18	0.10±0.02	0.99±0.05	0.980	0.07	(16)
	Ph	0.08±0.03	1.03±0.07	0.966	0.10	(17)
	CN	0.07±0.02	0.95±0.06	0.970	0.06	(18)

These equations appear particularly significant because they relate phenyl and cyano systems to the octadecyl column, whose retention behaviour has been widely described (4-8).
The intercepts obtained were near 0. For the pair phenyl - C-18 the slope values were near 1 when ACN and THF were the organic modifiers (eqs. 2, 3). According to Melander classification (11), this behaviour suggests a *homoenergetic* retention: identical retention energies are involved in the elution on the two stationary phases. When the retention values on the cyano phase were related to those on C-18 (eqs. 4-6), the slope values of 0.6 prove that the corresponding Gibbs energy for the two columns is not identical at a fixed temperature, rather it is proportional (*homeoenergetic* binding) (11): on the more polar CN phase, the retention energy is less than is found on the less polar C-18. A similar equation was obtained when Δ log k' - Δ log k' correlation was calculated for the pair Ph - C-18 when methanol was the organic modifier (eq. 1). This behaviour as *homeoenergetic* retention due to the solvent, can most likely be explained by considering that the solvent molecules participate with the stationary phase in determining solute retention. In fact, various retention models, describing the solute distribution between the mobile phase and the solvent-surface stationary phase, assume the stationary phase to be a thin layer of solvent, the composition of which is determined via molecular interactions with the solid support (12-15).
When the experimental Δ log k' values are compared with the calculated values (according to eqs. 1-6, Table VII) the resulting differences may measure the retention specificity of the given retention systems (14). Figures 2 and 3, reporting the difference values ($\Delta \log k'_{exp.} - \Delta \log k'_{calc.}$) for each substituent group, show that the most pronounced deviations, and therefore the strongest selectivity effects, were displayed by methanol as compared with acetonitrile and tetrahydrofuran. In particular, the stronger selectivity of the phenyl phase towards unsaturate compounds (higher positive deviations for substituent groups 9-11) was shown as well as the distinct specific retention of the C-18 phase towards the glycoside compounds (higher negative differences for substituents 12-14, 16). The positive deviations of unsatured compounds may be attributed to the increased specific interactions of these aromatic, more highly conjugated molecules with the phenyl groups of the stationary phase (16, 18). The dependence on the nature of the organic modifier may be explained on the basis of the existence of strong interactions (such as polar and hydrogen bonding) between the OH group of the adsorbed methanol and the polar solute molecules, also containing OH groups. Moreover, due to the restructuring of the stationary phase to form relatively ordered alkyl groups containing solvent molecules, the stationary phase seems to be more ordered in methanol than in other solvents, since methanol molecules are known to associate with one another by hydrogen bonding.
This peculiarity of methanol as organic modifier was further emphasized by the correlation between solvent pairs on each column (eqs. 10-18 in Table VII). The THF-ACN pair (eqs. 16-18) exhibited the best statistic parameters, intercept value near 0 and slope near 1. On the other hand, when acetonitrile and tetrahydrofuran were related to methanol (eqs. 10-15) the statistical quality of the correlation worsened and slope was at times different from 1. This means that

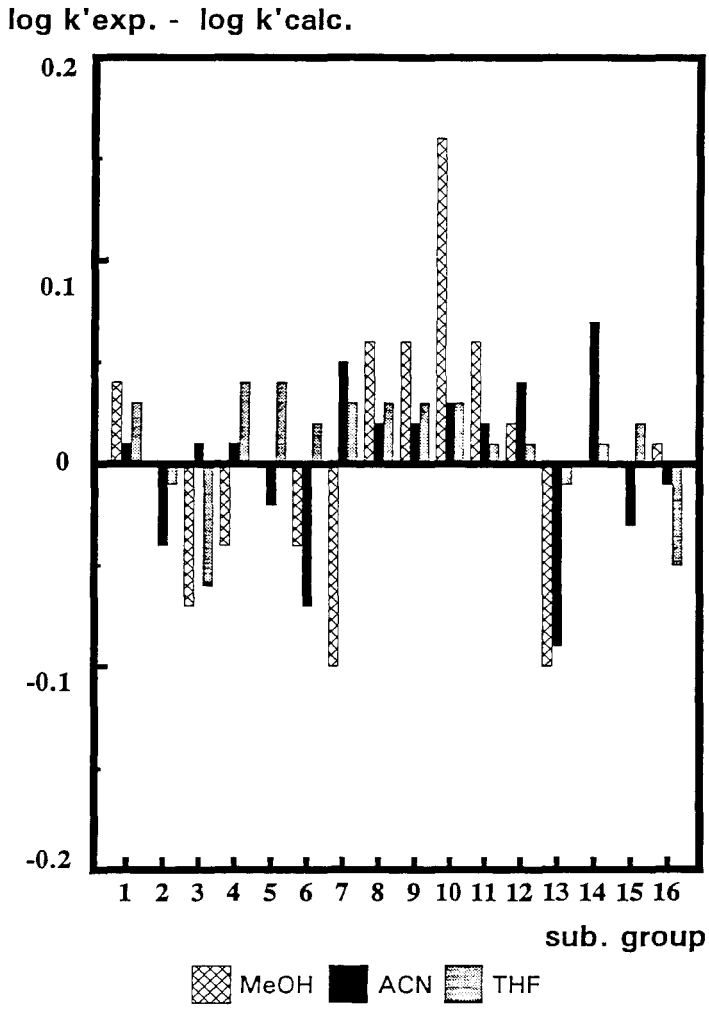


Figure 2: Δ log k'$_{experimental}$ - Δ log k'$_{calculated}$ difference values for various substituent groups calculated from the Δ log k' - Δ log k' correlation for the phenyl - C-18 column pair (eqs. 1-3 in Table VII).
Number of group contributions as in Table VI.

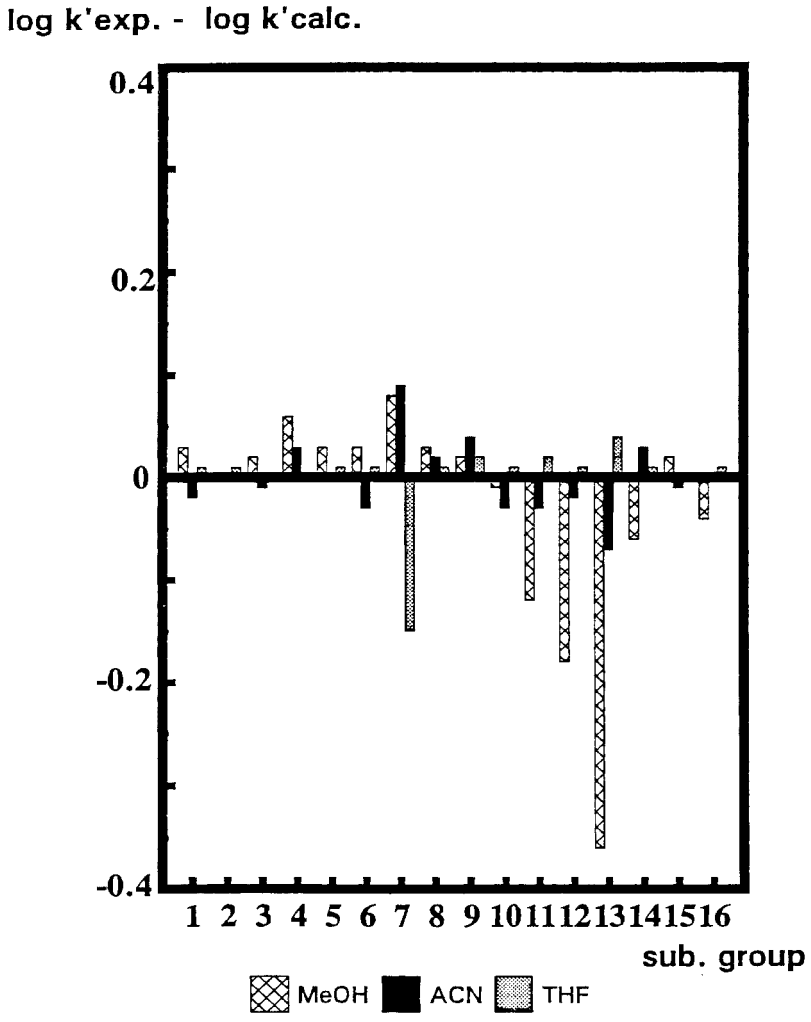


Figure 3: $\Delta \log k'_{experimental} - \Delta \log k'_{calculated}$ difference values for various substituent groups calculated from the $\Delta \log k' - \Delta \log k'$ correlation for the cyano - C-18 column pair (eqs. 4-6 in Table VII).
Number of group contributions as in Table VI.

pairing methanol elution with tetrahydrofuran and acetonitrile elutions, can provide two poorly correlated elution systems. This behaviour, valid on each column, is especially strong for the C-18 column.

CONCLUSIONS

This comparative study of retention properties of different RP chromatographic systems for flavonoid compounds made it possible to single out specific selectivity effects and their dependence on solute molecular structure. Detailed analysis of the selectivity of different column-solvent pairs would appear to be the guide-line for the choice of the most useful RP system to solve separation problems, when poorly correlated retention systems are required. In fact, when a complex mixture containing many flavonoid compounds is to be analyzed, overlapping retention is most likely on a single retention system, while the use of different, poorly correlated column-mobile phase different systems can reduce overlapping and improve detection and quantitation.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF GROUP 6 DIMETAL DECACARBONYL COMPLEXES SINGLY-BRIDGED BY DIPHOSPHINE LIGANDS

KAH-CHING TAN, T. S. ANDY HOR, AND HIAN KEE LEE*

Department of Chemistry
National University of Singapore
Kent Ridge
Republic of Singapore 0511

ABSTRACT

The normal-phase high-performance liquid chromatography of a series of Group 6 dimetal decacarbonyl complexes, of the form $(OC)_5M(P(CH_2)_nP)M(CO)_5$, where M = Cr, Mo and W, and $P(CH_2)_nP$ = diphenylphosphinyl bridge with n = 2-6, is reported. Chromatography was carried out on a stainless steel column packed with silica to which polar amino-cyano groups have been bonded; hexane-chloroform was used as mobile phase. The retention behaviour of these homometallic complexes is discussed.

INTRODUCTION

The first reported use of high-performance liquid chromatography (HPLC) for the separation of organometallic compounds in 1969 (1) has subsequently engendered widespread interest in, and applicability of, the technique in inorganic chemistry. Several reviews have been published in recent years, reflecting the amount of work done in the area (2-5). Metal carbonyl complexes, in particular, have received considerable attention (5).

*Author to whom correspondence should be addressed

Normal-phase and reversed-phase HPLC (in both isocratic and gradient elution modes) have been used for separating metal carbonyl complexes. Previously, we reported the separation of a series of *1,1'-bis*(diphenylphosphino)ferrocene (dppf)-substituted metal carbonyl compounds (6,7) on columns packed with silica particles bonded with polar amino-cyano groups. The bonded-silica stationary phase afforded a measure of selectivity to the separation of the aforementioned compounds which could not otherwise be resolved with columns packed with conventional silica. This paper reports further investigations on the use of such a stationary phase under normal-phase conditions in the separation of Group 6 dimetal decacarbonyl complexes.

The compounds considered in this work are of the form:

$$(OC)_5M(P(CH_2)_nP)M(CO)_5$$

where M = Cr, Mo and W, and $P(CH_2)_nP$ = *bis*(diphenylphosphinyl) bridge, *viz.* $[Ph_2P(CH_2)_nPPh_2]$ with n = 2 (dppe); 3 (dppp); 4 (dppb); 5 (dppp'), and 6 (dpph) (see Figure 1).

EXPERIMENTAL

Chromatographic separations were performed on a Shimadzu (Kyoto, Japan) Model LC-6A pump equipped with a Shimadzu Model SPD-6A variable wavelength UV spectrophotometric detector (with detection wavelength set at 254 nm), and a Whatman (Clifton, NJ, USA) Partisil 5 PAC (polar amino-cyano) column (5-μm particle size; 100 mm x 4.6 mm I.D.). Chromatographic data were collected and analysed on a Shimadzu Chromatopac C-R3A data processor. Eluent flow rate was 0.5 $mLmin^{-1}$. The binary mobile phase evaluated was hexane-chloroform, in the following compositions 93:7, 95:5 and 97:3 (%, v/v).

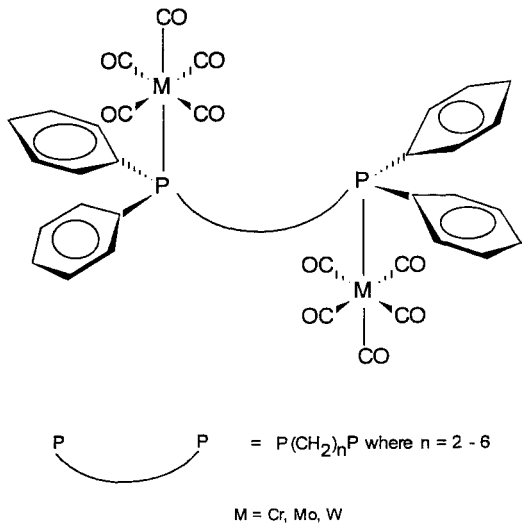

Figure 1 Structures of metal carbonyl complexes studied in this work.

All solvents (HPLC-grade, from various suppliers) were filtered through Millipore (Milford, MA, USA) membrane filters (0.45-μm pore size), and degassed by ultrasonication before use. Mobile phases were prepared by measuring exact volumes of the individual components and then mixing them to give the desired compositions.

Sample solutions were filtered before being introduced into the column using a Rheodyne (Cotati, CA, USA) Model 7125 injection valve. 5-10 μL of samples were injected. HPLC runs were carried out at least in triplicate. Reproducibility of retention times between runs was ±2% or better.

Typical procedures for the preparation of some of the compounds considered in this work follow the published method (8), and are briefly described below:

(a) $(OC)_5Mo_2(\mu\text{-dppf})$

Trimethylamine *N*-oxide (TMNO) (0.212 gm) was added to a suspension of $Mo(CO)_6$ (0.504 gm) in acetonitrile (20 mL) at 0°C. With vigorous stirring, a yellow colour steadily developed over 20 min. To this solution was added dppe (0.384 gm) and the resulting light-brown solution was kept at -15°C for 15 hr. The mixture was reduced in volume to about 10 mL and filtration yielded colourless crystals. The product was recrystallized from dichloromethane-methanol.

(b) $(OC)_5Cr(\mu\text{-dppb})Cr(CO)_5$

A mixture of $Cr(CO)_6$ (0.301 gm), dppb (0.263 gm) and TMNO (0.170 gm) in tetrahydrofuran (100 mL) was stirred for 2 hr. to give a green suspension. Evaporation to dryness gave a green residue which was redissolved in dichloromethane and applied onto a silica-coated TLC plate. Elution with dichloromethane-hexane (30:70) yielded the yellow product which was recrystallized from dichloromethane-methanol.

(c) $W(OC)_5W(\mu\text{-dpph})W(CO)_5$

$W(CO)_6$ (0.300 gm) and dpph (0.175 gm) were dissolved in tetrahydrofuran (100 mL). TMNO (0.099 gm) was then added. Completion of the reaction was indicated by the disappearance of the phosphine ligand when checked by TLC. The resulting yellow solution was evaporated to dryness under vacuum. The product was reconstituted in dichloromethane and precipitated by addition of excess methanol. The solid was purified by recrystallization from dichloromethane-methanol.

All the other complexes were synthesized under conditions slightly modified from those described.

Fresh solutions of the compounds (individual and in mixtures), shielded from light, were used for the HPLC. The integrity of the compounds (in solutions of chloroform), based on their carbonyl absorptions, was periodically checked by infrared spectroscopy.

For chromatographic analysis, solutions of all the complexes were prepared in hexane-chloroform (50:50). All the compounds considered exhibited acceptable and useful solubilities in only certain solvents or combinations of these solvents. Their insolubilities in solvents normally used for reversed-phase HPLC precluded their analysis by this mode.

RESULTS AND DISCUSSION

Three hexane-chloroform compositions (93:7, 95:5 and 97:3) were initially evaluated to optimize the separation of the complexes considered in this work. The 93:7 and 95:5 eluent mixtures did not provide satisfactory resolution of some of the components. Better separation was achieved by using the 97:3 mobile phase which was adopted for all subsequent separations. The capacity factors (k') of all the carbonyl complexes studied are listed in Table 1.

In a previous HPLC study (9) of related complexes, differences in electronegativity of the metal centres was used to rationalize the retention behaviour of the compounds. Basing this argument on the Allred-Rochow electronegativity scale (Cr = 1.56, W = 1.40, Mo = 1.30) (10), the order of increasing retention should be Mo-complex < W-complex < Cr-complex. This observation (9) was originally made with respect to $M_3(CO)_{12}$ (M = Fe, Ru and Os) and $M(CO)_6$ (M = Cr, Mo and W) complexes. On the basis of this aforementioned work, we attempted (6,7) to confirm a possible

TABLE 1
Capacity Factors (k') of Homometallic Diphosphine Complexes Obtained from Chromatography of Individual Components and Mixtures. Mobile Phase Composition: Hexane-Chloroform (97:3). Other Conditions as Given in Experimental Section. See Text for Explanation of Abbreviations.

	$(OC)_5M(dppx)M(CO)_5$		
	M = Cr	Mo	W
dppe	5.30	3.87	5.71
dppp	8.91	6.96	11.27
dppb	5.64	4.33	6.64
dppp'	7.03	5.58	8.77
dpph	6.40	4.98	7.87

electronegativity-retention correlation with related metal-carbonyl complexes possessing two or more metal centres and *1,1'-bis*(diphenylphosphino)ferrocene (dppf) as the ligand (the only structural variation being the type of metal centre involved), as in $(OC)_9Mn_2(dppf)M(CO)_5$ or $(OC)_5M(dppf)M(CO)_5$ (M = Cr, Mo, W). Our finding was that there was no unequivocal relationship; specifically, the W-dppf complexes exhibited greater retention than those of Cr and Mo. Similar observations were made in the present work for the $(OC)_5M(P(CH_2)_nP)M(CO)_5$ series (n = 2-6) as data in Table 1 show, *i.e.* in general, (reading along the rows), the W complexes exhibited the greatest retention followed by the Cr complexes, and finally the Mo analogues. The present observations thus argue against the exclusive use of metal electronegativities to account for the retention behaviour of these types of complexes.

Within a particular set of the five homometallic complexes, with each differing only in the number of CH_2 units, another possible parameter that could conceivably play a role in determining retention behaviour for the series of complexes considered in this work is molecular size. We have in the past speculated (7) on the influence this property might have in explaining retention characteristics of several metal-carbonyl

complexes with dppf as the ligand; however, we found the correlation tenuous. Similarly, when considering each of the three respective sets of homometallic species (*i.e.* where M is common) in the present work, absolute size may be ruled out as the sole contributor to retention behaviour as the capacity factors listed in Table 1 (reading down the table) show no obvious trend. Size, however, may play a secondary role, as explained below.

We next turned our attention to the consideration of relative polarities, as determined by overall dipole moments, of the complexes in the present study. Differences in polarity can best be illustrated by referring to Figure 2. In the complex with dppe as ligand, for example, the dipole moments along the (two sets of) methylene carbon and phosphorus bonds are equal and opposite to each other (*i.e.* the molecule has a centre of symmetry, *i*), producing a zero net dipole. When this is applied to the other diphosphine complexes, one can generalize that all the even-numbered CH_2 homologues show an *i* symmetry that nullifies the localised dipoles on the carbonyl and phosphine moeities. This results in these species possessing a zero dipole moment. On the other hand, the odd-numbered CH_2 homologues lack an *i* symmetry (although an approximate σ (mirror) plane exists in the less stable *syn* conformation), and thus have their dipole moments aligned in such a way that an overall dipole prevails for the molecule. The result of this is possession of polar character by the molecule. In effect, therefore, it is the relative polarity (based on *i* or lack of it thereof) of the complexes that appears to play the predominant role in determining retention behaviour. Accordingly, complexes with odd-numbered CH_2 homologues (*i.e.* where dppp, dppp' are the ligands) would be expected to be more retained under normal-phase HPLC conditions, than those with ligands containing even-numbered CH_2 units (*i.e.* dppe, dppb and dpph) which are fundamentally non-

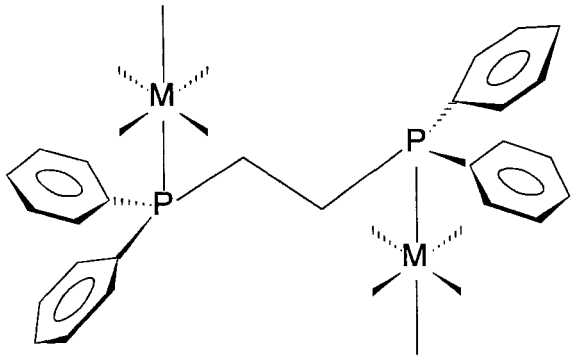

Figure 2 Dimetal decacarbonyl with *1,1'-bis*(diphenylphosphino)ethylene ligand.

polar. The data given in Table 1 support this explanation. While size (with respect to the length of the ligand methylene chain) contributes to retention behaviour (note the increase in retention corresponding with the increasing length of the even-numbered ligand chain), it is polarity, when it exists, that predominates. Note that the same order of retention is observed for different metal centres with the odd-numbered-CH_2 complexes exhibiting a sudden and sharper increase in retention compared to those containing ligands with even-numbered CH_2 units. Figure 3, which is a plot of $(OC)_5MPh_2P(CH_2)_nPPh_2M(CO)_5$ (n = 2-6) *vs.* capacity ratio with data from Table 1, makes clear this observation. We had previously observed (7), as pointed out above, that polarity played a more significant role than electronegativity, than hitherto surmised, in influencing retention behaviour for complexes of this nature. The results of the present study have provided a stronger case for this view.

It is evident from Figure 3 that the capacity factors of the dppp complexes show sharper increases than those of dppp'. With data from only two sets of odd-numbered CH_2 complexes, it is difficult to establish with certainty why this is so. However, it may

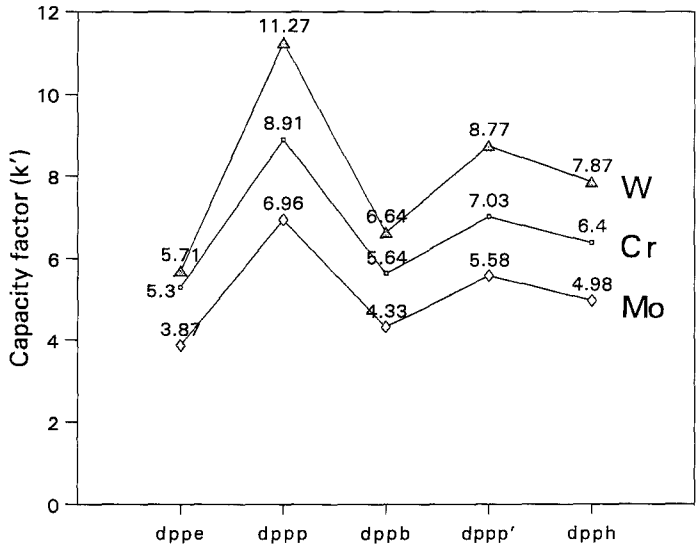

Figure 3 Plot of capacity factor (k') *vs.* $(OC)_5MPh_2P(CH_2)_nPPh_2M(CO)_5$ (M = Cr, Mo, W; n = 2-6)

be speculated that the dppp complex, with only three CH_2 units, has limited rotational motion, in contrast to the dppp' complex which has five CH_2 units. The bigger molecule could conceivably partake of a higher degree of twisting along the methylene linkage, providing individual dipoles within the molecule with the means of cancelling out. On the other hand, the smaller dppp complex, being more rigid, possesses a more definite and specific molecular dipole, elevating its relative polarity *vis-à-vis* the dppp' complex.

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SIMULTANEOUS DETERMINATION OF A NOVEL ANTIARRHYTHMIC AGENT, 7-BENZYL-3-THIA-7-AZABICYCLO[3.3.1]NONANE AND ITS SULFOXIDATION METABOLITE IN PLASMA AND URINE BY HPLC

C. L. CHEN[1], S. SANGIAH[1]*, H. CHEN[1], K. D. BERLIN[2], G. L. GARRISON[2], B. J. SCHERLAG[3], AND R. LAZZARA[3]

[1]*Department of Physiological Sciences and* [2]*Department of Chemistry*
Oklahoma State University
Stillwater, Oklahoma 74078
[3]*Department of Medicine*
OHSC
921 N.E. 13th Street
Oklahoma City, Oklahoma 73104

ABSTRACT

A HPLC method was developed for the simultaneous determination of the concentrations of 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane (I) as the hydrochloride and the corresponding sulfoxide (II), the major metabolite, in dog plasma and urine. Plasma and urine samples were alkalinized and extracted with chloroform. An aliquot was injected on to a HPLC system with a C6 reversed-phase column and an UV detector. Acetonitrile-methanol-37.5 m*M* phosphate buffer, pH 6.8 (28:28:44 v/v) containing 4.0 m*M* triethylamine was used as a mobile phase. The compounds I and II were detected at 261 nm. The extraction recovery for I and II was 85% and 94% from plasma and 89% and 91% from urine, respectively. Good linearity ($r>0.994$) was

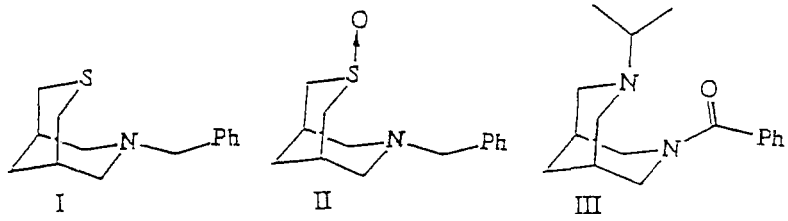
(*) Author to whom correspondence should be addressed.

observed throughout the range of 0.1-10.0 μg/ml for I and 0.04-10 μg/ml for II in plasma and in urine. Intra- and inter-assay variabilities were less than 8%. The accuracy of this method was > 95% for both compounds, and the limits of quantitation were 0.08 μg/ml for I and 0.03 μg/ml for II in plasma, and in urine, respectively. This method was applied to determine plasma and urine concentrations of I and II simultaneously in a dog treated with I.

INTRODUCTION

7-Benzyl-3-thia-7-azabicyclo[3.3.1]nonane (I) (Figure 1) has been demonstrated to have effective antiarrhythmic properties[1-4] and less proarrhythmic activity than those of lidocaine in dog models.[5] This drug could be classified as class Ib antiarrhythmic drug.[6,7] The significant elevation of systemic arterial blood pressure at the effective antiarrhythmic dose of I during sinus rhythm in dogs[2,4,5] could possibly be due to its inhibitory effects on myocardial Na^+,K^+-ATPase and Mg^{2+}-ATPase activities and its positive inotropic effects on atrial and papillary muscles.[8] Reduced proarrhythmic effects and little cardiac depressant actions make this compound a very promising candidate as an antiarrhythmic agent.

A HPLC method for determining the concentrations of 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane·$HClO_4$ in biological fluids has been developed.[9] The pharmacokinetics and plasma protein binding of I·$HClO_4$ in rats[10] and of I·HCl in dogs[11] have been characterized. In dogs, parent compound I·HCl was found to be extensively metabolized to form a major metabolite, 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane-3-oxide (II)[12] (Figure 1). However, no analytical methods are currently available to analyze II in biological fluids.



I II III

Figure 1. Chemical structure of I, II and III
I = 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane
II = 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane-3-oxide
III = 3-benzyol-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane

This paper describes a reversed-phase HPLC method for simultaneous determination of I and its major metabolite II in dog plasma and urine.

EXPERIMENTAL MATERIALS AND METHODS

Chemicals

The compounds I and II were synthesized by established methods.[1,13] All solvents used in this study were HPLC grade. All water used was purified through a Milli-Q™ water system (Millipore Corp., Marlborough, MA). Acetonitrile, methanol, chloroform, potassium phosphate monobasic (Fisher Chemicals, Fair Lawn, NJ), and triethylamine (Pierce Chemical Co. Rockford, IL) were used in this study. ß-Glucuronidase crude solution (G 0876) from *Helix pomatia* was purchased from Sigma Co. (St. Louis, MO). 3-Benzoyl-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane (III) was synthesized via a type of Mannich reaction starting from *N*-benzyl-4-piperidinone[14] and used as internal standard (I.S.).

Stock Solutions and Standards

Methanol stock solutions of I, II (1 mg/ml) and the I.S. (10 μg/ml) were stable for at least 2 months at 2-4°C. Known amounts of the solution were added to 0.25 ml of drug-free plasma and 0.50 ml of drug-free urine to obtain calibration curves in the range of 0.1-10 μg/ml for I and 0.04-10 μg/ml for II. Several standards were prepared for each curve.

HPLC Analysis

The HPLC system consisted of a Waters 501 HPLC pump, a Waters U6K universal liquid chromatography injector with a 2 ml injection loop, a Model 484 Tunable Absorbance Detector controlled by a Baseline 810 Chromatography Work Station with a NEC Powermate Sx plus a computer, and a NEC Pinwriter P5200 (Millipore, Milford, MA). A 250 x 4.6 mm Ultramex 5 C_6 (5 μm) and 30 x 4.6 mm Ultramex 5 C_6 guard column (5 μm) were purchased from Phenomenex (Torrance, CA). The mobile phase was acetonitrile-methanol-37.5 m*M* phosphate buffer, pH 6.8 (28:28:44 v/v) containing 4.0 m*M* triethylamine. The mobile phase was filtered through a 0.5 um Millipore filter and degassed before use. The column was eluted under isocratic conditions utilizing a flow rate of 1.2 ml/min at ambient temperature. The detection wavelength was 261 nm for I and II.

Animal Study

An adult, male mongrel dog (10.5 kg) was pre-conditioned for 7 days and was housed in a controlled environment (12-hour light/12-hour dark

photoperiod, 22±1°C, 60±10% relative humidity). The dog was allowed free access to food and water. Twenty-four hours before the experiments, food was withdrawn. The dog had free access to water before and during the experiments. The dog had polyethylene cannulas implanted in the right and left cephalic veins for collection of blood samples. The experiment was initiated between 0900 and 1100 h. Compound I was dissolved in water immediately before each experiment, and a dose of 20 mg/kg was administrated orally. Blood samples (ca. 5 ml) were collected via the cephalic vein catheter at 0, 5, 10, 30, 45 min and at 1, 2, 4, 6, 8, 12 hr after administration of I. After each sampling, the lost blood was replaced with an equal volume of sterile heparinized saline. Blood samples were heparinized, and centrifuged at 2000 x g for 10 min. Urine samples were collected from the dogs housed in a stainless steel metabolism cage at designated times, 0-2, 2-24, 24-48, 48-72 hr, via a urinary catheter. Both plasma and urine samples were stored at -20°C. Aliquots of 0.25 ml of plasma and of 0.5 ml urine were used for HPLC assay.

Extraction of I and II from Dog Plasma

Twenty five μl of 10 μg/ml of internal standard (III) were added to 250 μl of dog plasma. After alkalinization with 100 μl of 5 *M* NaOH, 5 ml of chloroform was added, and the mixture was mixed for 3 min. Following centrifugation (1000 x g 10 min), the organic phase was transferred into a clean test tube. The supernatant was reextracted with 1 ml of chloroform. The combined chloroform extracts were evaporated to dryness under a stream

of N_2. The solid residue was reconstituted in 50 μl of methanol, and 35 μl of this solution was utilized for HPLC analysis.

Extraction of Total (Free + Conjugated) I and II from Dog Urine

A crude solution of ß-glucuronidase (100,000 units of ß-glucuronidase and 1000-5000 units of sulfatase per ml) was used to hydrolyze the glucuronidate and sulfate of I and II. For the determination of total (free plus conjugated) I and II, aliquots of 0.5 ml of dog urine samples were subjected to enzymatic hydrolysis prior to extraction. The urine sample were adjusted to pH 5 with acetic acid, to which 0.1 ml of ß-glucuronidase crude solution was added, and then the solution was incubated at 37°C for 24 hr. The hydrolysate solution was adjusted to pH ±12 with NaOH (2 *M*) and then extracted with chloroform to yield total I and II.

Extraction Recovery

The samples (n = 5) were prepared to give final concentrations of 0.2 and 4 μg/ml in plasma and urine, respectively. Using the extraction procedure cited above, the samples were extracted in the absence of III (I.S). The ratio of the peak area of I and II extracted to that of unextracted equivalent concentrations of drugs under identical chromatographic conditions was calculated as extraction recovery.

Pharmacokinetic Analysis

Pharmacokinetic modelling and parameters were performed by PharmK program.[15]

RESULTS AND DISCUSSION

Extraction Efficiency

The same procedures described previously for extraction of I from biological fluids (9) was used to extract simultaneously I and II from plasma and urine. Alkalinization of plasma and urine samples increases extraction efficiency. The use of chloroform to precipitate proteins and to extract compounds I and II directly from plasma offered great advantage in that fewer pollutant peaks were found. Anticoagulants, such as EDTA and heparin, do not affect the extraction recovery. Extraction recoveries of compound I were 79-92% from plasma and 78-95% from urine and of compound II, 80-103% from plasma and 85-101% from urine, respectively (Table 1). The recoveries of I.S. were 85% from plasma and 90% from urine.

Chromatographic Separation

A modified chromatographic separation conditions[9] for I was used to separate I, II, and III (I.S). A minor change in the combinations of acetonitrile, methanol, buffer and triethylamine were evaluated as possible mobile phases. It was determined that the combination described in the method was found to be the most suitable for separating I and II. Under the described chromatographic conditions, a good separation of compound I, II and its internal standard was achieved. The retention times were 5.1 ± 0.3 and 10.3 ± 0.6 min for I and I.S., respectively. We were still unable to get a stable retention time for I,[9] but the retention time for I was determined to be 14-19 min. With these retention times, the drug I, metabolite II, and its I.S. were

TABLE 1
Linearity and recovery of compound I and II added to plasma and urine

Samples	Range (μg/ml)	n	Linear Correlation[a]	r	CV[b] (%)	m[c]
I in plasma	0.1-10	35	Y= 0.05484+11.461X	0.995	1.2-10.1	85
I in urine	0.1-10	35	Y= 0.11655+5.5174X	0.994	3.5-9.7	89
II in plasma	0.04-10	35	Y=-0.19556+3.1716X	0.997	2.8-11.2	94
II in urine	0.04-10	35	Y=-0.10697+3.0815X	0.995	1.8-9.7	91

[a] Y was drug recovered in μg/ml, X was peak area ratio (drug/I.S.).
[b] CV = coefficient of variance
[c] m = mean recovery

eluted without interference peak originating in blank plasma and urine (Figure 2 and 3). The effects of varying proportions of triethylamine and pH value in the mobile phase on separation were similar to that in the analysis of I and other 3,7-diheterobicyclo[3.3.1]nonane analogues. [9,16-18] The effect of these variables were not only on the retention time but also on the sharpness of the peaks for compounds I and II. Like other DHBCN (3,7-diheterobicyclo-[3.3.1]nonane) analogues,[9,16-18] neither acetonitrile nor methanol alone is suitable as a strong solvent for the separation of I and II. It was found that minor changes in the combination of acetonitrile, methonal, triethylamine and buffer (pH 6.8) could provide a very good mobile phase for analyzing several DHBCN analogues.[9,16-18]

Assay Validation

Linearity. Five consecutive standard curves for pure I and II analyzed on separate days demonstrated a good linear relationship between

concentration and peak area. The standard curves obtained from extraction of dog plasma and urine containing known amounts of I were linear ($r>0.996$) over the concentration ranges tested. The range of coefficient of variations was between 1-12%. The calibration curves were found to be linear (Table 1). The limits of quantitation of I and II were 80 ng/ml and 30 ng/ml for plasma and for urine, respectively. This sensitivity is adequate for use in the analysis of pharmacokinetics data of dog plasma and urine after administration of compound I or II.

Precision and accuracy. The results obtained indicate that intra- and inter-assay coefficient of variance (C.V.) in plasma and urine was less than 8%. The accuracy of this method was 89-97%. These results suggest that the proposed procedure is satisfactory with respect to both accuracy and precision.

Applications to Dog Samples

The internal standard was added to the dog plasma and urine samples, and samples were extracted as previously described. Representative HPLC profiles of the plasma and urine samples of a dog given I (20 mg/kg) orally are shown in Figures 2 and 3.

The plasma concentration-time profiles of I and II in one dog given an oral dose of 20 mg/kg are shown in Figure 4 and are fitted to a one compartment open model. There were 3.22% and 15.6% of I and II excreted into urine with respect to the oral dose. The area under the curve for II is 1.77 times of that of I, indicating that I undergoes extensive metabolism. A recent drug metabolism study[12] showed that I is present in the free state, while

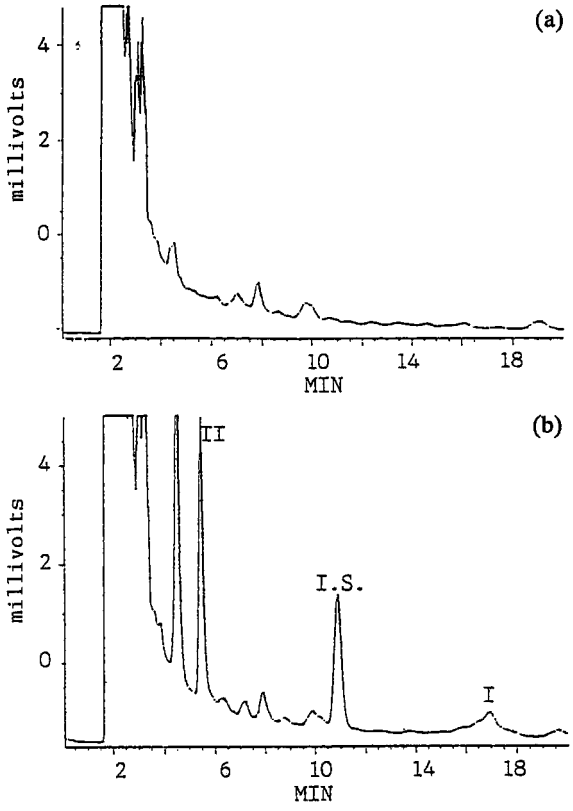


Figure 2. Representative chromatograms from (a) blank plasma and (b) plasma sample 30 min after oral dose of 20 mg/kg of I to a dog. See experimental for chromatographic conditions. The estimated concentration of I and II was 1.2 μg/ml and 3.5 μg/ml, respectively. I.S. = Internal Standard

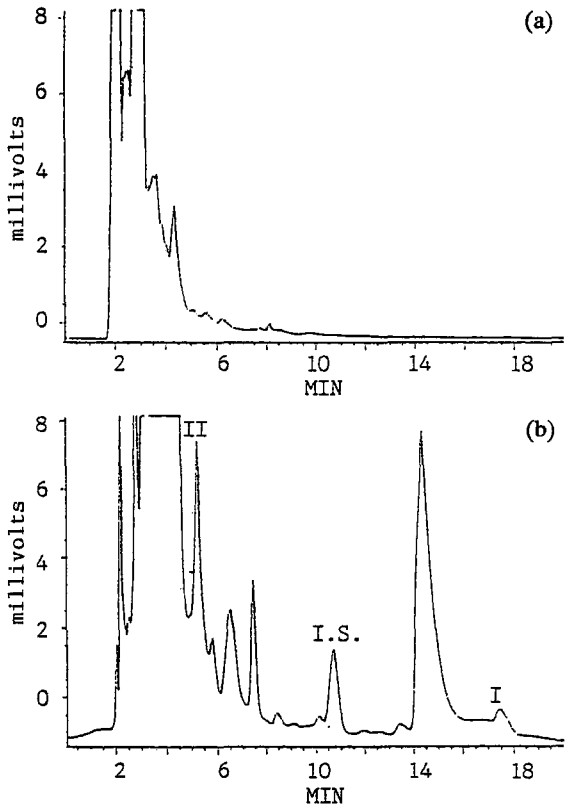


Figure 3. Representative chromatograms from (a) blank urine and (b) urine sample 1 hr after oral dose of 20 mg/kg of I to a dog. See experimental for chromatographic conditions. The estimated concentration (free + conjugated) of I and II was 3.1 μg/ml and 4.2 μg/ml, respectively. I.S. = Internal Standard

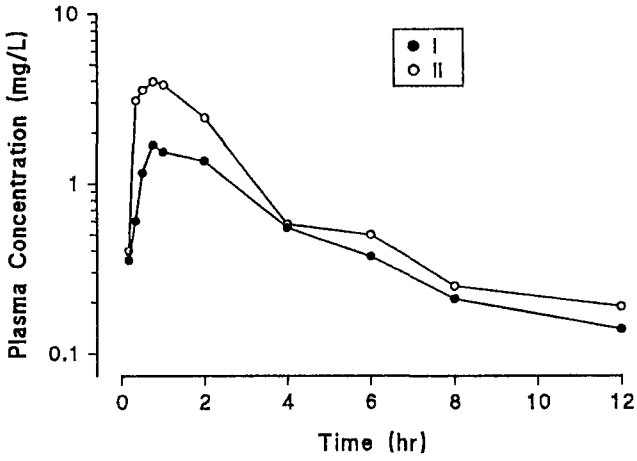


Figure 4. Plasma I and II concentration profile after oral dose of 20 mg/kg in one dog.

II is predominantly present in the form of glucuronide and/or sulfate conjugates in urine.

The results show that the HPLC method described above has a lower quantitation limit of 0.08 μg/ml for I and 0.03 μg/ml for II using a sample volume of 250 μl. As shown in this report, this method is suitable for simultaneous pharmacokinetic studies of this novel antiarrhythmic agent and its S-oxidation metabolite II. In addition, this method can also be used to isolate II and quantitate it from the biological samples containing only II.

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ENANTIOMER SEPARATION OF 2-ARYLPROPIONIC ACIDS ON AN ERGOT ALKALOID-BASED STATIONARY PHASE MICROBORE COLUMN APPLICATION

L. CASTELLANI[1], M. FLIEGER[2], AND M. SINIBALDI[1]

[1]Istituto di Cromatografia
CNR - Area della Ricerca di Roma
P.O. Box 10
I-00016 Monterotondo Stazione
Rome, Italy
[2]Institute of Microbiology
Academy of Sciences of the Czech Republic
Videnskà 1083
C-142 20 Prague 4, Czech Republic

ABSTRACT

The separation of enantiomers of underivatized ketoprofen, as well as a number of other non-steroidal anti-inflammatory drugs, was studied by high performance liquid chromatography using a recently developed chiral stationary phase packed in a microbore column. Electrostatic and π-π interactions are assumed to control the retention of the analytes. The enantioselectivity remains constant in a wide range of organic modifier concentration, and is restricted to a pH window ranging from 2.5 to 5.0.

INTRODUCTION

2-Arylpropionic acids (profens) represent an important class of non-steroidal anti-inflammatory drugs (2-APA-NSAIDs), all commercialised as

racemic mixtures, with the exception of naproxen, which is used as the pure (S)-isomer. It is well known that only the (+)(S)-form of 2-APA-NSAIDs exhibits biological activity (1). Recent pharmacodynamic studies have shown the (R)-isomer undergoes metabolic unidirectional chiral inversion to its pharmacologically active (S)-antipode (2-5). Use of optically pure profens should permit the reduction of the dosage as well as avoid any toxic effect arising from non-stereospecific mechanisms. Therefore separation methods of profen enantiomers are of growing interest, and chiral separations of these compound by HPLC have been in detail reviewed (6-8). Among such methods the direct ones appear to be of great applicability for the optical purity control in drug production, routine pharmacokinetic analyses, in the field of enzyme stereo-catalysed reactions, as well as in preparative scale separations. 2-APA-NSAIDs are well resolved by LC without derivatization on chiral columns based on α_1- acid glycoprotein (9), bovine and human serum albumin (10,11), ovomucoid (12), cyclodextrins (13), tris(3,5-dimethylphenylcarbamate) derivatives of cellulose and amylose (14). A CSP specially designed for the resolution of underivatized profens has been recently reported by Pirkle et al. (15).

We have recently described the synthesis of an ergot alkaloid based chiral stationary phase for the resolution of optical isomers by HPLC (16,17) and interpreted by ^{1}H NMR spectroscopy the enantioseparation in terms of electrostatic and π-π interactions (18). This CSP showed to provide good resolution for a series of carboxylic group containing compounds, including amino acid derivatives (19) and underivatized dicarboxylic and arylpropionic acids. We now report the use of this CSP for the resolution of underivatized NSAIDs on a microbore column with the aim of studying the effect of mobile phase parameters on the resolution.

EXPERIMENTAL

Materials and methods

The CSP was prepared using silica gel Exsil 100 (Scientific Glass Engineering, Milton Feynes, UK) (particle size 5 μm, average pore diameter 100 Å), 3-glycidoxypropyltrimethoxysilane (Serva, Heidelberg, Germany) and (+) 1-(3-aminopropyl)-(5R,8S,10R)-terguride (AMP-TER) according to the previously described procedure (16).

Conventional slurry procedure was used to pack a microbore column (500 x 1.1 mm i.d.).

Racemic suprofen was purchased from Sigma (St. Louis, MO, USA). Ibuprofen and flurbiprofen were extracted from commercially available drugs (Nurofen® and Froben®, respectively) using $CHCl_3$ of analytical grade. (R,S)-Fenoprofen, (R,S)-ketoprofen, and (R,S)- and (S)-naproxen were a kind gift of Prof. E. Cernia, Department of Chemistry, "La Sapienza" University of Rome. All solvents were of HPLC grade.

Instrumentation

The chromatographic apparatus consisted of a Perkin Elmer (Norwalk, CT, USA) Model Series 410 LC solvent delivery pump equipped with a Rheodyne Model 7520 internal loop injection valve (0.5μl), and connected to a Kontron (Milan, Italy) Model 433 UV capillary detector.

RESULTS AND DISCUSSION

The previously obtained results (17) led us to study in more detail the parameters influencing the enantioseparation of underivatized 2-APA-NSAIDs on AMP-TER based chiral stationary phase.

The influence of the organic modifier content in the mobile phase on the separation of naproxen enantiomers was examined under constant values of both, ionic strength and pH. The corresponding capacity and enantio-selectivity factors are reported in Table I. The plot of ln k' vs acetonitrile content (Fig. 1) indicates decreasing retention with increasing concent_ ration of organic solvent, which is in good agreement with reversed phase mechanism.

The influence of the buffer pH on the retention for six 2-APA-NSAIDs was examined and chromatographic data are summarised in Table II. Data show that chiral recognition operates in a pH range between 2,5 - 5.0, and is influenced by the nature of the aryl moiety and dissociation degree (pK_a) of the carboxylic acids. Separation of all profens was observed in the whole examined pH range, with the exception of ibuprofen and flurbiprofen, which have not been resolved at pH < 3. Separation factors (α) remain substantially constant for all compounds. An example of baseline enantio-separation of fenoprofen is given in Figure 2.

The plots of ln k' vs pH of the buffer for ibuprofen, fenoprofen and suprofen enantiomers are shown in Figure 3. All pairs of enantiomers eluted with similar retention trend (maximum at pH 4) which is the most probably the result of two interactions involved in the adsorption process. First one could be ascribed to the increased dissociation of the analyte acids, in accordance with the pK_a values. (9), resulting in a stronger electrostatic interaction with the protonated nitrogen atoms in the aminopropyl-terguride molecule. At pH > 4 electrostatic interaction becomes less significant owing to the lower protonation of the selector, and second interaction (hydrophobic) prevails thus resulting in an overall

TABLE 1
Effect of Acetonitrile Content in Mobile Phase on Capacity Factors (K'), Enantioselectivity (α) and Resolution (R) for Naproxen Enantiomers. Buffer, 0.05 potassium acetate, pH = 4.0.

% AcN	K_R'	K_S'	$\alpha^{\#}$	$R^{\varnothing}$
30	12.6	13.1	1.04	0.85
40	7.7	8.0	1.04	0.81
50	5.3	5.6	1.04	0.77
60	3.3	3.5	1.06	1.04

$^{\#}\ \alpha = K_S / K_R$, $^{\varnothing}\ R = \sqrt{N}/4) \times [(\alpha - 1)/\alpha] \times [K'/(K'+1)]$

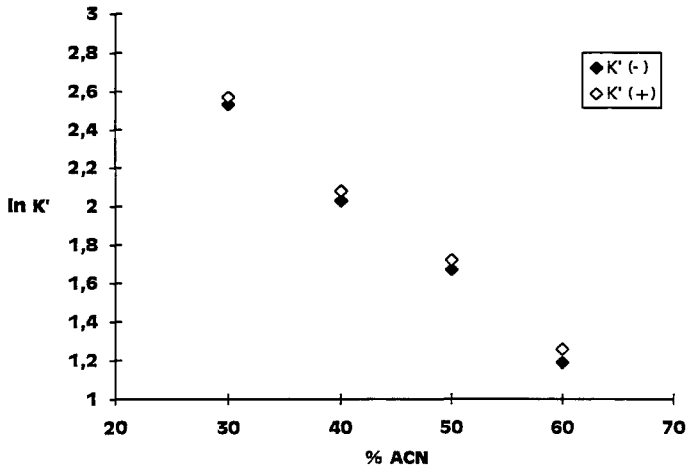

FIGURE 1 Plot of ln k' *vs* acetonitrile content of the mobile phase for naproxen enantiomers. Chromatographic conditions: column, 50 x 0.1 cm i.d.; buffer, 0.05 M potassium acetate at pH 4.0; flow rate, 80 µl / min; UV detector set at 254 mn.

TABLE 2

Capacity (K') and Enantioselectivity Factors (α) of NSAIDs Enantiomers as a Function of pH. Chromatographic Conditions: column, 50 X 0,1 cm I.D.; buffer, 0.05 M potassium acetate - acetonitrile (50:50 v/v); flow-rate, 80 µl/min; detection UV, 254 nm.

		K_R' (ln K_R')	K_S' (ln K_S')	α
	IBUPROFEN	1.9 (0.64)	1.9 (0.64)	1.00
	FENOPROFEN	3.5 (1.25)	3.7 (1.31)	1.06
pH = 2.9	NAPROXEN	3.9 (1.36)	4.0 (1.39)	1.03
	KETOPROFEN	5.8 (1.76)	6.1 (1.81)	1.05
	FLURBIPROFEN	5.7 (1.74)	5.7 (1.74)	1.00
	SUPROFEN	6.1 (1.81)	6.3 (1.84)	1.03
	IBUPROFEN	2.4 (0.87)	2.5 (0.92)	1.04
	FENOPROFEN	4.2 (1.43)	4.5 (1.50)	1.07
pH = 3.2	NAPROXEN	4.8 (1.57)	5.0 (1.61)	1.04
	KETOPROFEN	---	---	---
	FLURBIPROFEN	7.0 (1.94)	7.2 (1.97)	1.03
	SUPROFEN	8.8 (2.17)	9.1 (2.21)	1.03
	IBUPROFEN	2.7 (0.99)	2.9 (1.06)	1.05
	FENOPROFEN	---	---	---
pH = 3.6	NAPROXEN	5.4 (1.69)	5.7 (1.74)	1.05
	KETOPROFEN	---	---	---
	FLURBIPROFEN	6.7 (1.90)	6.9 (1.93)	1.03
	SUPROFEN	9.3 (2.23)	9.8 (2.28)	1.04
	IBUPROFEN	4.0 (1.37)	4.2 (1.42)	1.04
	FENOPROFEN	5.5 (1.70)	5.8 (1.76)	1.05
pH = 4.0	NAPROXEN	5.3 (1.67)	5.6 (1.72)	1.04
	KETOPROFEN	7.5 (2.01)	7.9 (2.07)	1.05
	FLURBIPROFEN	7.5 (2.01)	7.7 (2.04)	1.03
	SUPROFEN	9.9 (2.29)	10.3(2.33)	1.04
	IBUPROFEN	2.5 (0.92)	2.6 (0.95)	1.04
	FENOPROFEN	4.3 (1.46)	4.6 (1.53)	1.07
pH = 4.5	NAPROXEN	5.5 (1.70)	5.7 (1.74)	1.04
	KETOPROFEN	6.0 (1.79)	6.3 (1.84)	1.05
	FLURBIPROFEN	5.4 (1.69)	5.6 (1.72)	1.04
	SUPROFEN	5.8 (1.76)	6.0 (1.79)	1.03

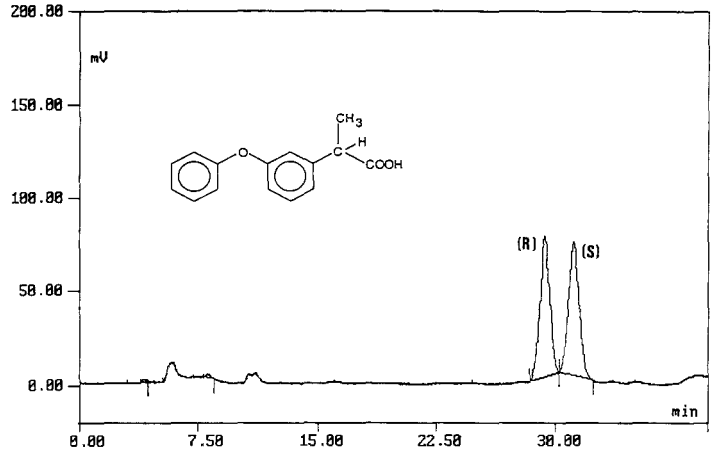

FIGURE 2 Chiral separation of (R)- and (S)- fenoprofen enantiomers. Chromatographic conditions: 0.05 M potassium acetate buffer (pH 4.6) / acetonitrile (60:40 v/v); flow-rate 80 µl/min; λ, 254 nm.

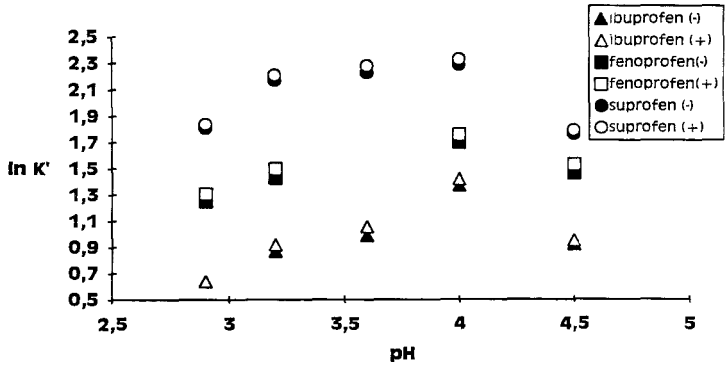

FIGURE 3 Plots of ln k' vs pH values of the acetate buffer for a serie of 2-APAs enantiomers. Chromatographic conditions as on figure 1.

decrease of retention. These observations are consistent with ^1H NMR spectroscopic data (18), obtained for aqueous equimolar mixtures of AMPTER and (R,S)- and (R)-naproxen, individualising the positively charged nitrogen N(6)-CH_3 and H(12), H(13),and H(14) of the ergine skeleton as the most significant sites of interactions. Accordingly, experimental results may be explained assuming the formation of diastereoisomer complexes. N(6) represents an anchoring point for the carboxylic group of the analyte, allowing to its aryl moiety a π-π stacking with the ergoline structure. Study in progress, separation of different derivatives of amino acids (19), should further confirm this proposed mechanism of chiral recognition process.

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SEPARATIONS OF MAJOR SOYBEAN PHOSPHOLIPIDS ON β-CYCLODEXTRIN-BONDED SILICA

S. L. ABIDI*, T. L. MOUNTS, AND K. A. RENNICK
Food Quality and Safety Research
National Center for Agricultural Utilization Research
Agricultural Research Service
U.S. Department of Agriculture
1815 North University Street
Peoria, Illinois 61604

ABSTRACT

The four major phospholipids (PL) phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA) found in soybean oil were separated by normal-phase high-performance liquid chromatography (HPLC) on β-cyclodextrin-bonded silica (CDS) with UV detection. Adequate base-line separations of the PL components were achieved by isocratic elution with mobile phases containing hexane, isopropanol, ethanol and water/tetramethylammonium phosphate (TMAP). The presence of TMAP in the mobile phases was critical to improve component resolution and enhance peak symmetry. Analyte retention and component separations were dramatically influenced by a small change in mobile phase compositions. Under the HPLC conditions employed, the elution order appeared to follow the order of increasing PL polarity with increasing retention times (PE < PC < PI < PA). The HPLC method was used in the qualitative analyses of selected

*Author to whom correspondence should be addressed.

commercial lecithin samples. Potential applicability of the HPLC-UV-CDS technique for the quantitative analysis of several crude oil samples derived from genetically modified soybean oil is demonstrated.

INTRODUCTION

Phospholipids (PL) are important physiological substances naturally occurring in animal and plant cell membranes. Our on-going research directed toward assessment of the impact of genetic engineering on quality of vegetable oils, required continuous development of new chromatographic techniques for the analysis of PL in oil samples. Accurate analysis of the polar lipid compositions and molecular species distributions in modified oils provides useful information on the stability and quality of the oil. Although there are many publications in the literature on analytical methodology for the quantification of PL in a wide variety of tissue samples derived from plants and animals, high-performance liquid chromatographic (HPLC) separations of PL mixtures are heavily dependent on experimental conditions employed, specific sample matrices used, and the nature and complexity of PL structures in the mixtures for particular studies. Therefore, methodological alternatives to published HPLC procedures continue to be of much interest to lipid scientists.

Mixtures containing various PL classes have been separated by normal-phase HPLC (1-23). Most PL

separations have been carried out on silica (SI) columns. To a lesser extent, some researchers have used columns of silica-based polar phases such as amino- (15, 19-21), diol- (22, 23) and benzene sulphonate-bonded silica (7) for PL analyses. In recent years, another type of polar stationary phase, cyclodextrin-bonded silica (CDS) columns have become available commercially and have been used extensively in chiral separations. However, the CDS phases have not been utilized for the separation of polar lipids. In view of the wide applicability of the CDS column for the analysis of a broad spectrum of compounds (24) including tocopherols (25), the normal-phase HPLC behavior of soybean PL components on CDS was studied under mobile phase conditions similar to the hexane-isopropanol-water solvent systems conventionally used in conjunction with a SI column. The results are reported in this paper.

EXPERIMENTAL

Materials:

Reference soybean PL standards phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), lyso-PC, lyso-PE, and egg phosphatidic acid (PA) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Commercial soybean lecithin samples were supplied by Lucas Meyer Inc. (Decatur, IL, USA). Soybean oil samples derived from

genetically modified soybeans (supplied by North Carolina State University, NC, USA) were subjected to silica gel column chromatography following a published procedure (26) to give analytical samples for soybean PL assays. Tetramethyl ammonium phosphate (TMAP) was prepared from a 25% aqueous solution of tetramethyl hydroxide (Aldrich Chemical Co., Milwaukee, WI, USA) by titration with 85% phosphoric acid (Fisher Chemicals, Fair Lawn, NJ, USA) until the solution reached Ph 6.3. Chromatography-grade hexane and isopropyl alcohol (ISP) were obtained from Fisher Chemicals. Ethyl alcohol (ETOH) was the product of U.S. Industrial Chemical Co. (Cincinnati, OH, USA). HPLC-quality water was obtained by purification of distilled water through a Milli Q water purifier (Millipore Corp., Bedford, MA, USA). All other reagents and solvents were purchased from J. T. Baker, Inc. (Phillipsburg, NJ, USA).

Methods:

In all HPLC experiments, a Spectra-Physics (San Jose, CA., USA) liquid chromatograph equipped with a Model SP8700 solvent delivery system was used. The LC instrument was interfaced with an LDC Analytical (Riviera Beach, FL, USA) SpectroMonitor D variable wavelength UV detector which was set at 208 nm for monitoring column effluents. Mobile phases employed

three solvent reservoirs containing (1) hexane, (2) ISP-ETOH, and (3) water/5 Mm TMAP (Ph 6.3). Solvent proportions were controlled by a built-in solvent programmer and a dynamic mixer of the LC instrument. Two ISP-ETOH solutions in ratios of (67:33, v/v) and (55:45, v/v) were used in this study to obtain reasonable separations of the PL components. Throughout the HPLC experiments, the mobile phase eluents were filtered through a 0.2 μm filter, degassed with a helium sparge, and pumped isocratically through a β-CDS column at a flow rate of 1 ml/min.

The β-CDS stationary phase was prepacked commercially with β-CDS of 5 μm spherical particles in a stainless steel column (250 x 4.6 mm ID) (Advanced Separation Technologies, Inc. Whippany, NJ, USA). Aliquots (0.5-1 μl) of samples in chloroform solutions (1-3 mg /ml) were injected onto the column via a Rheodyne (Cotati, CA, USA) Model 7125 injector fitted with a 10-μl loop. Capacity factors (k') were determined as $k' = t/t_0 - 1$, where t and t_0 represent respective retention times of a PL analyte and an unretained solute. Separation factors (α) for adjacent HPLC peaks were determined as $\alpha = k'_{c+1}/k'_c$, where subscript "c" represents a PL component.

Calibration curves for soybean PE, PI, and PC were constructed separately by plotting amounts (μg) of the

individual polar lipids against peak areas (cm^2). For PA quantitation, fractions containing pure soybean PA were collected from column effluents. The isolated PA samples were then subjected to phosphorus analysis following a published procedure (27). A linear calibration plot for PA was then prepared from the HPLC peak areas and the amounts of analytes as determined by phosphorimetry for phosphorus contents.

A mixture of PL standards and a genetically modified soybean oil sample were analyzed by normal phase-HPLC on an EM Separations (Gibbstown, NJ, USA) LiChrospher Si 60/II column (250 x 4 mm ID, 3μm spherical particles). The HPLC experiment was conducted using a linear gradient with mobile phase solvents (A) chloroform-t-butyl methyl ether (75:15) and (B) methanol-chloroform-ammonium hydroxide (92:1:7). The gradient solvent program was run as follows: 100% solvent (A) was gradually changed to 100% solvent (B) over a 30 min linear gradient, and was then held at solvent (B) for 10 min before returning to 100% solvent (A) in a 10 min linear gradient. The mobile phase flow rate was 0.5 ml/min. A Varex (Burtonsville, MD, USA) evaporative laser light scattering detector Model ELSD II was used for all gradient HPLC work. The ELSD instrument parameters used in this study were the same as those set for previous work (26).

RESULTS AND DISCUSSION

In the early phase of this work, attempts to separate the four major soybean PL (FIGURE 1) on a CDS column with a wide range of mobile phase systems were unsatisfactory. First, HPLC-UV of PL inevitably suffers from limitations of required low UV-absorbing solvents for mobile phases. As common practice, the UV detector is normally set at 208 nm in most HPLC-UV work on polar lipids. Accordingly, mobile phase solvents chosen initially for the present study were (1) a hexane-ISP-water system and, (2) an acetonitrile-methanol-water system because both were nearly transparent at the low UV range. HPLC of mixtures of standard PL samples with different combinations of hexane, ISP, and water without mobile phase additives led to either ill-defined peaks or unresolved broad bands. In addition, acetonitrile-methanol-water based solvent mixtures were too polar for the PL components to be adsorbed on the CDS phase. However, addition of TMAP to the hexane-ISP-water mobile phases significantly improved the separation of the polar lipids, although component peaks remained broad. The undesirable peak shape problem was solved by incorporation of ETOH into the hexane-ISP-water/TMAP system, which markedly sharpened the chromatographic peaks.

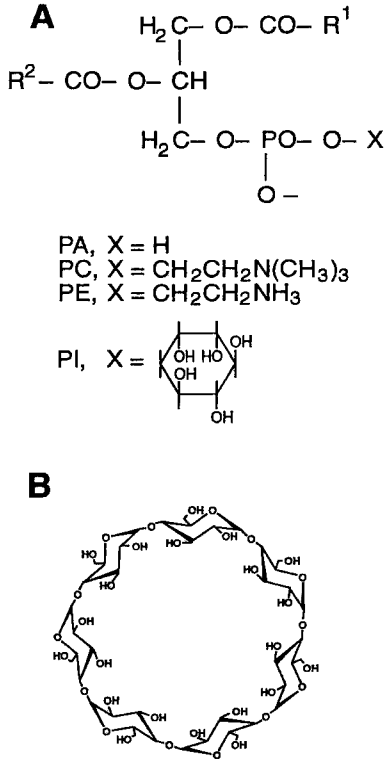

FIGURE 1. (A) Structures of soybean phospholipids PE, PC, PI and PA. (B) Structure of β-CD.

Unlike component separations in the reverse-phase mode where solutes interact with the CDS via inclusion complex formation (28), the retention behavior of the PL solutes in normal phase HPLC is believed to be due to solute adsorption to the hydroxyl-surface of the β-cyclodextrin entity (29) whose structure is depicted in FIGURE 1B. Accordingly, in HPLC of a standard soybean

PL mixture with a mobile phase of hexane-ISP-ETOH-water/5 Mm TMAP(Ph 6.3) (35 : 32.7 : 26.8 : 5.5), PI was found to be more strongly adsorbed to the CDS phase than PC (FIGURE 2A). Under the HPLC conditions employed, the PL components eluted from the CDS column in the order of increasing polarity and retention times: k'-PE < k'-PC < k'-PI < k'-PA (FIGURE 2A).

On the other hand, normal-phase HPLC-ELSD detection of a standard mixture of soybean PL on a SI column in the gradient mode with a mobile phase consisting of (i) chloroform/t-butyl methyl ether, and (ii) methanol/chloroform/ammonium hydroxide (see EXPERIMENTAL for details of the gradient elution program) led to an elution order (FIGURE 2B) that was different from that found in the HPLC-UV-CDS work described above. The PL components eluted from the SI column in accordance with the following order of increasing retention times: k'-PE < k'-PI < k'-PA < k'-PC. In this situation, PC was apparently more strongly adsorbed to the SI phase than both PI and PA.

TABLE 1 summarizes results obtained from HPLC-UV-CDS separations of a standard PL mixture studied under various mobile phase conditions. Examination of the retention data indicated that both k' and α values were sensitive to changes in the ratio of ISP-ETOH-water/5 Mm TMAP(Ph 6.3) solvents even though the hexane content

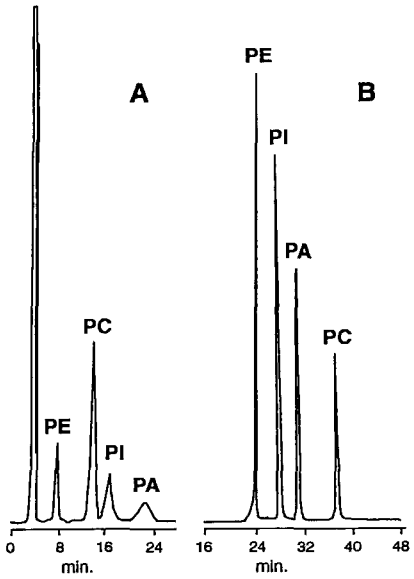

FIGURE 2. Separations of mixtures of PL standards (A) by HPLC-UV under isocratic elution on a β-CDS column; (B) by HPLC-ELSD under gradient elution on a SI column. Mobile phases: (A) [hexane-ISP-ETOH-water/5 Mm TMAP(Ph 6.3)] (35: 32.7: 26.8: 5.5); (B) See EXPERIMENTAL for gradient solvent systems.

was kept at a constant value of 35%. HPLC with a lower water content in the mobile phase [experiment (v), TABLE 1] tended to give more evenly dispersed component peaks with a smaller range of α values (1.42-2.01). However, the retention times (higher k' values) of the PL components were too long to be of any analytical value.

FIGURES 3A-D present typical examples of normal-phase HPLC-UV-CDS separations of PL in selected oil

TABLE 1
Effects of HPLC Mobile Phase Solvent Compositions [hexane-ISP-ETOH-water/5 Mm TMAP (35:x:y:z) on Capacity Factors, k', and Separation Factors, α, of The PL Components of Interest (UV detector at 208 nm; CDS column)

Mobile phase solvent ratio[a]	k'[b]						
	Component						
x : y : z	PE	(α)	PC	(α)	PI	(α)	PA
(i). 32.5:26.5:6.0	1.21	(2.36)	2.86	(1.52)	4.36	(1.11)	4.86
(ii). 32.7:26.8:5.5	1.71	(2.30)	3.93	(1.27)	5.00	(1.42)	7.09
(iii). 39.9:19.6:5.5	2.07	(1.79)	3.57	(1.27)	4.53	(2.05)	9.29
(iv). 40.5:20.0:4.5	3.14	(1.87)	5.86	(1.17)	6.86	(2.22)	15.2
(v). 40.9:20.1:4.0	4.86	(2.01)	9.79	(1.87)	18.3	(1.42)	26.0

[a]Values under the (x:y:z) column represent solvent ratios of ISP-ETOH-water/5 Mm TMAP(Ph 6.3) with hexane at a constant value of 35% for the mobile phases evaluated.
[b]Values in parentheses are separation factors, α, for adjacent component peaks. For definition of k' and α values, see EXPERIMENTAL.

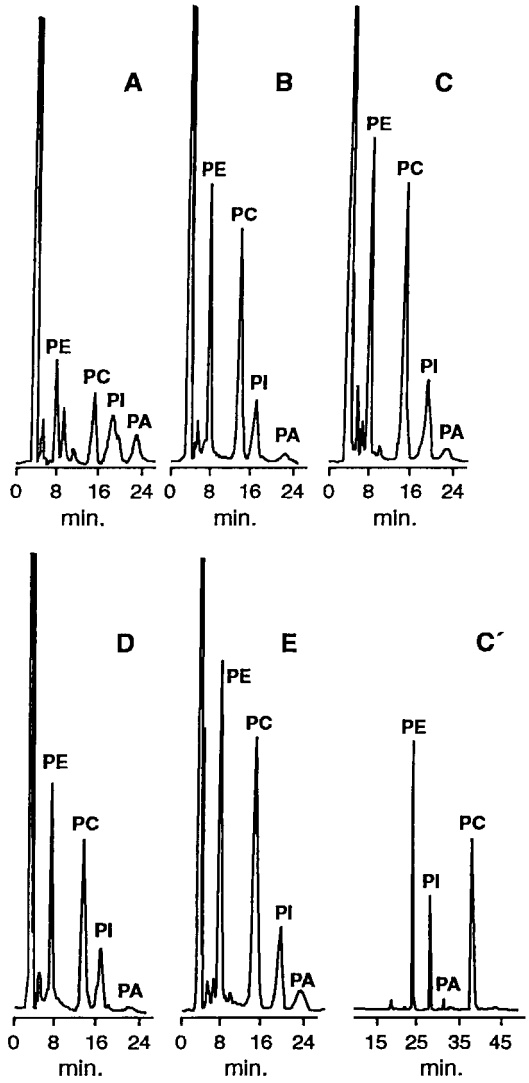

FIGURE 3. HPLC-UV-CDS separations of PL components in selected samples derived from genetically modified soybean oils (A, B, C,and D) along with a control unmodified soybean oil sample (E); mobile phase conditions are same as in FIGURE 2A. HPLC-ELSD-SI separation (C') of PL components was obtained from sample (C); mobile phase conditions are same as in FIGURE 2B.

samples derived from genetically modified soybeans. Along with the modified oil samples, an unmodified soybean oil sample was also analyzed (FIGURE 3E) by the same HPLC method for comparison. Depending on the nature of samples, variable degrees of separations of the sample components were obtained with a mobile phase of hexane-ISP-ETOH-water/5 Mm TMAP(Ph 6.3) (35: 32.7: 26.8: 5.50). Traditionally, hexane-ISP-water solvent mixtures have proved successful in gradient HPLC analyses of PL classes on SI columns. Nonetheless, the HPLC chromatograms shown in Fig's. 3A-E indicate that, in general, isocratic HPLC-UV-CDS experiments with soybean PL mixtures led to satisfactory separations of PL components within reasonable elution times of about 25 min. For the purpose of comparing the HPLC peak profiles obtained from the two detection methods with different stationary phases, the same sample was analyzed by the HPLC-UV-CDS and HPLC-ELSD-SI procedures to give clearly different patterns of component separations as shown in FIGURE 3C and FIGURE 3C', respectively. It must be pointed out here that the isocratic mobile phases employed in the present HPLC-UV-CDS work can not be used with the ELSD because of incompatibility of TMAP with the detector.

To demonstrate the general applicability of the HPLC-UV-CDS technique in practical qualitative

analyses of commercial samples, a standard reference soybean lecithin sample together with three experimental soybean lecithin samples were chromatographed (FIGURE 4). The experimentally modified lecithin samples derived from industrial sources were obtained by partially treating the reference standard lecithin with hydroxylating, acetylating, or hydrolyzing agents. Generally, the PL separations of the standard sample (FIGURE 4A) were similar to those of the three experimental samples (FIGURES 4B-D) with the exception of minor peaks present in the chromatograms of the latter samples. Thus, hydroxylation of standard lecithin led to few notable changes in HPLC peak pattern within the 25 min elution time of the product (FIGURE 4B). HPLC of the acetylated lecithin sample produced a diminished PE peak shouldered by a new unresolved peak (FIGURE 4C). An enzymatically hydrolyzed product of standard lecithin contained significant amounts of lyso-PE and Lyso-PC (FIGURE 4D). When the enzyme-treated lecithin sample was analyzed under different mobile phase conditions with somewhat lower hexane and water contents, PI was found to elute from the CDS column before PC (FIGURE 4D').

Because UV absorptions vary among the different PL classes within a sample as well as in samples derived

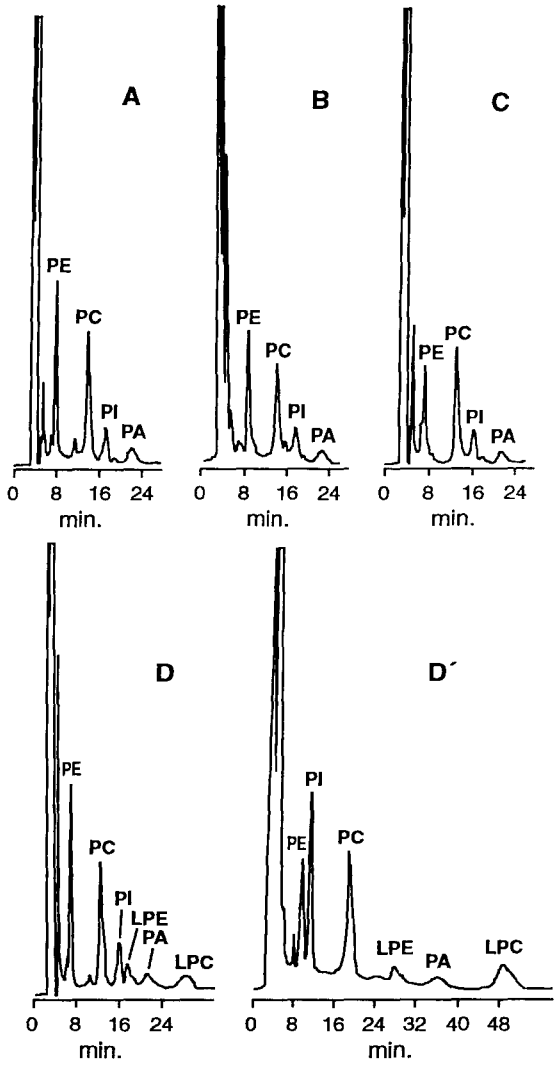

FIGURE 4. HPLC-UV-CDS separations of PL components in a commercial soybean lecithin sample (A) and experimental soybean lecithin samples (B, C, and D). HPLC-UV-CDS separation (D') of PL components was obtained from sample (D). Mobile phases: (A, B, C, and D) same as in FIGURE 2A. ; (D'), [hexane-ISP-ETOH-water/5 Mm TMAP(Ph 6.3)] (25: 38.5: 31.5: 5.0).

from different sources (plants or animals), HPLC-UV detector responses of samples require calibration with standards of the same origin as the sample prior to the quantitative estimation of the PL components present in a sample. Direct determinations of PL compositions can be accomplished by the HPLC-ELSD-SI. It was apparently fortuitous that the HPLC-UV-CDS peak intensities shown in FIGURE 3C are visually similar to those obtained by HPLC-ELSD-SI (FIGURE 3C') of the same sample.

A correlation study established linear relation between the injected amounts of soybean PL standards PE, PC, and PI and the corresponding HPLC-UV-CDS peak areas (TABLE 2). Because soybean PA standards are not commercially available, phosphorimetric PA analyses were necessary in order to eliminate intrinsic analysis errors caused by differences in molecular species distributions between soybean PA and egg PA. A calibration curve for PA analyses was constructed by plotting peak areas with amounts of PA obtained by phosphorimetry (TABLE 2). Inspection of the calibration data in the table showed that the slope (b) values of the correlation lines increase with decreasing retention times m (PE) > m (PC) > m (PI) > m (PA).

The results of quantitative HPLC-UV-CDS analyses of major soybean PL in four samples of genetically

TABLE 2
Calibration Data for Quantitative Analyses of Major Soybean PL By HPLC-UV Under HPLC Conditions Employed[a] (UV detector at 208 nm; CDS column)

PL	Slope (m) cm^2 /μg	Coefficient of variation (CV)[c]	Correlation coefficient (r)
PE	8.752	2.5	0.9955
PC	5.375	2.2	0.9969
PI	3.333	3.0	0.9941
PA[b]	0.232	3.2	0.9921

[a]See Experimental section for mobile phase conditions.
[b]Data obtained from phosphorimetry.
[c]CV values are based on mean values of three determinations of peak areas.

modified oil and a control unmodified oil sample are summarized in TABLE 3. The numerical values given in the table are mean values of triplicate determinations. Coefficients of variation of the sample analyses ranged between 1.8 - 6.9%. Since the UV absorbance of the polar lipids is dependent on the fatty acid composition, quantitative analyses were carried out with the assumption that the fatty acid composition of each PL class is nearly identical to that of the PL standard. As shown by the analytical data (TABLE 3), the percent compositions of individual PL classes in the modified oils decrease in the order % PC > % PE > % PI > % PA, which is same as that observed in the

TABLE 3
Quantitative Analyses of PL in Selected Samples of Soybean Oil By HPLC-UV With a CDS Column[a]

Sample No.[b]	Soybean PL component							
	PE		PC		PI		PA	
	A	B	A	B	A	B	A	B
#1	17.9	30.3	26.0	44.0	11.8	20.0	3.40	5.70
(CV)	(3.5)	(4.0)	(2.3)	(3.3)	(3.7)	(3.9)	(5.8)	(6.3)
#2	19.8	28.9	32.0	46.7	13.5	19.7	3.15	4.61
(CV)	(4.1)	(3.7)	(1.9)	(1.8)	(3.3)	(5.3)	(5.0)	(6.5)
#3	31.9	28.7	47.8	42.9	25.5	22.9	6.10	5.50
(CV)	(3.6)	(4.5)	(2.0)	(3.0)	(2.7)	(2.3)	(4.9)	(5.8)
#4	31.9	35.2	32.3	35.6	22.0	24.2	4.53	5.00
(CV)	(4.5)	(4.4)	(2.9)	(3.1)	(4.3)	(3.6)	(6.0)	(6.7)
#5	39.7	27.0	72.1	48.9	32.3	21.9	3.20	2.20
(CV)	(4.0)	(3.9)	(2.3)	(1.9)	(5.2)	(1.9)	(6.6)	(5.5)

[a]A = amount (mg) of a PL component present in 10 g of each soybean oil sample. B = percent composition of a PL component in each oil sample. These values are mean values of three determinations.
[b]Samples #1-4 represent genetically modified soybean oil; sample #5 represents control unmodified soybean oil. CV= coefficients of variation.

control oil sample. The PA levels in the modified oils (TABLE 3, samples # 1-4) appeared to be at least two times greater than that in the control sample (TABLE 3, sample #5). The analytical results obtained by HPLC-UV-CDS are generally in good agreement with those obtained by HPLC-ELSD-SI (30).

In conclusion, the results of this study represent the first report on the use of a CDS phase for the

separation of phospholipids. The four major PL (PE, PC, PI, and PA) found in soybean oil can be separated by normal-phase HPLC on β-CDS. The two minor PL (LPE and LPC) found in enzymatically treated soybean oil can also be separated from the major PL components. The HPLC-UV-CDS technique coupled with reference calibration and phosphorimetry can be used for the quantitative analysis of PL classes in soybean oil samples. The HPLC-UV-CDS method can be utilized for the qualitative analysis of industrial lecithin products. The method may be applicable to the separation of a wide range of PL by optimization of HPLC mobile phases. The HPLC technique may be used as a viable alternative to PL analysis in various sample matrices.

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A STABILITY INDICATING HPLC ASSAY FOR DIAMORPHINE IN AQUEOUS SOLUTION

DAVID A. BARRETT* AND P. NICHOLAS SHAW
Department of Pharmaceutical Sciences
University of Nottingham
Nottingham, NG7 2RD, United Kingdom

ABSTRACT

A method is described for the rapid and simultaneous determination of diamorphine, and its hydrolysis products, 6-acetylmorphine and morphine using high-performance liquid chromatography. The column used was a Hypersil BDS C18 (100 x 4.6 mm) and the mobile phase comprised 80% aqueous potassium hydrogen phosphate (0.05M), 20% acetonitrile, adjusted to pH 3.0 with orthophosphoric acid. The flow rate was 1.0 $ml.min^{-1}$ and the limit of detection of each compound was 0.01 $\mu g.ml^{-1}$. The assay was linear over the range of 0.01 to 100 $\mu g.ml^{-1}$. The inter and intra-assay reproducibilities were better than 4% over this range of concentrations. The assay method was used to determine the effect of temperature on the degradation of diamorphine at pH 7.4 in aqueous solution.

INTRODUCTION

Diamorphine (3,6-diacetylmorphine, heroin) is a potent synthetic opiate analgesic which is commonly used for the relief of acute and chronic pain in man as well as being a drug of abuse [1]. Aqueous solutions of diamorphine hydrochloride are much less stable than solutions of other opiates such as morphine and codeine. Previous studies have shown that the drug is rapidly deacetylated in aqueous solution at alkaline pH to form 6-monoacetylmorphine [2-6] and is further hydrolysed slowly to form morphine [7]. Hydrolysis of the 3-acetyl group takes place both in the dark and in the cold or on exposure to daylight [8]. Because of its instability in aqueous solution, the use of diamorphine in *in vitro* experiments requires the careful monitoring of its breakdown products.

(*to whom correspondence should be addressed)

A major problem in determining the stability of diamorphine in solution has been the lack of a specific assay to separate the 6-acetylmorphine and morphine. Early studies using quantitative TLC and GLC [9, 10] focussed on the breakdown of diamorphine but had difficulty in accurately measuring concentrations of the two hydrolysis products. HPLC methods have been more successful, but have often required derivatisation or dilution [4] or have required the use of a complex HPLC mobile phase containing ion-pairing agents or other additives [5,11-13].

In this study several types of HPLC column and mobile phase have been evaluated in order to develop a rapid and simple method for the separation of diamorphine and its hydrolysis products.

MATERIALS AND METHODS

Chemicals and reagents

Diamorphine hydrochloride and 6-acetylmorphine hydrochloride were purchased from Macfarlane Smith (Edinburgh, UK) and morphine hydrochloride was obtained from Boots Co. Ltd (Nottingham, UK). Acetonitrile, triethylamine and potassium dihydrogen orthophosphate were all HPLC grade (Fisons plc, Loughborough, UK). All other chemicals were of analytical grade or better.

Stock solutions

Diamorphine, morphine and 6-acetylmorphine stock solutions were prepared in acetonitrile : water (20:80 v/v) and stored at -30°C. Calibration standards were prepared by diluting the stock solutions of the drugs with HPLC mobile phase. A 10-point calibration line was used with concentrations in the range 0.01 to 100.0 $\mu g.ml^{-1}$ for all three compounds.

Chromatographic conditions

The HPLC apparatus consisted of two Gilson 305 pumps (Gilson Medical Electronics, Villiers-le-Bel, France), a Gilson 805 Manometric module, a Rheodyne model 7125 injector with 200 µl loop (Rheodyne, Cotati, CA, U.S.A.), a Gilson 115 UV detector, and a Gilson 712 data handling system. Several types of HPLC column packing materials were evaluated for an optimum separation of diamorphine and its hydrolysis products: Hypersil ODS, Hypersil BDS C18 and Hypersil CPS (Shandon HPLC, Runcorn, UK), Spherisorb ODS and Spherisorb CN (Phase Separations, Queensferry, UK). All the HPLC columns were 150 mm

x 4.6 mm i.d., with 5 μm particle size. For the BDS C18 column, the mobile phase consisted of 80 % (v/v) aqueous 0.05M potassium hydrogen phosphate (adjusted to pH 3.0 with orthophosphoric acid), and 20 % (v/v) acetonitrile. For the remaining three columns the mobile phase was 60 % (v/v) aqueous 0.05M potassium hydrogen phosphate (adjusted to pH 3.0 with orthophosphoric acid), and 39.9 % (v/v) acetonitrile containing 0.1% triethylamine. The mobile phase flow rate was 1.0 $ml.min^{-1}$ and the injection volume was 50 μl.

Assay validation

Standard calibration lines for each compound were generated from the HPLC analysis of a range of calibration standards (see stock solutions section). Peak-areas of all three compounds (diamorphine, 6-acetylmorphine and morphine) were recorded. Linear regression analysis was performed to determine the slope, intercept and the correlation coefficient of the calibration lines.

The intra-assay precision was evaluated by analysing six replicate samples at each of three different concentration levels for each compound, as shown in Table 1. The inter-assay precision was evaluated by analysing replicate samples, identical to those used for the intra-assay validation, on three different days. The precision of the assay is expressed as a coefficient of variation (CV%) at each concentration point.

Stability of diamorphine in aqueous solution

The stability of diamorphine (100 $\mu g.ml^{-1}$) in aqueous solution (pH 7.4) was assessed at temperatures of 4°C, 25°C and 37°C. Aliquots (1.0 ml) of freshly made up diamorphine solution in aqueous sodium phosphate buffer (0.1 M, pH 7.4) were sealed in amber glass vials, which were stored at 4°C, 25°C or 37°C. Vials were removed at appropriate time intervals for analysis over a period of 14 days. The HPLC system was calibrated at the start, during, and at the end of the stability study. The decrease in diamorphine concentration and the increase in 6-acetylmorphine and morphine concentrations were determined by interpolation from the appropriate averaged calibration line.

RESULTS AND DISCUSSION

Chromatographic separation and validation of assay

The order of elution of the compounds, for all the HPLC columns evaluated was: morphine, 6-acetylmorphine and diamorphine. The Hypersil ODS and Spherisorb ODS columns produced

TABLE 1

Intra- and inter-day reproducibilities for the analysis of diamorphine, morphine and 6-acetylmorphine

Compound	spiked conc ($\mu g.ml^{-1}$)	intra-day variation (n=6) CV%	inter-day variation (n=18) CV%
diamorphine	0.1	2.8	4.2
	1.0	2.3	3.7
	10.0	0.8	2.3
	100.0	1.2	2.8
6-acetylmorphine	0.1	2.9	3.8
	1.0	2.0	2.1
	10.0	0.9	2.5
	100.0	1.1	1.9
morphine	0.1	2.6	2.7
	1.0	1.4	2.0
	10.0	1.6	2.8
	100.0	0.8	1.7

a very long retention time for diamorphine (>60 min), which was required in order to separate morphine from the solvent front. The Spherisorb CN column gave broad and tailing peak shapes for the compounds, particularly for diamorphine. Optimum separations were obtained with the Hypersil CPS or the Hypersil BDS HPLC columns, both of which gave baseline separation of the three compounds with symmetrical peaks shapes, within a run time of 5 min. The Hypersil BDS column was chosen in preference to the Hypersil CPS since the addition of triethylamine was not necessary in order to obtain non-tailing peaks.

The retention times on the Hypersil BDS column for diamorphine, 6-monoacetylmorphine and morphine were 4.2 min, 3.4 min and 2.8 minutes respectively. All the validation analyses were performed using the Hypersil BDS column. The intra-day and inter-day precision (expressed as a coefficient of variation) for the assay was better than 4% (n=6) for each compound at concentrations of 0.1, 0.5, 1.0, 5.0, 10, 25, 50, 75, 100 $\mu g\ ml^{-1}$. Details of the validation at 0.1, 1.0, 10.0, and 100.0 $\mu g\ ml^{-1}$ are shown in Table 1 . The limit of detection was defined as three times the signal-to noise-ratio, and was found to be 0.01 $\mu g\ mL^{-1}$ for all three compounds. Calibration lines showed good linearity over the range of concentrations between 0.1 $\mu g\ ml^{-1}$ and 100 $\mu g\ ml^{-1}$ with the R^2 value being greater than 0.99 for each compound. A typical chromatogram showing the separation of diamorphine, 6-monoacetylmorphine and morphine is shown in Figure 1.

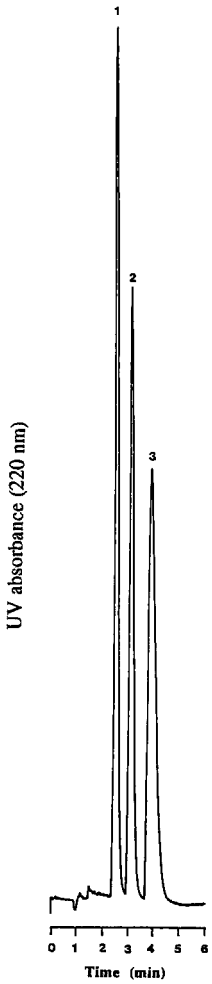

Figure 1: HPLC chromatogram of a standard solution containing 10.0μg.ml^{-1} of morphine (1), 6-acetylmorphine (2) and diamorphine (3).

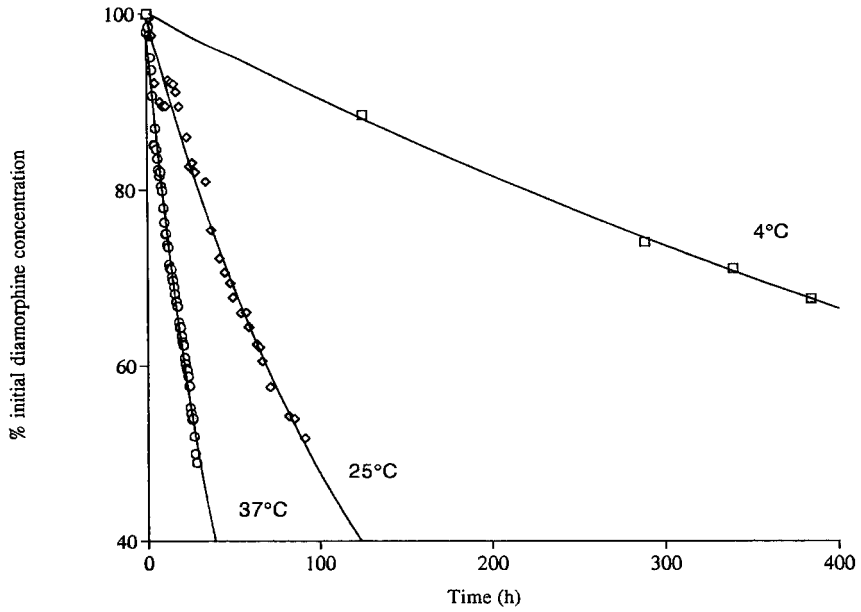

Figure 2: The effect of temperature on the stability of diamorphine in pH 7.4 buffer.

Assay application: stability of diamorphine in aqueous solution

The rate of deacetylation of diamorphine in aqueous solution was studied at 4°C, 25°C, 37°C. First order kinetics were apparent in all cases and the rate of degradation was substantially increased at higher temperature (Figure 2), with 6-monoacetylmorphine being the major degradation product and morphine a minor product. In all cases, the rate of appearance of 6-monoacetylmorphine mirrored the disappearance of diamorphine. The mean half-life of diamorphine in aqueous solution at pH 7.4 was 774 h at 4°C, 96.8 h at 25° C, 32.9 h at 37°C. A previous study of diamorphine stability at pH 4.9 in aqueous solution [2] indicated that the drug was considerably more stable than at neutral pH. Other studies of diamorphine stability at acid pH values have also concluded that a small but significant degradation of diamorphine will occur over the prolonged periods when the drug is made up in aqueous solution before use [13-16]. The data from this study suggests that there is a significant increase in the rate of hydrolysis of diamorphine at neutral pH compared with the acid pH values used in previous stability studies.

CONCLUSION

The method described provides a sensitive and specific assay for assessing the stability of diamorphine in aqueous solution. It has been successfully applied to study the stability of diamorphine over a range of temperatures.

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PARTITION COEFFICIENTS (log P) AND HPLC CAPACITY FACTORS (k') OF SOME Gd(III) COMPLEXES OF LINEAR AND MACROCYCLIC POLYAMINO CARBOXYLATES

KRISHAN KUMAR*, K. SUKUMARAN, S. TAYLOR, C. ALLEN CHANG, A. D. NUNN, AND M. F. TWEEDLE
The Bristol-Myers Squibb Pharmaceutical Research Institute
P.O. Box 4000
Princeton, New Jersey 08543-4000

ABSTRACT

The partition coefficients (log P) in n-butanol/water and n-octanol/water together with the HPLC capacity factors (k') in 2% and 4% acetonitrile (ACN) and aqueous buffered mobile phase were determined for 21 Gd(III) chelates of linear and macrocyclic polyamino carboxylates. A C_{18} reversed-phase (Nucleosil) column was used for determination of k'. The log P values were calculated (clog P) for the fully protonated form of the ligands. A reasonably good correlation (r = 0.76) between the measured log P values for the chelates in n-butanol/water and the calculated log P values for the ligands was observed. In general, the log P value in n-butanol/water for a chelate was 1.3 log unit different than that in n-octanol/water. The k' values in 2% and 4% acetonitrile/aqueous buffer were also correlated with measured log P values.

INTRODUCTION

Structure-activity relationships are useful in understanding certain biological effects of pharmaceuticals [1-3]. These include an understanding of biodistributions, effective dose, ED_{50}, and acute tolerance, LD_{50}. For a pharmaceutical to exhibit certain biological effects, it must interact with some cellular components of the site of action. The pharmaceutical must be transported through phase boundaries and undergo adsorption and desorption processes with

proteins and membranes, as well as partitioning between different liquid phases before it reaches the site of action. Hansch and coworkers [4-6] proposed a model for understanding biological activity of potential pharmaceuticals. According to this model the lipophilic-hydrophilic balance of a compound, which is expressed by a partition coefficient is critical for drug absorption and transport. The n-octanol/water partition coefficient is a recognized model for biological lipids and aqueous phases. As the determination of lipophilicity by the traditional shake-flask method has certain limitations, the reversed-phase HPLC capacity factor is a useful alternative to the n-octanol/water system [7].

Our interest in lipophilicity determination resulted from a need to develop structure-activity relationships (SARs) in magnetic resonance imaging (MRI). MRI contrast agents are a class of pharmaceuticals [8,9] which utilizes gadolinium chelates, GdL (where L is a chelating agent or ligand). Four such chelates, e.g. the complexes of diethylenetriaminepentaacetic acid (DTPA), the bis methyl amide of DTPA (DTPA-BMA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), and 10-(2-hydroxypropyl)-1,4,710-tetraazacyclododecane-1,4,7,10-tetraacetic acid (HP-DO3A), are being used clinically as extracellular contrast agents [9]. One of the requirements for intravenously administered gadolinium-based extracellular contrast agents is high hydrophilicity. This property is required to: (1) maintain water solubility, (2) minimize interaction of the pharmaceutical with plasma proteins, membranes, and other macromolecules, which allows for rapid equilibration of the complex in the extracellular space and efficient excretion, and (3) avoid permeation of the pharmaceutical through intact plasma membranes by diffusion. There is little published information on the lipophilicity (hydrophilicity) of this class of compounds [10].

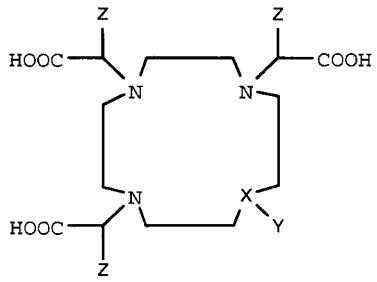
The objectives of this investigation were to determine the lipophilicity (log P values) or hydrophilicity of 21 potential MRI contrast agents by the traditional shake-flask method [4] and by the reversed-phase HPLC method. Calculated log P values (clog P) for the ligands were compared to the measured log P values of the complexes to evaluate the predictability of log P for unknown gadolinium complexes. A general structure of the macrocyclic framework is shown in Table I. The structures of other ligands, e.g. EDTA, DTPA, and DTPA-BMA are well known [11].

MATERIALS AND METHODS

Instruments

The HPLC system used was a two pump, Rainin Instrument or Beckman model 110, with a Rheodyne injection valve containing a 20 μL sample loop. A C_{18} reversed-phase Nucleosil (Jones Chromatography or Alltech) (25.0 x 0.46 cm, 5μm) column was used. After each working period,

Table I. Structural Formulas of Macrocyclic Polyamino Carboxylates.



S.N.	X	Y	Z	Name
1	N	H	H	DO3A
2	N	$-CH_3$	H	Me-DO3A
3	N	$-CH_2CHOHCH_3$	H	HP-DO3A
4	N	$-CH_2CONHCH_3$	H	MA-DO3A
5	N	$-CH_2CONHC_6H_5$	H	PA-DO3A
6	N	$-(CH_2)_2CN$	H	CE-DO3A
7	O	-	H	OX-DO3A
8	N	$-CH_2C_6H_5$	H	BZ-DO3A
9	N	$-(CH_2)_2COOH$	H	HO-DO3A
10	N	$-CH_2CHOHCH_2OH$	H	PG-DO3A
11	N	$-CH2CHOHCH_2OCH_3$	H	HM-DO3A
12	N	$-CH_2COH(CH_2OCH_2)_3CH_3$	H	HD-DO3A
13	N	$-CH_2CHOH(CH_2)_3CH_3$	H	HH-DO3A
14	N	$-CH_2CH_2CH_3$	H	NP-DO3A
15	N	$-CH_2CONH(C_6H_4)(CONHCHOHCH_2OH)_2$	H	HAA-DO3A
16	N	-H	CH_3	DO3MA
17	N	$-CH_2COOH$	H	DOTA
18	N	$-CH(CH_3)COOH$	CH_3	DOTMA

the column was flushed with acetonitrile and then water/methanol. The column was stored in methanol. Integration of peak areas was performed either by a Shimadzu RC-3A chromatopac/ plotter or by using a Dynamax software. A Beckman 170 radioisotope detector was used for radioactivity and a Hitachi F-1050 or F-2000 detector for fluorescence.

A Radiometer PHM-82 pH meter with a combination glass electrode was used for all pH measurements. An LKB-Wallace gamma counter was used for the determination of radioactivity in the sample. The window used to count ^{153}Gd radioactivity was machine channels 20-140 inclusive. This window measures the gamma radiation at 97.4 and 103.2 keV.

Materials

Ethylenediaminetetraacetic acid (EDTA) (Fisher), diethylenetriaminepentaacetic acid (DTPA) (Aldrich) and DTPA-BMA (STREM) chemicals were used without further purification.

The chelating agents and Gd(III) chelates were synthesized by literature procedures [12-16]. The purity of the chelating agents, ligands, and Gd(III) complexes was checked by elemental, mass spectral analysis, ^{1}H, ^{13}C NMR, and HPLC and the purity was >99% . HPLC grade methanol and acetonitrile were used for all chromatographic studies. Silica gel impregnated glass fiber sheets (ITLC-SG) were purchased from Gelman Sciences, Inc. (Ann Arbor, MI). The purity of $Gd(EDTA)^-$ was checked by using freshly prepared mobile phase, 10% ammonium acetate (Fisher) in 50% (v/v) aqueous methanol. This was prepared fresh daily. Stock solution of $GdCl_3$ was prepared from a solid sample of $GdCl_3$ purchased from Research Chemicals (Phoenix, AZ), and was used without further purification. The sample solution was standardized by a complexometric titration with xylenol orange as the indicator [17]. A stock solution of tris acetate buffered EDTA (Aldrich chemicals) was prepared for sample preparation and for the HPLC mobile phase. The tris acetate buffer was prepared from TRIZMA (Sigma) base and acetic acid (Fisher). Dilute solutions of hydrochloric acid and sodium hydroxide were used for adjustment of the pH. All other chemicals were reagent grade. Normal butanol and n-octanol (Aldrich) were greater than 99% pure. A sample of $^{153}GdCl_3$ ($t_{1/2}$ = 241.6 d) with a known specific activity and radioactivity concentration was purchased from the Oak Ridge National Laboratory, Oak Ridge (TN).

Methods

The samples of $^{153}Gd/GdL$ were either prepared by a true tracer radiolabeling or by an isotope-exchange method [18]. In a true tracer radiolabeling procedure, a carrier-added sample of $^{153}GdCl_3$ was reacted with the free ligand. The pH of the reaction mixture was raised very slowly to 8 to avoid any $Gd(OH)_3$ formation. Any precipitated $^{153}Gd/Gd(OH)_3$ was removed by filtration through a 0.2 μm filter. The samples were purified by High Performance Liquid Chromatography [18].

In an isotope-exchange method, a solution of approximately 100 mM GdL was prepared. An aliquot of $^{153}GdCl_3$ was added to it. The pH was adjusted to 2 with 5 N HCl and the solution was heated to 80°C. Heating times varied for different chelates. The complexes of macrocyclic polyamino carboxylates were heated for 4 h, while the complexes of linear polyamino carboxylates required only 1 h. After heating, the solutions were neutralized with sodium hydroxide very slowly. The samples were analyzed by an ITLC or HPLC method for free Gd^{3+} [18-21]. In most of the samples the percentage of free Gd^{3+} was < 0.1%. High purity of these chelates is very important in these studies as free Gd^{3+} or free ligand, H_2L^{n-} at pH 7.4, will be very hydrophilic and influence the log P measurements.

The log P values were determined by a traditional shake-flask method. In a typical experiment, a 10 μL sample of a radiolabeled gadolinium chelate, ^{153}GdL ($[Gd]_T$ = 0.1 mmol/mL

and radioconcentration = 10-20 μCi/mL), and 2.0 mL of 25 mM tris acetate (pH 7.4) were mixed in a test tube. Two milliliters of either n-butanol or n-octanol were added and the test tube was vortexed for 2 min. The test tube was centrifuged for 2 min at 3000 rpm to insure complete separation of the two layers. Five hundred microliters of each phase were counted in an LKB gamma counter. The partition coefficient (P) values were calculated as follows:

$$P = \text{counts in organic phase/counts in aqueous phase} \quad (1)$$

Although the chelates used in this study are not protonated significantly above pH 3 [22], the tris acetate buffer was used to insure the presence of one species. The HPLC capacity factors (k') were determined for each complex using a silica-based reversed phase column. ^{153}GdL or GdL was used for these experiments. The mobile phase buffer containing 50 mM tris acetate and 10 mM EDTA (pH 7.4) was combined with 2% or 4% acetonitrile (v/v). Each chelate was injected in triplicate. The HPLC capacity factors (k') were determined using eq. 2.

$$k' = (t_r - t_o) / t_o \quad (2)$$

Where t_r is the retention time of the chelate, and t_o, the void volume or the time required to the mobile phase to move from one end of the column to the other. Normally a weaker solvent or non retained compound is used to determine the void volume of the column [23]. Recent work from this laboratory [21] has demonstrated that the percentage of acetonitrile does not affect the retention time of $Gd(EDTA)^-$, indicating that it is not retained. Consequently the retention time of $Gd(EDTA)^-$ has been taken as t_o. The calculation of the log P values (clog P) for the protonated form of the ligands was performed using MEDCHEM Vers. 3.42 [24].

RESULTS AND DISCUSSION

Log P Values

The log P values for 21 potential extracellular MRI contrast agents were determined and are given in Table II. Impurities are obvious potential source of error in log P values measured by the shake-flask method. The samples used in this study were relatively pure and free from unbound Gd^{3+} or ligand. Free Gd^{3+} and H_2L^{n-} are more hydrophilic than the chelates and will affect the measurement considerably. All measurements were made in a buffered medium at pH 7.4 and there was <0.01% protonated chelate, GdL(H) [22]. Consequently the term log P rather than log D (distribution coefficient) was used. The data in Table II show that the log P values in n-octanol/water are always more negative than those in n-butanol/water indicating that all of the complexes are relatively hydrophilic. The linear-least squares fit of the plot of log P values in n-butanol/water vs. those in n-octanol/water gave a slope of 0.9 and an intercept of 1.3 with a

Table II. The Measured Values of Partition Coefficients (log P) in n-Butanol/Water and n-Octanol/Water Systems for Some Gd(III) Complexes, GdL, and the Calculated log P Values for Free Ligands

GdL	log P[a]	log P[b]	clog P[c]	GdL	log P[a]	log P[b]	clog P[c]
EDTA	-2.79	-3.91	-0.565	HO-DO3A	-2.84	d	0.527
DTPA	-3.16	d	-0.553	PG-DO3A	-2.22	-3.82	0.202
DTPA-BMA	-2.13	-4.14	-1.875	HM-DO3A	-1.99	-3.68	0.490
DO3A	-2.15	-3.86	-2.45	HD-DO3A	-1.60	-3.50	0.574
Me-DO3A	-1.94	-3.62	0.698	HH-DO3A	-0.81	-2.17	2.015
HP-DO3A	-1.98	-3.68	0.736	NP-DO3A	-1.42	-2.99	1.851
MA-DO3A	-2.28	-4.19	-0.038	HAA-DO3A	-2.62	d	-1.119
PA-DO3A	-1.22	-2.83	1.36	DOTMA	-2.21	d	1.69
CE-DO3A	-2.46	-3.97	0.403	DO3MA	-1.51	-3.31	-1.526
OX-DO3A	-2.76	d	0.169	DOTA	-2.87	-4.26	0.458
BZ-DO3A	-1.61	d	2.62				

[a]in n-butanol/water, [b]in n-octanol/water, and [c]calculated log P values for the protonated form of the ligand, [d]did not have enough counts in n-octanol layer to determine log P value.

correlation coefficient (r) of 0.92 (Figure 1). Thus the following equation can be written to correlate log P data in n-butanol/water and in n-octanol/water for this series of Gd(III) complexes:

$$\log P_{\text{n-butanol/water}} = (0.9 \pm 0.2)\ \log P_{\text{n-octanol/water}} + (1.3 \pm 0.6) \qquad (3)$$

The correlation given in eq. 3 is very useful for highly hydrophilic compounds, as in some cases it is not possible to determine the log P value in the n-octanol/water system. Consequently, the log P determination in n-butanol/water is an alternative to n-octanol/water system. For example, from the correlation, the log P value in n-octanol/water system can be estimated as -4.9, -4.54, -4.73, -3.45, -4.3, and -4.12 for Gd(III) complexes of DTPA, HO-DO3A, OX-DO3A, BZ-DO3A, HAA-DO3A, and DOTMA, respectively, for which log P values could not be determined in n-octanol/ water. The n-octanol/water values were less accurate due to the lower number of counts in the n-octanol layer.

Correlation of the Measured log P Values for GdL and clog P for the Ligand

The log P values of the protonated form of the ligands were calculated by Hansch's fragmentation method (Table II) [24]. Deprotonation of the protonated form of the ligand is a

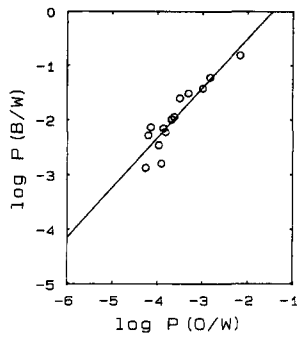


Fig. 1. Plot of log $P_{n\text{-butanol/water}}$ vs. log $P_{n\text{-octanol/water}}$.

requirement for its complexation to Gd(III) (eq. 4). Five protons from DTPA, four protons from EDTA, DOTA, and some derivatives of DO3A and DOTA, and three protons from DTPA-BMA and most DO3A ligands are released upon reacting with Gd^{3+}. This variation in the overall

$$H_nL^o + Gd^{3+} <=====> GdL^{3-n} + n\,H^+ \qquad (4)$$

charge of the Gd(III) complexes might be expected to cause deviations from a linear relationship between the calculated and the measured lipophilicity unless a correction is made for the variable charge. However, the correction, clog P = 4.13 units for each unit of charge [5-7], is insignificant relative to the large clogP values and the existing scatter. As can be seen from Fig. 2, there is a linear relationship (eq. 5 and 6) between calculated and the measured log P values (in n-butanol/water and n-octanol/water systems) without any correction for charge.

$$\log P_{n\text{-butanol/water}} = (0.5 \pm 0.1)\ \text{clog P} - (2.5 \pm 0.1) \quad r = 0.74 \qquad (5)$$

$$\log P_{n\text{-octanol/water}} = (0.7 \pm 0.1)\ \text{c log P} - (4.1 \pm 0.1) \quad r = 0.84 \qquad (6)$$

It is apparent from equations 5 and 6 that measured n-octanol/water and n-butanol/water data correlate equally well with clog P values. It is possible to estimate the log P values for most of the gadolinium complexes of linear and macrocyclic polyamino carboxylates from clog P and using equations 5 and 6. However, the measured log P values for DTPA-BMA, DO3A, and DO3MA complexes were 2-3 orders of magnitude less hydrophilic than predicted by the correlation. Our measured log P value in n-butanol/water for Gd(DTPA-BMA) is in excellent agreement with the literature value [11].

The HPLC Capacity Factors

The compounds given in Table II were chromatographed on a silica based reversed-phase Nucleosil column with C_{18} support. The HPLC capacity factors (k') were determined using either

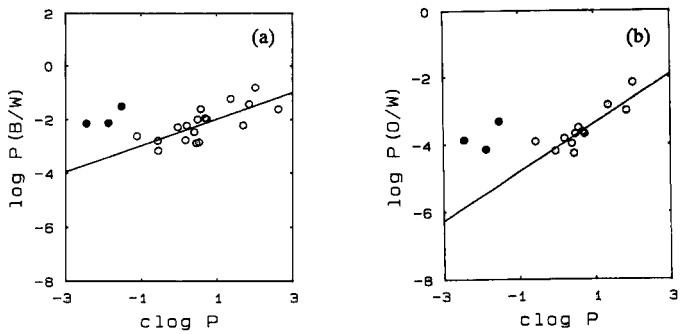


Fig. 2. A correlation of measured log P values (a) in n-butanol/water (b) n-octanol/water with clog P. The chelates GdL (where L = DTPA-BMA, DO3A and DO3MA and plotted by solid points) do not fall on the line.

Table III. HPLC Capacity Factors (k') in the Buffered Mobile Phase (2 and 4% ACN And 98 and 96% 10 mM EDTA and 50 mM tris acetate, respectively at pH 7.4).

GdL	k'(2% ACN)	k'4%(ACN)	GdL	k'(2% ACN)	k'4%(ACN)
EDTA	0.000	0.000	PG-DO3A	1.35	0.90
DTPA	0.09	0.000	HM-DO3A	3.67	1.75
DTPA-BMA	1.47	0.670	HD-DO3A	a	a
DO3A	1.35	0.76	HH-DO3A	a	a
Me-DO3A	1.88	0.94	NP-DO3A	a	3.86
HP-DO3A	3.09	1.77	HO-DO3A	1.21	0.65
MA-DO3A	0.89	0.57	HAA-DO3A	9.84	3.07
PA-DO3A	a	5.977	DOTMA	13.96	5.30
CE-DO3A	1.32	0.81	DO3MA	a	7.85
OX-DO3A	0.61	0.49	DOTA	0.71	0.44
BZ-DO3A	a	a			

[a]did not elute within 30 min.

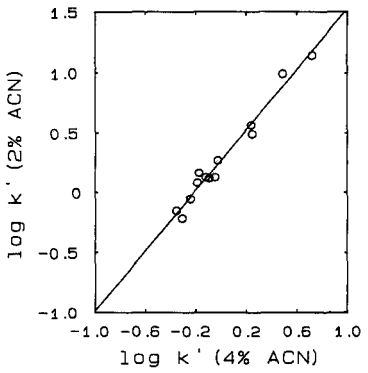


Fig. 3. A Plot of HPLC Capacity Factor log k' in 2% vs. log k' in 4% Acetonitrile.

GdL or ^{153}GdL and eq. 2. The retention times of Gd(EDTA)$^-$ and GdL were either determined separately or a sample of GdL was spiked with a known amount of free Gd^{3+} and the mixture was injected onto a Nucleosil C_{18} reversed-phase column. The rate of the reaction of Gd^{3+} with the protonated form of EDTA (eq. 7) is rapid [25]; under the HPLC conditions the reaction of Gd^{3+} will be complete in $<10^{-6}$ s. As expected from the reverse-phase chromatography, the HPLC

$$Gd^{3+} + H(EDTA)^- \text{ <====> } Gd(EDTA)^- + H^+ \qquad (7)$$

capacity factor (k') in 4% acetonitrile (ACN) was always smaller than the one obtained in 2% ACN. When the capacity factor values (log k') at 2% acetonitrile (y-axis) and 4% (x-axis) ACN were plotted, an excellent linear correlation resulted (eq. 8, Fig. 3) with a slope of 1.26 ± 0.06 and an intercept of 0.26 ± 0.02 with r= 0.99.

$$\log k' (2\%) = (1.26 \pm 0.06) \log k' (4\%) + (0.26 \pm 0.02) \qquad r = 0.99 \qquad (8)$$

Correlation Between Log P Values and HPLC Capacity Factors

Plots of log k' vs. log P values were attempted (Fig. 4). Only results for log k' vs. log P (B/W) are shown. Linear least squares fits of the plots of log k' vs. log P values in n-butanol/water and n-octanol/water gave the following results.

2% ACN: $\log k' = (0.5 \pm 0.1) \log P(B/W) + (1.8 \pm 0.3)$ r=0.77 (9)

$\log k' = (0.8 \pm 0.2) \log P(O/W) + (3.3 \pm 0.8)$ r=0.83 (10)

4% ACN: $\log k;' = (0.7 \pm 0.1) \log P (B/W) + (1.6 \pm 0.2)$ r =0.89 (11)

$\log k;' = (0.8 \pm 0.1) \log P (O/W) + (3.2 \pm 0.4)$ r =0.91 (12)

Some anomalies exist. For example, $Gd(DTPA)^{2-}$ is more hydrophilic than Gd(EDTA)$^-$, however, the k' value for the later is smaller than the former. Probably the presence of additional

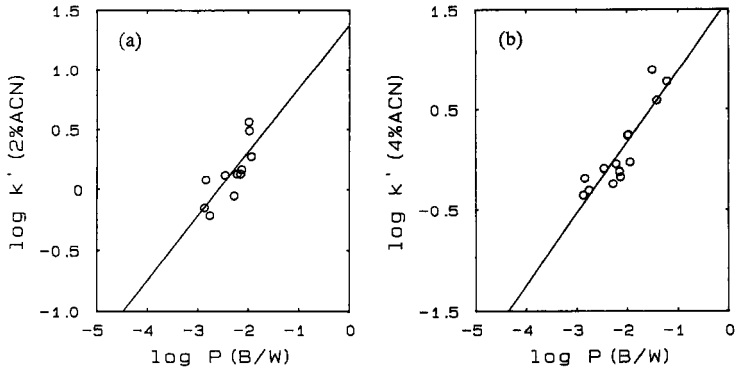


Fig. 4. Plot of log k' (a) in 2% and (b) 4 % acetonitrile vs. log P in n-butanol/water.

negative charge on $Gd(DTPA)^{2-}$ encourages interaction with silanol groups of the column. Coordinated carboxylate groups in lanthanide polyamino carboxylate complexes are also more labile than coordinated amines and may dissociate briefly and interact with the silanol groups [22]. A similar rationalization can be made for the complexes of HAA-DO3A and DOTMA. Either alcoholic groups on HAA-DO3A or carboxylate groups on DOTMA interact with the stationary phase of the column.

SUMMARY

We conclude that with some exceptions, the log P values of Gd(III) chelates can be predicted from the calculated log P of the free ligand and that the log P in n-butanol/water and n-octanol/water can be calculated from the HPLC capacity factors. These data will be useful in designing future MRI contrast agents, the physical and biological properties of which are influenced by log P values.

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DEVELOPMENT AND VALIDATION OF A CHIRAL HPLC METHOD FOR THE QUANTITATION OF METHOCARBAMOL ENANTIOMERS IN HUMAN PLASMA

WENG NAIDONG[1]*, JEAN W. LEE, AND JAMES D. HULSE
Harris Laboratories, Inc.
624 Peach Street
Lincoln, Nebraska 68501

ABSTRACT

An isocratic chiral HPLC method was developed and validated for the quantitation of methocarbamol enantiomers in human plasma. Methocarbamol and an internal standard were extracted with ethyl ether. Chiral separation was achieved on coupled Spherisorb CN and Chiralcel OD columns with a mobile phase of ethanol - hexane (30:70, v/v). The detection was by UV at 272 nm. Linearity was established at 0.5 - 50 μg/ml ($r > 0.998$). Interday precision and accuracy of the calibration standards were demonstrated by 0.8 to 9.4% coefficients of variance (C.V.) and -5.2 to +3.8% relative error (R.E.). Quality controls showed interday precision and accuracy of 4.4 to 7.2% C.V. and +0.4 to +5.5% R.E. Recovery of methocarbamol enantiomers was 77 - 84%. No interconversion of the methocarbamol enantiomers was observed during process of storage, extraction nor chromatograph. Reproducibility and stability of the analytical columns were demonstrated.

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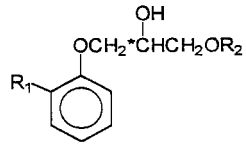
* To whom correspondence should be addressed.
[1] Present address : ESi Pharmaceuticals, 2 Esterbrook Lane, Cherry Hill, NJ 08003, U.S.A.



R_1	R_2	
$-OCH_3$	$-OCNH_2$	Methocarbamol
$-OCH_3$	-H	Guaifenesin
$-CH_3$	-H	Mephenesin (I.S.)

Figure 1. Chemical structures of methocarbamol, guaifenesin and mephenesin (I.S.). The asterisk denotes the location of the chiral center.

INTRODUCTION

Methocarbamol (MET) is a carbamate derivative used for the relief of discomfort. Guaifenesin (GUA), also biologically active, is the major degradation compound of MET [1]. The chemical structures of MET and GUA are shown in Figure 1. MET is administered as the racemate. The mechanism of the pharmacological action of MET in vivo has not been established [2]. Information is lacking for pharmacokinetics and pharmacodynamics of each of the MET enantiomers.

Previous methods using UV spectrophotometry after derivatization [3] and HPLC [4] for the quantitation of racemic MET lacked the sensitivity and selectivity. A more sensitive HPLC method for the routine quantitation of racemic MET in human plasma was developed and validated in our laboratory [5]. With this method, the lower limit of quantitation is 1 μg/ml with a signal to noise ratio of 20.

Alessi-Severini and co-workers have published a chiral HPLC method to determine MET enantiomers in biological fluids [6]. This method consists of a lengthy derivatization procedure (12 hours) and a long run time (50 minutes). Degradation of MET occurred during derivatization. Their results showed

differences in the pharmacokinetics of the enantiomers in the rat; the ratio of the two enantiomers (S/R) was 2.40. The ratio was 1.19 in one human plasma sample and 1.14 in one human urine sample.

A simple chiral HPLC method was developed and validated to facilitate the pharmacokinetic investigations of MET enantiomers. Separation of MET enantiomers from matrix interferences was achieved on coupled Spherisorb CN and Chiralcel OD columns with a mobile phase consisting of ethanol and hexane.

Very recently, Demian has reported using a Chiralcel OD column to separate enantiomers of several compounds including methocarbamol for the purpose of optical purity determination [7].

EXPERIMENTAL

Materials and Reagents

MET and GUA were from the United States Pharmacopeia and the internal standard (I.S.) mephenesin was from Aldrich (Milwaukee, WI, U.S.A.). The chemical structure of mephenesin is also shown in Figure 1. Dehydrated alcohol (ethanol) was purchased from Quantum (Newark, NJ, U.S.A.). All other organic solvents were of HPLC grade and were from Fisher (Fair Lawn, NJ, U.S.A.). Deionized water was purified by a NANOpure™ system from Barnstead (Dubuque, IA, U.S.A.). Control human sodium fluoride plasmas were drawn in our laboratory from healthy volunteers.

Standards and Quality Controls

Two primary MET stock solutions prepared from separate weighings were used to prepare standards and quality control samples (QCs). Water solutions of MET primary stock and substocks were prepared and stored at 4°C in polypropylene tubes. Working standards were prepared fresh daily by spiking 100 µl 4-fold concentrated solutions into 400 µl of blank control plasma using Gilson pipettors from Rainin (Woburn, MA, U.S.A.). The pipettors were calibrated daily.

The concentrations of calibration standards of each enantiomer were 0.5, 1, 2.5, 5, 12.5, 25, 37.5 and 50 μg/ml. Three levels of QCs, 1.5, 10, and 35 μg/ml for each enantiomer were prepared, aliquoted, and stored frozen at - 20°C with the clinical samples to be analyzed.

Instrumentation

The HPLC system consisted of a Waters 501 HPLC pump (Milford, MA, U.S.A.), a Perkin-Elmer ISS-100 autosampler (Norwalk, CT, U.S.A.), a Waters 484 UV detector set at 272 nm and a VG™ Multichrom data system for VAX™/VMS (Manchester, England). The HPLC autosampler tray was cooled at 4°C by a Brinkman RM6 cooling system (Westbury, NY, U.S.A.).

A Spherisorb CN column, 5 μm, 25 cm x 0.46 cm i.d. (Phase Separation Inc., Norwark, CT, U.S.A.) and a Chiralcel OD column, 5 μm, 25 cm x 0.46 cm i.d. purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.) were coupled in series. The columns were used at room temperature. The mobile phase was ethanol - hexane (30:70, v/v). The flow rate was 2.0 ml/min and the run time was 15 min.

Data Treatment

Chromatograms were measured using a VG™ Multichrom data system. The raw data output was acquired on a VG™ Chromserver and then transferred to the VAX™/VMS. A weighted 1/y linear regression was used to determine slopes, intercepts and correlation coefficients, where y = the ratio of the compound peak height to the I.S. peak height. The resulting parameters (y-intercept and slope) were used to calculate concentrations from the equation:
concentration = [y - (y-intercept)]/slope.

Extraction Procedure

To 400 μl plasma sample, 100 μl of I.S. water solution (200 μg/ml) was added. After mixing, 5 ml of ethyl ether was added to extract the compounds of interest by shaking for 15 min. The ethyl ether layer was decanted to another tube after

freezing the aqueous layer in a dry ice - acetone bath. The ethyl ether extract was evaporated to dryness under a stream of nitrogen and the residue reconstituted in 200 µl of ethanol - hexane (30:70, v/v). Fifty µl was injected onto the chiral HPLC system.

RESULTS AND DISCUSSION

HPLC separation

Figure 2 shows the chromatograms of unextracted enantiomers of MET, GUA, and I.S. Excellent chiral separations of MET and GUA enantiomers were achieved. The I.S. enantiomers were partially separated. Figure 3 shows chromatograms of extracted blank control plasma, the standard at limit of quantitation (L.O.Q) and a QC (35 µg/ml) for the chiral assay. The reproducibility of the HPLC method was demonstrated on Figure 2(B) showing chromatograms of the same samples obtained on another lot of the analytical columns.

The stereo-configuration of each enantiomer peak was not identified because the reference substances of each MET enantiomer are not available. MET enantiomer peaks were designated as MET-E1 and MET-E2 based on their elution order on the chromatogram. The enantiomers of the IS were partially separated from each other and the first peak (I.S.-E1) was used as the internal standard peak. Using several different mobile phases we were unable to separate MET-E1 from a matrix interference peak by using Chiralcel OD column alone. Figure 4 shows a chromatogram obtained on a Chiralcel OD column alone with a mobile phase ethanol - hexane (30:70, v/v). Coupling a CN column in front of the Chiralcel OD enable MET-E1 complete separation from the matrix peak as shown in Figure 3. Six control lots of control plasma were screened and none of them interferred the quantitation of the MET enantiomers as shown in Figure 5.

The GUA enantiomers had retention times of 5.2 min (GUA-E1) and 6.7 min (GUA-E2) on the chiral HPLC system. GUA-E2 coeluted with MET-E1. However, this should not cause any quantitation problem because GUA was less than 0.4% of the MET as determined by the previously developed achiral method [5]. The same proportion of GUA was also presented in an unextracted,

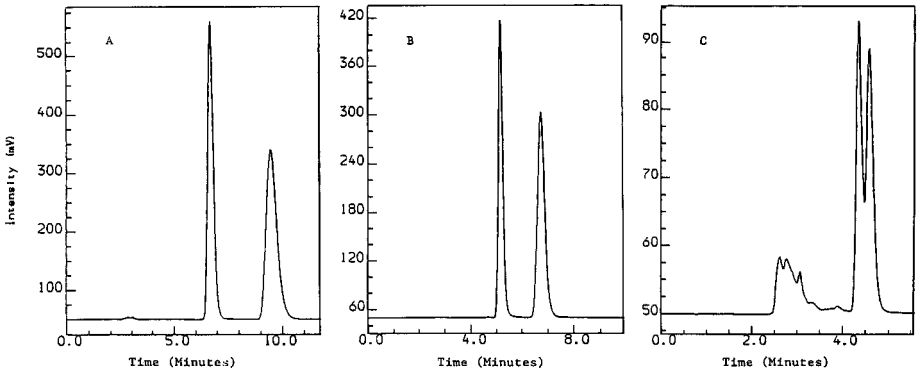


Figure 2. Chromatograms of chiral separation of unextracted methocarbamol (A), guaifenesin (B), and I.S. (C).

Columns: Spherisorb CN, 5μm, 25 x 0.46 cm i.d. and Chiralcel OD, 5 μm, 25 x 0.46 cm i.d. in series. Mobile phase: ethanol - hexane (30:70, v/v). Flow rate: 2.0 ml/min. Detection: UV at 272 nm.

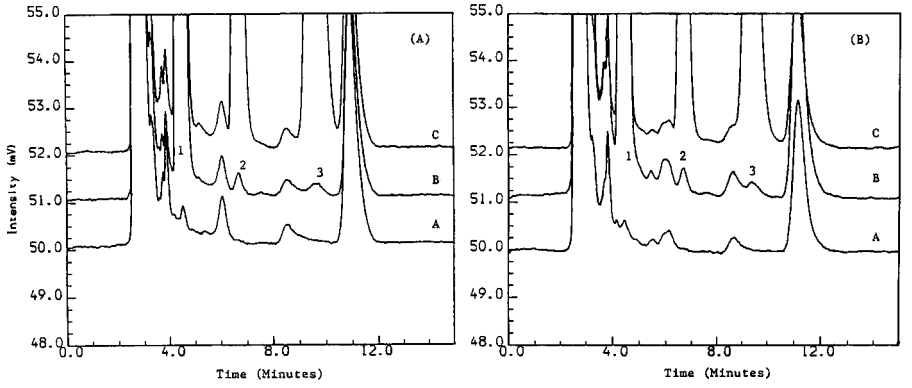


Figure 3. Chromatograms of chiral separation of methocarbamol enantiomers in human plasma on two lots (A and B) of Spherisorb CN and Chiralcel OD column.

A: blank control plasma, B: standard at 0.5 μg/ml for each enantiomer and C: QC at 35 μg/ml for each enantiomer. Columns: Spherisorb CN, 5μm, 25 x 0.46 cm i.d. (Lot # A and B) and Chiralcel OD, 5 μm, 25 x 0.46 cm i.d. (Lot # A and B) in series. Mobile phase: ethanol - hexane (30:70, v/v). Flow rate: 2.0 ml/min. Detection: UV at 272 nm. Peak identification: 1 = mephenesin (I.S.) enantiomers, 2 = methocarbamol enantiomer 1 (MET-E1), 3 = methocarbamol enantiomer 2 (MET-E2).

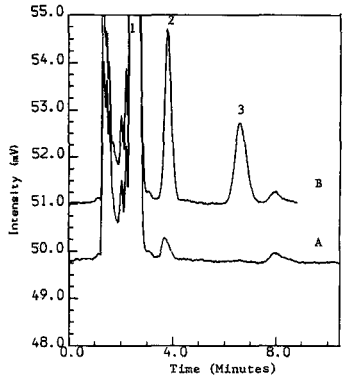


Figure 4. Chromatograms of chiral separation of methocarbamol enantiomers in human plasma on a Chiralcel OD column alone.

A: blank control plasma, B: standard at 2.5 μg/ml for each enantiomer. See Figure 3 for peak identification.

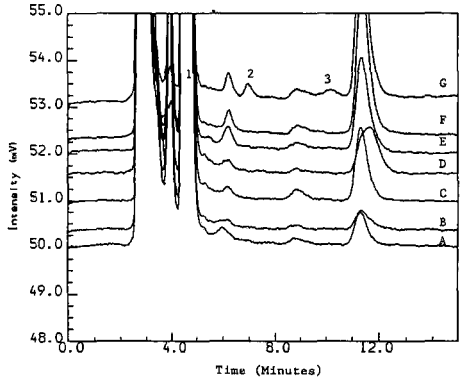


Figure 5. Chromatograms of screening six lots of blank control human plasma for the chiral separation of methocarbamol enantiomer.

A-F: six lots of control human plasma spiked with the I.S., G: standard at 0.5 μg/ml for each enantiomer. See Figure 3 for peak identification.

freshly-prepared MET reference solution, indicating that GUA was not formed upon storage, extraction or injection procedures [5].

Figure 6 shows that after 280 injections, a slight decrease in the resolution of I.S. enantiomers was observed for the Chiralcel OD column. The I.S. enantiomer resolution changed from 0.82 to 0.76 and the resolution of MET-E1 and MET-E2 changed from 4.2 to 4.1. The calculated plate number per column, calculated from MET-E1, decreased from 3070 to 2710 after 280 injections. This slight loss of performance did not affect the accurate determination of MET enantiomers.

We compared the results of using racemic I.S. to those of using only one enantiomer, I.S.-E1, on two validation curves. The C.V. values and mean results were analyzed by an F-test and a Student's t-test ($P = 0.05$) [8]. In no instance were the difference by the F-test or the t-test significant. The single I.S. enantiomer was prepared by injecting 100 µl of racemic I.S. (20 mg/ml in mobile phase) onto the chiral HPLC system described here with a mobile phase ethanol - hexane (5:95, v/v). The fractions corresponding to I.S.-E1 (retention time = 17.5 min) and I.S.-E2 (retention time = 20.0 min) were collected and evaporated to dryness under nitrogen. The enantiometric purities for I.S.-E1 and I.S.-E2 were 99.9% and 99.5% as determined by the afore-mentioned HPLC. The I.S.-E1 residue was taken up by 10 ml of water and this solution was used as the working I.S. in these two comparative curve runs. I.S.-E1 was stable for at least 9 days at 4 °C. No I.S.-E2 was formed through the extraction and chromatography process. Since the pure I.S.-E1 was not commercially available, we considered it was easier to use the racemate as the I.S.

Extraction

We developed a simple procedure to extract MET and the I.S. from plasma. The recoveries of MET from human plasma were close to 100% using ethyl acetate as an extraction solvent [5]. However, for the chiral assay, extraction using ethyl acetate lead to down field peaks which interfered with the subsquent chromatographic run. A less polar organic, ethyl ether, was therefore used for the extraction. The recoveries at 0.5, 25, and 50 µg/ml of each enantiomer were 79% ($n = 2$, C.V. = 8.1%), 84% ($n = 2$, C.V. = 1.7%) and 84% ($n = 2$, C.V. = 7.5%) for

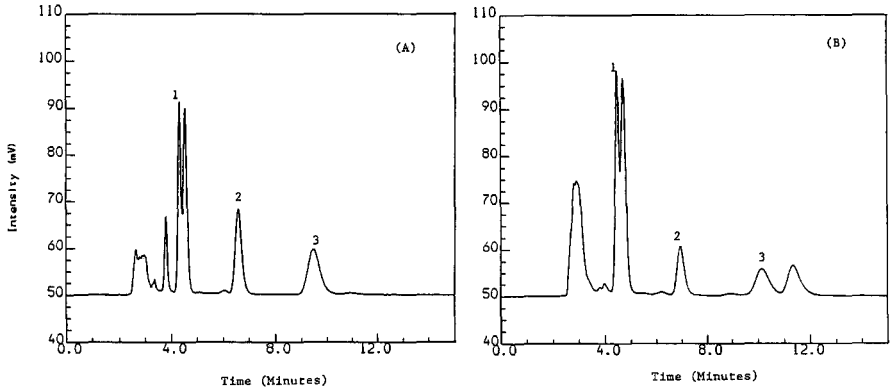


Figure 6. Chromatograms of injections # 15 (A) and # 280 (B) of chiral separation of methocarbamol enantiomers.

Peak identification: 1 = I.S. enantiomer 1 (I.S.-E1), 2 = methocarbamol enantiomer 1, 3 = methocarbamol enantiomer 2.

MET-E1, 77% (n = 2, C.V. = 3.7%), 84% (n = 2, C.V. = 0.8%) and 84% (n = 2, C.V. = 7.5%) for MET-E2. The I.S.-E1 recovery was 98% (n = 6, C.V. = 7.0%).

Validation performance

Five validation curves were run on five separate days. We observed consistent slopes and good correlation coefficients ($r > 0.9983$) through out these validation runs. Table 1 shows the interday linearity and precision data of each standard concentration. The L.O.Q. was 0.5 μg/ml (signal to noise ratio = 10 for MET-E1 and 7 for MET-E2). Tables 2 shows the interday and intraday accuracy and precision of QCs. The accuracy and precision data show that the chiral method is consistent and reliable with low error and imprecision for standards and QCs at the entire concentration range. The current standard curve range is suitable for pharmacokinetic studies.

Stability

In order to mimic the analysis of authentic clinical samples, stabilities of processing (freeze-thaw, benchtop), chromatography (on-system and re-injection)

TABLE 1
Interday Precision and Accuracy of Methocarbamol Enantiomer Standards

Standard curve number	Methocarbamol enantiomer (μg/ml) 0.50	1.00	2.50	5.00	12.5	25.0	37.5	50.0	r
				MET-E1					
A	0.49	1.03	2.61	4.95	11.9	25.0	36.9	51.3	0.9990
B	0.48	1.15	2.52	4.76	11.9	24.9	37.2	51.2	0.9992
C	0.50	0.96	2.42	5.19	13.2	25.3	37.0	49.5	0.9996
D	0.45	0.98	2.82	4.93	12.9	25.5	37.3	49.2	0.9995
E	0.45	1.07	2.53	5.01	12.4	25.3	37.3	49.3	0.9995
Mean	0.47	1.04	2.58	5.01	12.4	25.3	37.3	50.1	
C.V. (%)	4.9	7.3	5.8	4.0	4.9	1.2	0.8	2.1	
R.E. (%)	-5.2	+3.8	+3.2	+0.3	-0.8	+1.0	-0.7	+0.2	
				MET-E2					
A	0.48	1.07	2.64	4.96	11.8	24.7	36.7	51.8	0.9983
B	0.60	1.01	2.31	4.52	12.2	25.4	37.1	51.0	0.9993
C	0.51	0.92	2.41	5.20	13.4	25.7	37.1	48.9	0.9988
D	0.50	0.86	2.90	4.98	12.6	25.4	37.1	49.7	0.9997
E	0.49	0.98	2.49	5.19	12.3	25.7	38.0	48.8	0.9991
Mean	0.52	0.97	2.55	4.97	12.5	25.4	37.2	50.0	
C.V. (%)	9.4	8.4	9.0	5.5	4.8	1.6	1.3	2.6	
R.E. (%)	+3.2	-3.2	+2.0	-0.6	-0.3	+1.5	-0.8	+0.1	

TABLE 2
Precision and Accuracy of Methocarbamol Enantiomers Quality Controls

	MET-E1 (μg/ml) 1.50	10.0	35.0	MET-E2 (μg/ml) 1.50	10.0	35.0
Interday (n = 30)						
Mean	1.58	10.6	35.7	1.51	10.5	35.9
C.V. (%)	5.4	5.2	7.2	7.7	4.4	7.2
R.E. (%)	+5.2	+5.5	+2.0	+0.4	+5.4	+2.5
Intraday (n = 6)						
Mean	1.59	10.2	34.3	1.60	10.1	34.0
C.V. (%)	1.8	1.8	2.7	1.6	1.5	4.6
R.E. (%)	+5.2	+1.7	-2.1	+6.8	+1.0	-2.9

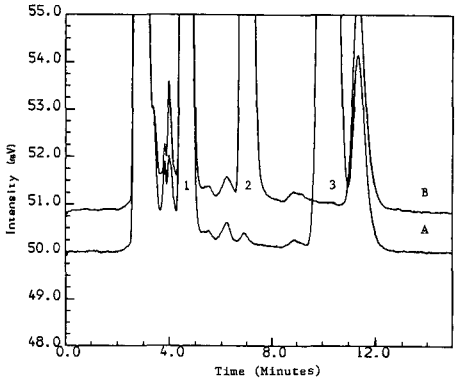


Figure 7. Chromatograms of methocarbamol enantiomer 1 (A) and enantiomer 2 (B). The samples have gone through a storage period of 9 days at -20°C, three cycles of freeze-thaw, 3 hours on bench prior to extraction and 32 hours on the autosampler before injection.

See Figure 3 for peak identification.

and sample storage were established [5]. No degradation of MET was observed after those stability tests [5]. Stability of single enantiomer was also examined. Small amounts of pure MET enantiomers were isolated by the chiral method described here. Figure. 7 shows the chromatograms of single MET enantiomers through nine days of storage in plasma at -20 °C, three cycles of freeze-thaw, two hours on bench-top at room temperature prior to extraction and 32 hour on an autosampler before injection. Interconversion of the enantiomers was insignificant. There were only 0.1% of MET-E2 in MET-E1 and 0.3% of MET-E1 in MET-E2 in these stability test chromatograms.

CONCLUSIONS

A simple chiral HPLC method was developed for the analysis of methocarbamol enantiomers in human plasma. This chiral HPLC method is suitable for pharmacokinetic studies of drug interaction as well as dose-efficacy of formulations with the single methocarbamol enantiomer. Stability of methocarbamol and its

enantiomers during storage, extraction and injection has been established. The analytical column demonstrated good robustness and no column batch to batch variability was observed.

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FLUOROMETRIC DETERMINATION OF AMINOCARB AND MEXACARBATE AND SOME OF THEIR METABOLITES BY LIQUID CHROMATOGRAPHY: INFLUENCE OF STRUCTURAL FACTORS ON FLUORESCENCE INTENSITY

K. M. S. SUNDARAM AND J. CURRY
Natural Resources Canada
Canadian Forest Service
Forest Pest Management Institute
Sault Ste. Marie, Ontario, P6A 5M7, Canada

ABSTRACT

A direct and sensitive high performance liquid chromatographic method with fluorescence detection is reported to identify aminocarb and mexacarbate and some of their metabolites. The observed detection limits were compared by linking the liquid chromatograph to a variable wavelength UV detector. The separation system consisted of an RP-8 OS (10 μm) 20 cm x 4.6 mm I.D. column and acetonitrile-phosphate (pH 7.2) buffer. The excitation and emission wavelengths of the fluorescence detector were set respectively at 200 and 370 nm. The UV detector was set at 242 and 200 nm for aminocarb and mexacarbate, respectively. The sensitivity in fluorescence detection was not superior to UV method because of the influence of substituents on the aryl ring on fluorescence intensity. Both methods were found to be adequate for the determination of most of the analytes from natural water at nanogram levels after necessary extraction and cleanup procedures.

INTRODUCTION

In previous papers, methods based on high performance liquid chromatography (HPLC) using ultraviolet (UV) absorbance detection (UVD) and post-column derivatization (PCD) to form fluorescent derivatives were reported to quantify two carbamate insecticides, aminocarb (4-dimethylamino-3-methylphenyl N-methylcarbamate) and mexacarbate (4-dimethylamino-3,5-xylyl N-methylcarbamate), used in forest insect control programs in Canada, and some of their common metabolites (1,2). The inherent sensitivity and selectivity of fluorescence detection (FD) in residue analysis has not been explored fully for these compounds although some sporadic attempts have been made earlier (3,4). Aminocarb and mexacarbate and some of their metabolites have intrinsic fluorescence and exhibit measurable fluorometric intensity provided that they are sufficiently excited at a suitable wavelength with a light source, such as a deuterium lamp. The emission wavelength is specific to each molecular species and is measurable using a spectrofluorometric detector. In this paper, we report a simple and direct fluorometric method, without any derivatization, to detect and quantify aminocarb and mexacarbate and some of their metabolites using a fluorescence detector linked to a liquid chromatograph. We have also examined the structural factors of the analytes chromatographed, which influenced, either positively or negatively, the sensitivity and specificity of fluorometric detection. In addition, we have compared the fluorometric sensitivity with the commonly used UVD method. Finally, we used both methods to quantify the analytes in fortified natural water samples to examine the suitability of the FD over UVD method and to evaluate the limits of quantification (LOQ) of the analytes and possible interferences in the chromatographic profiles obtained by both methods.

MATERIALS AND METHODS

Materials

Analytical grade standards (purity > 98.5 %) of aminocarb and mexacarbate and 14 of their metabolites (7 for each insecticide) used in this study

were supplied respectively by Union Carbide Agricultural Products Co., Inc., Research Triangle Park NC, and Mobay Chemical Corporation, Kansas City, MO. The chemical names of the compounds and abbreviations used for them in this paper are given in Table 1.

Working standards were prepared by diluting 1000 μg/mL stock solutions of the analytes in acetonitrile with aqueous acetonitrile ($CH_3CN:H_2O$ = 1:2) to appropriate concentrations. Separate standards were prepared for each analyte to determine its sensitivity and linear range. Mixed standards were prepared consisting of aminocarb and its seven metabolites and mexacarbate and its seven metabolites to study the response of the detectors. The standards were prepared in low actinic stained volumetric flasks and stored at −20°C until use. All standards were thoroughly filtered (Acrodisc® LC13 PVDF, 0.2 μm, Gelman Sciences, Ann Arbor, MI U.S.A.) prior to injection into the HPLC system.

All solvents and water were HPLC grade supplied by Canlab, Mississauga, Ontario and were tested for their spectral purity prior to use. They were filtered through appropriate Millipore® filters and degassed before use. Alumina (activity 1, type WN-6 neutral), sodium sulphate (anhydrous), potassium dihydrogen phosphate (KH_2PO_4) and sodium hydroxide were of analytical grade and obtained from BDH Chemicals, Canada Ltd., Toronto, Ontario. The buffer solution was prepared by adjusting the pH to 7.2 by adding dropwise 0.10 M sodium hydroxide to 1.0 L of 0.01 M phosphate solution, under magnetic stirring. It was filtered (0.45 μm Millipore filter) and degassed prior to use.

Equipment

A Hewlett Packard (HP)(model 1084B) variable wavelength (190-600 nm UV detector) liquid chromatograph interfaced with a variable volume injector (HP 79842 A) and autosampler (HP 79842) was used. It is also equipped with a microprocessor and an electronic integrator linked to an LC terminal (HP 79850 B) to provide the area, area %, retention time (RT), etc. for each chromatographic peak. The instrument also had automatic degassing system, dual solvent system

TABLE 1

List of Aminocarb and Mexacarbate and Their Carbamate Metabolites Used in the Study

Chemical Name	Abbreviation
4-dimethylamino-3-methylphenyl N-methylcarbamate	A
4-methylformamido-3-methylphenyl N-methylcarbamate	MFA
4-methylamino-3-methylphenyl N-methylcarbamate	MA
4-formamido-3-methylphenyl N-methylcarbamate	FA
4-amino-3-methylphenyl N-methylcarbamate	AA
4-dimethylamino-3-methylphenol	MP
4-methylamino-3-methylphenol	MAP
4-amino-3-methylphenol	AMP
4-dimethylamino-3,5-xylyl N-methylcarbamate	M
4-methylformamido-3,5-xylyl N-methylcarbamate	MFM
4-methylamino-3,5-xylyl N-methylcarbamate	MAM
4-formamido-3,5-xylyl N-methylcarbamate	FM
4-amino-3,5-xylyl N-methylcarbamate	AM
4-dimethylamino-3,5-xylenol	DMAX
4-methylamino-3,5-xylenol	MAX
4-amino-3,5-xylenol	AX

and dual pump heads with common drive to give stable and reproducible flows. A prepacked HP RP-8 OS (10 μm) stainless steel separation column (20 cm x 4.6 mm I.D.) was used in conjunction with an HP RP-8 guard column (3 cm x 4.6 mm I.D. x 10 μm) throughout this work. The column temperature was kept at 30°C to maintain RT reproducibility and the injection volume used was 100 μL. The fluorescence detector was a Kratos FS 970 LC fluorometer (Kratos Analytical Instruments, Ramsey, NJ) equipped with a 10 μL flow cell and automatic overload reset (FSA 986) with variable excitation wavelengths (GM 970 monochrometer) and fixed wavelength emission filters. Additional operating parameters were as follows:

Mobile system:	Acetonitrile - 0.01 M phosphate buffer (pH 7.2)	
Flow rate:	1.0 mL/min	
Run time:	60 min	
Gradient:	Time(min)	% CH_3CN
	0	0
	25	30
	35	50
	45	50
	55	0
Wavelengths (nm):	Aminocarb	Mexacarbate
UV (sample:reference)	242:430	200:430
FD (excitation:emission)	200:370	200:370

Although the excitation and absorption spectra of many compounds in dilute solutions are nearly identical (5), the use of different wavelengths in absorption and fluorescence for aminocarb was necessary, as a compromise, to enhance the detection sensitivity of some of its metabolites. Also it is observed generally that the use of short excitation wavelength (200 nm) in the fluorometric detection of aminocarb and mexacarbate resulted in higher emission intensities, thus more sensitive detection limits for the analytes studied. In addition, the

percent composition of the mobile phase constituents in the gradient system was adjusted after repeated trials to give optimum resolution of the peaks.

Method validation

With all system components in place in the UVD system, the column was equilibrated with mobile phase at a flow rate of 1 mL/min until a steady baseline was obtained. The same was repeated for the FD system and intensities are recorded for solvent responses at the chosen wavelengths for necessary corrections. To evaluate sensitivity, individual standard solutions of aminocarb, mexacarbate and their metabolites were injected five times, first with UVD and then with the FD system. Mixed standard solutions were then injected to obtain good resolution of the peaks, reproducible peak area measurements and retention times under the chosen experimental conditions. The coefficient of variation (CV) between injections ranged from 1.7 % to 3.4 % for UVD and 2.7 % to 5.6 % for FD, depending on the type of analyte. Replicate analysis of mixed standards of the insecticides at four day intervals gave good reproducibility (av. CV < 5 %) in both the detector systems. To establish linearity of the detectors, the individual analytes, ranging in concentration from 0.005 μg/mL to 10.0 μg/mL in acetonitrile, were chromatographed, each in triplicate, using both the detectors successively. The linearity of each compound was established by linear regression analysis of peak area responses versus concentration. The correlation coefficients ranged from 0.97 to 1.00 indicating that UVD and FD methods are suitable to analyze the compounds listed in Table 1.

Recovery of analytes from stream water

The procedure used was similar to the one reported recently (2). In brief, water samples (100 mL) fortified with the analytes (0.5 and 5 μg/mL) were serially extracted with dichloromethane, dried with anhydrous sodium sulphate, flash-evaporated to dryness and the residue in acetonitrile was partitioned with hexane. After flash evaporation of the polar layer, the crude residue in ethyl

acetate was cleaned up over an alumina minicolumn, eluted with ethyl acetate or ethyl acetate/methanol solution depending on the type of analyte, the volume was then adjusted and analyzed by HPLC using the UV and fluorescence detectors. The retention times and peak areas were compared with those of the standard solutions.

Unfortified water samples and reagent blanks were extracted and analyzed following the described method. No interfering peaks corresponding to any of the analytes studied were found.

RESULTS AND DISCUSSION

Optimization of chromatographic response

The LOQ in HPLC depends to a large extent: (a) on the type of detector; (b) the wavelength used for detection (UV) or excitation (FD) and (c) the chromatographic column. Fluorescence is often 10- to 100-fold more sensitive than absorption, provided the analytes have appreciable native fluorescence (6). However due to low quantum efficiency of these molecules, the FD measurements have nearly the same sensitivity as in UV detection. The use of 242 nm for aminocarb and 200 nm for mexacarbate, found by ultraviolet scans, as optimum absorption wavelengths, gave good chromatographic response to the parent materials and their metabolites. Any alteration in the wavelengths resulted in reduced sensitivity (especially for the metabolites) and/or increased background noise due to impurities in the mobile phase. The same is true for the selection of 200 nm, as the excitation wavelength for FD, which resulted in reasonably high emission intensities for the fluorescent analytes and low background fluorescence.

Based on our earlier studies (1,2), the HP RP-8 10 μm - 20 cm column gave better resolution of chromatographic peaks, which are symmetrical, compared to the shorter columns with 5 μm column packing. The use of acetonitrile - phosphate buffer as the mobile phase increased chromatographic efficiency (better resolution of peaks) compared to either methanol - water or acetonitrile - water

used previously (1,2). The buffer system was also necessary to reduce tailing of the phenol metabolites which were not previously studied (1,2). The system chosen was also sufficiently selective to resolve, within the chromatographic run time of 60 minutes, most of the analytes (except FA/AA and AM/MAX) belonging to each insecticide.

Chromatograms of the analytes

Figures 1 and 2 illustrate the separation achieved for aminocarb and its metabolites using the UV and fluorescence detectors respectively. Figures 3 and 4 illustrate the same for mexacarbate and its metabolites. The concentration shown in the figures is 1.0 μg/mL for all the analytes. This is below the LOQ in FD for AMP, MAP, AX and MAX. Their retention times only, without their chromatographic response, are depicted in figures 2 and 4. Mean RT (min) obtained for each individual compound after replicate determinations (n>5) using the UVD are given in the caption of figures 1 and 3. The variation in RT was less than 3 % for all the analytes.

The elution patterns of both insecticides show that more hydrophilic analytes, especially the phenols with some exceptions, and aldehydes, eluted sooner (low RT) compared to the hydrophobic moieties, such as the parent insecticides. The elution region of FA and AA in the UVD are nearly similar, therefore it is not possible to detect and quantify them from the mixed standards unless the analytes were injected separately. However FA is non-fluorescent, therefore the analysis of AA in the mixed standard by fluorescent detection is feasible with direct sample injection. A similar situation is also encountered between AM and MAX. MAX does fluoresce, but its LOQ is 1000 ng, compared to 20 ng for AM, so the interference from MAX is minimal. The aldehyde metabolites, MFA, FA, MFM and FM are quantified only by using the UV absorbance detector, because fluorescence detection is not possible for them, due to lack of sufficient quantum efficiency. The metabolites MAP, AMP, MAX and AX all have low fluorescent signals and the use of FD to quantify them is not

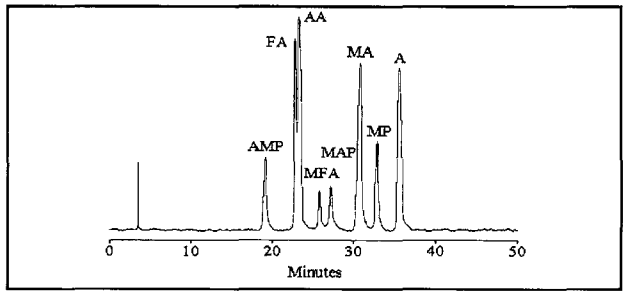

Figure 1: Chromatogram of aminocarb and metabolites by UVD with RT (min): A=35.94; MFA=25.83; MA=30.90; FA=23.80; AA=24.22; MP=32.44; MAP=27.12; AMP=19.06.

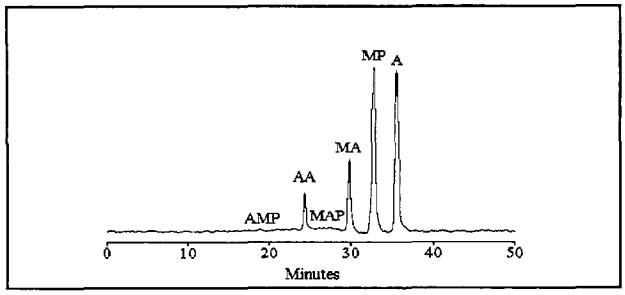

Figure 2: Chromatogram of aminocarb and its metabolites by FD. Retention times set to match with UVD values.

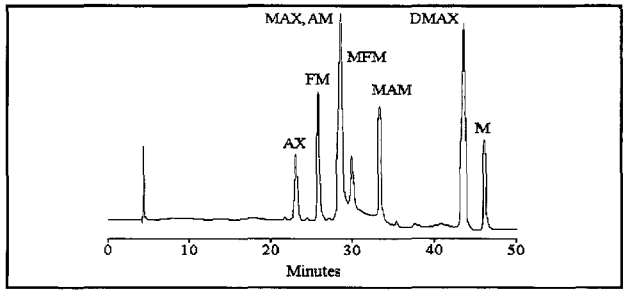

Figure 3: Chromatogram of mexacarbate and metabolites by UVD with RT (min): M=46.11; MFM=30.02; MAM=33.11; FM=25.85; AM,MAX=28.86; DMAX=43.43; AX=23.02.

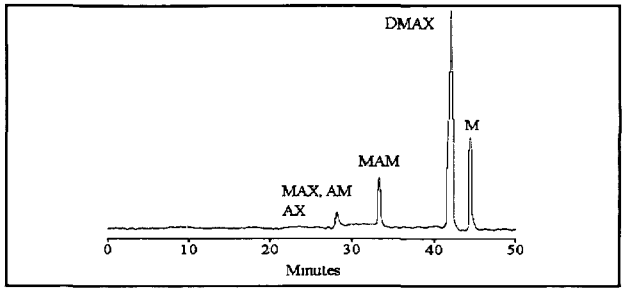

Figure 4: Chromatogram of mexacarbate and its metabolites by FD. Retention times set to match with UVD values.

desirable. Among the analytes studied, DMAX was highly sensitive to the HPLC-fluorescence technique, giving a sharp symmetrical peak even at the 1 ng level.

The LOQ (taken arbitrarily as five times signal/noise ratio) for aminocarb and its metabolites for ultraviolet detection, in the standard solutions, ranged from 5 to 50 ng, whereas for mexacarbate the values ranged from 5 to 20 ng. However, in the fluorometric method, the LOQ for the fluorescent moieties ranged from 5 to 1000 ng for aminocarb and 1 to 1000 ng for mexacarbate. Therefore, it is apparent that fluorometric quantification for these series of compounds is less sensitive than UV detection, unless a post-column derivatization technique (2) is adapted to improve the situation.

Structural factors affecting fluorescence

Aminocarb and mexacarbate and some of their metabolites listed in Table 1 exhibit weak native fluorescence due to the interaction among delocalized π orbital electrons of the aryl (Ar) ring, lone electron pairs on the N atom of NMe_2 group and O atom of the C_{Ar}-O-C group. In addition, the methyl (Me) and NMe_2 groups attached to the Ar ring are electron donors, thus increasing the electron density on the Ar ring, which facilitates fluorescence. On the other hand, the

presence of Me groups (one in aminocarb and two in mexacarbate) in the *o*-position to the NMe_2, interfere sterically and inhibit the overlap of lone pair electrons on the NMe_2 group with the π electrons of the Ar ring, reducing fluorescence intensity (7).

The four compounds, MFA, FA, MFM and FM, did not show any measurable fluorescence, because of the presence of the -CHO group, which is a strong electron withdrawer from the Ar ring. Thus fluorescence detection is not feasible for these molecules with direct sample injection. Successive demethylation to form NHMe and NH_2 from NMe_2 also diminished the electron density on the AR ring, leading to higher LOQ levels, increasing progressively from MAM (5 ng) to MA (10 ng) and finally to AA, AM (20 ng).

Among the six phenols, DMAX exhibited maximum fluorescence, probably due to the formation of highly conjugated phenoxide ion by the loss of proton accelerated by the presence of electron donating Me and NMe_2 groups on the Ar ring. However, the fluorescence intensity diminished (or the fluorometric LOQ increased) from DMAX to MP, due to the absence of electron donating Me groups in the latter. On the other hand, the other four phenols, MAX, AX, MAP and AMP, all show low quantum efficiency or low fluorescence intensity (LOQ 1000 ng), although the phenolic group is an electron donor. The reasons for this are not clear, however we can speculate the formation of a protonated structure, such as $Me_2H\text{-}N^+\text{-}Ar\text{-}O^-$ established by the interaction between the non-bonding electron pairs on the N atom and the proton from phenolic OH, thus preventing lone pair - π electron interactions, eventually reducing fluorescence intensity (6). This aspect requires further investigation, but it is apparent that the molecular environment of these molecules has profound effect on their fluorescence intensity.

Recoveries of aminocarb and mexacarbate and their metabolites from water

Percent recoveries of aminocarb, mexacarbate and their metabolites from stream water are given in Table 2 along with their standard deviation (SD) and CV. Corresponding chromatograms are given in Figures 5A and B (A - UV

TABLE 2

Average Percent Recoveries (n=3) of Aminocarb and Mexacarbate and Their Metabolites from Natural Water by UV Absorbance and Fluorometric Methods

Analyte	Percent Recovery ± SD (CV)			
	Fortification level			
	0.5 µg/mL		5.0 µg/mL	
	UVD	FD	UVD	FD
A	98.1 ± 4.8 (4.9)	99.8 ± 7.9 (7.9)	99.4 ± 5.2 (5.2)	101.3 ± 9.1 (9.0)
MFA	81.1 ± 7.1 (8.8)	-	83.9 ± 7.3 (8.7)	-
MA	101.3 ± 6.1 (6.0)	98.8 ± 8.4 (8.5)	97.7 ± 3.8 (3.9)	102.1 ± 8.4 (8.2)
FA	80.6 ± 7.8 (9.7)	-	81.1 ± 9.2 (11.3)	-
AA	93.9 ± 3.3 (3.5)	94.9 ± 6.4 (6.7)	96.2 ± 4.9 (5.1)	96.8 ± 9.1 (9.4)
MP	78.2 ± 6.6 (8.4)	81.0 ± 7.7 (9.5)	73.9 ± 7.2 (9.7)	74.3 ± 8.8 (11.8)
MAP	77.7 ± 8.1 (10.4)	80.2 ± 8.7 (10.8)	78.9 ± 8.1 (10.3)	82.2 ± 9.3 (11.3)
AMP	79.3 ± 7.7 (9.7)	76.8 ± 9.3 (12.1)	81.2 ± 3.9 (4.8)	80.8 ± 8.1 (10.0)
M	98.9 ± 2.9 (2.9)	103.2 ± 7.2 (7.0)	98.3 ± 4.4 (4.5)	102.6 ± 7.3 (7.1)
MFM	80.0 ± 6.2 (7.8)	-	83.1 ± 7.1 (8.5)	-
MAM	92.4 ± 5.1 (5.5)	98.8 ± 8.3 (8.4)	97.3 ± 6.1 (6.3)	103.2 ± 10.1 (9.8)
FM	80.8 ± 7.2 (8.9)	-	77.6 ± 8.1 (10.4)	-
AM	87.1 ± 8.2 (9.4)	86.0 ± 6.9 (8.0)	86.7 ± 7.0 (8.1)	86.2 ± 8.2 (9.5)
DMAX	79.9 ± 6.6 (8.3)	84.2 ± 9.1 (10.8)	83.3 ± 6.1 (7.3)	84.1 ± 9.0 (10.7)
MAX	74.7 ± 7.4 (9.9)	78.1 ± 9.9 (12.7)	77.4 ± 5.9 (7.6)	81.2 ± 8.8 (10.8)
AX	71.7 ± 6.9 (9.6)	73.2 ± 8.4 (11.5)	76.6 ± 7.2 (9.4)	80.0 ± 9.4 (11.8)

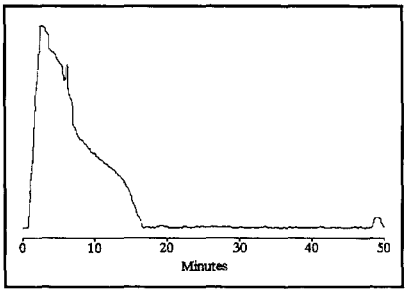

Figure 5A: Chromatogram of stream water blank by UVD, after column cleanup.

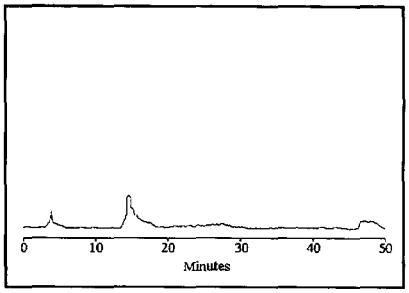

Figure 5B: Chromatogram of stream water blank by FD, after column cleanup.

absorbance, B - fluorometric)(water blank after cleanup), 6A and B (aminocarb and its metabolites in water at 0.5 μg/mL fortification level) and 7A and B (mexacarbate and its metabolites in water at 0.5 μg/mL fortification level). Recoveries of both insecticides at the two fortification levels were quantitative (> 98 %) indicating that the parent materials are amenable to analysis by both UVD and FD methods. Although the recoveries by fluorometric method seem to be higher, the CV is rather high (range 6.7 to 12.7 %) compared to UVD (range 2.9 to 11.3 %). Nevertheless, both techniques are suitable to analyze the parent materials from natural water.

The recoveries of MA, AA, MAM and AM are reasonably good (range 86.0 to 103.2 %) at both fortification levels by the UVD and FD methods. However, we have to point out that, because of the high LOQ (about 10 to 20 ng, except MAM), the FD method is not suitable if the analyte concentrations are very low in water. Compared to the parent materials, the recoveries of the four aldehydes (MFA, FA, MFM and FM) by the UVD method averaged only about 81.0 ± 1.9 %. As mentioned earlier, no quantification by FD was possible for them due to their poor quantum efficiency. Similarly the recoveries of six phenols (MP, MAP, AMP, DMAX, MAX and AX) by both UVD and FD methods

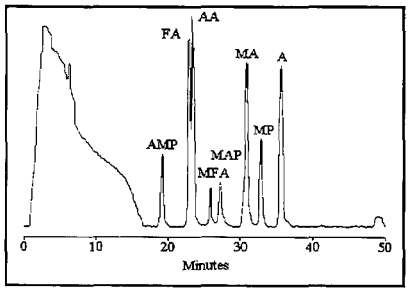

Figure 6A: Chromatogram of water by UVD, fortified with aminocarb and its metabolites each at 0.5 µg/mL.

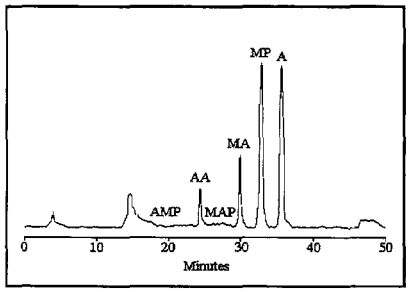

Figure 6B: Chromatogram of water by FD, fortified with aminocarb and its metabolites each at 0.5 µg/mL.

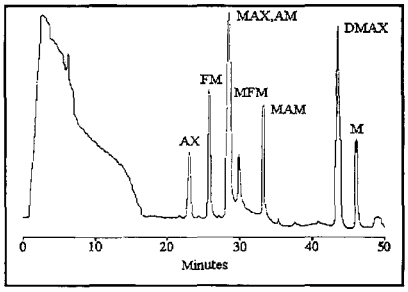

Figure 7A: Chromatogram of water by UVD, fortified with mexacarbate and its metabolites each at 0.5 µg/mL.

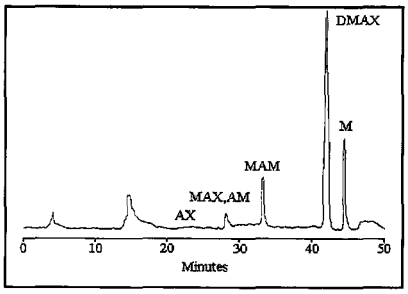

Figure 7B: Chromatogram of water by FD, fortified with mexacarbate and its metabolites each at 0.5 µg/mL.

averaged only about 78.7 ± 3.4 %. The low recoveries of aldehydes and phenols could be due to their high polarity and enhanced water solubility, preventing quantitative partition from aqueous to organic phase. The LOD for each analyte from water calculated as three times the SD of the blank response (8) was in the range of 1 to 10 ng in UVD and 0.2 to 200 ng in FD, depending on the type of analyte. The LOQ was five times the SD and ranged from 5 to 50 ng in UVD

and 1 to 1000 ng in FD. The LOQ values found here agreed with the values reported earlier for the standards using the two techniques.

CONCLUSION

The results presented in this study indicate that UV and fluorometric methods are suitable for isolating aminocarb and mexacarbate and some of their metabolites from natural waters. Contrary to the high sensitivity observed in other compounds with strong native fluorescence, most of the analytes in the present study have marginal quantum efficiency. Consequently, their sensitivities in FD are not high. In addition, the FD method has some inherent limitations such as: (a) the fluorescence intensity varied among the analytes studied and (b) the four aldehydes did not fluoresce at all. In such cases, the fluorescence detection is not feasible with direct injection unless a post-column treatment, such as derivatization is introduced (2) to improve the situation. The UVD is straight-forward and sensitive enough to quantify the analytes. It is applicable to all compounds, except for those solutions containing the AM and MAX or FA and AA together, because the elution regions of AM and FA overlap with the elution regions of MAX and AA.

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RAPID ASSAY FOR MONITORING RESIDUES OF ENROFLOXACIN IN MILK AND MEAT TISSUES BY HPLC

VÍCTOR HORMAZÁBAL AND MAGNE YNDESTAD
Division of Food Hygiene
Norwegian College of Veterinary Medicine
P.O. Box 8146-Dep.
N-0033 Oslo 1, Norway

ABSTRACT

A high-performance liquid chromatography method for the determination of enrofloxacin in milk and meat is presented. After homogenization of the milk/tissue, the fat was separated by extraction with organic solvents and the aqueous phase analysed by HPLC. The method is simple and robust, having a limit of quantification of 3 ng/ml and 5 ng/g enrofloxacin in milk and meat respectively. The recovery rate was 86-87% from milk and 89-93% from meat.

INTRODUCTION

Enrofloxacin (EF) belongs to the quinolone carboxylic acid group of antibiotics with a broad antibacterial spectrum. EF has been found to have a satisfactory effect in the treatment of severe intestinal and respiratory infections in turkeys (1), and is useful in the treatment of calves and pigs (2), dogs and cats (3), and poultry (4).

This wide application represents a potential hazard to consumers due to the persistence of residues in milk and meat. Few methods for the determination of EF in biological samples have been published. Waggoner at al. (5) published a spectrofluorometric method for the determination of EF residues in poultry tissues, Tyczkowska et al. (6) an HPLC-method for the simultaneous determination of EF and its metabolite ciprofloxacin in canine serum and prostatic tissue, Rogstad et al. (7) an HPLC method for the determination of EF in fish serum and tissues, Hormazábal et al. (8) a rapid assay for monitoring residues of EF and sarafloxacin (SF) in fish tissues by HPLC, and Steffenak et al. (9) a rapid assay for the determination of SF in fish serum by HPLC.
This study is a continuation of our recent work on EF with the aim of developing sensitive methods for the determination of EF in milk and meat.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and solvents were of analytical or HPLC grade. EF was supplied by Bayer Norge A.S (Oslo, Norway), whereas sarafloxacin (SF), (internal standard) was donated by Abbott Laboratories (Chicago, IL, USA). Stock solutions (1mg/ml) of EF and SF were prepared in 0.03 M sodium hydroxide, and working standards were prepared by dilution with 0.002M phosphoric acic/acetonitrile/methanol (65:27:8). The solutions of 1-heptanesulfonic acid were obtained from Supelco Inc.Supelco Park, Bellefonte, USA.
Spin-X centrifuge filter units from Costar (Cambridge, MA, USA) were used.

Samples of cow`s milk and cow-beef

Whole milk (pasteurized, 3.8% fat) and cow-beef were purchased from local grocery stores, for use as control material and for spiking with EF to conduct recovery experiments.

Chromatographic Conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 410 Bio solvent delivery

system, an ISS 100 sampling system equipped with a Lauda RMT6 cooler (14°C) from Messgeräte Werk Lauda, (Lauda Königshafen, Germany), and a LS 240 fluorescence detector (Perkin-Elmer, Norwalk, Conn., USA). The detector was operated at an excitation wavelength of 278 nm and emission wavelength of 440 nm, and with a response of 5 and an attenuation factor of 256. The integration was carried out using the software programme Omega-2 (Perkin-Elmer), wich was operated on an Olivetti M300 personal computer connected to a Bj-330 printer (Canon).
The analytical column (stainless steel, 150 x 4.6 mm I.D.) and guard column (stainless steel, 5.0 x 3 mm I.D.), were packed with 5 µm particles of PLRP-S polymer adsorbent (Polymer Laboratories, Amherst, MA, USA).
The mobile phase was a mixture of three solutions, A, B and C (65:27:8). Solution A was 0.02 M heptane sulphonate and 0.002 M phosphoric acid, made by dissolving 4.45 g/l 1-heptane sulphonic acid sodium salt (Supelco) in ca. 750 ml of 0.002 M phosphoric acid when preparing 1 litre of solution, the solution being made up to volume with 0.002 M phosphoric acid. Solution B was acetonitrile, and solution C was methanol. The flow-rate was 0.7 ml/min for 6 min followed by 1.0 ml/min for 3 min. The samples were injected at intervals of 10 min. Aliquots of 15 and 10 µl for milk and meat, respectively, were injected onto the column for the determination of EF.

Sample pretreatment

Milk. The pretreatment of milk samples is shown in Fig. 1. To 1 ml milk was added 100 µl of internal standard solution (SF, 2.5 µg/ml), 100 µl water (or standard), 0.5 ml 1M NaOH, and 3 ml acetonitrile. The sample was mixed for approx. 5 sec. and 5 ml diethyl ether - hexane (3:2) then added. The sample was again mixed for 5 sec. and was then centrifuged for 4 min. (4000 rpm.). The upper layer of organic solvents was discarded. 850 µl of the aqueous solution (corresponding to 0.5 ml milk) was pipetted into a glass-stoppered centrifuge tube, and 250 µl phosphoric acid (1M) and 6.9 ml methanol were added. The sample was again mixed for 5 sec. and then centrifuged for 3 min. (4000 rpm.).

Meat. The sample pretreatment of tissues is shown in Fig.2, and is based upon the method by Hormazábal et al. (8) for the determination of EF in fish tissue. The tissue sample, 3g of ground muscle, was weighed

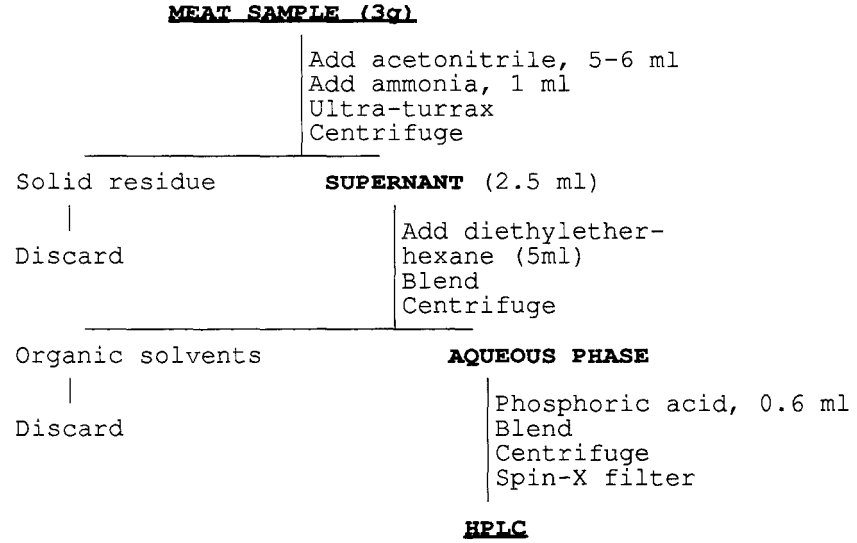

FIGURE 1
Extraction and Clean-up Procedure for Enrofloxacin from Meat Tissue.

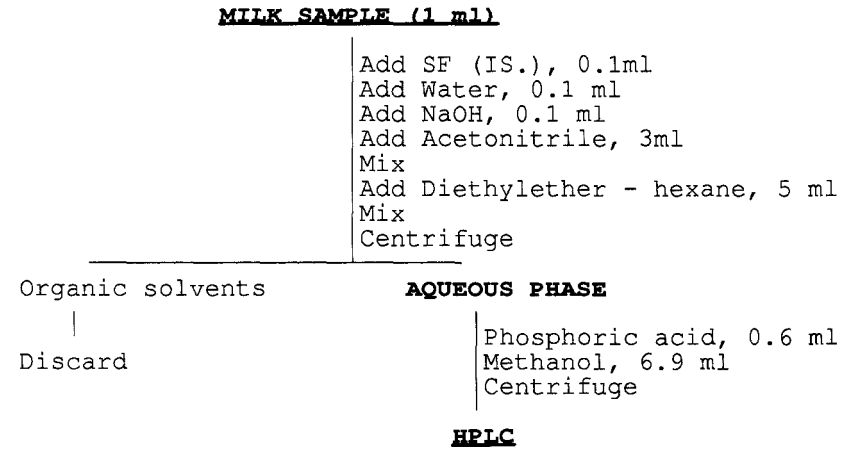

FIGURE 2
Extraction and Clean-up Procedure for Enrofloxacin from Milk.

into a 50 ml centrifuge tube with a screw cap (NUNC, Roskilde, Denmark). Volumes of 300 µl of internal standard solution (SF, 2.5 µg/ml), 1 ml ammonia and 5-6 ml acetonitrile were added. The total volume of added solvents should amount to 7 ml. The mixture was homogenized for approx. 6 sec. in an Ultra-Turrax TP 18/2 (Janke & Kunkel KG, Ika Werk, Staufen, F.R.G.). After centrifugation (3 min. at 4000 rpm.), 2.5 ml of the supernatant was pipetted into a glass-stoppered centrifuge tube and 1 ml NaCl-solution (1M), and 5 ml diethyl ether - hexane (3:2) were added. The mixture in the tube was blended well and centrifuged (2 min. at 3000 rpm.). The upper layer of organic solvents was discarded, and the aqueous solution was acidified using 0.6 ml phosphoric acid (5M). The sample was mixed and centrifuged for 3 min at 3000 rpm. Approximatly 500 µl were then filtered through a Costar Spin-X centrifuge filter unit with 0.2 µm nylon membrane, and centrifuged for 4 min. at 10000 rpm. (5600g).

Validation of the pretreatment procedure

The precision, recovery and linearity of the pretreatment procedure were determined by analyses of spiked milk and meat in the concentration range 3-200 ng/ml and 5-200ng/g, respectively.
The spiked samples were extracted using the above procedures. Duplicated samples were used. The recovery rates were determined by comparing results of analysis of the spiked milk and muscle sample with those of standard solution. The linearity of the standard curves for EF in milk and meat was tested using peak-height measurements and internal standard.

RESULTS AND DISCUSSION

The chromatographic system that had appeared to be efficient for analysis of EF in fish tisssue is not applicable to milk and meat. Minor modifications of the aqueous phase/organic phase ratio of the mobile phase did not improve the separation of the peaks of the drugs from those of the endogenous compounds. However, the problem was overcome by adding an anion-pairing agent to the mobile phase.
Chromatograms of extracts of blank samples, and spiked samples from milk and meat are shown in Fig. 3.
The limit of quantification was 3 ng/ml and 5 ng/g for EF in milk and meat, respectively. However, the

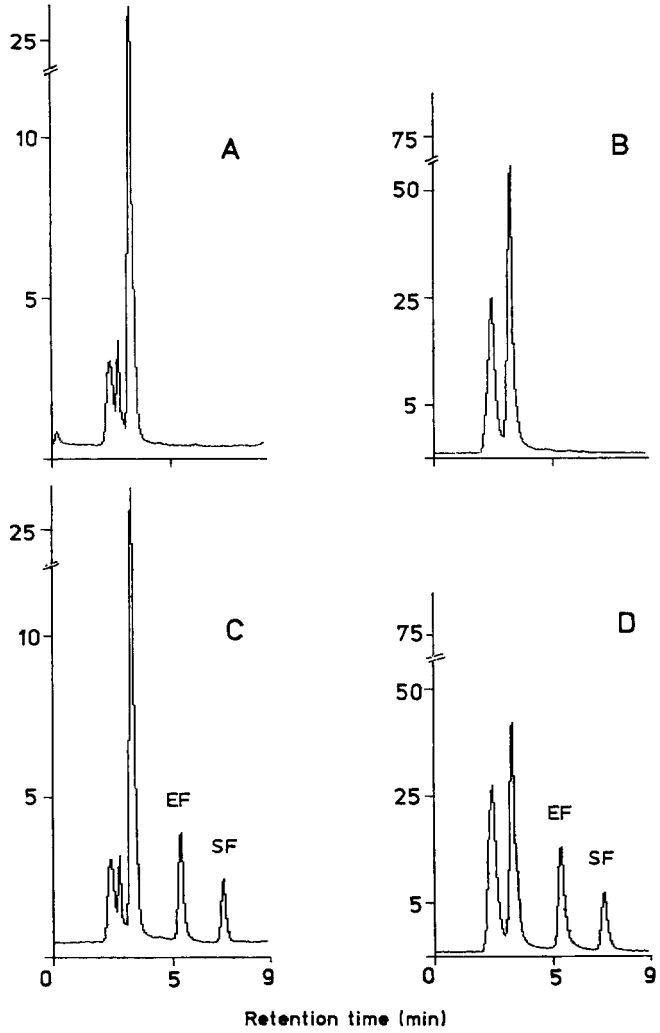

FIGURE 3

Chromatograms of extracts from milk and meat. **A**: drug-free milk, **B**: drug-free meat, **C**: milk spiked with enrofloxacin (100 ng/ml), **D**: meat spiked with enrofloxacin (100 ng/g).

TABLE 1.

Recovery and repeatability for enrofloxacion from spiked samples of milk and meat.

Sample	No of Samples	Amount of drug added (ng/ml or g)	Recovery % EF Mean	SD*
Milk (1ml)	8	10	86	2.8
	8	100	87	0.9
Meat (3g)	8	10	89	0.5
	8	50	93	2.0

*SD-standard deviation

sensitivity may be enhanced by using a larger amount of sample.

The extraction procedures were validated, and the results are shown in Table 1, showing good recovery of EF in milk and meat. The average recoveries for EF over the concentration range of the standard curve were 86.5% for milk and 91% for meat.

The precision and recovery of the internal standard were calculated, the average recovery of SF being found to be 85.5% from milk (standard deviation (SD)= 1.9%) and 90% from meat (SD= 1.65%).

The linearity of the standard curve was 0.9996 for EF both in milk and meat when using the internal standard method. The external standard method of calculation gave a linearity coefficient of 0.9990 and 0.9993 for EF in milk and meat, respectively.

The results also showed that the precision and accuracy of the quantification of EF are good.

The simplified extraction and clean-up procedure makes it possible to monitor drug concentrations in 30 or 40 samples of meat or milk, respectively per day.

CONCLUSIONS

This study has shown that EF levels in milk and meat can be determined after the samples have been subjected

to certain very simple clean-up steps. Good recovery, precision and sensitivity were obtained.

ACKNOWLEDGEMENT

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APPLICATION OF HPLC WITH DIODE ARRAY DETECTION IN TRIBOLOGY

J. LEHOTAY AND D. OKTAVEC
Department of Analytical Chemistry
Slovak Technical University
Radlinskeho 9
812 37 Bratislava, Slovakia

ABSTRACT

A strategy for the analysis of engine oils is described based on the correlation between previously developed methods and HPLC. The areas of some chromatographic peaks of oil samples were linearly correlated to: covered kilometres, the kinematic viscosity, the amount of insoluble compounds in heptane, Conradson's carbonised residue, the number of alkalinity and the carbonyl number.

INTRODUCTION

Structural study of engine oils or their residues is difficult because of the extreme complexity of these mixtures. Methods of separating these materials according to chemical classes have been reported, but even within each separate group, one finds a complex mixture of compounds (1). For many of these mixtures, spectroscopic measurements especially ^{1}H and ^{13}C NMR indicate the presence of alkyl plus aromatic

and heterocyclic rings (2). However, obtaining more detailed structural information from NMR is difficult due to the large variety of compounds and the superposition of peaks from different chemical structures. To obtain some information about the composition of oils, any authors have exploited the HPLC method especially adsorption chromatography (3,4) and reversed phase chromatography with chemical bonded phase (5-9).

In this paper we report on a HPLC study of the degradation of oils during their use in an engine. The detection is performed by a diode array detector, simultaneously providing structural information and quantitative data. The results are compared with other analytical methods, which are used for the evaluation of the oil quality. The main aim of this work was to investigate a number of parameters to find correlation between HPLC results and another parameters that characterised the properties of oil wear.

EXPERIMENTAL

The liquid chromatograph used was a Waters equipped with a 10 ml loop, a diode array detector (Waters Model 990) and a high-pressure pump (Model 510). The oil samples (1 g) were extracted with 10 ml of methanol and the clear solution was injected into the chromatographic column (Separon C-18, 0.32 x 15 cm, 5 mm particle size, Tessek Prague). A solution of 80% methanol in water was used as the mobile phase. The mobile phase was first filtered with a 0.2 mm membrane and degassed. The flow-rate was held at 0.5 ml/min and all experiments were carried out at room temperature. All solvents were twice distilled and were of an analytical reagent grade.

The sample set selected for this study consists of two typical engine oils of different origin: MT 16p - Russia and M6W/20D - Slovakia.

RESULTS AND DISCUSSION

Care must be taken to optimise HPLC conditions for the detection and the separation process. Using a diode array detector, a suitable wave length for detection can be found.

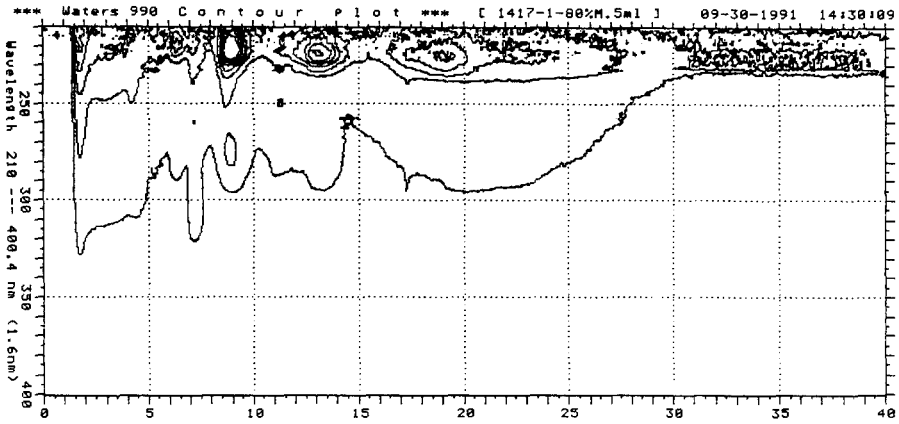

Fig.1. Contour chromatogram of oil extract MT 16p after 266 Km.

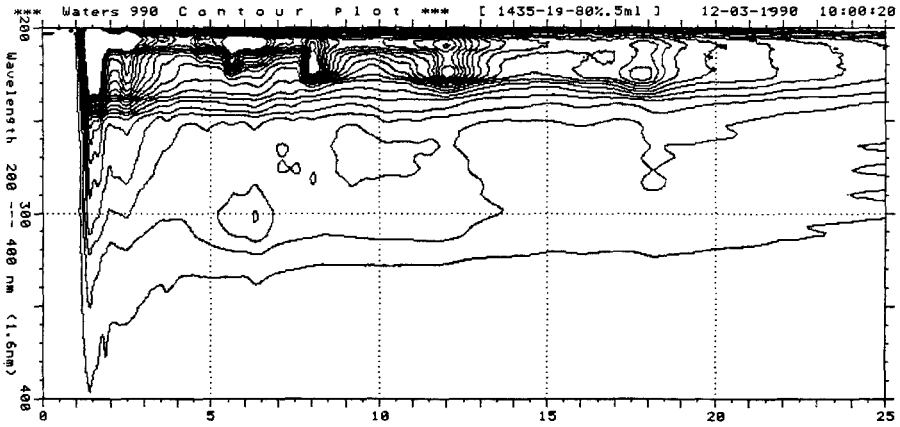

Fig.2. Contour chromatogram of oil extract M6W/20D after 1963 Km.

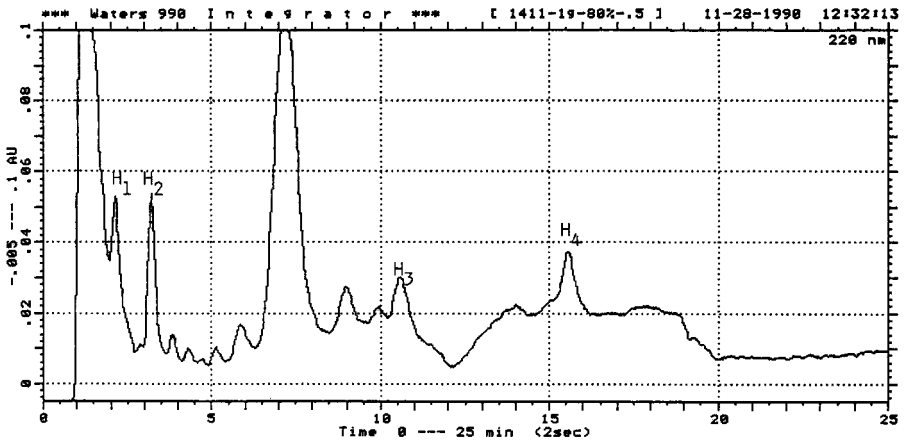

Fig. 3. Chromatogram of oil extract MT 16p, detection at 220 nm.

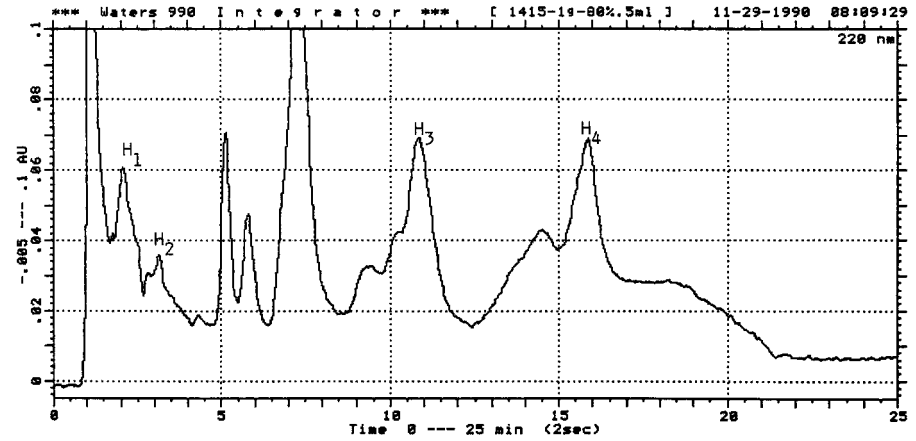

Fig. 4. Chromatogram of oil extract MT 16p after 1392 Km. Detection at 220 nm.

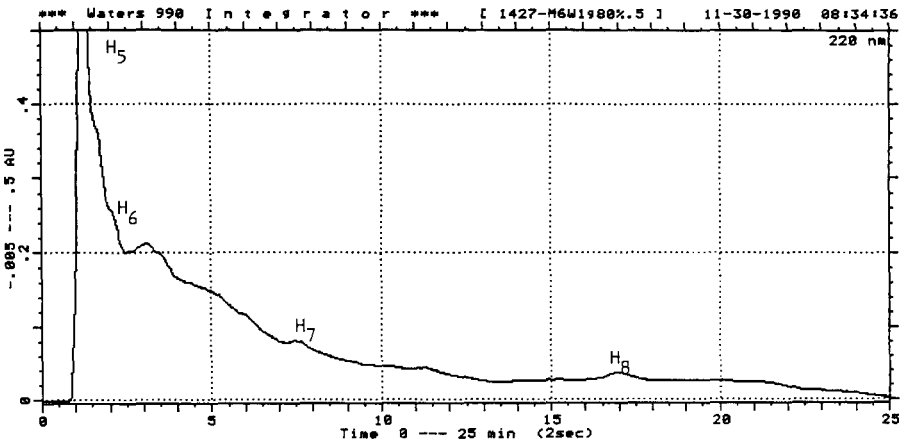

Fig. 5. Chromatogram of oil extract M6W/20D. Detection at 220 nm.

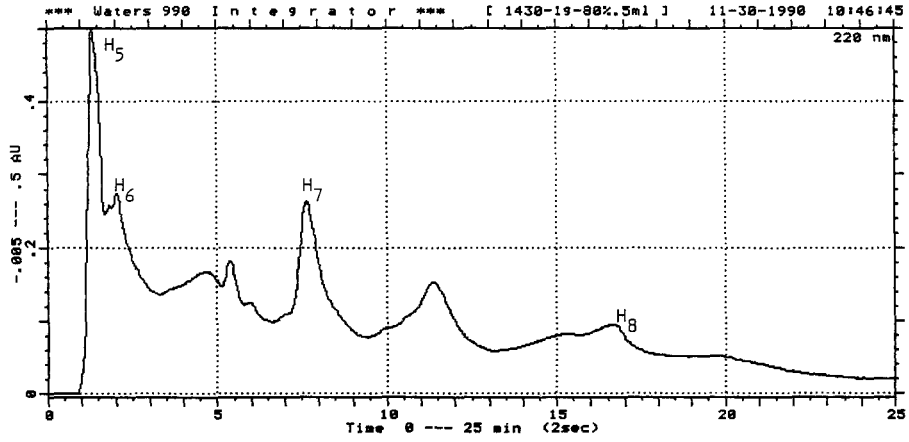

Fig. 6. Chromatogram of oil extract M6W/20D after 898 Km. Detection at 220nm.

Table 1. The results of oil analyses.

Oil MT-16p.

	KM	VIS	AIS	CCR	ALK	CN	H_1	H_2	H_3	H_4
1	0	15.2	0.38	0.1	2.05	0.01	1.6	5.0	2.4	4.6
2	499	14.7	0.57	1.10	1.47	0.06	2.5	1.7	1.4	8.0
3	1072	14.8	0.81	1.29	2.09	0.16	3.4	1.5	0.5	9.1
4	1392	14.9	0.92	1,28	1.99	0.20	3.8	0.8	1.1	9.6
5	2200	15.7	1.89	1.56	1.90	0.23	3.8	1.4	0.6	10.9
6	2660	15.6.	1.24	1.49	1.98	0.29	4.9	0.8	0.3	10.4
7	3240	17.3	1.52	1.63	1.90	0.24	5.3	0.6	0.5	12.2
8	3723	17.2	1.72	1.50	2.24	0.28	5.7	2.0	1.1	10.4
9	4172	17.9	1.74	1.63	2.28	0.31	4.6	0.2	0.8	11.7
10	4724	18.2	1.78	1.69	2.05	0.31	5.9	0.9	0.2	13.0
11	4953	16.7	1.74	1.55	2.06	0.23	4.7	1.9	0.7	9.0

Oil M6W/20D.

	KM	VIS	AIS	CCR	ALK	CN	H_5	H_6	H_7	H_8
1	0	20.4	0.40	2.93	14.21	0.01	96.1	0.2	0.1	1.0
2	500	16.2	0.87	2.90	12.37	0.24	70.6	3.2	11.4	2.2
3	898	16.1	0.85	3.25	11.19	0.13	61.3	1.5	16.7	2.2
4	1231	15.8	0.95	3.68	13.53	0.08	35.5	0.4	19.0	2.2
5	1480	16.2	1.01	3.56	13.85	0.12	36.5	0.3	15.8	4.3
6	1713	15.8	1.08	3.77	13.88	0.05	44.6	0.2	15.1	3.4
7	1963	16.2	1.12	3.75	14.24	0.01	54.6	0.0	15.6	4.1
8	2212	16.6	1.18	3.88	14.62	0.01	59.0	0.0	12.9	4.5
9	2462	16.2	1.08	3.91	14.68	0.02	52.2	0.0	12.8	4.4

KM - covered kilometres VIS - kinematic viscosity in $mm^2\ s^{-1}$

CN - carbonyl number ALK - number of alkalinity in mass %

Table 1 (continued).

CCR - Conradson's carbonised residue IR, 1720 cm^{-1}

AIS - amount of insoluble compound in heptane, 0.45 micrometer Mill.

H_1- area of peak at 2.1 min, H_2 - area of peak at 3.2 min.

H_3- area of peak at 10.7 min H_4 - area of peak at 15.8 min.

H_5- area of peak at 1.2 min. H_6 - area of peak at 2.1 min.

H_7- area of peak at 7.5 min. H_8 - area of peak at 16.9 min.

To obtain a robust method, single wavelength detection should be performed at an optimised bandwidth to monitor simultaneously a large group of compounds with high sensitivities. Most of the analytes has their maximum absorbance in the range of 200 - 220 nm (Figs. 1 and 2) and as it can be seen from the contour chromatographic records, the detection at 220 nm is the best. The conditions of the separation were chosen so that in the chromatogram the most numbers of peaks were observed.

To examine the correlation between HPLC results and other parameters, some peaks from chromatograms were selected. The areas of the chromatographic peaks of oil samples were correlated to: covered kilometres (KM), the kinematic viscosity (VIS), the amount of insoluble compounds in heptane (AIS), Conradson's carbonised residue (CCR), the number of alkalinity (ALK), and the carbonyl number (CN). In Figs. 3-6 representative chromatograms of methanol extract analysed by HPLC are shown. Covered kilometres, peak areas and other results of analyses of oils are summarised in Table 1. If the value of a correlation coefficient is more than 0.8, it indicates a good agreement between considered parameters. The correlation coefficients are presented in Table 2.

In the case of MT 16p, oil a good correlation between the peak areas H_4 (elution time 15.8 min.) and two parameters was found. It can be seen that the compounds, which are eluated at 15.5 min., have carbonyl group (the value of correlation coefficient is 0.82) and high Conradson's carbonised residue. The similar conclusion can be made for the peak H_1

Table 2. Correlation coefficients.

Oil MT 16p.

	H_1	H_2	H_3	H_4
	0.79	-0.13	-0.31	0.70
VIS	0.84	-0.32	-0.56	0.69
AIS	0.91	-0.59	-0.79	0.92
CCR	-0.11	-0.53	-0.15	-0.14
ALK	0.82	-0.71	-0.82	0.82
CN	0.88	-0.31	0.54	0.73
KM	0.88	-0.31	0.54	0.73

Oil M6 W/20D.

	H_5	H_6	H_7	H_8
VIS	0.83	-0.16	-0.94	-0.55
AIS	-0.75	-0.23	0.75	0.88
CCR	-0.71	-0.70	0.57	0.82
ALK	-0.09	-0.76	-0.26	0.50
CN	-0.72	0.91	0.25	-0.29
KM	-0.63	-0.55	0.53	0.92

KM - covered kilometres VIS - kinematic viscosity in mm^2 s^{-1}

CN - carbonyl number ALK - number of alkalinity in mass %

CCR - Conradson's carbonised residue IR, 1720 cm^{-1}

AIS - amount of insoluble compound in heptane, 0.45 micrometer Mill.

H_1- area of peak at 2.1 min, H_2 - area of peak at 3.2 min.

H_3- area of peak at 10.7 min H_4 - area of peak at 15.8 min.

H_5- area of peak at 1.2 min. H_6 - area of peak at 2.1 min.

H_7- area of peak at 7.5 min. H_8 - area of peak at 16.9 min.

but these compounds have more polar properties (according to the correlation coefficient H_1-AIS) and their concentration increase according to covered kilometres (H_1-KM). These substances must be formed during the degradation of oil. The expected agreement was found between H_1 and AIS. From the chromatographic point of view the more polar compounds are less soluble in heptane. On the other hand, the correlation was not found between the number of alkalinity and HPLC results. It can be assumed that the eluated compounds have neutral properties and they have no influence on the alkalinity. The area of H_3 correlated only with carbonyl number and it indicates that the compounds eluated at10.7 min. contain carbonyl group and their concentration decreased with covered kilometres (negative value of correlation coefficient). The area of H_2 peak has no significant influence on the followed parameters and it can by assumed that these compounds are not changed during the experiment.

A similar conclusion can be made in the case of M6W/20D oil. Good correlation between H_8 and number of covered kilometres was found. It indicates that these compounds are decomposed during the experiments. The correlation between H_8 and the content of insoluble compounds in heptane was not expected. This phenomena can be explained on the base of decomposition of substances and the formation of new less polar compounds. According to the values of correlation coefficients H_7-VIS and H_5-VIS, it can be assumed that the compounds are formed from the substances that have a viscostatic function in oil and they are eluated at 1.2 min.

To summarise, the HPLC method represents a useful analytical approach for the characterisation of the oil degradation during their use in engines. Naturally, every kind of oil must be evaluated separately. The main advantage of the method is the speed of analysis and the possibility to substitute some another methods.

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RAPID SIMPLIFIED ANALYSIS OF PYRAZINAMIDE IN RAT PLASMA BY HPLC

J. S. KAKA
Department of Clinical Pharmacy
College of Pharmacy
King Saud University
P.O. Box 2457
Riyadh 11451, Saudi Arabia

ABSTRACT

An HPLC method for quantitative estimation of pyrazinamide in rat plasma has been developed. The procedure consists of precipitation of plasma proteins with methanol containing 5-fluorouracil as an internal standard. The mixture was vortexed for 1 min. and centrifuged at 4000 rpm for 10 min. Twenty-five microliters of the supernatant was eluted on μ-Bondapack C_{18} column at 269 nm. The mobile phase consisted of 2% v/v acetonitrile in 5 mM potassium phosphate buffer at pH 4. The flow-rate was set at 1 ml per min. The retention times for 5-fluorouracil and pyrazinamide were 4 and 9 min. respectively with a detection limit of 0.5 μg/ml for pyrazinamide.

INTRODUCTION

The increase in acquired immunodeficiency syndrome (AIDS) has made tuberculosis an increasing worldwide problem(1,2). The effectiveness of modern chemotherapy

is also blunted by high incidence of primary drug resistance, especially in developing countries(3-5). Since 1952, pyrazinamide has been used in the treatment of tuberculosis(6). Excessive doses led to hepatic toxicity, which discontinued its use (7,8). Recently, there has been renewed interest in pyrazinamide use against Mycobacterium tuberculosis and Mycobacterium leprae(9,10). In AIDS and tuberculosis, a combination of drugs are to be used in conjunction with pyrazinamide for an effective outcome(9,11). In addition, pyrazinamide is to be used for at least six months for successful treatment(11,12).

Limited numbers of HPLC analytical methods have been reported for pyrazinamide in biological fluids. Lacroix and Yamamotos' methods require a fluorimetric apparatus(13,14). The Acocelli et al. procedure involves extraction, evaporation and at least 0.5 ml of plasma for analysis(15). The method described by Woo et al. requires extraction, a gradient programmable pump and one of the effluents require 60% acetonitrile (16). The procedure by Hammiche et al. is similar but requires filtration and 5% acetonitrile as mobile phase(17).

The purpose of the present report is to describe a simple and sensitive HPLC technique for quantitative determination of pyrazinamide in rat plasma.

MATERIALS

Pyrazinamide was purchased from Wilson Labs., Bombay, India and 5-florouracil was obtained from H. Roche, Basel, Switzerland. Acetonitrile, methanol and di-potassium hydrogen phosphate were purchased from BDH Chemicals Ltd., Poole, U.K.

METHODS

Stock Solutions:

Pyrazinamide solution was prepared by dissolving 10 mg in 10 ml of methanol. 5-Fluorouracil (5 mg) was dissolved in 100 ml of methanol. Potassium phosphate buffer (5 mM) was prepared and adjusted to pH 4 with phosphoric acid. All stock solutions were stored at $4^{o}C$.

Chromatographic conditions:

The HPLC unit consisted of a Waters Assoc. Model M-45 solvent delivery system, a Waters Assoc. Model 450 variable wavelength detector, a Waters Assoc. Model 46K universal liquid chromatograph injector and BBC Goerz Metrawatt SE120 recorder. The column used was a stainless steel Waters Assoc. μ-Bondapack C_{18} (30 cm x 8mm I.D., 10 μm particle size) column. The mobile phase was 5 mM potassium phosphate buffer-acetonitrile mixture at pH 4, with a final acetonitrile concentration of 2% v/v. The flow-rate was set at 1 ml/min. The

effluent was monitored at 269 nm with a detection scale of 0.1 or higher as needed. All measurements were performed at room temperature.

Sample Preparation:

To a 100 μl of plasma aliquot, 100 μl of methanol containing 5-fluorouracil was added. The mixture was vortexed for 1 min and centrifuged at 4000 rpm for 10 min. Twenty-five microliters of supernatant was injected onto the column.

Standard Calibration Curve:

Blank rat plasma aliquots were spiked with a pyrazinamide standard solution to get concentrations ranging from 2.0 to 40 μg/ml. 5-Fluorouracil in methanol was used as an internal standard. Each point was an average of four replicates.

Precision studies:

Rat plasma samples at concentrations 2, 20, and 40 μg/ml were analyzed on five separate days in order to determine the between day-to-day coefficient of variation. For elucidation of the within-day variability, five specimens of the same sample were analyzed on each day at the above mentioned concentrations.

Animal study:

Pyrazinamide disposition was assessed in a pilot study, where a single rat received pyrazinamide (25

mg/kg) by iv administration via the tail vein. A polyethylene cannula (1 mm O.D.) was implanted in the left femoral artery and exteriorized from the neck of the animal with enough length for safe handling. Approximately, 0.25 ml of blood was taken before and 10, 20, 30, 45 min. and 1, 2, 3, 4, 6, and 8 hour, respectively after iv administration. Heparinized blood was centrifuged, plasma separated, and aliquots were kept at -20°C until analysis.

RESULTS AND DISCUSSION

Using the chromatographic procedure described, the separation of pyrazinamide and 5-fluorouracil in rat plasma is shown in Fig. 1. The retention times for 5-fluorouracil and pyrazinamide were 4 and 9 min. respectively, with no interfering peaks from the plasma in this region. There was a good separation between 5-fluorouracil and pyrazinamide. Each sample was completely eluted in 10 min.

The calibration curve for pyrazinamide was linear over the range investigated with a correlation coefficient of 0.997. The limit of sensitivity was at least 0.5 μg/ml which was defined as a measure of peak height of 0.5 mm of pyrazinamide by the described procedure. Similar limits of sensitivity were reported by others authors. There is no extraction process in this method, thus recovery studies were not needed.

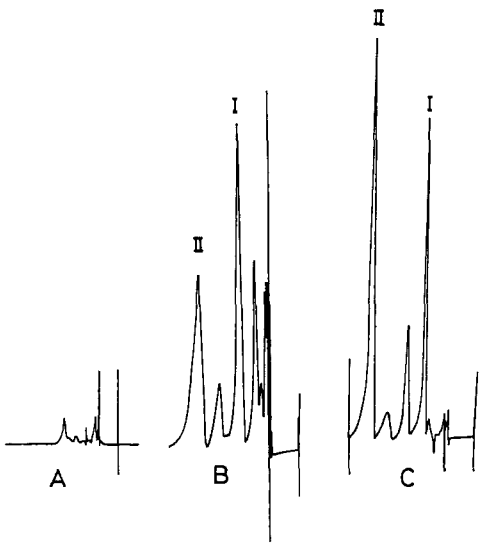

Figure 1. High-pressure chromatographs for a blank rat plasma (A), standard sample (B) containing 5-fluorouracil (I) and pyrazinamide (II) and rat sample (C) after 5 hours of pyrazinamide i.v. administration containing 5-fluorouracil (I) and pyrazinamide (II).

The precision of pyrazinamide assay was assessed by five replicate aliquots of rat plasma spiked with pure pyrazinamide at concentrations 2, 20, and 40 μg/ml respectively. The coefficient of variation for the within-day assay was less than 5%. The coefficient of variation for the day-to-day assay over a week at the above mentioned concentrations were less than 6%.

The assay was applied to determine pyrazinamide plasma concentrations obtained from a pilot

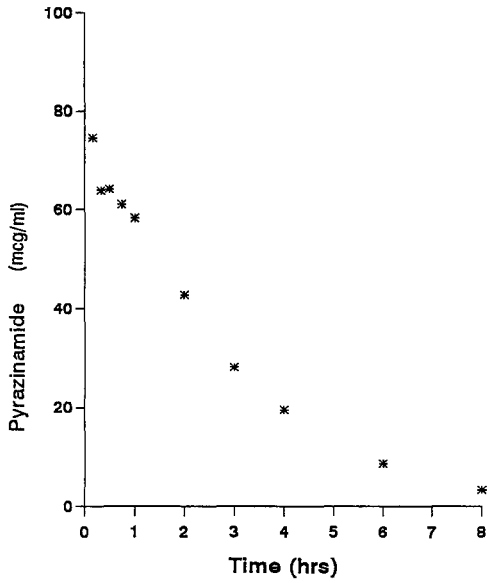

Figure 2. Plot of plasma pyrazinamide concentration after iv administration of pyrazinamide (25 mg/kg) to rat.

pharmacokinetic rat study. Fig. 2. shows the plot of pyrazinamide plasma concentrations following iv administration.

The advantages of this assay over other earlier published methods are that it involves no extraction process, only 2% v/v acetonitrile mobile phase at 1.0 ml/min, and requires only 100 μl of rat plasma without compromising the minimum detectable limits for pyrazinamide.

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A SIMPLE AND RAPID METHOD FOR DETERMINATION OF FLUCONAZOLE IN HUMAN PLASMA SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

F. J. FLORES-MURRIETA, V. GRANADOS-SOTO, AND E. HONG

Sección de Terapéutica Experimental
Departamento de Farmacología y Toxicología
Centro de Investigación y de Estudios Avanzados
del Instituto Politécnico Nacional
Apartado Postal 22026
14000 México, D.F., Mexico

ABSTRACT

A rapid and simple method for determination of fluconazole in plasma samples by high performance liquid chromatography was developed. The method includes a single extraction of alkalinized plasma with ethyl acetate. Plasma extracts were analyzed on a reverse-phase column eluted with a mixture of acetonitrile and 0.05 M sodium monohydrogen phosphate (pH 4) and detected by absorbance at 210 nm. Retention times for fluconazole and the internal standard were 7 and 13 min respectively. The method was linear in the range of 0.1 to 5 μg/ml and the detection limit of the method was 20 ng/ml. This method is suitable for determination of fluconazole after administration of the drug at therapeutic doses, and it could be used in pharmacokinetic studies of the drug in humans.

INTRODUCTION

Fluconazole, [2-(2,4-difluorophenyl)-1,3-bis-(1-(1-1,2,4-triazol-1-yl)-propan-2-ol], is an agent with an important antifungic activity (1,2). That compound has been shown to be effective in the treatment of peripheral and systemic mycoses (3,4). To our knowledge, only two methods for its quantitative determination in body fluids are available, one by gas chromatography (GC) with electron capture detection and one by high performance liquid chromatography (HPLC) (5,6). The methods reported have some disadvantages. In the GC method the column has to be pre-treated with benzoyl chloride to avoid that fluconazole is adsorbed to the column and, additionally, a time consuming extraction procedure is employed. On the other hand, the HPLC method has the disadvantage of employing a detection wavelength where sensitivity is very poor and the compound has to be detected at 0.002 absorbance units full scale (AUFS) and consequently, an important noise is present. An additional disadvantage is that a time consuming extraction procedure was performed. In this paper, a simple and rapid method for determination of fluconazole in plasma samples by HPLC is described with enough sensitivity for realization of pharmacokinetic studies after administration of therapeutic doses of the drug.

MATERIAL AND METHODS

Reagents and solutions

Fluconazole was provided by Laboratorios Senosiain, S.A. de C.V. (Mexico City). TR3318, 5-phenyl-5-(2-pyridyl)hydantoin (fig. 1), the internal standard, was

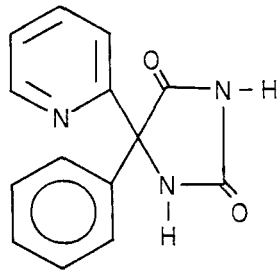

Fig. 1. Chemical structure of the internal standard used in the determination of fluconazole.

provided by the Sección de Terapéutica Experimental del Centro de Investigación y de Estudios Avanzados del I.P.N. (Mexico City). Acetonitrile chromatographic grade was purchased from Merck (Darmstadt, Germany). Deionized water was obtained through a Milli Q system (Continental Water Systems, El Paso, TX, USA). All other reagents were of analytical grade.

Stock solutions of fluconazole corresponding to 1 mg/ml were prepared in the mobile phase (see under), and of the internal standard corresponding to the same concentration was prepared in a mixture of methanol-water (60:40, v/v). Standard solutions were prepared by diluting the stock solutions ranging from 0.1 to 100 μg/ml in mobile phase. A standard solution of the internal standard was prepared at a fixed concentration of 10 μg/ml in mobile phase. Sodium borate (0.025 M), providing a pH of 9.0, was prepared in deionized water.

Extraction procedure

Plasma samples (1 ml) were pipetted into 15 ml conical glass tubes and 1 μg of the internal standard were added. Plasma was alkalinized by addition of 0.5 ml

of 0.025 M sodium borate (pH 9). 5 ml of ethyl acetate were added and samples were extracted by agitation in vortex at maximum speed for 1 min. To separate the layers, samples were centrifuged at 4000 g for 10 min and the organic layer was transferred to another conical glass tube. The organic layer was evaporated to dryness in a water bath at 50°C under nitrogen stream. Dry residue was redissolved in 200 μl of mobile phase (see under) and aliquots of 80 μl were injected into the chromatographic system.

Chromatographic system

The chromatographic system was manufactured by Waters (Waters Assoc., Milford, MA, USA) and was formed by a model 510 solvent delivery system, an U6K injector, a model 490 detector. Chromatograms were recorded in a model 4270 integrator (Varian, Palo Alto, CA, USA). Separation of the compounds was performed on a C_8 Novapak column of 150 mm X 3.9 mm I.D. of 4 μm particle size eluted by a mixture of 0.02 M sodium monobasic phosphate adjusted to pH 4 with *o*-phosphoric acid with acetonitrile (88:12, v/v). To evaluate the optimum wavelength for detection of fluconazole, an ultraviolet absorbance spectrum was obtained (fig. 2). As a good absorbance was obtained at 210 nm, an evaluation of the signal to noise ratio was performing comparing against 260 nm and it was found that the best signal to noise ratio was at 210 nm. Therefore, detection was performed at that wavelength using a sensitivity of 0.05 AUFS.

Calibration

The method was calibrated by addition of known amounts of fluconazole and the internal standard to drug-free plasma samples (1 ml). Calibration curves were established for fluconazole concentrations ranging from

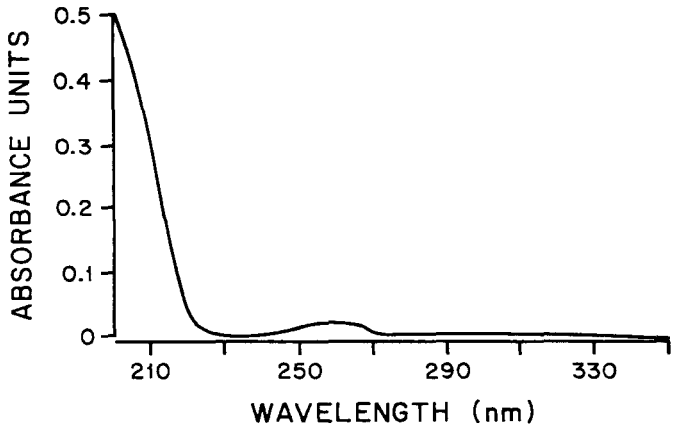

Fig. 2. Ultraviolet absorbance spectrum obtained with a concentration of 111 μg/ml of fluconazole.

0.1 to 5 μg/ml. The internal standard was used at a fixed concentration of 1 μg/ml. The actual sample concentration of fluconazole was calculated by determination of the peak-height ratios of fluconazole to the internal standard.

RESULTS

Typical chromatograms obtained after injection of plasma extracts into the chromatographic system are shown in fig. 3. Retention times for fluconazole and the internal standard were 7 and 13 min respectively. No interfering peaks occurred at these times. Any endogenous contaminants remaining in the extracts were eluted before the fluconazole, and samples could be injected immediately after elution of the internal standard. A linear relationship was obtained when height ratios of

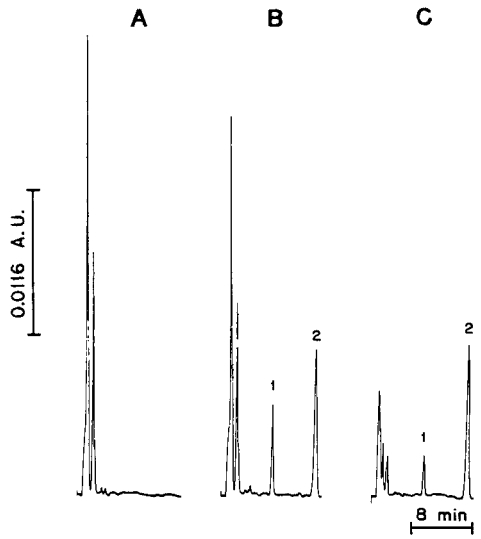

Fig. 3. Typical chromatograms resulting of the injection of plasma extracts into the chromatographic system. A) Drug-free plasma, B) plasma spiked with 1 μg/ml of fluconazole (1) and 1 μg/ml of the internal standard (2), and C) plasma obtained from a subject 72 h after the administration of a capsule of 100 mg fluconazole.

fluconazole to the internal standard were plotted against fluconazole concentration ranging between 0.1 and 5 μg/ml ($r = 0.9998$), as shown in fig. 4.

The recoveries of fluconazole and the internal standard from plasma samples were similar and ranged between 90 and 100%, by comparison of peak heights from plasma extracts with those from standard solutions. The accuracy and precision of the method were evaluated by adding known amounts of fluconazole to drug-free plasma and analyzing the samples. An accuracy of 100.89 ± 2.42 was obtained for concentrations ranging between 0.1 to 10 μg/ml and the intra-assay and inter-assay coefficient of variation of the method ranged between 14% for 0.1 μg/ml and 4.5% for 0.5 μg/ml.

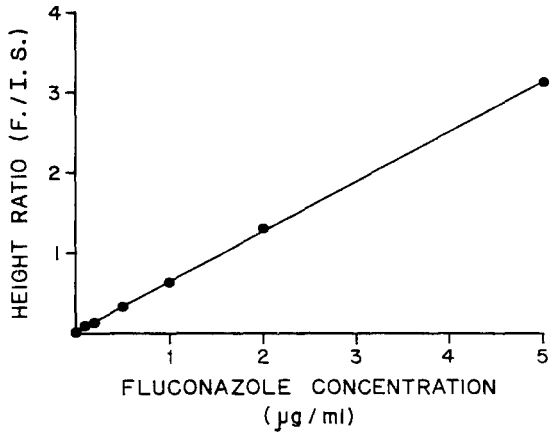

Fig. 4. Calibration curves of fluconazole in human plasma established in the range of 0.1 to 5 µg/ml. Data are expressed as mean of 6 determinations. In all cases the s.e.m. did not exceeded the symbol size.

To evaluate the usefulness of the method for determination of fluconazole after therapeutic doses of the drug, a human volunteer received an capsule of 100 mg of fluconazole p.o. and plasma samples were obtained at selected times. Temporal course of fluconazole concentrations are shown in the fig. 5. Pharmacokinetic parameters obtained in that subject were maximal concentration (C_{max}) of 2.49 µg/ml, time to reach the maximal concentration (t_{max}) of 3 h, area under the plasma level against time curve (AUC) of 112.77 µg·h/ml and half-life ($t_{1/2}$) of 27.15 h.

DISCUSSION

A new method for determination of fluconazole in plasma samples has been developed. This method is more

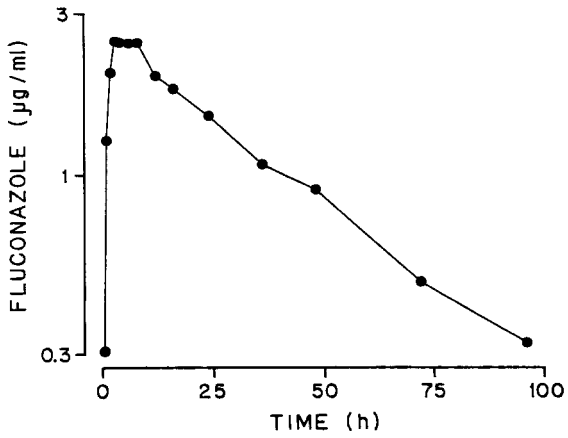

Fig. 5. Temporal course of fluconazole plasma levels in one male volunteer that received a single capsule of 100 mg of fluconazole.

rapid and simple than those previously reported by GC (3) and HPLC (4), since a single extraction is used. Additionally, it is more sensitive than that reported by HPLC, since a wavelength where the drug has a better signal to noise ratio was used. It is important to note that solvents used in the chromatographic system here reported have to be of a good quality, because the wavelength used in this method is very low and some interferences could appear if solvents of poor quality are used. As the method is rapid and simple, over 30 samples can be analyzed by one analyst in a working day. Results of this study allow us to conclude that the method here reported is suitable to carry out pharmacokinetic or bioavailability studies after administration of therapeutic doses of the drug.

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DETERMINATION OF FREE ESTRIOL IN AMNIOTIC FLUID BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

S. U. SHEIKH AND J. C. TOUCHSTONE
University of Pennsylvania
School of Medicine
Philadelphia, Pennsylvania 19104

ABSTRACT

The estrogen levels of amniotic fluid and maternal plasma may be altered in those cases with a fetus which presents Down syndrome. This paper describes the methodology for determination of free estriol in amniotic fluid. Fresh amniotic fluid was extracted using solid phase extraction techniques. Analtech C18 Spice cartridges were used to extract estriol (E3) prior to chromatography. After elution of the analyte E_3 from the cartridge, the solvent was evaporated under nitrogen in a warm water bath at 60°C. The extract was reconstituted in methanol and E3 separated by high-performance liquid chromatography. A reversed phase C18 Partisil ODS-3 (4.6 mm X 25 cm) column was developed with acetonitrile - methanol (65:35 v/v) as mobile phase. Subambient temperature (-20°C) improved the resolution and aided separation from extraneous material. Quantitation followed intrapolation against a standard curve prepared under identical conditions. Measurement of amniotic fluid E_3 may provide insight into the reported alteration of maternal plasma E_3 level in Down syndrome. Results so far indicate that in Down syndrome the amniotic fluid E_3 levels wee below the detection limits.

INTRODUCTION

Estriol (E_3) levels in blood serum in Down syndrome and normal patients is a controversial issue. No report indicates the level of estriol in blood and amniotic fluid in Down syndrome, and Trisomy - 18. Three components in serum - α-fetoprotein, free estriol (E_3) and human chorionic gonadotropin (β-hCG) have been analyzed in order to detect Down syndrome. A report by Carrick, et al, showed that maternal serum "free" estriol (E_3) was low in Down Syndrome (1). Greenberg et al also reported lower values for these constituents in maternal plasma (2). The

concentration of the "unconjugated" estriol increases approximately 20 percent per week in the second trimester. Maternal estriol E_3 is mostly conjugated, while the unconjugated estriol is of fetal origin.

Merkatz et al (2) showed that a low maternal serum a-fetoprotein is associated with Down fetuses. Several other studies (3,4) have confirmed this finding. The fetal liver is the source for this protein and the measurement of it is used on prenatal screening for neural tube defects. Approximately 20% of mothers with affected fetuses and 5% of mothers with unaffected fetuses have a level below 0.5 multiples of the median as compared to mothers of Down fetuses with 0.7 multiples of the median. There have been several studies on the relationship of free E_3 of the maternal plasma (3,4) to Down Syndrome. These have indicated that levels of (E_3) in the unconjugated form may correlate with the genetic status of the fetus.

The present report indicates that the free estriol (E3) in amniotic fluid may also be indicative of Down Syndrome. Undetectable levels of free estriol were found in these cases. In some cases of "normal" pregnancies the levels of free estriol were also below detection limits. Considering that the fetus is a major source of the estriol it might be more appropriate to assess estriol in the amniotic fluid rather than the maternal blood. A description of the methods used and the results obtained in normal and affected pregnancies are presented. Considering that amniotic fluid is routinely drawn for genetic studies and the small amounts required for the method as described the utility of amniotic fluid estriol merits further investigation.

MATERIAL AND METHODS

All specimens were from 15-18 weeks gestation. Fresh or frozen amniotic fluid samples were extracted using solid phase extraction (SPE) techniques; then aliquots of reconstituted samples were subjected to HPLC at subambient column temperature (-20°C) to resolve E_3.

All solvents were EM Omnisolv, purchased from E.M. Science. Steroids were from Sigma Chemical Company, USA. Water was demineralized, filtered and degassed before use. Sample solutions were made in methanol and kept at 4°C. All solvents were degassed by filteration.

A modular liquid chromatograph (LC) equipped with LDC pump and a variable wavelength UV Spectroflow 773 detector, was interfaced with a Hewlett Packard HP 3385A integrator. The column was a Whatman Partisil 10 ODS (250 x 4.6 mm). The mobile phase was acetonitrile-methanol (65:35 (v/v)). Detection was at 280 nm. Flow rate was 1 ml/min.

The column was cooled to -20°C using a cooling system provided by U.S. Coolers (Quincy, Ill). Quantitation followed intrapolation against a standard curve prepared from serial amounts of estriol under identical conditions as the samples.

Extraction procedure

Amniotic fluids are difficult to analyze by chromatography without specimen cleanup. The samples were prepared by extraction using solid phase extraction cartridges obtained from Analtech (Newark, DE) The C18 Spice cartridges were processed as outlined in Table 1. The procedure was carried out under vacuum of a water aspirator with the cartridges placed in a vacuum manifold. (Analtech, Newark, DE).

After the E3 fraction was eluted, the methanol was evaporated at 60°C using a stream of nitrogen. The residue so obtained was reconstituted in 10 µl of methanol for injection into the high performance liquid chromatograph. Recovery of 3H-estriol carried thru this procedure was routinely 91-93%.

TABLE 1

Solid Phase Extraction of Estriol from Amniotic Fluid

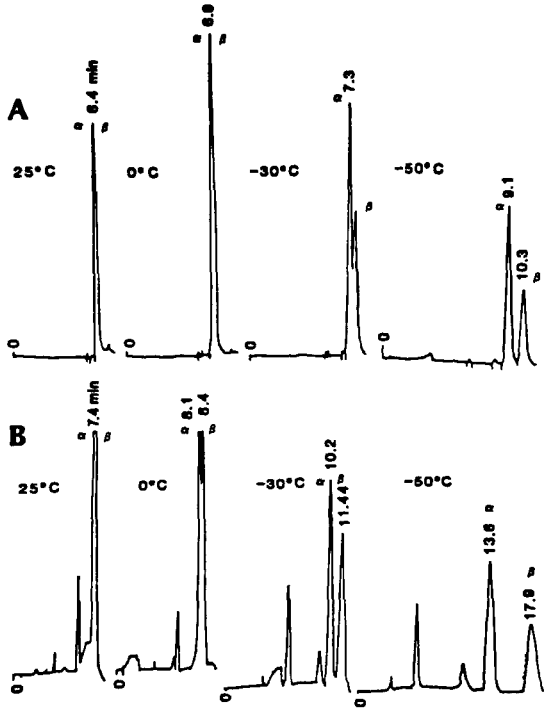
1. Conditioning - wash in sequence as follows:
   a. 2 ml of acetone - water (20:80)
   b. 2 ml of methanol
   c. methanol
2. Add 1 ml of amniotic fluid directly to the column.
3. Washing - add in sequence as follows:
   a. 2 ml of acetone-water (20:80)
   b. 2 ml of water
4. Drying - under vacuum aspirate air through the cartridge.
5. Elution of estriol follows:
   a. 1 ml of methanol
6. Evaporate in warm water bath (60°C) under nitrogen flow
7. Subject to HPLC.

Chromatography

The chromatography was carried out by injection of 5 µl of extract using a loop injector (Reodyne). For reproducible results the loop injector was necessary. The column cooled to -20°C gave improved separation of the estriol with a reasonable retention time (6).

RESULTS AND DISCUSSION

The use of subambient temperatures for separation of steroids has been described in previous work from this laboratory (6). It was deemed desirable to follow these procedures to assure confidence in the identity of the separated materials. Figure 1 shows how subambient temperatures facilitated separations of isomers of estradiol and of androstenediols (7). Figure 2 gives the results of HPLC of

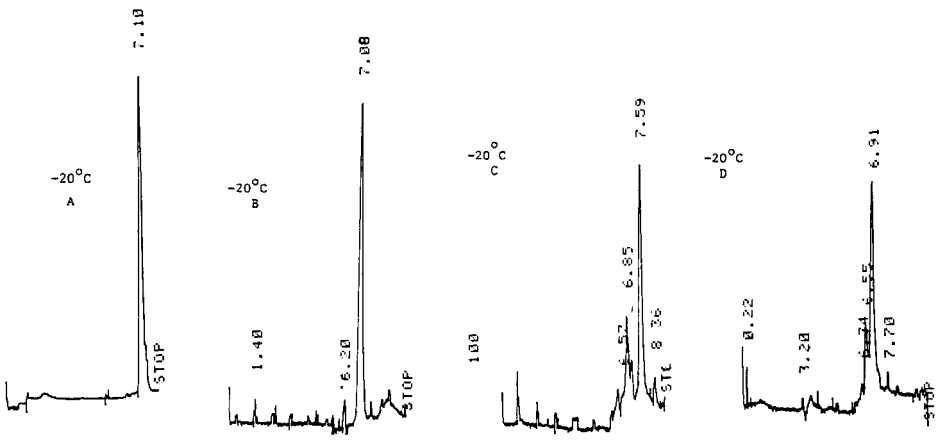


(A) Estradiol-17α and estradiol -17 β
(B) Androst-5ene-3β,17α-diol and androst-5ene-3β,17β-diol
Detection: Estrogen (280 nm) and androgen (200nm)

Figure 1: A: estradiol - 17α and estradiol - 17β
B: androst-5ene-3β, 17α-diol and androst-5ene-3β, 17β-diol

Detection: estrogen (280 nm) and androgen (200 nm)

extracts of normal amniotic fluid and those of Down syndrome as well as Trisomy - 18. The chromatograms in each case indicate clear separation of estriol. The Down syndrome and Trisomy - 18 samples did not show the presence of free estriol. The identity of the peaks separated in the chromatograms which presumably are not estriol is not as yet known. Since these peaks are not present in the normal samples,



Estriol resolved at subambient temperature (-20°C) by HPLC. Chromatograms shown are A: Standard Estriol (E_3) peak with retenion time $t_R E_3$= 7.10; B: Normal Amniotic fluid, C: Down's Synodrome; D: Trisomy-18.
Detection of E_3 = 280 nm.

Figure 2: Estriol resolved at subambient temperature (-20°C) by HPLC. Chromatograms shown are
A: Standard estriol (E_3) peak with retention time $t_R E_3$=7.10
B: Normal Amniotic fluid
C: Down syndrome
D: Trisomy-18

Detection of E3=280 nm

it might be appropriate to attempt identification and determine whether there is a relationship if any to the genetic defect in question.

As seen in Table 2 the levels of free estriol in normal amniotic fluid were variable. In some cases only trace amounts were seen. However, no free estriol was seen in the amniotic fluid from the 20 cases diagnosed as Down syndrome or the 5 Trisomy - 18 cases.

The studies carried out in this work involved only the "free" estriol. Whether an investigation of a possible role of the "conjugated" estriol would provide further

Table II

Estriol In Amniotic Fluid[1]

Normal*					Down's Syndrome			
Sex	Age weeks	No of Specimens	E_3 Conc. ng/mL	$t_R E_3$	No of Specimens	E_3 Conc. ng/mL	$t_R X$
F	15	5	245, T, T, T,	7.08	(Trisomy-21)[2] 20	Not detected	7.59
M	15	5	T, 146, 48, T, T	"			
F	16	5	77, 99, 266.2, 306, 80.1	"			
M	16	5	61.3, 110.2, 28, 150.5, T	"			
F	17	5	T, T, 485.8, 264.9, T	"			
					(Trisomy-18) 5	-	-

T = trace, tRE_3 = retention time, E_3 = estriol, - = not detected, X = unknown compound, F = female, M = male

[1]Method involving Solid phase extraction and high-performance liquid chromatography.

[2]All sexes.

*Normal reported E3 level in plasma = 93 - 560 ng/mL

Gurpide, E., Giebenhain, M.E., Tseng, L and Kelly, W.G. Am. J. Obstet. Gyne. 109:897 (1971)

insight to the genetic defect of Down syndrome is not known. A comparison of the ratios of the "free" to the "conjugate" levels might provide useful information. In conjunction with concurrent estimation of α-fetoprotein and gonadotropin the amniotic estriol could provide the clinician with additional information of the status of the fetus.

CONCLUSION

The present method is comparable with other micro analytical methods. Solid phase extraction methodology and HPLC at subambient temperatures is applicable to other biologically important compounds.

REFERENCES

1. Canick, J.A., Knight, G.J., Palomaii, G.E., Haddow, J.E., Cuckle, H.D., Wald, N.J. (1988) Low second trimester maternal serum unconjugated oestriol in pregnancies with Down syndrome. Br. J. Obstet. Gynaecol., 95:330-333.

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3. Darnule, A., Schmidt, D., Weyland, B., Greenberg, F., Rose, E., Alpert, E. (1990) Serum HCG, AFP and unconjugated estriol levels in trisomy 19 pregnancies in mide-trimester. Am. J. Hum. Genet., 47 [Suppl]:A272.

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6. Sheikh, S.U., Touchstone, J.D. (1987) HPLC of Steroids in non-aqueous mobile phases at subambient temperatures. J. Liquid Chromatogr. 10, 2489.

7. ibid, (1987) Separation of steroid diasteriomers by HPLC at subambient temperature. Anal. Chem. 59, 1472.

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Accepted: June 20, 1994

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(17), 3821 (1994)

ANNOUNCEMENT

CALL FOR NOMINATIONS
CHROMATOGRAPHY FORUM OF THE DELAWARE VALLEY

STEPHEN DAL NOGARE AWARD
FOR EXCELLENCE AND SIGNIFICANT CONTRIBUTIONS
IN THE FIELD OF CHROMATOGRAPHY

This award will be given to the recipient during the 1995 calendar year. All nominations should consist of one or more letters of nomination and a biographical sketch listing experience and significant contributions to the advancement of the chromatographic field.

Deadline for nominations is December 31, 1994. Previous years' nominations can be renewed or appended with an updating letter of nomination.

Nominations should be sent to:

Mary Ellen MacNally, Ph.D.
E. I. du Pont de Nemours & Company
Du Pont Agricultural Products
Experimental Station
Wilmington, DE 19880-0402

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(17), 3823-3829 (1994)

LIQUID CHROMATOGRAPHY CALENDAR

1994

OCTOBER 3 - 4: Course on Capillary Electrophoresis, Budapest, Hungary. Contact: Dr. F. Kilar, Central Research Laboratory, Medical School, University of Pecs, Szigeti ut 12, H-7643 Pecs, Hungary.

OCTOBER 5 - 7: 9th International Symposium on Capillary Electrophoresis, Budapest, Hungary. Contact: Dr. F. Kilar, Central Research Laboratory, Medical School, University of Pecs, Szigeti ut 12, H-7643 Pecs, Hungary.

OCTOBER 9 - 14: 186th Meeting of the Electrochemical Society. Contact: Electrochem. Soc., 10 S. Main St., Pennington, NJ 08534-2896, USA.

OCTOBER 16 - 19: 46th Southeastern Regional Meeting, ACS, Birmingham, Alabama. Contact: L. Kispert, Chem Dept, Univ of Alabama, Box 870336, Tuscaloosa, AL 35115, USA.

OCTOBER 17 - 19: 3rd Int'l Symposium on Supercritical Fluids, Strasbourg, France. Contact: Int'l Soc for the Advancement of Supercritical Fluids, 1 rue Grandville, B. P. 451, F-54001 Nancy Cedex France.

OCTOBER 18 - 22: 30th ACS Western Regional Meeting, Sacramento, California. Contact: S. Shoemaker, Calif. Inst of Food & Agricultural Res., Univ. of Calif-Davis, 258 Cruss Hall, Davis, CA 95616-8598, USA.

OCTOBER 23 - 27: Annual Meeting of the American Association of Cereal Chemists, Nashville, Tennessee. Contact: J. M. Schimml, AACC, 3340 Pilot Knob Road, St. Paul, MN 55121-2097, USA.

OCTOBER 24 - 26: 2nd Internat'l Conference on Chemistry in Industry, Bahrain, Saudi Arabia. Contact: SAICSC-ACS, P. O. Box 1723, Dhahrain 31311, Saudi Arabia.

OCTOBER 25 - 26: Frederick Conference on Capillary Electrophoresis, Holiday Inn @ FSK Mall, Frederick, Maryland. Contact: Margaret L. Fanning, PRI, NCI-FCR&DC, P. O. Box B, Frederick, MD 21702-1201, USA.

NOVEMBER 2 - 5: 29th Midwestern Regional Meeting, ACS, Kansas City, Kansas. Contact: M. Wickham-St. Germain, Midwest Res. Inst, 425 Volker Blvd, Kansas City, MO 64110, USA.

NOVEMBER 3: Anachem Symposium, Dearborn, Michigan. Contact: Paul Beckwith, Program Chairman, Detroit Edison Co., 6100 W. warren, Detroit, MI 48210, USA.

NOVEMBER 7 - 10: 13th Int'l Conference on the Application of Accelerators in Research & Industry, Denton, Texas. Contact: J. L. Duggan, Univ. of N. Texas, Dept. of Physics, P. O. Box 5368, Denton, TX 76203, USA.

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

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

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

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

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

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

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

1995

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

1996

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

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

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

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

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

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

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

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

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

1997

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

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

1998

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

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

1999

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

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

2000

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

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

2001

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

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

2002

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

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

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2. **Title & Authors:** The entire title should be in capital letters and centered within the width of the typing area, located at least 2 inches (5.1 cm) from the top of the page. This should be followed by 3 lines of space, then by the names and addresses of the authors, also centered, in the following manner:

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F. D. Pierce and H. R. Brown
Utah Biomedical Test Laboratory
520 Wakara Way
Salt Lake City, Utah 84108

3. **Abstract:** The title **ABSTRACT** should be typed, capitalized and centered, 3 lines below the addresses. This should be followed by a **single-spaced,** concise abstract. Allow 3 lines of space below the abstract before beginning the text of the manuscript.

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INTRODUCTION
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RESULTS
DISCUSSION
ACKNOWLEDGEMENTS
REFERENCES

These **major headings** should be separated from the text by two lines of space above and one line of space below. Each major heading should be typed in capital letters, centered and underlined.

Secondary headings, if any, should be placed flush with the left margin, underlined and have the first letter of main words capitalized. Leave two lines of space above and one line of space below secondary headings.

5. The first word of each **paragraph** within the body of the text should be indented five spaces.

6. **Acknowledgements**, sources of research funds and address changes for authors should be listed in a separate section at the end of the manuscript, immediately preceding the references.

7. **References** should be numbered consecutively and placed in a separate section at the end of the manuscript. They should be typed single-spaced, with one line space between each reference. Each reference should contain names of all authors (with initials of their first and middle names); do not use *et al.* for a list of authors. Abbreviations of journal titles will follow the American Chemical Society's Chemical Abstracts List of Periodicals. The word **REFERENCES** should be capitalized and centered above the reference list.

Following are acceptable reference formats:

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

Book:

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

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

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

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

9. Only standard symbols and nomenclature, approved by the International Union of Pure and Applied Chemistry (IUPAC) should be used.

10. Material that cannot be typed, such as Greek symbols, script letters and structural formulae, should be drawn carefully with dark black India ink. Do not use any other color ink.

Additional Typing Instructions

1. The manuscript must be prepared on good quality **white bond paper**, measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). The typing area of the first page, including the title and authors, should be 5½ inches wide by 7 inches high (14 cm x 18 cm). The typing area of all other pages should be no more than 5½ inches wide by 8½ inches high (14 cm x 21.6 cm).

2. The **title, abstract, tables and references** are typed single-spaced. All other text should be typed 1½-line spaced or double line spaced.

3. It is essential to use **dark black** typewriter or printer ribbon so that clean, clear, **solid characters** are produced. Characters produced with a dot/matrix printer are not acceptable, even if they are "near letter quality" or "letter quality." Erasure marks, smudges, hand-drawn corrections and creases are not acceptable.

4. **Tables** should be typed on separate pages, one table to a page. A table may not be longer than one page. If a table is larger than one page, it should be divided into more than one table. The word **TABLE** (capitalized and followed by an Arabic number) should precede the table and should be centered above the table. The title of the table should have the first letters of all main words in capitals. Table titles should be typed single line spaced, across the full width of the table.

5. **Figures (drawings, graphs, etc.)** should be professionally drawn in **black** India ink on separate sheets of **white** paper, and should be placed at the end of the text. They should not be inserted in the body of the text. They should not be reduced to a small size. Preferred size for figures is from 5 inches x 7 inches (12.7 cm x 17.8 cm) to 8½ inches by 11 inches (21.6 cm x 27.9 cm). **Photographs** should be professionally prepared ***glossy*** prints. A typewriter or lettering set should be used for all labels on the figures or photographs; they may not be hand drawn.

Captions for figures should be typed single-spaced on a separate sheet of white paper, along the full width of the type page, and should be preceded with the word FIGURE and an Arabic numeral. All figures and lettering must be of a size that will remain legible after a 20% reduction from the original size. Figure numbers, name of senior author and an arrow indicating "top" should be written in light blue pencil on the back of the figure. Indicate the approximate placement for each figure in the text with a note written with a light blue pencil in the margin of the manuscript page.

6. The **reference list** should be typed single-spaced. A single line space should be inserted after each reference. The format for references should be as given above.