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STUDY OF POLYSTYRENE-BLOCK-POLY(METHYL METHACRYLATE) MICELLES BY SIZE EXCLUSION CHROMATOGRAPHY/LOW ANGLE LASER LIGHT SCATTERING. INFLUENCE OF COPOLYMER COMPOSITION AND MOLECULAR WEIGHT

ZLATKA GRUBIŠIC-GALLOT[1], YVES GALLOT[1], AND JAN SEDLÁČEK[2]

[1]*Institut Charles Sadron, (CRM-EAHP)(CNRS-ULP)*
6, rue Boussingault
67083 Strasbourg, Cedex, France
[2]*Dept. of Physical and Macromolecular Chemistry*
Faculty of Science, Charles University
Albertov 2030, Prague, Czech Republic

ABSTRACT

Results of size exclusion chromatography study of the micellar systems polystyrene-*block*-poly(methyl methacrylate) in the mixed solvent 1,4-dioxane/cyclohexane are reported and the behaviour of copolymers with different overall molecular weight and chemical composition is compared. At higher content of cyclohexane, the effect of the solute trapping in the column due to the adsorption of the unimer on the packing was observed. This effect was more pronounced for the lower molecular-weight copolymers as compared with the higher molecular-weight ones and was found to depend significantly on the thermodynamic quality of solvent (eluent). Experiments in 1,4-dioxane/70 vol.% cyclohexane showed strong influence of unimer - micelles re-equilibration in the column on the resulting chromatograms, especially for the micellar systems of the lower molecular-weight copolymers. For the copolymers of higher molecular-weight, the micelles dissociation in the column was reduced probably due to the more entangled and compact cores of micelles.

INTRODUCTION

The formation of copolymer associates - micelles in dilute solutions of block copolymers in selective solvents (i.e. thermodynamically good solvent for one block and at the same time poor solvent for the other block) is well-known. The formation of these micelles is described by the "closed association" model. This model is characterized by an equilibrium between the micelles (M), with a narrow molecular-weight and size distribution, and the molecularly dissolved copolymer - unimer (U).

$$n\,U \rightleftarrows M \qquad (1)$$

where n is the association number. The equilibrium, (i.e. the association number and concentrations of unimer and micelles) and the dynamics of its achievement depend upon several parameters such as the nature of the copolymer and the selectivity of solvent (usually controlled by the composition of a solvent mixture or/and by the temperature) [1,2].

Various techniques are currently used for the study of micellar systems e.g. light scattering [2,3], sedimentation [4], size exclusion chromatography (SEC) [5]. The application of SEC to the separation and characterization of individual components for a micellar system is usually complicated by the unimer $\rightleftarrows$ micelles association and dissociation which takes place in the SEC column. Chromatograms obtained with micellar systems reflect two competing processes: (i) the separation of the solute in the column; and (ii) the disturbing and subsequent re-establishment of the unimer $\rightleftarrows$ micelles equilibrium (Eq. 1). The shape of the corresponding chromatograms depends on the relative rates of these two processes as proposed theoretically [6] and confirmed by SEC studies on various block copolymer micellar systems [5,7,8]. Additional complications in the use of SEC to study of micellar systems often arise due to the loss of the solute in the column during the SEC separation. The adsorption of the unimer form of the copolymer is most frequently given as the reason for this phenomenon [7-9].

ERRATUM

Z. Grubišic-Gallot, Y. Gallot, and J. Sedláček, "Study of Polystyrene-block-poly(methyl Methacrylate) Micelles by Size Exclusion Chromatography/Low Angle Laser Light Scattering. Influence of Copolymer Composition and Molecular Weight," *J. Liquid Chrom.*, 18(12), 2291-2307 (1995).

Due to a printing error, the third author's name in the above-mentioned article was misspelled in the table of contents. It should have appeared as J. Sedláček, not J. SedláÒek.

These effects, the strong influence of the unimer $\rightleftarrows$ micelles re-equilibration on the shape of chromatograms and the significant loss of the solute in the course of the SEC separation were observed in previous work [10]. This latter study used SEC coupled with a low-angle laser light scattering (LALLS) detector applied to a polystyrene-*block*-poly(methyl methacrylate) (PS-PMMA) in a mixed 1,4-dioxane/cyclohexane solvent. In this solvent, micelles with PMMA core and PS shell are formed at higher contents of cyclohexane. Use of the LALLS detector was found to be very advantageous for the subsequent interpretation of the elution curves and for the determination of the molecular weight characteristics of the micelles; molecular weights of micelles determined by this detector were found to be in agreement with those determined independently by static light scattering.

This article focuses on the influence of the composition and overall molecular weight of PS-PMMA on the behaviour of the micellar system in 1,4-dioxane/cyclohexane. The same techniques, i.e. SEC/LALLS and the static light scattering, were applied.

EXPERIMENTAL

Copolymers

Samples of polystyrene-*block*-poly(methyl methacrylate) (PS-PMMA) having different overall molecular-weight and composition were synthesized by anionic polymerization using phenylisopropyl-potassium as initiator. The details of syntheses are described elsewhere [10]. The chemical composition of copolymers was determined by elemental analysis. For the determination of weight-average molecular weight ($\bar{M}_w$) and number-average molecular weight ($\bar{M}_n$), size exclusion chromatography/low angle laser light scattering was used.

Size exclusion chromatography

Waters 150 C apparatus with two detectors coupled on line: a low-angle laser light scattering (LALLS) photometer (Chromatix CMX-100) and a standard Waters differential refractometer (DR) was applied [11]. For the characterization of the copolymer samples five column in series (PL gel) having upper permeability limits of 10^6, 10^5, 10^4, 10^3 and $5x10^2$, respectively, and tetrahydrofuran (THF) as eluent with a flow rate of 1 mL/min were used. The SEC study of the micellar systems was carried out with one column packed with μ-styragel (10^5). Different 1,4 dioxane/cyclohexane mixtures were employed as the mobile phase at a temperature of 25°C. The copolymers were always dissolved in a given solvent mixture for 24 h at a temperature of 25°C prior to analysis. All the SEC experiments were carried out at a flow rate of 1 mL/min, using the concentration of the injected sample (100μL) of 10 mg/mL.

Static light scattering

SEM-633 apparatus from SEMATECH (wavelength λ = 632.8 nm) was used. The weight-average molecular weights of micelles, $\bar{M}_w^{(M)}$ were obtained at 25°C using the relation:

$$Kc/R_o = 1/\bar{M}_w^{(p)} + 2A_2c \qquad (2)$$

where K is the optical constant, R_o is the Rayleigh ratio extrapolated to zero angle, c is the copolymer concentration and A_2 is the second virial coefficient. $\bar{M}_w^{(p)}$ is the weight-average molecular weight of all scattering particles in solution, which can be considered as the value of $\bar{M}_w^{(M)}$ provided that the equilibrium concentration of unimer is small in comparison with the concentration of micelles [2]. The estimates of the critical micelle concentration (CMC) were obtained from the shape of the dependencies Kc/R_o vs c. The details on $\bar{M}_w^{(M)}$ and CMC determination from the static light scattering data are available elsewhere [2,10].

RESULTS AND DISSCUSION

Four samples of polystyrene-*block*-poly(methyl methacrylate) (PS-PMMA) with different molecular-weights and contents of PS and PMMA were synthesized. Their characteristics are given in Tab. 1. Applying solvent mixture 1,4-dioxane/cyclohexane at various compositions as the eluent, SEC analyses of these copolymers were carried out. The region of solvent composition where PS-PMMA micelles were detected by SEC/LALLS was determined for each copolymer (Tab. 1). For all solvent mixtures where micelles were detected, good unimer-micelle resolution was achieved in the chromatograms of all copolymer samples. The peaks of micelles (detected by DR) were found to increase in intensity with the decrease in thermodynamic quality of solvent, i.e. with the increasing cyclohexane content. Just the opposite trend was observed for the peaks of unimer. The loss of some of the solute in the SEC column was observed for all copolymers at higher contents of cyclohexane. This trapped solute was always completely eluted by a zone of good solvent (an injection of 100 μl of 1,4-dioxane). In the previous article [10] an explanation of this phenomenon was proposed based on the adsorption of the unimer form of the PS-PMMA copolymer on the column packing. The amount of solute lost on the column was found to depend significantly on the thermodynamic quality of the solvent (eluent) and on the molecular weight and composition of the copolymer as shown in Fig. 1. This figure shows the mass fraction of copolymer, w, which passed through the column as a function of the solvent composition. Values of w represent the mass of the copolymer detected by DR (both forms: unimer and micelles) divided by the mass of copolymer injected into the column. All dependencies in Fig. 1 pass through a minimum and the following explanation can be proposed. There are at least two important aspects of the solute loss in the column affected by the solvent composition: (i) the affinity of the unimer to the adsorption on the column packing, which would be expected to increase with the decrease in the thermodynamic

TABLE 1

Weight-average molecular weight ($\bar{M}_w$), polydispersity index ($\bar{M}_w/\bar{M}_n$) and chemical composition in wt.% of polystyrene (PS) of polystyrene-*block*-poly(methyl methacrylate) copolymers (PS-PMMA). Composition range of 1,4-dioxane/cyclohexane mixed solvent (in vol.% of cyclohexane) where micelles of PS-PMMA were detected by size exclusion chromatography/low angle laser light scattering (SEC/LALLS). Weight-average molecular weight of micelles ($\bar{M}_w^{(M)}$) determined by SEC/LALLS and by static light scattering (SLS), polydispersity index of micelles ($\bar{M}_w^{(M)}/\bar{M}_n^{(M)}$) by SEC/LALLS and critical micelle concentration (CMC) by SLS in 1,4-dioxane/70 vol.% cyclohexane.

No.	Characteristics of copolymers			Selective solvent composition[a]	Characteristics of micelles in 1,4-dioxane/70vol.% cyclohexane			
					SEC/LALLS		SLS	
	$10^{-3}\times\bar{M}_w$	$\bar{M}_w/\bar{M}_n$	composition wt.% of PS	vol.% of cyclohexane	$10^{-6}\times\bar{M}_w^{(M)}$	$\bar{M}_w^{(M)}/\bar{M}_n^{(M)}$	$10^{-6}\times\bar{M}_w^{(M)}$	CMC in g/mL
I	96	1.04	64.0	80 - 85	--	--	1.8	1×10^{-4}
II	162	1.03	66.5	65 - 80	20	1.08	18	$<1\times10^{-5}$
III	91	1.04	52.6	60 - 80	26	1.11	11	$<1\times10^{-5}$
IV	172	1.05	51.2	60 - 75	71	1.13	77	$<5\times10^{-6}$

[a] Composition range of 1,4-dioxane/cyclohexane mixed solvent where micelles were detected by SEC/LALLS.

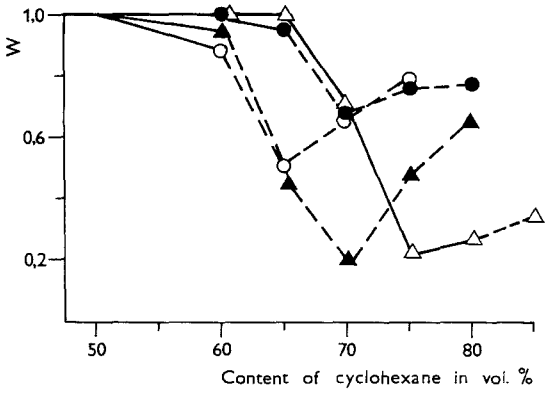

FIGURE 1. Weight fraction, w, of PS-PMMA samples which passed through SEC column as function of the content of cyclohexane in the 1,4-dioxane/cyclohexane mixed solvent used as the eluent. Concentration of the injected solution c = 10 mg/mL; injection volume: 100 μL; flow rate: 1 mL/min. Overall weight-average molecular weight of copolymers and content of PS block in the copolymers, respectively: 96 000; 64.0 wt.% (sample *I*) (△), 162 000 66.5 wt.% (sample *II*) (●), 91 000; 52.6 wt.% (sample *III*) (▲), 172 000; 51.2 wt.% (sample *IV*) (○). Dashed line: solvent compositions where micelles were detected by SEC.

quality of the solvent for a given copolymer, i.e. with the increase in content of cyclohexane; and (ii) the equilibrium concentration of the unimer in the column and the rate of the re-establishment of this equilibrium concentration (i.e. the rate of the dissociation of micelles) in the course of unimer-micelles separation. The equilibrium concentration of unimer is generally reported as decreasing with the decrease in the thermodynamic quality of solvent. A similar decrease might be assumed for the rate of dissociation of micelles in the column because more compact micelle cores may be expected in the thermodynamically worse solvent, which hinders a release of the unimer from the micelles. Considering (i) and (ii) the position of minima in Fig. 1 may represent a solvent composition (the quality of

solvent) for which the combined effect of both the adsorption affinity of the unimer and its equilibrium concentration are the highest. Although a further drop in the thermodynamic quality of the solvent should cause an increase in the affinity of the unimer to the adsorption, it is simultaneously accompanied by a drop in the concentration of unimer and the latter effect seems to predominate leading, in fact, to the rise in w as shown in Fig. 1. A similar observation was reported by Price et. al. in the SEC study of the micellar solutions of polystyrene-*block*-poly(ethylene-*co*-propylene). The authors observed negligible adsorption for the thermodynamically bad solvents but the complete loss of the solute on the column in the thermodynamically better solvents where less stable micelles were present [8].

If we compare the behaviour of copolymers having similar molecular weight but differing in chemical composition (sample *I* vs. *III*, sample *II* vs. *IV*) we observe that the position of the minimum is shifted towards the thermodynamically worse solvent (i.e. higher cyclohexane content) for copolymers with a shorter insoluble (PMMA) block. A similar phenomenon is observed if the solvent composition corresponding to the last point in the dependencies in Fig. 1 is considered. This composition represents the highest content of cyclohexane in 1,4-dioxane/cyclohexane mixed solvent in which a given copolymer was found to be still soluble (solvent composition was changed in steps of five vol.%).

Fig. 1 also offers a comparison between copolymers having similar composition but differing in molecular weight (sample *I* vs. *II* and sample *III* vs. *IV*). Both the position of the minima and the position of the last points in the dependencies in Fig. 1 are shifted towards thermodynamically better solvent for the copolymers with the higher molecular weight. This seems to be ascribable to the longer insoluble blocks (PMMA) in the copolymers having the higher molecular weight (sample *II* and *IV*).

It is also seen from Fig. 1 that the highest losses of the solute in the column were detected for the lower molecular-weight

copolymers (compare the depth of the minima in Fig. 1). This finding can be easily explained if we take into account the conformation of the unimer form of copolymer chains in a selective solvent mixture. It may be assumed that this conformation is similar to that known as unimolecular micelles [12] i.e. the insoluble PMMA block slightly collapsed and partially protected by a better soluble PS block which is forming a shell. From this point of view the longer soluble PS block of the higher molecular-weight samples should provide a better protection of the collapsed insoluble PMMA block and thus the unimer adsorption affinity could be reduced to some extent. The effect of the equilibrium concentration of unimer and the rate of its re-establishment must also be considered. The re-equilibration process might be expected to be slowed down for the higher molecular weight copolymers in comparison with the lower molecular-weight ones because of the more difficult disentanglement processes in cores of micelles of the former. All of the above effects probably contribute to the observed dependence of w on the molecular-weight of copolymers (Fig. 1).

For the detailed study of the PS-PMMA micellar systems the selective solvent mixture 1,4-dioxane/70 vol.% cyclohexane was chosen. Solutions of the copolymers in this solvent mixture were studied by means of static light scattering; the results are shown in Fig. 2 where the dependencies of Kc/R_o (extrapolated to zero angle) on the overall concentration of copolymer in solution, c, are given. The dependencies exhibit a shape typical of associating systems and suggest that all the copolymers form micelles from low copolymer concentrations. The estimates of values of critical micelle concentration (CMC) (the copolymer concentration at which micelles are just detected by a given method) are given in Tab. 1. In this table the values of weight-average molecular weight of micelles $\tilde{M}_w^{(M)}$ obtained from the static light scattering measurements are also given. Values of $\tilde{M}_w^{(M)}$ were obtained extrapolating the Kc/R_o values from the horizontal parts of

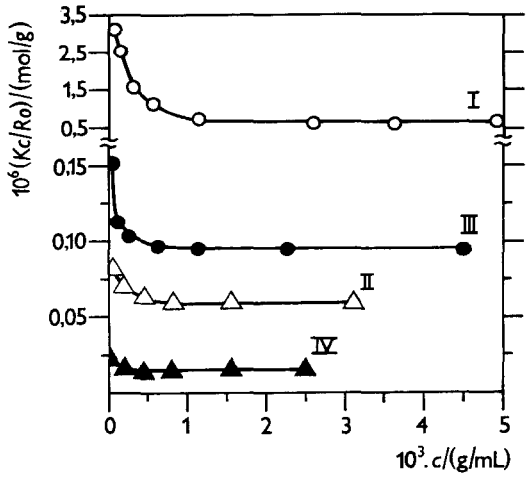

FIGURE 2. Concentration dependence of Kc/R_o from static light scattering measurements for solutions of samples of PS-PMMA in the mixture 1,4-dioxane/70vol.% cyclohexane at 25°C. Overall weight-average molecular weight of copolymers and content of PS block in the copolymers, respectively: 96 000; 64.0 wt.% (sample *I*), 162 000; 66.5 wt.% (sample *II*), 91 000; 52.6 wt.% (sample *III*), 172 000; 51.2 wt.% (sample *IV*)

dependencies in Fig. 2 to c=0 (Eq. 2). Although this process leads to the weight-average molecular weight of all scattering particles (i.e. so-called particles molecular weight $\bar{M}_w^{(p)}$) this value can be considered as $\bar{M}_w^{(M)}$ in the case of all copolymers because the unimer contribution to the measured value $\bar{M}_w^{(p)}$ is negligible. This is a result of the very low values of CMC (Tab. 1) (CMC is close to the equilibrium concentration of unimer in a micellar system) as already discussed in the previous article [10].

In Fig. 3 the chromatograms resulting from SEC experiments carried out with the copolymers in 1,4-dioxane/70 vol% cyclohexane are shown. In the concentration profiles (DR response) the peaks with a lower elution volume (V_e= 9.1 mL Fig. 3b, V_e= 8.9 mL Fig. 3c, V_e= 8.6 mL Fig. 3d) for which high LALLS detector responses

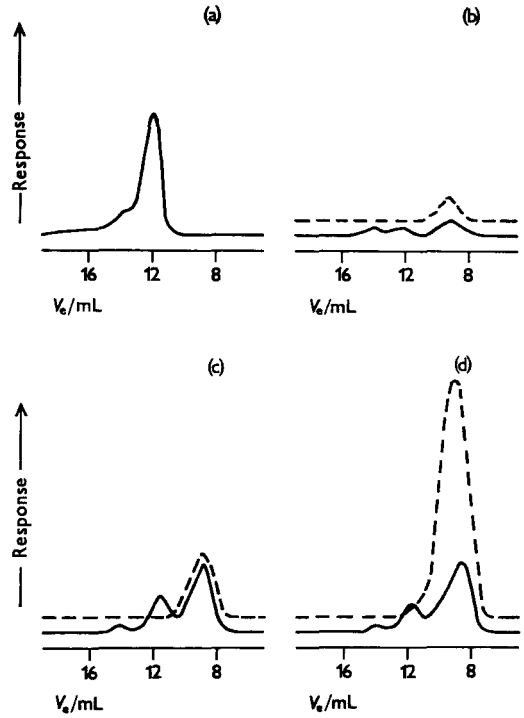

FIGURE 3. SEC curves of PS-PMMA samples in mixture 1,4-dioxane/ 70 vol.% cyclohexane at 25°C. Overall weight average molecular-weight of copolymers and content of PS block in the copolymers, respectively: 96 000; 64.0 wt.% (sample *I*)(a), 91 000; 52.6 wt.% (sample *III*)(b), 162 000; 66.5 wt.% (sample *II*)(c), 172 000; 51.2 wt.% (sample *IV*)(d). Concentration c= 10 mg/mL; injection volume: 100 μL; flow rate: 1 mL/min. Full line: DR response; dashed line: LALLS response. (V_e: elution volume)

were obtained were ascribed to micelles. The peaks at higher elution volume (V_e= 11.6-12.0 mL) correspond to the unimer in the case of all copolymers studied. The small peaks at V_e=13.8-14.2 mL (at the permeation limit of the column) correspond to the SEC system peaks and probably reflect the selective solvation of the solute [10,13].

As mentioned previously, the values of CMC (see above and Tab. 1) are, according to the closed association model (Eq. 1), very close to the equilibrium concentration of the unimer in the micellar system. It can therefore be deduced that for all copolymer samples in the 1,4-dioxane/70 vol.% cyclohexane the unimer $\rightleftarrows$ micelles equilibrium was significantly shifted in favour of the micelles in the solutions injected on the SEC column (c = 10 mg/mL). For example, in the injected solution of sample No. *I*, ca 99 wt.% of copolymer was in the form of micelles. This fraction was even higher for the samples *II*, *III* and *IV*. Considering the values of (1.0 - w) for the 1,4-dioxane/70 vol.% cyclohexane solvent mixture from Fig. 1 (which represent the fraction of unimer trapped in the column) and the intensities of unimer peaks in Fig. 3 as well, it is clear that the amount of micelles detected after the passage through the column does not reflect the micellar equilibrium concentration in the injected sample for any copolymer investigated. This is due to the dissociation of micelles in the course of the SEC experiment, as already described in ref [10]. As soon as the micelles are separated from the unimer in the column (as a result of different hydrodynamic volumes) the equilibrium (Eq. 1) is disturbed in the zone of micelles and a certain amount of the micelles tends to dissociate to re-establish the unimer $\rightleftarrows$ micelles equilibrium. As seen in Fig. 3 this process is in 1,4-dioxane/70 vol.% cyclohexane more pronounced for the micellar systems of the lower molecular-weight copolymers and in case of sample No. *I* it even leads to the total disappearance of the micelles from the system (Fig. 3a). The fact that the fraction of the micelles which dissociate in the column is reduced in the case of the higher molecular-weight copolymers (Fig. 3c, d) might be explained by a lower rate of dissociation of these micelles due to the more entangled and compact micelles cores as already proposed above. Also the values of CMC (Tab. 1) seem to suggest that the equilibrium concentration of unimer is lower in the micellar

systems of higher molecular-weight copolymers. Unfortunately the exact comparison is not possible since only upper estimates of CMC were available for the samples *II*, *III* and *IV* due to the shift of the onset of micellization to very low copolymer concentrations.

It is clear that for the exact interpretation of chromatograms in Fig. 3 other effects should also be considered - in particular the unimer - micelle SEC separation efficiency for each micellar system and the unimer trapping on the column packing. In our case the SEC process deals with the separation of particles (unimer and micelles) for which a significant difference in hydrodynamic volume is expected. This difference is probably not the same for all of the micellar systems studied. We suppose however that this fact has only little influence on the results in Fig. 3. The trapping of the unimer on the column packing is probably more important. The overall influence of this phenomenon is however difficult to evaluate. On the one hand, it might promote the separation of micelles from unimer (a certain amount of unimer which is being formed by the dissociation of micelles in the SEC column is continuously drawn off by adsorption, which can be thus regarded as an additional separation mechanism to SEC process). On the other hand, the trapping of unimer contributes to the reduction of the concentration of the unimer in the micellar zone and thus supports the dissociation of the micelles.

Good separation of peaks of micelles in chromatograms in Fig. 3 b-d enabled direct determination of $\bar{M}_w^{(M)}$ and polydispersity index $\bar{M}_w^{(M)}/\bar{M}_n^{(M)}$ of micelles from LALLS and DR detectors responses; the results are summarized in Tab. 1. As already stated in the previous article [10], in the case of copolymer sample No. *I* 80 vol.% of cyclohexane in 1,4-dioxane/cyclohexane mixed solvent was necessary for the micelles to be detected by SEC. For the micelles of this copolymer, the value of $\bar{M}_w^{(M)}$ was determined to be 7.9×10^6 by SEC/LALLS and 8.5×10^6 by static light scattering in 1,4-dioxane/80 vol.% cyclohexane [10].

There seems to be satisfactory agreement between the values of $\bar{M}_w^{(M)}$ determined by SEC/LALLS and by static light scattering for all copolymers except for the sample No. *III* (Tab. 1). For this sample the static light scattering provided a significantly lower value of $\bar{M}_w^{(M)}$ as compared with SEC/LALLS. Both values were reproducible and the differences between them cannot be explained by errors associated with the methods used. We suppose that this discrepancy might be caused by the effect of anomalous micellization. This phenomenon has been reported in connection with the study of micellization of various block copolymers in different selective solvents [14-16]. In these systems two kinds of micelles differing in size and molecular weight were observed: (i) the expected spherical regular micelles with a lower size and molecular weight and (ii) particles with a higher size and molecular weight: the anomalous micelles. Often only a small fraction of copolymer was found to be in the form of anomalous micelles. The formation of anomalous micelles by equilibrium association of regular ones was proposed for the systems studied by Price et. al. [15] and by Tuzar et. al. [14]. Provided that there exist two kinds of micelle in our micellar system of copolymer No. *III* in 1,4-dioxane/70 vol.% cyclohexane, the anomalous ones might be assumed to be more difficult to disentangle (due to their complexity), i.e. their rate of dissociation into the unimer may be lower compared with the rate of the dissociation of the regular ones. In the course of the SEC separation of this sample the relative fraction of anomalous micelles in the micellar zone should therefore increase continuously as a result of a more rapid dissociation of the regular micelles. The micellar peak in Fig. 3 b should thus be enriched with the anomalous micelles. Since this peak (DR response) seems to be symmetrical and since a certain SEC separation between regular and anomalous micelles should be expected (leading at least to the deformation of the shape of this peak) it might be even assumed that this peak is formed

predominantly or even exclusively by the anomalous micelles. It might be concluded therefore, that the value of $\bar{M}_w^{(M)}$ resulting from SEC/LALLS is significantly affected by the presence of anomalous micelles. It is even possible that this value is very close to the weight average molecular-weight of anomalous micelles, provided that a major part of regular micelles had dissociated during the SEC separation process. The value of $\bar{M}_w^{(M)}$ resulting from the static light scattering represents the weight-average molecular weight of all scattering particles in solution, i.e. the weight-average molecular weight of regular and anomalous micelles, since the contribution of unimer to this value is negligible (see above). If the fraction of anomalous micelles in the solution is small compared to the fraction of regular ones, $\bar{M}_w^{(M)}$ should be close to the weight-average molecular weight of regular micelles.

Further research is in progress aimed at the SEC separation of regular and anomalous micelles. It should be noticed that the SEC results confirmed the anomalous behaviour of the copolymer No. *III* in 1,4-dioxane/cyclohexane over a wide range of solvent composition investigated; however no clear evidence of anomalous micellization was found by the static light scattering so far (see also Fig. 2). Results will be published in a forthcoming article devoted exclusively to this phenomenon.

The anomalous behaviour of copolymer No. *III* complicates the correlation between the molecular weight of micelles and the type of the copolymer tested. If however, the value of $\bar{M}_w^{(M)}$ determined by static light scattering for the micelles of copolymer No. *III* is considered as an upper estimate for the weight-average molecular weight of the regular micelles, and provided that this estimate is not very far from the real value, the increase in the weight-average molecular weight of the regular micelles with increasing length of the insoluble PMMA block in the copolymers may be deduced for the mixed solvent 1,4-dioxane/70 vol.% cyclohexane (Tab. 1).

CONCLUSION

For all PS-PMMA copolymers solute trapping due to the adsorption of unimer in the column packing was observed in 1,4-dioxane/cyclohexane solvent mixture for higher contents of cyclohexane. This effect was more pronounced for copolymers with lower overall molecular weights. The SEC results obtained in 1,4-dioxane/70 vol.% cyclohexane showed that the rate of the re-establishment of the unimer $\rightleftharpoons$ micelles equilibrium in the zone of micelles (leading to the decrease in the amount of the micelles detected after the passage though the column) was certainly not negligible in comparison with the rate of the SEC separation process. A higher fraction of micelles was detected in the case of copolymers having higher overall molecular weights. This is probably due to the higher stability of these micelles and the lower values of CMC. The molecular weight of micelles formed in 1,4-dioxane/70 vol.% cyclohexane was found to increase with the increasing length of the insoluble (PMMA) block.

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SEPARATION OF OCTYLPHENOL POLYETHER ALCOHOLS SURFACTANTS BY CAPILLARY COLUMN SFC AND HPLC

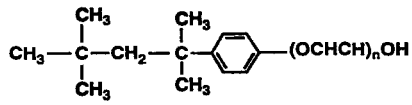
MING Y. YE, RON G. WALKUP, AND KIM D. HILL
ManTech Environmental Research Services Corporation
P.O. Box 1198
Ada, Oklahoma 74820

ABSTRACT

Separation of nonionic octylphenol polyether alcohols (OPA) by supercritical fluid chromatography (SFC) and HPLC is described. Using a density programming and a 50-μm i.d. capillary column, a total of 18 group oligomers was separated. The effects of the operating parameters, such as temperatures of mobile phase, flame ionization detector (FID) and injector, on SFC analysis were investigated. The results demonstrated that the separation efficiency is better when mobile phase temperature is above 100 °C, which must be due to the higher volatility of the oligomers. The results also demonstrated that the change of FID temperature from 300 °C to 420 °C has no effect on the total peak response. The study shows that injector cooling is not necessary when the analyte is in methanol. The total peak response was lower when the injector temperature was changed from 21 °C to 10 °C. A group of polar oligomers was found in OPA in HPLC analysis, not found in SFC. The result indicates that the polar oligomers present in OPA were not soluble in supercritical fluid carbon dioxide and, therefore, not observed in SFC.

INTRODUCTION

There has been considerable interest in using surfactants to enhancesubsurface remediation. [1, 2, 3] Because surfactants increase the solubility of hydrocarbon contaminants in ground-water systems, they could potentially greatly



$\overline{n}$ = 9 - 10

Figure 1 The molecular structural formula of octylphenol polyether alcohols (OPA)

reduce the number of pore volumes to be pumped in a cleanup effort. [3] Nonionic octylphenol polyether alcohols (OPA) is under consideration for this application. OPA, as other alkylphenol polyethoxylates such as nonylphenol and linear alkylbenzenesulphonates, is used as a detergent ingredient, [4] which may be discharged into municipal and industrial wastewater, eventually entering natural waters. [5, 6] These surfactants have been studied with respect to their behavior in wastewater treatment and their environmental impact. [7]

OPA, similar to other commercial surfactants such as T-MAZ, [8, 9] CS-330 [10] and Dowfax, [11] is a complex mixture of many isomers, homologues and oligomers. OPA is an aromatic nonionic surfactant and prepared by the reaction of octylphenol with ethylene oxide. The structural formula is shown in Figure 1. The complexity of this chemical is mainly due to the various lengths of ethylene oxide units $(OCHCH)_n$. According to the manufacturer, the average number of ethylene oxide units is 9 to 10, which corresponds to an average molecular mass of 584 to 626.

Because of the low volatility and high molecular weight, OPA is difficult to analyze by gas chromatography (GC). High temperature GC was used to analyze sucrose fatty acid ester fractions, [12] however, a derivatization procedure has to be performed prior to analysis.

Capillary supercritical fluid chromatography has been demonstrated as a very effective technique for the separation of nonionic surfactant polymers. [8, 13, 14, 15] Capillary supercritical fluid chromatography (SFC) performance is very much dependent on operating parameters, such as temperatures of mobile phase, injector and flame ionization detector. It is important to choose the proper operating parameters to optimize SFC performance. Although capillary SFC has been used to analyze nonionic surfactants such as alcohol ethoxylates, T-MAZ and Triton surfactants previously, [8, 13, 14, 15, 16] there are relatively few publications on the detailed investigations of the effects of the operating parameters on SFC analysis of nonionic surfactants. This work provides a detailed study of the effects of these operating parameters on SFC separation of OPA. The results have significance not only to the analysis of OPA, but also to optimization of SFC conditions for other nonionic polymers.

Reverse phase chromatography has been used to separate polymers previously. [9, 17] There are several advantages using HPLC for the polymer analysis: aqueous samples can be directly injected into an HPLC system, eliminating the need for sample extraction procedures; sample injection for HPLC system in our lab is automated, therefore more samples can be analyzed per 24 hour period. The separation was performed using reverse phase chromatography to see how the SFC separation of the oligomers differs from HPLC.

MATERIALS AND METHODS

Supercritical carbon dioxide (Scott Specialty Gases, Inc., Plumsteadville, PA, USA) was used as the mobile phase. OPA was purchased from Union Carbide

Chemicals and Plastics Company Inc. (Danbury, CT, IL, USA). Polyethylene glycol standards with molecular masses of 440, 600 and 960 were from Polymer Laboratories Ltd (Foster City, CA, USA). All solutions were made with methanol (Burdick and Jackson, Baxter Healthcare Corporation, Muskegon, MI, USA).

SFC

SFC analysis was performed on a Dionex (Dionex Corporation, Sunnyvale, CA, USA) 600-D Supercritical Fluid/Gas Chromatography system which includes a syringe pump to generate the high-pressure fluid flow, a chromatograph oven for temperature control, flame ionization detector (FID), and a data acquisition and processing system. All chromatographic conditions, including density programming, are computer-controlled. To obtain maximum sensitivity, the hydrogen/air ratio (1/10) was optimized and the detector operated in the most sensitive range. The flow rates were H_2: 30 ml/min, air: 300 ml/min and N_2: 25 ml/min. The detector body was heated to 390 °C. Samples were introduced into the chromatographic system using a Valco injector (Valco, Houston, TX, USA). The injection mode used was time split with the injection duration of 0.1 second. 1 µl of the sample was loaded to the injector loop which has a volume of 0.5 µl. With 0.1 second time split injection, 0.3 µl (60% of 0.5 µl) sample was loaded to the SFC column. The injector was cooled at 10 °C with a NesLab constant temperature circulator (NesLab, Portsmouth, NH, USA).

Separations were accomplished using a 10 meter SB-Biphenyl-30 capillary column from Dionex which has a 50-µm i.d. coated with approximately 0.25-µm film thickness. Prior to detection the supercritical fluid was decompressed, and the mobile phase linear velocity was controlled to approximately 1.5 cm/sec by connecting the

terminal end of the capillary column to a frit restrictor attached through a fused butt connector.

HPLC

Instrumentation was from Waters (Waters Associates, Milford, MA, U.S.A.), which included a 484 tunable absorbance detector, a 600E multisolvent delivery system and a 717 autosampler. Separations were accomplished using a Waters NovaPak C18 stainless steel column (3.9 x 150 mm). The mobile phase was 25% acetonitrile. The injection volume was 100 µl at an eluent flow rate of 1.0 ml/min. Data acquisition and processing was accomplished with a Waters Maxima 820 chromatography workstation, which included a system interface module and a NEC PowerMate SX/16 computer.

RESULTS AND DISCUSSION

SFC Separation

The density of the mobile phase is the primary factor involved in the partitioning of a solute between the stationary and mobile phase in SFC, and density is not directly proportional to pressure near the critical point. [18] Therefore linear density programming is preferred over pressure programming for resolving components of mixtures with a wide molecular mass range.

The chromatogram of OPA, Figure 2a, was obtained under the following conditions: (1) 0.3392 g/ml (150 atm, at 100 °C), held for 6 minutes and ramped to

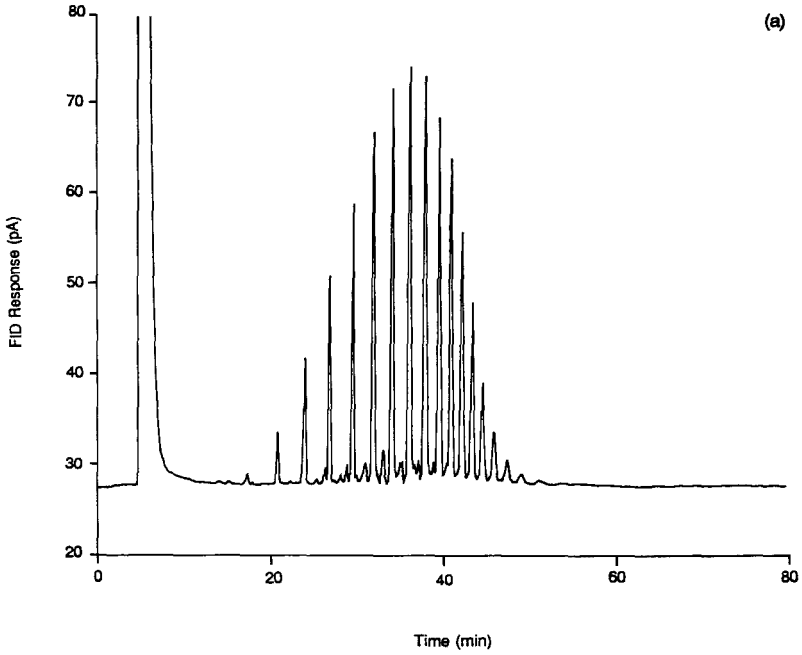


Figure 2 SFC chromatograms of OPA and PEG standards

Conditions: CO_2 mobile phase at 100 °C; FID at 390 °C; injector temperature: 21 °C. Linear isothermal density program: (1) 0.3392 g/ml (150 atm, at 100 °C), held for 6 minutes and ramped to 0.7100 g/ml (340 atm, at 100 °C) at 0.01 g/ml/min and held for 20 minutes; (2) from 0.7100 g/ml to 0.7430 g/ml (380 atm, at 100 °C) at 0.01 g/ml/min and then held for 10 minutes. Samples: (a) OPA, 43 µg/µl; (b) PEG 600, 70 µg/µl.

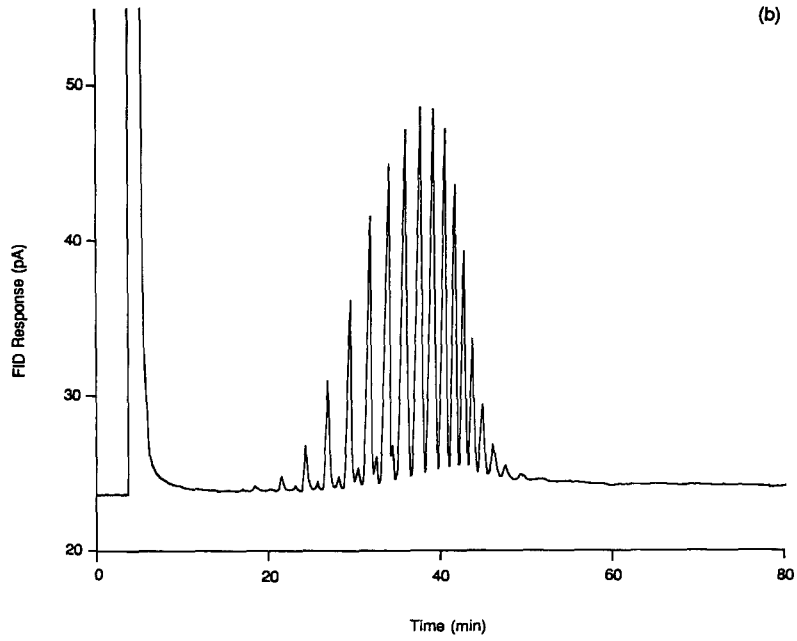


Figure 2 (continued)

0.7100 g/ml (340 atm, at 100 °C) at the rate of 0.01 g/ml/min and held for 20 minutes; (2) 0.7100 g/ml to 0.7430 g/ml (380 atm, at 100 °C) at 0.01 g/ml/min and held for 10 minutes.

Polyethylene glycol standards were used to estimate the average molecular mass of OPA as was done previously for T-MAZ. [8] The chromatogram shown in Figure 2b is a PEG standard with an average molecular mass of 600 using identical SFC conditions as in Figure 2a. Two chromatograms (Figures 2a and 2b) showed significant similarity in retention times and peak distribution, which demonstrated that the average molecular mass of OPA is about 600, confirming that of the range of 584

to 626 from the manufacturer. In addition, it also demonstrated that the oligomers of OPA and PEG have very similar solubilities at the same density of the mobile phase and the SB-Biphenyl-30 capillary column has almost no selectivity in polarity difference between molecules of OPA and PEG.

Two other PEG standards with average molecular mass of 440 and 960 were also analyzed. As expected, oligomers in PEG 440 elutes earlier than OPA and PEG 600, because of their smaller molecular mass than OPA and PEG 600. Oligomers in PEG 960 elutes later than OPA and PEG 600, which is due to the higher molecular mass.

Effect of Mobile Phase Temperature

The effect of mobile phase temperature on the separation was examined by changing column oven temperature. The isothermal separation was performed at 80 ^{o}C, 100 ^{o}C, 120 ^{o}C and 150 ^{o}C with density programming started at 150 atm and ended at 380 atm. Resolution between the oligomers is much higher at 100 ^{o}C, 120 ^{o}C and 150 ^{o}C than that at 80 ^{o}C. Integrated from 12 to 60 minutes, the total peak response of the injection of 52 μg/μl standard is essentially constant at 80 ^{o}C, 100 ^{o}C, 120 ^{o}C and 150 ^{o}C (Table 1), however, the heights of the major peaks at 150 ^{o}C are 10% to 92% higher than at 80 ^{o}C. This indicates that the efficiency is better at higher temperatures which is due to the change in the volatility of the oligomers. [19]

There are several additional advantages of operating the column at higher temperature, when possible. First, it will prevent condensation in FID restrictor of

TABLE 1 Average Peak Area and Relative Standard Deviation at Various Mobile Phase Temperatures of 52 µg/µl OPA

Mobile Phase Temperature (°C)	Peak Area [a] (%RSD)
80	8923836 (4.4)
100	8833640 (3.2)
120	9053170 (4.3)
150	8873713 (3.9)

[a] Averaged from 3 experiments

RSD: Relative Standard Deviation = 100 x (Standard Deviation / Average Peak Area)

compounds with high molecular mass as indicated in the previous studies. [8, 14] Second, when operating at higher column temperature, [16] it is expected to result in an increase in the solute diffusion coefficients and an improvement in the linearity of the mobile phase density-pressure isotherm. This results in faster optimum velocities, and more even peak spacing in density-programmed chromatograms of oligomers.

Effect of FID Temperature

Analysis was performed at various FID temperatures: 300 °C, 350 °C, 390 °C and 420 °C. As shown in Table 2, the total peak response remained basically unchanged. The indication is that OPA, with an averaged molecular mass of 584 to 626, does not condense in the restrictor at FID temperature as low as 300 °C. The condensation in the restrictor was significant when polymers with high molecular masses, such as T-MAZ (MW, 1300) and alkyl ethoxylated alcohols were analyzed at FID temperature of 350 °C. [8, 14]

TABLE 2 Average Peak Area and Relative Standard Deviation at Various FID Temperatures of 43 µg/µl OPA

FID Temperature (°C)	Peak Area [a] (%RSD)
300	7610953 (3.8)
350	7349170 (4.1)
390	7448399 (2.9)
420	7782887 (4.7)

[a] Averaged from 3 experiments.

RSD: Relative Standard Deviation = 100 x (Standard Deviation / Average Peak Area)

TABLE 3 Peak Area and Peak Area Ratio at Injector Temperatures of 10 °C and 21 °C

Concentration (µg/µl)	Injector Temperature (°C)	Peak Area [a]	Area (10 °C) / Area (21 °C) [b]
25	10	4339909	0.91
52	10	7448399	0.84
25	21	4787291	
52	21	8833640	

[a] Averaged from 3 experiments.

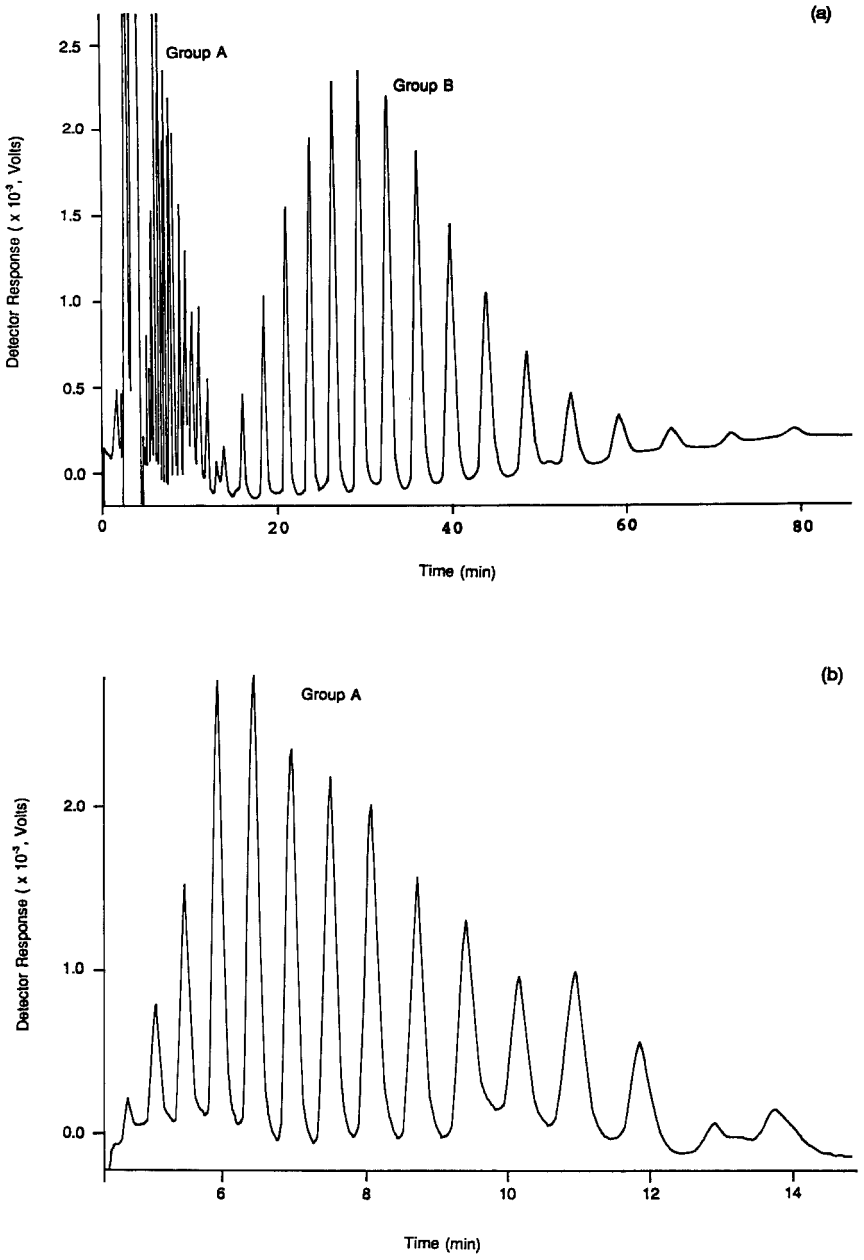
[b] Area (10 °C) / Area (21°C) : The ratio of peak areas at injector temperature 10 °C vs. that at 25 °C.

Effect of Injector Temperature

Experiments were carried out with the injector at 10 °C (cooled by a water bath) and at room temperature (21 °C). OPA standards of 25 and 52 μg/μl were loaded into the sample loop and immediately injected. Integrated as one peak group from 12 to 50 minutes, the responses were 9% and 16% lower for the 25 and 52 μg/μl standards when the injector temperature was at 10 °C than that at 21 °C (Table 3). This can be attributed to the precipitation of OPA in the sample loop, which became significant at high concentration. The result indicates that injector cooling is not needed when the analyte is in methanol. However, if methylene chloride is used as a solvent, vapor bubbles can enter into the sample loop when the injector is not cooled, due to the high volatility of methylene chloride. [8, 14]

HPLC Separation

As shown in Figure 1, since molecules in OPA have a benzene ring, a hydrocarbon chain, oxyethylene groups, and hydroxyl group, a C_{18} column offers desirable interactions between the stationary phase, OPA molecules and the mobile phase. Figures 3 show the HPLC separation of 1000 ppm OPA. Figure 3b shows the peak group A in Figure 3a. These chromatograms again demonstrate the complicity of this industrial chemical as it was shown in the SFC analysis. The peak group A was not found in the SFC separation (Figure 2a). The oligomers in peak group A must be more polar and smaller than oligomers in peak group B, because polar and small molecules elute faster than less polar and larger ones in the reverse phase chromatography. Polar oligomers may not soluble in supercritical fluid carbon dioxide,



Figures 3 HPLC chromatograms of OPA

(a) 1000 ppm OPA standard; (b) group A in Figure 3(a).

which explains peak group A was not observed in SFC separation. A total of 15 oligomers was found in the peak group A and 16 in the peak group B as found in SFC separation (Figure 2a). We also used a Waters NovaPak C_8 column and the separation of peak group A was not as good as using a C_{18} column. The study demonstrate HPLC is a very valuable technique for polymer analysis as we previously indicated, [9] especially for polar polymers. [10]

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DISCLAIMER

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QA/QC REQUIREMENTS

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

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CHEMICALLY REMOVABLE DERIVATIZATION REAGENT FOR CHROMATOGRAPHY. II. 2-(1-NAPHTHYL)ETHYL 2-[1-(4-BENZYL)-PIPERAZYL]ETHANESULFONATE DIHYDROCHLORIDE

H. S. KOU, H. L. WU*, S. H. CHEN, AND S. M. WU
Graduate Institute of Pharmaceutical Sciences
Kaohsiung Medical College
Kaohsiung 807, Taiwan, Republic of China

ABSTRACT

A sulfonate reagent, 2-(1-naphthyl)ethyl 2-[1-(4-benzyl)piperazyl]ethanesulfonate dihydrochloride, was synthesized for analytical derivatization in liquid chromatography. The reagent has two main functions, one with a chromophore (naphthyl) for detection and the other with a substituted piperazine moiety for being removable after derivatization. The reagent was preliminarily applied to the derivatization of iodide anion. The results indicated that the reagent can be readily removed by acid after derivatization; this favorably avoids the interference of the excess reagent with the resulting iodide derivative for analysis.

INTRODUCTION

Analytical derivatization coupled with chromatography[1-4] has found a wide range of application in the fields of chemical and biochemical sciences. Based on analytical derivatization, a highly responsive tag such as chromophore, electrophroe or fluorophore can be incorporated with an analyte for trace analysis. Usually, much higher concentration of a derivatization reagent as compared to that of an analyte is required for the derivatization of the analyte at trace levels. As a consequence, the excess reagent may seriously interfere in the separation of the target derivative for sensitive detection, especially in case of a derivatization reagent itself also being very responsive to a detector. To solve this problem, common approaches used includes the removal of excess reagent by nitrogen purge[5] and by additional column clean up[6]; these treatments are usually tedious and time consuming. Therefore, attempts for devising the derivatization reagents with removable properties after reaction were made and resulted in the syntheses of 2-(N-phthalimido)ethyl 2-(dimethylamino)ethanesulfonate[7] and 2-(1-naphthyl)ethyl 2-[1-(4-benzyl)piperazyl]ethanesulfonate dihydrochloride (NEBPES • 2HCl) (Fig. 1). Using iodide as a model analyte, NEBPES can derivatize iodide into a chromopheric derivative, 2-(1-naphthyl)ethyl iodide in a biphasic system, for chromatographic analysis. The excess reagent, can be easily removed as water soluble salt after derivatization by protonation (acid treatment) of the tertiary nitrogens of its piperazine moiety. This presents a simple approach for the removal of the unwanted reagent for the analytical derivatization.

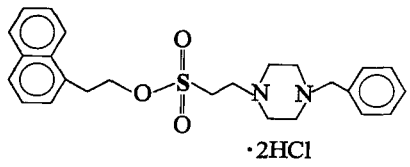

FIGURE 1. Structure of 2-(1-naphthyl)ethyl 2-[1-(4-benzyl)piperazyl]ethanesulfonate dihydrochloride

METHODS

Materials and Reagent

Benzyldimethylphenylammonium chloride (BDMPAC), 2-(1-naphthyl)ethanol, 2-chloroethanesulfonyl chloride, N-benzylpiperazine and 1,2,4,5-tetrachlorobenzene (TCB) (TCI, Tokyo, Japan), tetra-n-amylammonium chloride (TAAC) (Wako, Osaka, Japan), tetra-n-butylammonium chloride (TBAC) (Fluka, Buchs, Switzerland), tetra-n-butylammonium hydrogen sulfate (TBAHS) (Aldrich, Milwaukee, USA), trimethylamine (45%, w/v), toluene, dichloromethane, methanol, sodium carbonate, potassium hydroxide, potassium iodide, hydrochloric acid (37%, w/v), sulfuric acid and silica gel 60 (70-230 mesh) (E. Merck, Darmstadt, Germany), acetonitrile (Fisher, Fair Lawn, NJ, USA) were used without further treatment. All other chemicals were of analytical-reagent grade. Solution of TCB was prepared by dissolving the appropriate amounts of the compound in toluene. Solutions of potassium iodide, potassium hydroxide, sodium carbonate, NEBPES · 2HCl, TAAC, TBAC, TBAHS and BDMPAC were prepared by dissolving the appropriate

amounts of the respective compounds in distilled and deionized water.

HPLC Conditions

A Waters-Millipore LC system with a U6K injector, a Model 510 pump and a Model 486 UV-Vis detector was used. A Nova-Pak C_{18} column (150 x 3.9 mm I.D., 4 μm) and a mobile phase consisting of 65% (v/v) acetonitrile in water at a flow-rate of 0.8 mL/min were used. The column eluate was monitored at 282 nm. The solvents were pretreated with a vacuum filter for degassing.

Synthesis of 2-(1-Naphthyl)ethyl 2-[1-(4-Benzyl)piperazyl]-ethanesulfonate Dihydrochloride (NEBPES • 2HCl)

1. Synthesis of 2-(1-Naphthyl)ethyl Ethenesulfonate (NEES)

2-(1-Naphthyl)ethanol (8.61g, 50.00 mmol), 2-chloroethane-sulfonyl chloride (15.78 mL, 150.04 mmol) and trimethylamine (31.00 mL, 200.00 mmol) were added successively to a 100-mL reaction flask containing 50.00 mL of dichloromethane pre-cooled in an ice bath. The solution was magnetically stirred at 0°C for 2 h, then the resulting mixture was washed with cold sodium carbonate solution (10%, v/v) (50 mL × 2) and water (50 mL). The separated organic layer was dried with anhydrous sodium sulfate (ca. 2.50 g) and the filtrate was concentrated by a rotary evaporator. The liquid residue was purified by column chromatography (40 × 4 cm I.D.) on silica gel 60 (ca. 200 g) with ethyl acetate - n-hexane (1:3, v/v) as an eluent to give NEES (8.02 g, 30.61 mmol, 61.22% yield) as colorless

oily liquid. Element analysis (%) calculated for $C_{14}H_{14}O_3S$: C, 64.12; H, 5.35; O, 18.32; found: C, 64.07; H, 5.47; O, 18.25. MS (EI): m/z 262 (M^+); 141 (naphthylmethyl), a basal peak.

2. Synthesis of NEBPES · 2HCl

NEES (7.86 g, 30.00 mmol) and N-benzylpiperazine (6.34 g, 36.04 mmol) were added to a 150-mL reaction flask containing dichloromethane (40.00 mL) pre-cooled in an ice bath. The mixed solution was magnetically stirred at 0℃ for 0.5 h, then the resulting solution was concentrated by a rotary evaporator. The liquid residue obtained was purified by column chromatography (40 × 4 cm I.D.) on silica gel 60 (ca. 200 g) with ethylacetate - n-hexane (1:2, v/v) as an eluent, to give NEBPES (11.56 g, 26.40 mmol, 88.00% yield) as colorless oily liquid, ^1H NMR ($CDCl_3$): δ 2.35 (br, 8H, piperazyl H); 2.67 (t, 2H, N-$\underline{CH_2}$-CH_2-SO_2); 3.10 (t, 2H, N-CH_2-$\underline{CH_2}$-SO_2); 3.45 (s, 2H, N-$\underline{CH_2}$-phenyl); 3.51 (t, 2H, $\underline{CH_2}$-naphthyl); 4.53 (t, 2H, CH_2-$\underline{CH_2}$-OSO_2); 7.29-8.02 (m, 12H, aromatic H). Analysis (%) calculated for $C_{25}H_{30}N_2O_3S$: C, 68.49; H, 6.85; N, 6.39; O, 10.96; found: C, 68.28; H, 6.97; N, 6.27; O, 10.90. MS (FAB): m/z 439 ($M^+ + 1$); 347 (M^+ minus benzyl); 283 (M^+ minus naphthylethyl); 203 [1-(4-benzyl)piperazylethyl], a basal peak; 155 (naphthylethyl); 91 (benzyl).

NEBPES · 2HCl was prepared by adding hydrochloric acid (12 mL, 121.64 mmol) gradually to a cold solution of NEBPES (10.95 g, 25.00 mmol) in methanol (100 mL). The resulting mixture was concentrated and the residue obtained was recrystallized from ethanol - n-hexane (8:1, v/v) to give a white needle crystal (8.85 g, 17.32

mmol, 69.28% yield), m.p. 183.8-184.5 ℃ ; analysis (%) calculated for $C_{25}H_{32}N_2O_3SCl_2$: C, 58.71; H, 6.26; N, 5.48; O, 9.39; found: C, 58.91; H, 6.38; N, 5.52; O, 9.54.

Derivatization Procedure

A 0.1-mL aliquot of iodide solution (0.25 μM) was added to a 10-mL glass stoppered test-tube containing 0.10 mL TAAC solution (PTC) (0.20 M) and 0.50 mL TCB solution (I.S., 0.37 μM), then 0.70 mL NEBPES • 2HCl solution (28.57 μM) and 0.10 mL potassium hydroxide solution (0.50 M) were added. The reactants were shaken at 95 ℃ for 2 h. After cooling, the reaction mixture was added with 1.0 mL of H_2SO_4 aqueous solution (1.00 M) and the test tube was vortexed for 30 s. An aliquot of the toluene layer (0.10 mL) was five-fold diluted with acetonitrile for compatibility with reversed-HPLC. The resulting solution was used for HPLC analysis (25 μL).

RESULTS AND DISCUSSION

The chemical removability and reactivity of the new reagent (NEBPES • 2HCl) were studied, using iodide anion as a test model. For optimization of the derivatization conditions for iodide at an amount of 25 nmol, several parameters including reaction solvent, phase-transfer catalyst (PTC), concentrations of KOH, molar ratio of NEBPES • 2HCl to iodide, reaction temperature and reaction time were evaluated by measuring the peak-area ratio of the iodide derivative to the I.S.

Removability of Derivatizing Reagent after Derivatization

NEBPES • 2HCl was used in large excess to derivatize iodide (25 nmol), equivalent to a molar ratio of about 800 as indicated in the derivatization procedure. After derivatization, the reaction mixture was either treated with or without H_2SO_4 solution (1.00 mL, 1.00 M). The results are shown in Fig. 2. A broad tailing peak overlaped with the peaks of the iodide derivative and the I.S., resulting from the derivatization without further treatment with the acid solution. On the other hand, the interfering NEBPES peak can be easily removed after derivatization with a simple treatment of the reacted solution with the acid solution, favorably resulted from the protonation of the tertiary amino functions of NEBPES. This leads to a water soluble quaternary ammonium species of NEBPES; it was easily removed from the organic toluene layer after derivatization, also shown in Fig. 2.

Effect of Reaction Solvent

The effects of various organic solvents (0.50 mL) on the derivatization of iodide anion in aqueous phase (1.00 mL) were studied according to the derivatization procedure but at different temperatures in order to prevent the solvents from boiling. The immiscible solvents tested included toluene at 95°C, benzene at 70°C, chloroform at 50°C and dichloromethane at 30°C. Toluene was found to be the best solvent for the derivatization of iodide.

Effect of Phase-Transfer Catalyst

The effects of quaternary ammonium compounds (each with 0.10 mL) at suitable concentration (based on their aqueous solubility) on

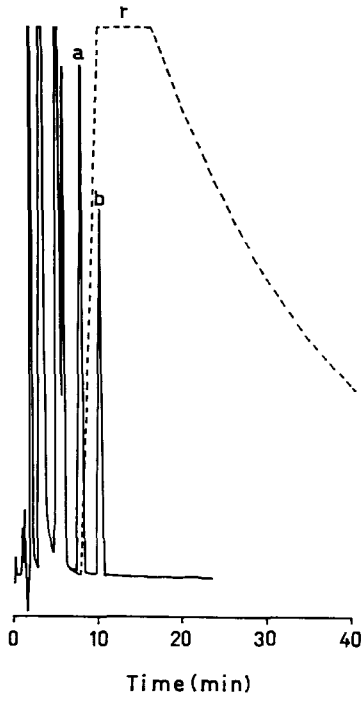

FIGURE 2. Composite liquid chromatogram of iodide (25 nmol) derivatized with NEBPES • 2HCl with acid treatment (solid line) and without acid treatment (dotted line) after derivatization: peaks a, the derivative of iodide; b, 1,2,4,5-tetrachlorobenzene (I.S.) and r, the excess reagent. See text for conditions.

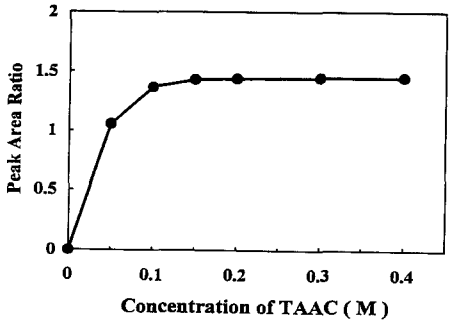

FIGURE 3. Effect of concentration of TAAC on the formation of iodide derivative. See text for conditions.

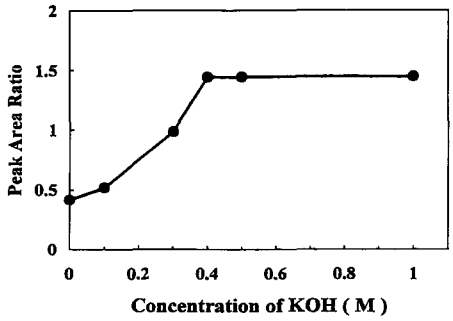

FIGURE 4. Effect of concentration of KOH on the formation of iodide derivative. See text for conditions.

the transfer of iodide from the alkaline aqueous phase to the toluene organic phase for reaction with NEBPES were cursorily studied, including TAAC (0.20 M), TBAC (0.20 M), TBAHS (1.00 M) and BDMPAC (0.10 M). The results revealed that TAAC was the best choice for the reaction system. Therefore, the effect of TAAC at various concentrations on the derivatization of iodide was further examined. The results (Fig. 3) indicated that the plateau formation of the derivative required TAAC (0.10 mL) at concentration $\geqq$ 0.15 M.

Effect of Base

Suitable amount of base (KOH) is needed to partition NEBPES • 2HCl in aqueous phase as a free amine to the organic layer for the derivatization of iodide. The effect of KOH (0.10 mL) at various concentrations on the derivatization of iodide was studied. The results (Fig. 4) indicated that the concentration of KOH were suitable over a range of 0.40-1.00 M tested.

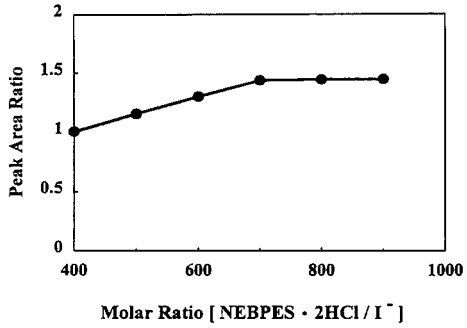

FIGURE 5. Effect of molar ratio of NEBPES · 2HCl to iodide on the formation of iodide derivative. See text for conditions.

Effect of the Derivatization Reagent

The molar ratio of NEBPES · 2HCl to iodide required for the derivatization of iodide (25 nmol) to a steady state of the derivative was studied. The results (Fig. 5) indicated that the suitable ratio is above 700.

Effect of Reaction Time

The effects of reaction time at 70 °C and 95 °C on the derivatization of iodide are shown in Fig. 6. For derivatization at 95°C, 1.5 h was needed to reach an equilibrium; with reaction at 70 °C, plateau formation of the derivative was not attainable in 4 h and resulted in lower yields as compared with reaction at 95°C.

Analytical Calibration

Based on the optimum derivatization conditions, the derivatization procedure for iodide was formulated under the

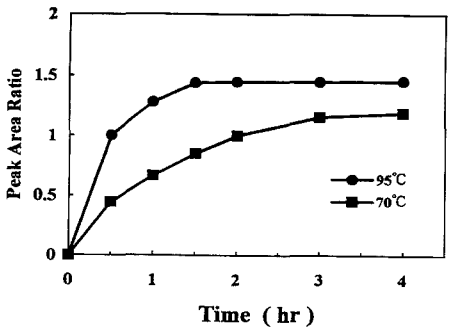

FIGURE 6. Effect of reaction time on the formation of iodide derivative. See text for conditions.

METHOD section. The quantitative application of the method to the determination of iodide was evaluated at six different amounts of iodide over the range of 0.5-25 nmol. The calibration graph was established with the peak-area ratio of the derivative to I.S. as ordinate (y) vs the amount of iodide in nmol as abscissa (x). A linear regression equation, $y = (0.0575 \pm 0.0012)\,x + (0.0047 \pm 0.0017)$, was obtained with a correlation coefficient 0.999 ($n = 6$), indicating good linearity of the method. The detection limit (as signal to noise ratio of 5) of iodide was about 5 pmol per injection (25 μL).

Mass Spectral Analysis of Iodide Derivative

The derivative of iodide was synthesized by scaling up the amount of iodide (1.00 mmol) with similar procedure as indicated in the derivatization procedure. The resulting derivative (colorless oily liquid) was examined by FAB-MS. The mass spectrum obtained

exhibited a pseudomolecular ion of m/z 283 ($M^+ + 1$), and a basal peak of m/z 155, corresponding to a naphthylethyl moiety. This suggests that the resulting derivative is 2-(1-naphthyl)ethyl iodide. The retention time of peak a in Fig. 2 is identical to that of the synthesized derivative.

In conclusion, a sulfonate reagent NEBPES • 2HCl was synthesized and its preliminary application to the derivatization of iodide in a biphasic system was studied. The results indicated that the excess reagent can be easily removed after derivatization by a simple acid treatment, leading to the clear separation of the iodide derivative from the interferent reagent. This signals a potential approach to using chemically removable reagent for analytical derivatization. A series of sulfonate reagents with various detection-oriented tags and acid protonable functions are being developed.

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DEVELOPMENT OF PRACTICAL HPLC METHODS FOR ANALYSIS AND QUALITY ASSESSMENT OF THE NOVEL CARBONIC ANHYDRASE INHIBITOR MK-0507 AND THE ACETAMIDOSULFONAMIDE INTERMEDIATE

ANGELOS DOVLETOGLOU*, SCOTT M. THOMAS, LORRIE BERWICK, DEAN K. ELLISON, AND PATRICIA C. TWAY

Analytical Research Department
Merck Research Laboratories
Merck & Co., Inc.
P.O. Box 2000
Rahway, New Jersey 07065-0914

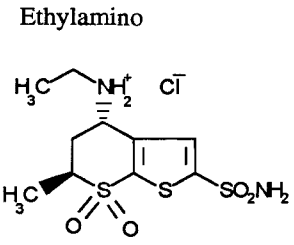
ABSTRACT

MK-0507 (Dorzolamide HCl, (4S,6S)-4-Ethylamino-5,6-dihydro-6-methyl-4*H*-thieno-[2,3-*b*] thiopyran-2-sulfonamide 7,7-dioxide hydrochloride) is the first topically active, water-soluble, carbonic anhydrase inhibitor to be developed for the treatment of glaucoma and ocular hypertension. Dorzolamide HCl is an effective and well tolerated agent as monotherapy for patients who cannot tolerate ophthalmic beta-blockers, and for those who need add-on therapy to beta-blockers.[1] The steps taken in the development and validation of a fast and rugged reverse-phase HPLC method for the analysis and quality assessment of MK-0507 drug substance and acetamidosulfonamide intermediate are described. Four columns were used during the development: Du Pont Zorbax C-18, Rainin Microsorb C-8, Perkin Elmer CR-C8 and YMC4. The last two columns showed excellent resolution, peak shape, and precision. The selected HPLC method for acetamidosulfonamide, using the Perkin Elmer CR-C8 column, was validated with respect to linearity, limit of detection (LOD) and limit of quantitation (LOQ). The selected HPLC method for the MK-0507 drug substance using the Perkin Elmer CR-C8 column was validated with respect to linearity, LOD, LOQ, precision on an injection-to-injection basis, and on a day-to-day basis, selectivity and accuracy.

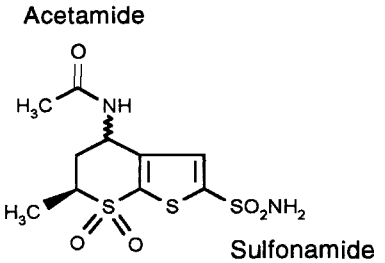
The method is rugged with respect to solution stability, variations in buffer concentration, flow rate, column-to-column variability and column temperature. The relative response factor for the *cis*-isomer of MK-0507 and the desethyl impurity were also determined in order to quantitatively measure the levels of these impurities in MK-0507. There are three possible stereoisomers of MK-0507 (4S,6S): the enantiomer 4R,6R and the *cis*-diastereoisomers 4S,6R and 4R,6S. The chiral HPLC method separates the two *trans*-diastereoisomers, and the two *cis*-diastereo-isomers; however, in the same chiral method the 4R,6R and 4R,6S stereoisomers coelute.

INTRODUCTION

A number of sulfonamides have been separated by using reverse- or normal-phase HPLC methods.[2] To our knowledge, there is no documentation of any separation of sulfonamide that contains a sulfone and/or an acetamide moiety. MK-0507 (I) is a sulfonamide that contains a sulfone and ethylamino group. Acetamidosulfonamide (II) contains a sulfone and an acetamide group.



(I)



(II)

The goals of analytical development of the HPLC method for the MK-0507 drug substance and acetamidosulfonamide were to provide methodology for characterization of purity and impurities during process development, stability studies, pilot plant campaigns and manufacturing. Also, the benefits of applying a single chromatographic method to monitoring completion of reactions were evaluated.

แบบ กท.3.2

แบบขอยืมเอกสารไปถ่ายสำเนา

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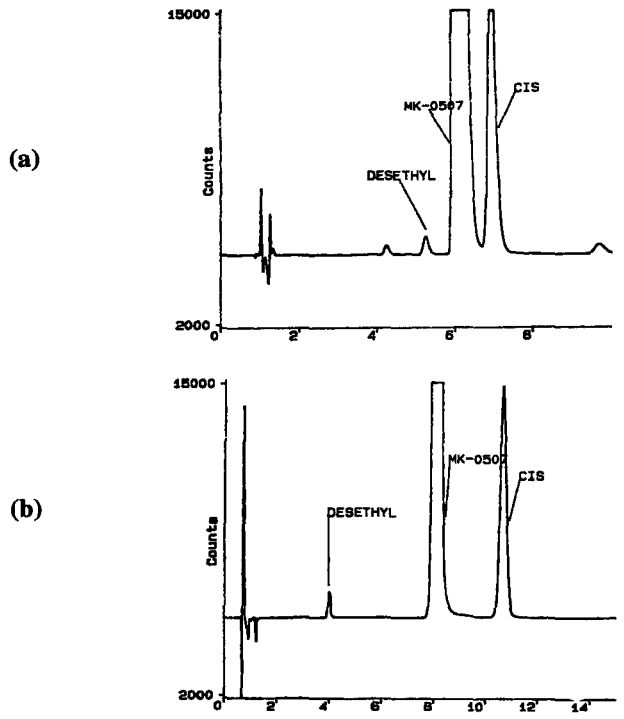


FIGURE 1. HPLC chromatograms of a mixture of 90:10:1 (w/w/w) MK-0507: *cis*-isomer of MK-0507:Desethyl Using (**a**) Microsorb C-8 and (**b**) Perkin Elmer CR-C8 Column.

The initial HPLC impurity profile involved a reverse-phase column (Du Pont Zorbax C-18) with gradient elution (aqueous acetate buffer (pH=5.0)/CH_3CN mobile phase). A second HPLC method was implemented using a Microsorb C-8 column (aqueous acetate buffer (pH=4.5) /CH_3CN mobile phase) (Figure 1a). The latter system showed improved peak shape and demonstrated better resolution of MK-0507 and the *cis*-isomer (R_s = 2.2 - 2.4). Both of these reverse-phase methods resulted in broad peaks (T_f >1.5), due to presence of two

amine groups (sulfonamide and ethylamino group). In a typical reverse-phase system, it is known that the interactions of amine groups with the surface Si-OH groups (silanols) cause tailing.[3] A new HPLC assay was developed by switching to a better end-capped column (Perkin Elmer CR-C8) and by adding triethylamine (TEA) to the mobile phase. These changes resulted in better peak shape (T_f=1.0-1.2) and greatly improved resolution (R_s = 5.6 - 6.4) between the two isomers (Figure 1b). The chromatographic performance of a second column (YMC4) was fully evaluated and compared to the Perkin Elmer CR-C8 column, and equivalent results were obtained (R_s = 6.0 - 7.0, T_f = 1.0-1.5).

MK-0507 contains two chiral centers with four possible stereoisomers: MK-0507 (4S,6S), the enantiomer 4R,6R and the diastereoisomers 4S,6R and 4R,6S.[4] The normal-phase chiral HPLC method was originally designed to control the level of the 4R,6R enantiomer.[5] Also, to date, no experimental evidence exists to demonstrate that both *cis*-isomers (4R,6S and 4S,6R) are controlled by the reverse-phase HPLC test. In this work it is determined that the stereochemical configuration is controlled by a combination of the normal-phase chiral HPLC and reverse-phase HPLC methods.

EXPERIMENTAL SECTION

Materials

MK-0507 was prepared in the *Departments of Medicinal Chemistry and Process Research, Merck Research Laboratories.*[6] The 4R,6R diastereoisomer of MK-0507, was provided by Mr. P. Sohar (*Process Research, Merck Research Laboratories*). The two enantiomers of *cis*-isomer of MK-0507, *cis*(+), 4S,6R: L-685,973 and *cis*(-), 4R,6S: L-685,974, were synthesized by Dr. Ponticello and his group (*Medicinal Chemistry, Merck Research Laboratories*). The chiral derivatizing reagent, (*S*)-(-)-α-methylbenzyl isocyanate, was purchased from Aldrich. All the solvents (Fischer Scientific) used were HPLC grade.

Solutions, Sample Preparation and Chromatographic Conditions

A 60 mg sample of MK-0507 (CAS Number: 130693-82-2) was dissolved into 100 mL of 20:80 (v/v) MeOH:H_2O. Using the Perkin Elmer CR-C8 and YMC4 columns, the mobile phase composition is: A: 1.0 mL of HPLC grade $(CH_3CH_2)_3N$ (TEA) and 1.0 mL of HPLC grade acetic acid (HOAc) into 1 L of HPLC grade H_2O, and B: HPLC grade CH_3CN. This method is very sensitive to changes in pH. The pH of solution A should be between 4.4 and 4.6. MK-0507 and acetamidosulfonamide were analyzed using the PE CR-C8 column with a mobile phase of 100% A for 10 min and then changing to 50% A in 20 minutes, and with UV detection at 254 nm. The injection volume was 10 μL, with a flow rate of 1.5 mL/min at ambient temperature. The data points collected for three samples indicate that MK-0507 is stable in the recommended diluent for at least seven days at room temperature. The Du Pont Zorbax C-18 is used with 98:2 (v/v) 0.02 M aqueous acetate buffer ($AcONH_4$) (pH=5.0):CH_3CN mobile phase at 2.0 mL/min. The Microsorb C-8 column is used with 98:2 (v/v) 0.02 M aqueous acetate buffer ($AcONH_4$) (pH=4.5):CH_3CN mobile phase at 1.8 mL/min.

Prior to an injection on a silica column, the two enantiomers of the *cis*-isomer of MK-0507 were derivatized with (*S*)-(-)-α-methylbenzyl isocyanate to form the urea derivatives 4S,6R(S) and 4R,6S(S). After the chiral derivatization, the mixture of 4R,6S(S) and 4S,6R(S) were separated under non-chiral chromatographic conditions (normal-phase Zorbax SIL column) with a *tert*-butyl methyl ether/acetonitrile/heptane mobile phase. The chromatographic conditions for the chiral assay of the *cis*-isomer of MK-0507 are: Zorbax SIL column (250 x 4.6 mm) and mobile phase of 65:35 (v/v) methyl tert-butyl ether (containing 3.0% CH_3CN and 0.3% H_2O):heptane. The flow rate is 2 mL/min, λ = 254 nm, injection volume is 10 μL, sample concentration is 2.0 mg/mL at ambient temperature, and retention times are 4S,6R(S) ~12 min, and 4R,6S(S) ~16 min.

Chromatographic/system suitability parameters, such as tailing factor (T_f) and resolution (R_s), etc., were calculated using Nelson Access*Chrom GC/LC Data System suitability software <USP method>. Digital spectra, from 200 nm to 440 nm, were taken using a diode array UV detector. The spectra for four points across the main peak were baseline corrected and the first and second derivatives were taken. The baseline-corrected spectra were normalized and the derivative plots were overlayed.

Instrumentation and Measurements

The HPLC system consisted of a SP8700 Spectra-Physics gradient pump, a Spectroflow 757 (Kratos Analytical) UV-Vis detector or a Applied Biosystems 759A UV-Vis detector and a SP8775 Spectra-Physics autosampler using a Perkin-Elmer CR C-8, particle diam 3 μm, pore diam 60 Å, length 8 cm, ID 3.0 mm column; YMC4, particle diam 3 μm, length 15 cm, ID 4.6 mm column; Du Pont Zorbax C-18, particle diam 5 μm, pore diam 120 Å, length 8 cm, ID 4.6 mm column; and Rainin Microsorb C-8, particle diam 5 μm, length 15 cm, ID 4.6 mm columns.

Chemical and Physical Stress Conditions

Acetamidosulfonamide: A 500 mg sample was stressed at 40 °C/75% relative humidity for 1 mo, and three 100 mg samples were thermally stressed at 40, 60 and 100 °C for 24 h. Four solutions of the stressed materials were prepared (1 mg/mL) and analyzed using diode array detection. The spectra for four points across the main peak were baseline corrected and the first and second derivatives were taken. The baseline-corrected spectra were normalized and the derivative plots were overlayed.

MK-0507: *Acid Stress:* MK-0507 was stored in 2*N* HCl solution at ambient temperature for 48 h. *Base Stress:* A sample of MK-0507 was stored in 2% (w/w) NaOH solution at ambient temperature for 48 h. *Peroxide Stress:* MK-

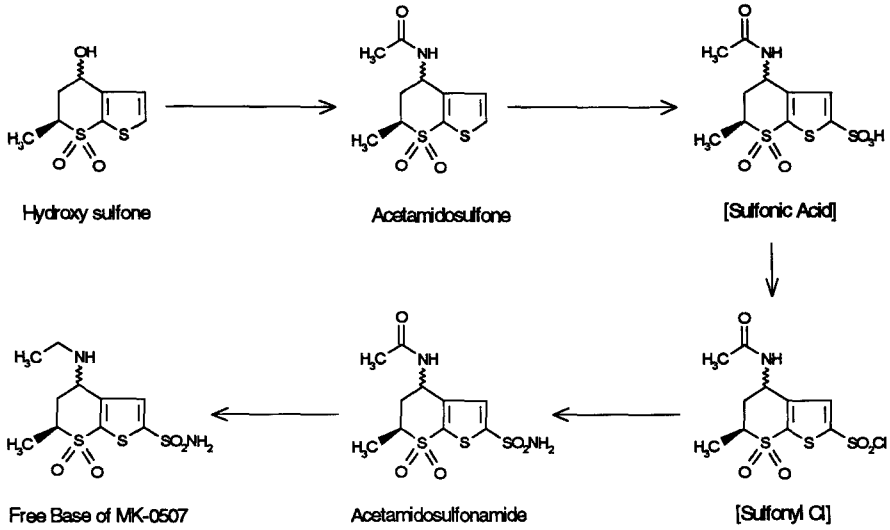
0507 was stored in a concentrated peroxide solution at ambient temperature for 2 h. *Thermal Stress:* A sample of MK-0507 was thermally stressed at 150 °C for 12 h. *UV Light Stress and White Light Stress:* Using a Rayonet Photochemical Reactor model RPR-200, equipped with nine ultraviolet bulbs (24 W, 90% in the 3500 angstrom range) or equipped with eight fluorescent bulbs (8 W, cool white light, Sylvania Fluorescent cat# F85T/CW), MK-0507 was exposed for 24 h. *Final Crystallization Solvent Stress:* A 1% aqueous solution of MK-0507 was prepared and stored at 80 °C for 18 h.

RESULTS AND DISCUSSION

In-process HPLC methods were developed for the synthesis of MK-0507.[7] All the in-process tests were designed to determine the impurity profile and the completion of reaction. The starting material for the synthesis is hydroxy sulfone ((6S)-5,6-Dihydro-4-hydroxy-6-methyl-4H-thieno[2,3-b]thiopyran-4-ol-7,7-dioxide, HS). The complete synthesis is shown in Scheme I.

Acetamidosulfonamide Validation

The assay for acetamidosulfonamide was validated and the detector response was found to be linear over the concentration range of 0.0001 to 0.96 mg/mL. We estimate the detection limit to be 0.0001 mg/mL or 1.45 ng (0.02% relative to the recommended sample concentration 0.5 mg/mL sample concentration). This estimate is based on data collected from the linearity study and a typical chromatogram with 1.45 ng injected on-column. The signal-to-noise level is 4:1 at this concentration. We estimate the limit of quantitation to be 0.0002 mg/mL (0.06% relative to the 0.5 mg/mL sample concentration). This estimate is based on data collected from the linearity study. Injection precision was demonstrated by dissolving a sample of acetamidosulfonamide in the recommended diluent and injected 11 consecutive times, resulting in a relative standard deviation of 0.2%.



Scheme I

Day-to-day precision was demonstrated by preparing three weighings of the reference standard and two weighings of acetamidosulfonamide on each of three days, resulting in a relative standard deviation of <0.5%. Acetamidosulfonamide is stable over a four day period in the proposed diluent. The method is selective, as judged by photo diode array detection and as demonstrated by the resolution of the *cis*- and *trans*-acetamidosulfonamide.

The method is rugged to variations in mobile phase composition and flow rate. The influence of mobile phase variations in the separation of *cis*- and *trans*-acetamidosulfonamide was examined. By varying the mobile phase composition, it was found that the optimum ratio is 6% CH_3CN and 94% buffer solution. Excellent k' and T_f values were obtained at flow rates of 1.0, 1.5 and 2.0 mL/min. The optimum resolution and number of theoretical plates was observed at 1.5 mL/min.

Using the PE CR-C8 and YMC4 column, the typical retention times of the in-process intermediates shown in Figure 2 are: 3-positional isomer of acetamidosulfonamide (3-AASA, ~6 min), *cis*-acetamidosulfonamide (*cis*-AASA), *trans*-acetamidosulfonamide (*trans*-AASA) and bis-acetamidosulfonimide (dimer). The *cis*-acetamidosulfonamide has a slightly higher response factor (1.07) than *trans*-acetamidosulfonamide.

Stress Studies of Acetamidosulfonamide

During the stress studies of acetamidosulfonamide diode array detection found no indication of impurities with a different UV chromophore coeluting with the main peak. A sample of acetamidosulfonamide was stressed at 40 °C/75% relative humidity for 1 mo, and three samples of acetamidosulfonamide were stressed at 40, 60 and 100 °C for 24 h. No new impurities were formed and levels of existing impurities remained the same. The selectivity of the method was demonstrated by using diode array detection to analyze (thermally stressed) samples of acetamidosulfonamide. No change in the physical appearance of the samples was observed and diode array detection revealed no evidence of the formation of thermal degradates.

MK-0507 Drug Substance Validation

Using the PE CR-C8 column, the detector response for the MK-0507 peak is linear over the concentration range of 0.0001 to 1.46 mg/mL. The correlation coefficient over this range is 0.999997. The sample concentration of 0.5 mg/mL is well within the linear range of this method and there is no bias toward low level impurities. The detection limit is 1.45 ng (0.02% relative to the recommended 0.5 mg/mL sample concentration). The signal-to-noise level at 1.45 ng on-column injection level is 4:1. Based on the data collected in the linearity study, the limit of quantitation is 0.0003 mg/mL (0.06% relative to the 0.5 mg/mL sample concentration). Three consecutive injections of the 0.0003 mg/mL solution averaged 3028 area counts with an RSD of 5.8%. The method

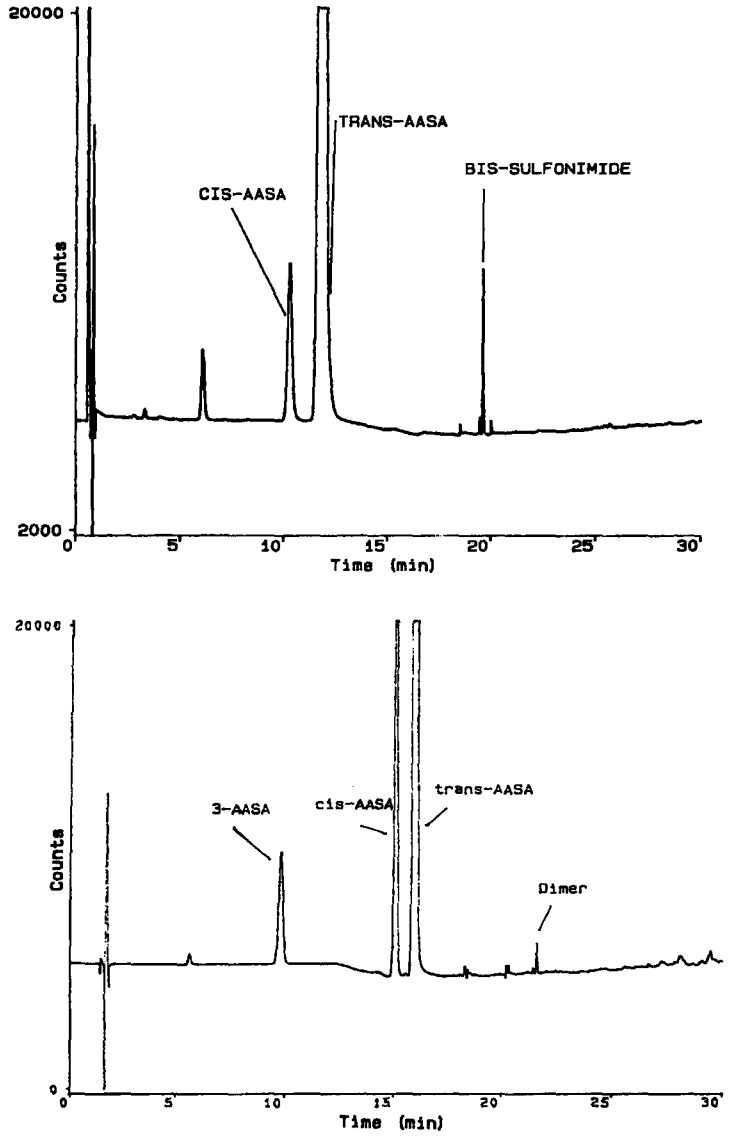


FIGURE 2. HPLC chromatograms of Acetamidosulfonamide Using (a) Perkin Elmer CR-C8 and (b) YMC4 Column.

has acceptable sensitivity to low-level impurities and has a limit of quantitation that meets our requirement of 0.10% minimum. The HPLC assay of a typical lot of the drug substance prepared in the pilot plant (99.8%) is accurate compared to the silver nitrate titration (99.9%), perchloric acid titration (99.9%), and phase solubility analysis (100%, Slope = 0.0 +/- 0.1).

The method is precise on an injection-to-injection and on a day-to-day basis. MK-0507 was injected 11 consecutive times, resulting in a relative standard deviation of 0.1% for the MK-0507 peak. Three samples were prepared on three different days, and each solution was injected in triplicate each day. The overall precision is acceptable, with an RSD of < 0.5% for the MK-0507 peak.

The effect of buffer concentration, pH, flow rate, temperature, and column-to-column variability on the method was studied. The mobile phase was studied as a function of the triethylamine (TEA) and acetic acid (AcOH) concentrations. The concentration of TEA and AcOH varied by 0.05% from 0.05% to 0.20% (v/v) each. No significant change in any of the chromatographic parameters was observed (Table I).

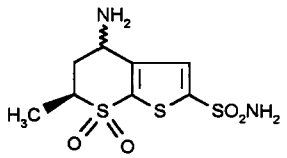
The pH of the mobile phase varied over the range of 3.8 to 5.0 (Table I). We found that the pH significantly influences the capacity factor (k'), number of theoretical plates (N) and peak tailing factor (T_f), (Table I). Changes were observed in the resolution between MK-0507 and *cis*-isomer of MK-0507, and also between the MK-0507 and the desethyl impurity (a known impurity formed during the MK-0507 process). In all cases, the resolution was acceptable (Table I).

The resolution of MK-0507 was studied as function of the flow rate over the range of 1.0 to 2.0 mL/min. High values of k' were obtained at 1.0 mL/min (MK-0507, k'=17.7) and high back pressure (>3000 psi), and increased peak tailing was observed at 2.0 mL/min (MK-0507, T_f = 2.0). The optimum flow rate was 1.5 mL/min.

TABLE I: Chromatographic Parameters of Desethyl (des), MK-0507 and *cis*-isomer of MK-0507 (*cis*) as a Function of Buffer Concentration and pH Using the PE CR-C8 Column (Flow Rate = 1.5 mL/min).

Conc.[1] Vol%	(pH)	k' des	k' 0507	k' *cis*	T_f 0507	N 0507	R_s[2]	R_s[3]
0.05		4.8	11.0	14.8	1.0	7344	>10	6.1
0.10	(4.5)	4.8	10.3	14.1	1.0	7406	>10	6.2
0.15		4.6	9.8	13.4	0.9	7400	>10	6.3
0.20		4.9	10.0	13.7	0.9	7527	>10	6.4
	(3.8)	3.5	5.1	7.8	1.2	5829	5.9	7.1
	(5.0)	7.4	24.2	30.4	0.9	8266	>10	4.8

1- TEA and AcOH each
2- Resolution between desethyl (des) and MK-0507
3- Resolution between MK-0507 and *cis*-isomer of MK-0507 (*cis*)



desethyl

The resolution of MK-0507 was studied as function of column temperature over the range of 25 to 40 °C. A decrease in k' (10.8 to 8.3) and R_s (7.1 to 5.2) for MK-0507 was observed as the column temperature increased, but the k' and R_s remained within the recommended operating parameters. The number of theoretical plates remained constant (~10,000 +/- 200).

The relative response factor for desethyl relative to MK-0507 is 1.20 and for *cis*-isomer of MK-0507 relative to MK-0507 is 0.98. The level of these impurities can be reliably measured using this assay.

The pH of the mobile phase has a significant influence in the retention of MK-0507 and *cis*-isomer of MK-0507. As the pH increases, the *N* decreases. The retention of the desethyl impurity is influenced, but to a lesser degree. The peak shape and the number of theoretical plates are also pH dependent.

Stress Studies of MK-0507 Drug Substance

Selectivity of the method was demonstrated by resolving impurities formed under the following stress conditions: 1) acid stress, 2) base stress, 3) peroxide stress, 4) severe thermal stress, 5) UV light stress, 6) white light stress and 7) stress of final crystallization solvent. In all of the stress studies listed below, no impurities were detected, by diode array detection, to have different UV chromophore coeluting with the main peak. Under acid stress and peroxide stress, the impurity formed at relative retention time (RRT) 0.06 (in solvent front) has a significantly different chromophore than that of MK-0507. Under base stress the impurity formed at RRT 1.35 is the *cis*-isomer of MK-0507. During thermal stress, a change in the physical appearance of the sample was observed: the color of the stressed sample was light tan as opposed to the white non-stressed material. A 1 mg/mL solution of the stressed material was prepared and analyzed using the diode array system and no decomposition was observed. No change in the physical appearance of the sample was observed under UV light stress, white light stress and final crystallization solvent (H_2O) stress. A 1 mg/mL solution of the stressed material was prepared and analyzed using the diode array system and no decomposition was observed.

MK-0507 Process Intermediates

A sample of MK-0507 was spiked with desethyl and with all the isolated process intermediates. All the compounds are well resolved from the main peak

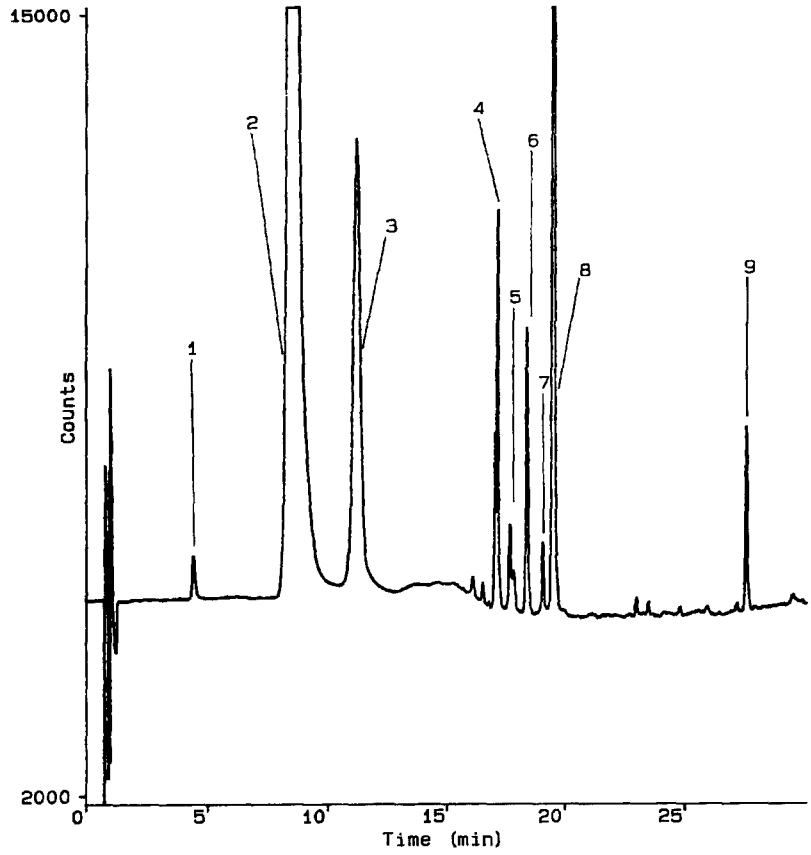


FIGURE 3. Resolution of Process Intermediates and Process Impurities Using PE CR-C8. 1 -Desethyl (RRT 0.52); 2 -MK-0507 (RRT 1.00); 3 -*cis*-isomer of MK-0507 (RRT 1.32); 4 -*cis* & *trans*-Sulfonic Acid (RRT 2.01) 5 -*cis*-Hydroxysulfone (RRT 2.08); 6 -*trans*-Hydroxysulfone (RRT 2.16); 7 -cis-Acetamidosulfone & *cis*-Acetamidosulfonamide (RRT 2.24); 8 -*trans*-Acetamidosulfone & *trans*-Acetamidosulfonamide (RRT 2.30); 9 -*trans*-Sulfonyl chloride (RRT 3.23)

(Figure 3). Each of the desethyl solutions was diluted 1000X to simulate a 0.1% level impurity in a typical MK-0507 sample preparation.

Stereoisomers

The chiral separation of MK-0507 (4S,6S) and its enantiomer (4R,6R) has been validated (see experimental section). This method also separates the two derivatized enantiomers (4R,6S(S), 4S,6R(S)) of *cis*-isomer of MK-0507. The order of elution and peak assignmentswere established by injecting authentic samples of the four derivatized stereoisomers. The chromatographic behavior of the two *cis*-enantiomers is in good agreement with the system suitability test currently used in the manufacturing of MK-0507 to demonstrate resolution between MK-0507 and *cis*-isomer of MK-0507. Early in the development of the chiral test, evidence suggested that either the 4R,6S diastereoisomer or the 4S,6R diastereoisomer co-elutes with the 4R,6R enantiomer. An attempt to separate the mixture of all four stereoisomers (4S,6S, 4S,6R, 4R,6S and 4R,6R) under the same conditions resulted in chromatography with three peaks. The first peak is MK-0507, the second is 4S,6R and the third is both the 4R,6S and 4R,6R stereoisomers. The *cis*-isomer level can be determined directly from the reverse-phase HPLC method and the percent 4S,6R level can be determined directly from the chiral HPLC method. The percent 4R,6S can be calculated by subtracting the percent 4S,6R from the *cis*-isomer level.

CONCLUSION

A simple, accurate and reproducible HPLC method was developed for separating sulfonamides that contain a sulfone and/or an acetamide and/or ethylamino moiety. The limit of quantitation is <0.1%, and the limit of detection is 0.03% of the sample concentration. The method is capable of separating all nine starting materials, process intermediates and the final product. The same method can be used to successfully monitor all the steps for the synthesis of

MK-0507. A combination of methods is required to monitor the stereoisomer levels.

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ANALYTICAL PEPTIDE MAPPING OF A COMPLEX YELLOWFIN TUNA MYOGLOBIN PEPTIC HYDROLYSATE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

C. LE COEUR[1], Q. ZHAO[1], I. GARREAU[1], F. SANNIER[1], M. MAURICE[2], P. DURAND[2], AND J. M. PIOT[1]*

[1]*Laboratoire de Génie Protéique*
Pôle des Sciences et Technologies, Université de la Rochelle
Av. Marillac, 17042- La Rochelle Cedex 1, France
[2]*Laboratoire de Valorisation des Produits de la Mer*
IFREMER, Rue de l'Ile d'Yeu, BP 1049
44037 Nantes Cedex 01, France

ABSTRACT

A method is described for a real identification of any peptides isolated from a complex peptic Yellowfin tuna red muscle myoglobin hydrolysate. A combination of size exclusion and reversed phase high performance liquid chromatography have proved to be a useful strategy for fractionation of such a mixture. This technique enable a large number of pure peptides from the total hydrolysate to be obtained. Peptides were identified and located on the known myoglobin sequence from their amino acid content determined by the Pico-Tag method and a second order derivative spectroscopic method. Location of the peptides allowed us to define effective cut sites of the porcine pepsin. The procedure described in this study will be useful for acquiring a better knowledge of such an hydrolysate.

INTRODUCTION

Proteins which have nutritional qualities as food additives or health qualities as cosmetics additives, are of high valorization potential. In addition to the proteins obtained from conventional sources (terrestrian mammals) which have been largely used these past few years, a new field of molecules issued from non conventional sources has emerged. Thus, recent studies carried out on terrestrian vegetables, seaweeds, and fishes allowed to considere these proteins as good candidates for the replacement of the first ones. For instance, casein hydrolysates, which constitute one of the most classical protein source [1, 2], may be replaced by soybean, wheat or fishes hydrolysates for animal feeding and nutritional therapy [3-6]. Furthermore, it was demonstrated that many edible proteins could lead to biologically active peptides when they are digested by gastrointestinal proteolytic enzymes [7-9]. Thus, it was suggested that food proteins could both constitute a nitrogen source and exert a biological role.

In this paper, tuna fish myoglobin was investigated. Tuna fish is an important source of myoglobin considering the high concentration found in tuna red muscle [10]. In addition, large amounts of tuna red muscle are available. Until now, tuna red muscle valorization was limited to pet food and other feeding manufacturing products. In myoglobin, the protein entity is associated to heminic iron. So, it could constitute not only a potential source of peptides, which have been demonstrated to be the best nitrogen intestinal absorption way [11-12], but also a source of hemin which is the most bioavailable iron form [13-14]. Moreover, as far as biologically active peptides or heminic peptides are concerned, none of them have ever been characterized from a myoglobin enzymatic hydrolysate. Up to now, hydrolyses have only been performed to determine the protein sequence and to understand myoglobin functional properties as oxygen carrier [15-17]. The

purpose of our work was to characterize a myoglobin hydrolysate prior to further studies as to its nutritional or bioactive peptides. A physiological enzyme, pepsin, was employed to hydrolyse yellowfin-tuna myoglobin. Peptides of this hydrolysate were purified by a chromatographic method associating size exclusion HPLC (SE-HPLC) and reversed phase HPLC (RP-HPLC) [18]. The identification and the location of the different peptides in the known myoglobin structure were then realized by aminoacid analysis completed with a second order derivative spectroscopic method to detect aromatic amino acids [19]. Moreover, we compared the specificity of pepsin towards peptidic bonds with that previously described in the literature [20].

MATERIALS AND METHODS

Materials

Red squeletal muscle of yellowfin-tuna was supplied from Paulet Society (Douarnenez, France). Porcine pepsin (EC.3.4.23, 496 Anson unity) was purchased from Sigma. Amino acid analysis reagents were obtained from Pierce. Acetonitrile was of HPLC grade. Other chemicals were of analytical grade. All aqueous HPLC eluants were filtered prior to use on 0.45 µm filters, and degassed with helium during analysis.

All HPLC analysis were performed with a Waters 600E gradient controler-pump module, a Waters Wisp 717 automatic sampling device, and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC image 466 computer. Millenium software was used to plot, acquire and treat chromatographic data.

Purification of Yellowfin-tuna Myoglobin

Myoglobin was isolated from the red squeletal muscle of yellowfin-tuna using a method adapted from Suzuki and Sugawara's procedure [21]. The tissue was homogeneized in distilled water (1:1, w:v) in an ice-cold waring blendor. The resulting suspension was centrifuged, (3000 g, 15 min), at 4°C and solid ammonium sulfate was added to the aqueous extract to give 60% saturation. After centrifugation (3000 g, 15 min at 4°C) the pellet, almost free from myoglobin, was discarded. Ammonium sulfate was then added to the supernatant to reach 80% saturation, and the resulting solution was centrifuged (3000 g, 15 min). The whole pellet including myoglobin was dissolved in water and then ultrafiltered and diafiltered (PTGC type membrane, Millipore, 10 KD cut-off) against water. The crude resulting solution, containing myoglobin, but also hemoglobin and other water soluble proteins, was loaded on a D.E.A.E. Sephacel column (32x3cm i.d.) previously equilibrated with 50mM TRIS-HCl buffer (pH 8.6) according to Brown [22]. The flow rate was 60 ml/h.

Enzymatic Hydrolysis

Myoglobin (1g) was dissolved in water (100ml), and the pH was adjusted at 2.0 by HCl. Enzymatic digestion by porcine pepsin (40mg) was performed in batch at 40°C for 3h. The pH was maintained at 2.0 by HCl using a Methrom pHstat. The degree of hydrolysis (DH) was deduced from the volume of HCl consumed during the reaction [23, 24]. In order to stop the reaction, the pH was adjusted to 6.5 with ammonium hydroxide. Insoluble core (proteins partly hydrolysed, large peptides and heme) was removed from the peptic digest by

centrifugation (10000g, 30min). The soluble peptidic mixture was then resolved by size exclusion and reversed phase HPLC.

SE-HPLC

Before hydrolysis, myoglobin purity was checked by SE-HPLC with a TSK G2000 SWG analytical column (600x 7.5mm i.d.). The column was equilibrated with 5mM ammonium acetate/acetic acid buffer, pH 6.0. The proteins were eluted with the same buffer at a flow rate of 0.9 ml/min.

SE-HPLC of peptides generated from the myoglobin peptic digestion was performed using a semi-preparative TSK G 2000 SWG column (600x21.5 mm i.d.). The column was equilibrated with 3mM ammonium acetate/acetic acid buffer, pH 6.0. The peptidic mixture was filtered through 0.22µm filters before being applied to the column and peptides were eluted with the equilibrating buffer at a 6ml/min flow rate. Absorbance was monitored at 215 nm. Peptidic fractions, determined on the basis of UV absorbance, were manually collected and then freeze dried before being applied to the RP-HPLC.

RP-HPLC

Analysis of the peptidic fractions issued from SE-HPLC was carried out on a Delta Pak C18 reversed phase column (300x3,9mm i.d.) previously equilibrated with 10mM ammonium acetate/acetic acid buffer, pH 6.0 (eluant A). Peptidic fractions were reconstituted in eluant A, and filtered through 0.22µm filters before being injected on the column. The analysis was performed with a linear gradient of acetonitrile (eluant B) optimized for each fraction. The flow rate was

1.5ml/min. UV absorbance of the peptides was monitored at 215 nm. The pure peptides obtained from the RP-HPLC column were then freeze dried.

Amino Acid Analysis

Each peptide purified by RP-HPLC was hydrolysed with 6M hydrochloric acid containing 1% phenol, for 24 h at 110°C in sealed glass tubes, using a Waters Pico-Tag station. Amino acids were then analyzed on a Waters RP-Pico-Tag column (150x3.9mm i.d.). Precolumn derivatization of amino acids with phenyl isothiocyanate and HPLC separation of derivatized amino acids was performed according to Bidlingmeyer *et al* [25].The detection wavelength was 254 nm and the flow rate 1ml/min.

Tryptophan could not be detected by this method, because it was destroyed during acid hydrolysis. So, the presence of tryptophan (Trp) and other aromatic amino acids, tyrosine (Tyr) and phenyl alanine (Phe) in peptides was determined by a derivative spectroscopic method [19]. In fact, it has been demonstrated that the second order derivative spectrum allowed to detect aromatic amino acid since their second order derivative spectra exhibited a minimum at 258.5, 283.5, and 289.5 nm for Phe, Tyr and Trp respectively.

RESULTS AND DISCUSSION

Myoglobin was extracted from tuna squeletal red muscle and purified by successive ammonium sulfate fractionation, and ion exchange chromatography. Myoglobin was first eluted with 50mM TRIS-HCl buffer, pH 8.6 (fig. 1). Hemoglobin and other contaminants were subsequently eluted with the same

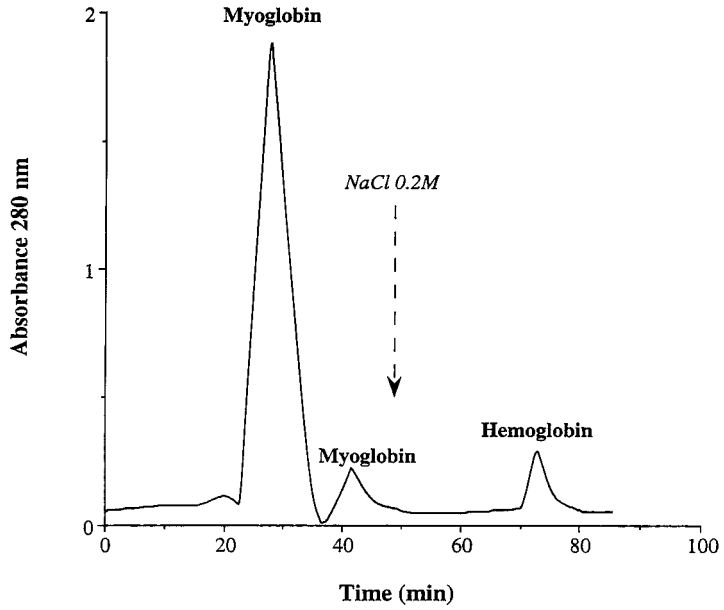

Figure 1. Preparative chromatography on a D.E.A.E. Sephacel column, (320 x 30 mm i.d) of a Yellowfin tuna red muscle extract. Equilibrating buffer: Tris/ HCl 50mM, pH 8.6. Eluting buffers: Tris/ HCl 50mM, pH 8.6 and Tris/HCl 50mM, pH 8.6, NaCl 0.2M. Flow rate: 1.6ml/min.

buffer containing 0.2M NaCl. After extraction and purification, the yield of myoglobin was approximately 56% (w,w).

Yellowfin tuna myoglobin was digested by pepsin for three hours, resulting in a DH of 26% (fig. 2). The generated peptides were resolved by size exclusion HPLC. Fifty injections were performed on the basis of 20mg of total hydrolysate per run. The same elution pattern was obtained for each injection. The chromatogram exhibits seven fractions, numbered I to VII (fig. 3). These fractions were manually collected, pooled, and freeze dried. They were then

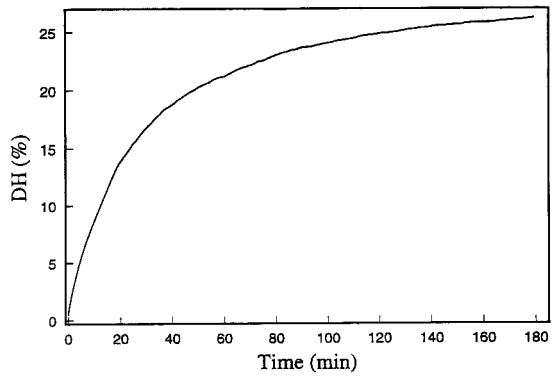

Figure 2. Peptic hydrolysis of Yellowfin tuna myoglobin.
Myoglobin (1g/100ml) was digested by porcine pepsin (40mg/100ml) in a batch at pH 2.0 and 40°C. DH (Degree of Hydrolysis) was measured according to the method of Adler-Nissen [24]

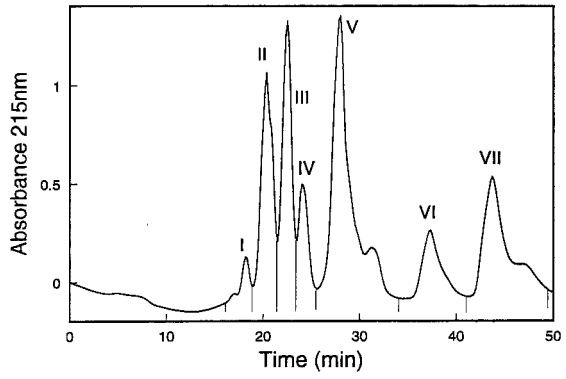

Figure 3. Elution profile of peptic digest of myoglobin on a TSK G2000 SWG semi-preparative column (600x21.5 mm i.d.) equilibrated and eluted with 3.0 mM ammonium acetate / acetic acid buffer, pH 6.0. Injection volume: 100µl (20mg of total hydrolysate). Flow rate: 6.0ml/min.

TABLE 1
Acetonitrile/Ammonium Acetate Buffer Gradient Applied on a RP-HPLC Column, Optimized for Each Fraction. (Eluant A: 10mM Ammonium Acetate/Acetic Acid Buffer, pH 6.0, Eluant B: Acetonitrile).

Fraction	Time (min)	Flow rate (ml/min)	Eluant A %	Eluant B %
I, II	0	1.5	100	0
	6	1.5	94	6
	56	1.5	70	30
	60	1.5	100	0
III, IV	0	1.5	100	0
	56	1.5	72	28
	60	1.5	100	0
V	0	1.5	100	0
	5	1.5	92	8
	55	1.5	74	26
	58	1.5	100	0
VI	0	1.5	100	0
	45	1.5	70	30
	47	1.5	50	50
	50	1.5	100	0
VII	0	1.5	100	0
	5	1.5	90	10
	50	1.5	65	35
	52	1.5	50	50
	55	1.5	100	0

dissolved in 10mM ammonium acetate buffer pH 6.0 to a final peptide concentration of 10mg/ml, and applied to a Deltapak C18 column (1mg per run). In order to improve optimal separation of peptides having largely different hydrophobicities, a specific gradient of acetonitrile/ammonium acetate buffer (10mM, pH6,0) was applied for each fraction (Table 1).

The chromatograms issued from the RP-HPLC separation are presented in figure 4. Four injections of each fraction (I to VII) were performed and individual resulting peaks were collected separately. They have been denoted according to the process of isolation and are represented by Arabic numerals following the Roman numerals. Each peak was checked for homogeneity by RP-HPLC. Thus, eighty-three peptides were collected and freeze dried before amino acid analysis.

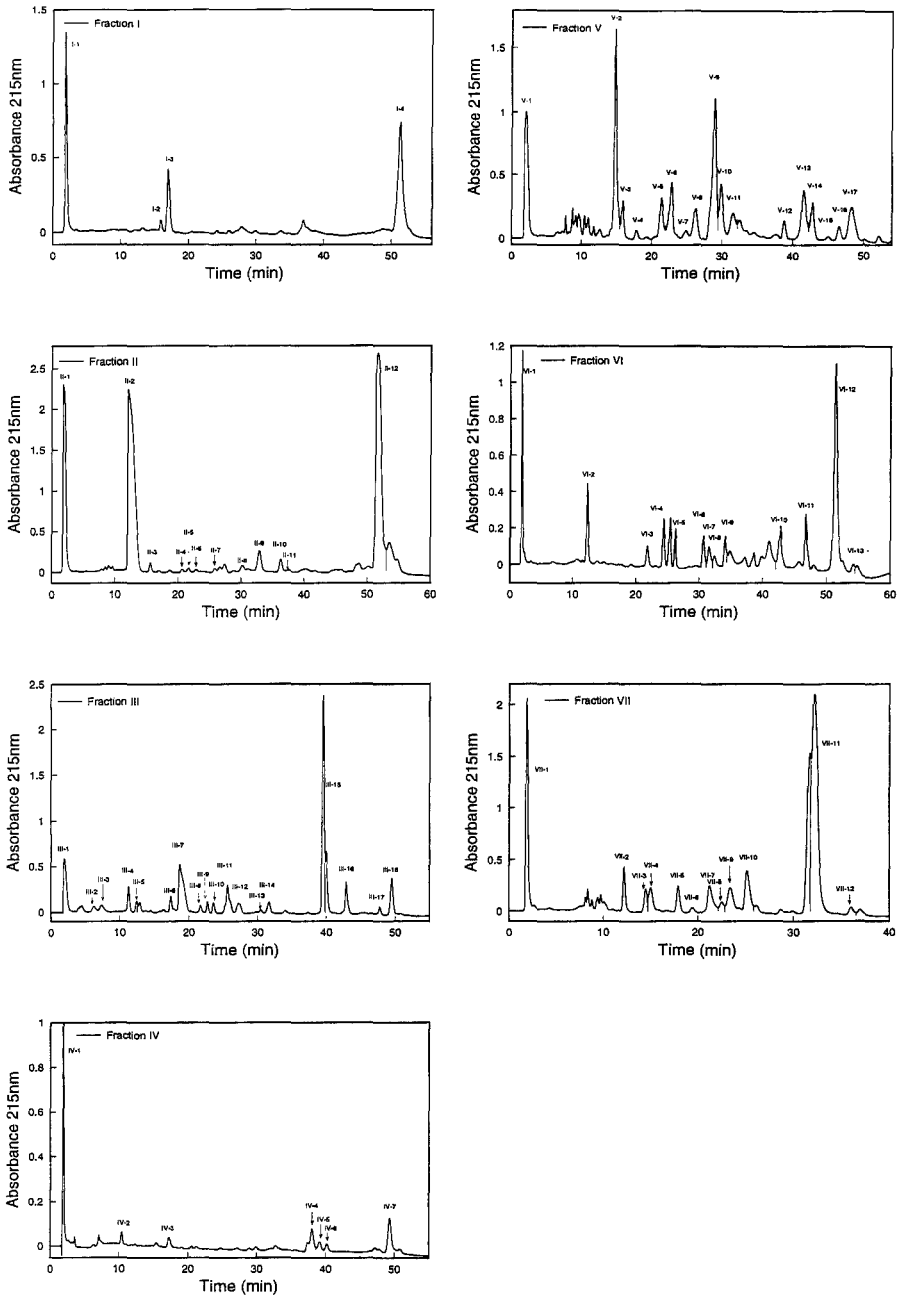

Figure 4. Purification by RP-HPLC on a Deltapak C18 column of the fractions I to VII issus from SE-HPLC. Injection volume: 100µl (1mg peptides). Flow rate: 1.5ml/min.

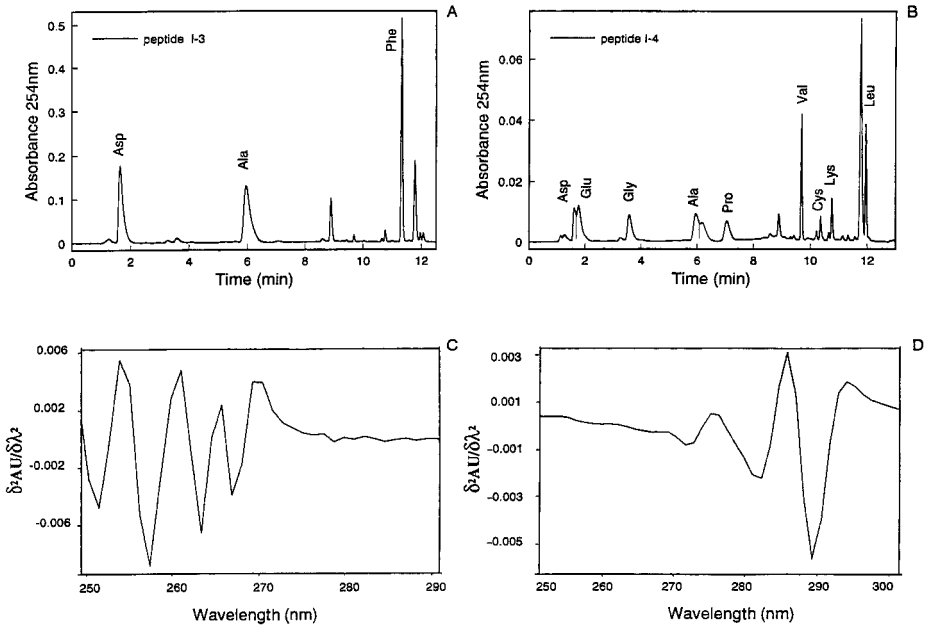

Figure 5. Amino acid analysis of the peptides I-3 and I-4.
Top: elution profiles of PTC-amino acids for the peptides I-3 (A) and I-4 (B). Peptides amino acids were converted into their PTC derivatives and separed by RP-HPLC on a Pico-Tag column.
Bottom: second order derivative spectra of the peptides I-3 (C) and I-4 (D).

Amino acid analysis of each peptide was performed on the Waters Pico-Tag column after converting amino acids into their PTC (phenylthyocarbamyl) derivatives. This analysis was completed with the second order derivative spectroscopic method [19]. As an example, the amino acids analysis profiles and the second order derivative spectra of the peptides I-3 and I-4 are shown in figure 5. The amino acid analysis allowed us to detect the presence of the amino acids Ala, Asp, Phe in peptide I-3 (fig. 5a) and Leu, Lys, Cys, Gly, Pro, Val, Glu, Ala,

Asp in peptide I-4 (fig. 5b). The presence of Phe in the first peptide was confirmed by the second order derivative spectroscopic method since a minimum was observed at 259 nm (fig. 5c). For the second peptide, a minimum at 289,5 nm revealed the presence of tryptophan, which could not be detected by classical amino acid analysis (fig. 5d). Thus, the identification and location of each peptide purified by RP-HPLC was carried out by the comparison of amino acid composition with the known myoglobin sequence [16] (Table 2).

Peptides molecular weights were deduced from amino acid compositions. So, by peptic hydrolysis of myoglobin, fragments ranging from about 200 to 2000 Daltons (2 to 20 amino acids) were generated. As far as peptides size is considered in SE-HPLC, it can be observed that there was no correlation between retention time and molecular weight. Thus, after TSK gel filtration, most of the largest peptides were eluted in fractions V and VII (fig. 6). Many factors such as hydrophobicity and electric charges can affect the behavior of the peptides during size exclusion chromatography [26]. Owing to this complexity, size exclusion chromatography could be performed only to get a rough separation of peptidic fractions for further RP-HPLC analysis and also to access the reproductibility of the hydrolysate. Taking the complexity of the hydrolysate into account, this two steps separation was particularly effective for the purification of most of the peptides released by myoglobin peptic digestion. In fact, even for the complex peptidic fractions III, V and VI, the high resolution allowed to identify unambiguously the major but also the minor peptides.

More than 50% of the peptides identified in the myoglobin hydrolysate were constituted by less than 7 amino acids residues. This result is in good agreement with the known characteristics of pepsin [27] which is one of the physiological

TABLE 2

Identification of Peptides from an Yellowfin Tuna Red Muscle Myoglobin Peptic Hydrolysate Isolated by SE-HPLC followed by RP-HPLC.

Fractions	Peptide N° from RP-HPLC	Corresponding Myoglobin fragment	Sequence	Aromatic amino acid detected by second order derivative spectrum
I	I-1	112-115	Glu-Lys-Ala-Gly	
	I-2	1-4	Ala-Asp-Phe-Asp	Phe
	I-3	1-3	Ala-Asp-Phe	Phe
	I-4	7-16	Leu-Lys-Cys-Trp-Gly-Pro-Val-Glu-Ala-Asp	Trp
II	II-1	133-134 or 1-2	Ala-Asp	
	II-2 as I-3	1-3	Ala-Asp-Phe	Phe
	II-3	14-17	Glu-Ala-Asp-Tyr	Tyr
	II-4	129-133	Gly-Ile-Ile-Ile-Ala	
	II-5	43-50	Ala-Gly-Ile-Ala-Gln-Ala-Asp	
	II-6	45-52	Ile-Ala-Gln-Ala-Asp	
	II-7	112-117	Glu-Lys-Ala-Gly-Leu-Asp	
	II-8	10-16	Trp-Gly-Pro-Val-Glu-Ala-Asp	Trp
	II-9	10-13	Trp-Gly-Pro-Val	Trp
	II-10	136-146	Glu-Ala-Asn-Tyr-Lys-Glu-Leu-Gly-Phe-Ser-Gly	Tyr
	II-11 as I-4	7-16	Leu-Lys-Cys-Trp-Gly-Pro-Val-Glu-Ala-Asp	Trp
	II-12	7-15	Leu-Lys-Cys-Trp-Gly-Pro-Val-Glu-Ala	Trp
III	III-1	14-16 or 47-49 or 136-138	Glu-Ala-Asp or Gln-Ala-Asp or Glu-Ala-Asn	
	III-2	133-135	Ala-Asp-Leu	
	III-3	15-17 or 137-139	Ala-Asp-Tyr or Ala-Asn-Tyr	Tyr
	III-4	136-140	Glu-Ala-Asn-Tyr-Lys	Tyr
	III-5	46-50	Ala-Gln-Ala-Asp-Ile	
	III-6	5-7	Ala-Val-Leu	
	III-7	4-7	Asp-Ala-Val-Leu	
	III-8	135-139	Leu-Glu-Ala-Asn-Tyr	Tyr
	III-9	130-135	Ile-Ile-Ile-Ala-Asp-Leu	
	III-10	136-143	Glu-Ala-Asn-Tyr-Lys-Glu-Leu-Gly	Tyr
	III-11	102-106	Ile-Ser-Glu-Val-Leu	
	III-12	44-49	Gly-Ile-Ala-Gln-Ala-Asp	
	III-13	14-22	Glu-Ala-Asp-Tyr-Thr-Thr-Met-Gly-Gly	Tyr
	III-14	130-134	Ile-Ile-Ile-Ala-Asp	
	III-15 as II-10	136-146	Glu-Ala-Asn-Tyr-Lys-Glu-Leu-Gly-Phe-Ser-Gly	Tyr,Phe
	III-16	130-135	Ile-Ile-Ile-Ala-Asp-Leu	
	III-17	129-135	Gly-Ile-Ile-Ile-Ala-Asp-Leu	
	III-18	8-16	Lys-Cys-Trp-Gly-Pro-Val-Glu-Ala-Asp	Trp
IV	IV-1	112-116	Glu-Lys-Ala-Gly-Leu	
	IV-2	14-19	Glu-Ala-Asp-Tyr-Thr-Thr	Tyr
	IV-3 as III-7	4-7	Asp-Ala-Val-Leu	
	IV-4 as II-10	136-146	Glu-Ala-Asn-Tyr-Lys-Glu-Leu-Gly-Phe-Ser-Gly	Tyr,Phe
	IV-5	10-15	Trp-Gly-Pro-Val-Glu-Ala	Trp
	IV-6	79-81	Ala-Ile-Leu	
	IV-7	10-16	Trp-Gly-Pro-Val-Glu-Ala-Asp	Trp

(continued)

TABLE 2 (continuation)

Identification of Peptides from an Yellowfin Tuna Red Muscle Myoglobin Peptic Hydrolysate Isolated by SE-HPLC followed by RP-HPLC.

Fractions	Peptide N° from RP-HPLC	Corresponding Myoglobin fragment	Sequence	Aromatic amino acid detected by second order derivative spectrum
V	V-1	145-146	Ser-Gly	
	V-2	17-22	Tyr-Thr-Thr-Met-Gly-Gly	Tyr
	V-3	29-38	Phe-Lys-Glu-His-Pro-Glu-Thr-Gln-Lys-Leu	Phe
	V-4	140-146	Lys-Glu-Leu-Gly-Phe-Ser-Gly	Phe
	V-5	59-64	His-Gly-Ala-Thr-Val-Leu	
	V-6	23-24 or 24-25	Val-Leu or Leu-Val	
	V-7	130-132	Ile-Ile-Ile	
	V-8	54-66	Ala-Ala-Ile-Ser-Ala-His-Gly-Ala-Thr-Val-Leu-Lys-Lys	
	V-9	58-69	Ala-His-Gly-Ala-Thr-Val-Leu-Lys-Lys-Leu-Gly-Glu	
	V-10	139-146	Tyr-Lys-Glu-Leu-Gly-Phe-Ser-Gly	Tyr,Phe
	V-11	129-146	Gly-Ile-Ile-Ile-Ala-Asp-Leu-Glu-Ala-Asn-Tyr-Lys-Glu-Leu-Gly-Phe-Ser-Gly	Tyr,Phe
	V-12	118-128	Ala-Gly-Gly-Gln-Thr-Ala-Leu-Arg-Asn-Val-Met	
	V-13	101-120	Leu-Ile-Ser-Glu-Val-Leu-Val-Lys-Val-Met-His-Glu-Lys-Ala-Gly-Leu-Asp-Ala-Gly-Gly	
	V-14	107-125	Val-Lys-Val-Met-His-Glu-Lys-Ala-Gly-Leu-Asp-Ala-Gly-Gly-Gln-Thr-Ala-Leu-Arg	
	V-15	100-119	Lys-Leu-Ile-Ser-Glu-Val-Leu-Val-Lys-Val-Met-His-Glu-Lys-Ala-Gly-Leu-Asp-Ala-Gly	
	V-16	14-24 or 13-23	(Val)-Glu-Ala-Asp-Tyr-Thr-Thr-Met-Gly-Gly-Leu-(Val)	Tyr
	V-17	1-14	Ala-Asp-Phe-Asp-Ala-Val-Leu-Lys-Cys-Trp-Gly-Pro-Val-Glu	Phe,Trp
VI	VI-1	113-115	Lys-Ala-Gly	
	VI-2	25-27 or 26-28	(Leu)-Thr-Arg-(Leu)	
	VI-3	125-128	Arg-Asn-Val-Met	
	VI-4	125-129	Arg-Asn-Val-Met-Gly	
	VI-5	140-142	Lys-Glu-Leu	
	VI-6	64-69 or 140-143	Leu-Lys-Lys-Leu-Gly-Glu or Lys-Glu-Leu-Gly	
	VI-7	140-144	Lys-Glu-Leu-Gly-Phe	Phe
	VI-8	68-70	Gly-Glu-leu	
	VI-9	63-71	Val-Leu-Lys-Lys-Leu-Gly-Glu-Leu-Leu	
	VI-10	143-146	Gly-Phe-Ser-Gly	Phe
	VI-11	107-116	Val-Lys-Val-Met-His-Glu-Lys-Ala-Gly-Leu	
	VI-12	33-44	Pro-Glu-Thr-Gln-Lys-Leu-Phe-Pro-Lys-Phe-Ala-Gly	Phe
	VI-13	2-14	Asp-Phe-Asp-Ala-Val-Leu-Lys-Cys-Trp-Gly-Pro-Val-Glu	Phe,Trp
VII	VII-1	28-41	Leu-Phe-Lys-Glu-His-Pro-Glu-Thr-Gln-Lys-Leu-Phe-Pro-Lys	Phe
	VII-2	74-87	Lys-Gly-Ser-His-Ala-Ala-Ile-Leu-Lys-Pro-Leu-Ala-Asn-Ser	
	VII-3	72-79	Lys-Ala-Lys-Gly-Ser-His-Ala-Ala	
	VII-4	66-71	Lys-Leu-Gly-Glu-Leu-Leu	
	VII-5	28-41 or 36-39	Leu-Phe-Lys-Glu or Gln-Lys-Leu-Phe	Phe
	VII-6	84-95	Leu-Ala-Asn-Ser-His-Ala-Thr-Lys-His-Lys-Ile-Pro	
	VII-7	88-104	His-Ala-Thr-Lys-His-Lys-Ile-Pro-Ile-Asn-Asn-Phe-Lys-Leu-Ile-Ser-Glu	Phe
	VII-8	80-85	Ile-Leu-Lys-Pro-Leu-Ala	
	VII-9	36-41	Gln-Lys-Leu-Phe-Pro-Lys	Phe
	VII-10	39-44	Phe-Pro-Lys-Phe-Ala-Gly	Phe
	VII-11	29-44	Phe-Lys-Glu-His-Pro-Glu-Thr-Gln-Lys-Leu-Phe-Pro-Lys-Phe-Ala-Gly	Phe
	VII-12	34-46	Glu-Thr-Gln-Lys-Leu-Phe-Pro-Lys-Phe-Ala-Gly-Ile-ala	Phe

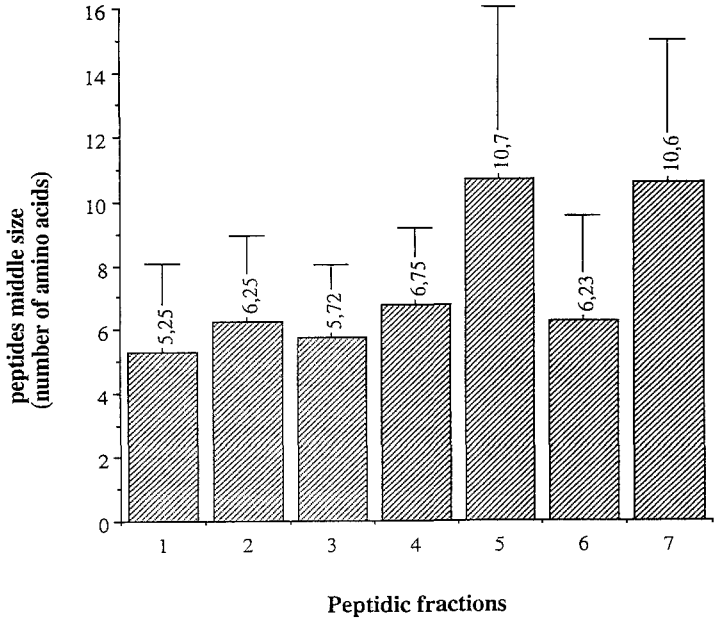

Figure 6. Peptides middle size distribution of each peptidic fraction obtained by SE-HPLC (in amino acids).

proteases exhibiting the lowest specificity. It could be observed that some peptides seemed to be broken down into other peptides, e.g. peptide V-11 is partly converted into III-17 and II-10 and peptide III-6 into III-7. The location of these peptides in the myoglobin structure allowed us to specify the pepsin cut sites towards this protein in our hydrolysis conditions. Effective pepsin cut sites on myoglobin and theoretical pepsin cut sites found in the literature were indicated in fig. 7. If some cleavage sites previously described were confirmed, new sites appeared in our study. This corroborated the weak specificity of pepsine and illustrated how it could be difficult to predict peptidic composition of such an hydrolysate.

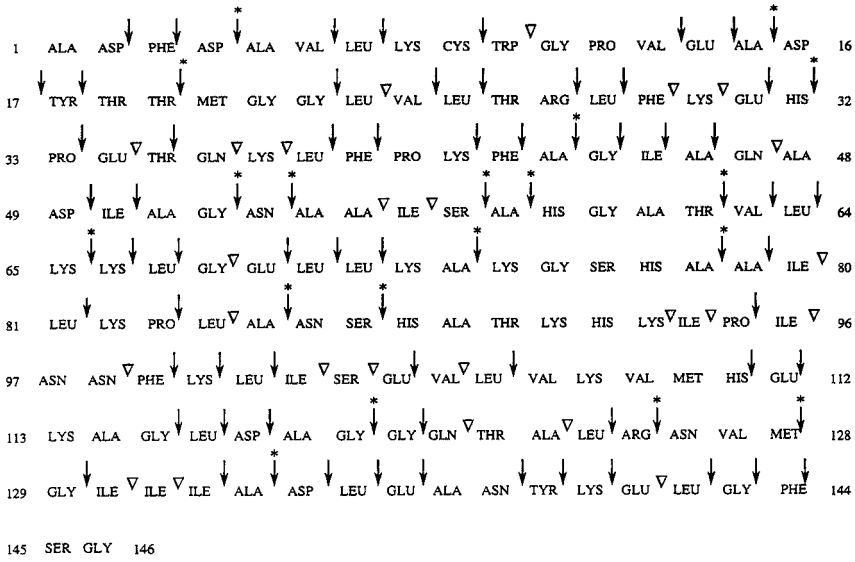

Figure 7. Yellowfin tuna myoglobin sequence determined by Rice *et al.* [16]
↓: pepsin cut sites observed during myoglobin hydrolysis in accordance with those described in the literature.
↓: pepsin cut sites observed during myoglobin hydrolysis and not described in the literature.
∇: pepsin cut sites described in the literature and not observed during myoglobin hydrolysis.

It is well known that the end products of digestion, i.e. short oligopeptides and amino acids are not absorbed in a similar mode [28]. It was previously demonstrated that enteral infusions containing small peptides (di or tri peptides) were more efficiently assimilated than corresponding free amino acid mixture and displayed better nutritional value [29]. In other respects, nutritional studies have been carried out to provide a better understanding of the mechanisms involved in iron absorption. Different pathways could be concerned depending on

whether the iron is heme-iron or free-iron. It has been observed in previous studies that hemoglobin-iron and myoglobin-iron [14] were absorbed more efficiently than inorganic iron. Furthermore, it was established that the iron could enter intestinal cells as heme [14]. Moreover, the importance of the protein was suggested since it has been observed that the degraded globin increased heme absorption probably by binding the coordinating bonds of heme and preventing its polymerization [14]. More recently, it was suggested that small peptides could facilitate iron absorption into the intestinal mucosal cells [30].

In this work, we have demonstrated that peptic hydrolysis of yellowfin tuna myoglobin generated mostly small peptides. Eighty-three peptides have been purified and identified in the complex peptidic mixture. Most of them consisted of less than seven amino acids. It should be interesting to study the nutritional importance of this hydrolysate both for the nitrogen absorption as small peptides and iron absorption as iron-heme or peptide-iron-heme. Moreover, it would be a real chalenge to investigate the potential biological activity of these peptides. In fact, it has been established that peptic digestion of many proteins, especially of hemoglobin [31, 32], results in the production of subtances that were found to have opioïd-like activity.

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OPTIMIZATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION FOR PROGESTOGENIC, ESTROGENIC, AND ANDOGENIC STEROIDS USING FACTORIAL DESIGN

YEONG SHUN GAU, SHAO WEN SUN, AND RUSSEL RHEI-LONG CHEN*
School of Pharmacy
National Taiwan University
College of Medicine
No. 1 Jen Ai Road, Ist Section
Taipei, Taiwan 100, Republic of China

ABSTRACT

A 2^3 factorial design has been used in an isocratic HPLC system to study the effect of methanol and acetonitrile composition of the eluents as well as different columns on the retention and resolution of steroids. By factorial design and computer simulation, six steroids of sex hormones and contraceptives were simultaneously separated within 18 min and 28 min using RP-8 column and RP-18 column, respectively.

INTRODUCTION

Norethindrone and mestranol are synthetic steroids showing progestogenic activity. They are usually combined in use as oral concentraceptives. According to therapeutic uses, progesterone, medrogestone, testosterone propionate and fluoxymesterone are categorized in sexual hormones. Various functional group

substitutions in the chemical structures of these six steroids make them show different chromatographic retention characteristics. Many high-performance liquid chromatographic (HPLC) methods have been described for analysis of similar steroids [1-3]. Optimization of separations by a trial and error approach is time-consuming and likely to fail owing to the many variables involved and their interactions. Therefore, there has been much interest in finding a simplified and systematic means of optimizing HPLC separations. Many optimization methods such as sequential simplex method [4], window diagrams [5], overlapping resolution mapping method [6], Plackett-Burman design [7] and factorial design [8], have been employed for the selection of optimal mobile phase compositions in HPLC. In this study, a factorial design and a computer program has been used for the optimization of the retention and separation of the afore mentioned steroids by an isocratic RP-HPLC system. The optimization procedure was focused on selecting the optimum mobile phase composition in the different column.

EXPERIMENTAL

Materials

Norethindrone, progesterone and testosterone propionate were purchased from Sigma (St. Louis, MO, U.S.A.). Mestranol was purchased from Aldrich (Milwaukee, WI, U.S.A.). Fluoxymesterone was from MS Chemicals (Milano, Italy) and Medrogestone was from Diosyth BV (Oss, the Netherlands). Methanol and Acetonitrile (L.C. grade) were purchased from Mallinckrodt (Paris, KY, U.S.A.). The compound numbers, names and concentrations of the steroids are listed in Table 1. Standard solutions were prepared in methanol-water (1:1, v/v).

HPLC

The chromatographic system consisted of a JASCO 880-PO HPLC pump, a JASCO 875-UV variable wavelength detector (Hachioji, Japan) and a SIC Chromatocorder 11 recorder (Tokyo, Japan). UV detection at 230 nm was set for all optimization procedures. Columns used were 250 - 4 mm LichroCART C_{18} (7 mm) and 250 - 4 mm LichroCART C_8 (7 mm) (Merck, Darmstadt, Germany). The

TABLE 1. Compound numbers, names and concentrations of the steroids

No.	Compound name	Concentration
1	Fluoxymesterone	20 mg/ml
2	Norethindrone	20 mg/ml
3	Progesterone	20 mg/ml
4	Mestranol	120 mg/ml
5	Medrogestone	120 mg/ml
6	Testosterone propionate	30 mg/ml

TABLE 2. A 2^3 factorial design : experimental presentation

Variable	Experiment No.							
	1	2	3	4	5	6	7	8
X_1	1	1	1	1	−1	−1	−1	−1
X_2	1	1	−1	−1	1	1	−1	−1
X_3	1	−1	1	−1	1	−1	1	−1
X_1 = Methanol-Water			+1 = 46:54 (v/v)			−1 = 40:60 (v/v)		
X_2 = Acetonitrile-Water			+1 = 38:62 (v/v)			−1 = 32:68 (v/v)		
X_3 = Column			+1 = RP-8			−1 = RP-18		

flow rate of mobile phase was 1 ml min^{-1}, the injection volume was 20 ml. The dead time was measured with the first peak resulting from the injection of 0.5 % NaCl solution (w/v).

Factorial design [9]

The mobile phase was a ternary system including methanol, acetonitrile and water. The fractions of methanol and acetonitrile in the solvent were assigned as variables X_1 and X_2, and the different column as variable X_3, respectively in the factorial design (Table 2). Two values denoted by +1 (the upper level) and −1 (the lower level) were given for each variable to define the experimental domain. All experiments were performed in a random order.

Computer simulation

A computer program was written in QBASIC to predict chromatograms as systematically changing the three variables X_1, X_2 and X_3 within certain ranges. The maximum t_R was as a measure of analysis time. The predicted t_R was calculated using the following equation:

$$t_R = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_4 X_1 X_2 + \alpha_5 X_1 X_3 + \alpha_6 X_2 X_3 + \alpha_7 X_1 X_2 X_3 \quad (1)$$

where t_R is the retention time in minutes of an individual steroid under a given set of conditions and X_1, X_2 and X_3 are values of the three variables as indicated above. The coefficients α_1, α_2, α_3, α_4, α_5, α_6 and α_7 were estimated by Yates algorithm [12]. The predicted t_R were used to predict resolution (Rs) between each pair of successively eluted steroids, using the equation:

$$R\text{s} = 2(t_{R_{i+1}} - t_{R_i})/(W_{i+1} + W_i) \quad (2)$$

where $t_{R_{i+1}}$ and t_{R_i} were the retention times of i+1 and i peaks, and W_{i+1} and W_i were the peak widths of i+1 and i peaks.

RESULTS AND DISCUSSION

In the preliminary studies, tetrahydrofuran was chosen as a mobile phase component for the separation of steroids in the literature [8]. The high column pressure caused by the heavy proportion of tetrahydrofuran rendered its use impraticable. Furthermore, commercially available tetrahydrofuran contains an antioxidant which might cause interference during detection [10]. In the steroid analysis, acetonitrile was claimed to increase the peak sharpness and column efficiency [11]. For these reasons, acetonitrile was used instead of tetrahydrofuran. In the following study, the composition of methanol and acetonitrile in the mobile phase were used as variables. Many papers reported RP-C_{18} column could be used as the stationery phase of steroid separation [1,8,12]. However, it is worthwhile testing other RP columns of shorter hydrocarbon chains to reduce the analysis time while maintaining the same resolution on the basis of a similar retention mechanism.

TABLE 3. Retention times (min) of six steroids while changing the mobile phase according to TABLE 2

Steroid	Experiment No.							
No.[a]	1	2	3	4	5	6	7	8
1	3.29	3.26	3.62	3.86	3.49	3.64	4.10	4.41
2	3.29	3.46	3.95	4.27	3.84	4.07	4.71	5.15
3	4.19	5.51	5.49	8.09	5.27	7.47	7.34	11.23
4	4.19	6.24	5.95	10.45	5.56	9.20	8.40	15.55
5	4.79	7.18	6.91	11.71	6.55	10.58	10.00	17.47
6	5.13	8.47	7.81	14.99	7.36	13.43	11.91	23.91

[a] The corresponding steroids are listed in Table 1

In this study, a RP-C_8 column was tested and the retention times were compared with those of the RP-C18 column.

The retention times of each compound and parts of the chromatograms were shown in Table 3 and Fig. 1 for the eight experiments of the factorial design. In all eight experiments, the elution order of the steroids remained as fluoxymesterone, norethindrone, progesterone, mestranol, medrogestone and testosterone propionate, which is essentially in accordance with the decreasing polarity of these compounds [13]. The peaks of fluoxymesterone and norethindrone, and the peaks of progesterone and mestranol, were overlapped respectively in some experimental conditions (Table 3 and Fig. 1). The nature of organic solvent (the ratio of methanol-acetonitrile) affected the resolution of steroids both on the selecitvity and the column efficiency [11]. In acetonitrile-rich eluents, resolution was mainly dependent on the change in column efficiency better provided by acetonitrile than mathanol (Fig. 1(B)). In acetonitrile-lean eluents, separation was largely influenced by the higher selectivity of methanol compared to acetonitrile; however, the column efficiency decreased with the increase of methanol concentration (Fig. 1(A)).

The main effects and interaction effects of variables X_1, X_2 and X_3 on the t_R of an individual steroid are indicated by α_1, α_2, α_3, α_4, α_5, α_6 and α_7 (Table 4).

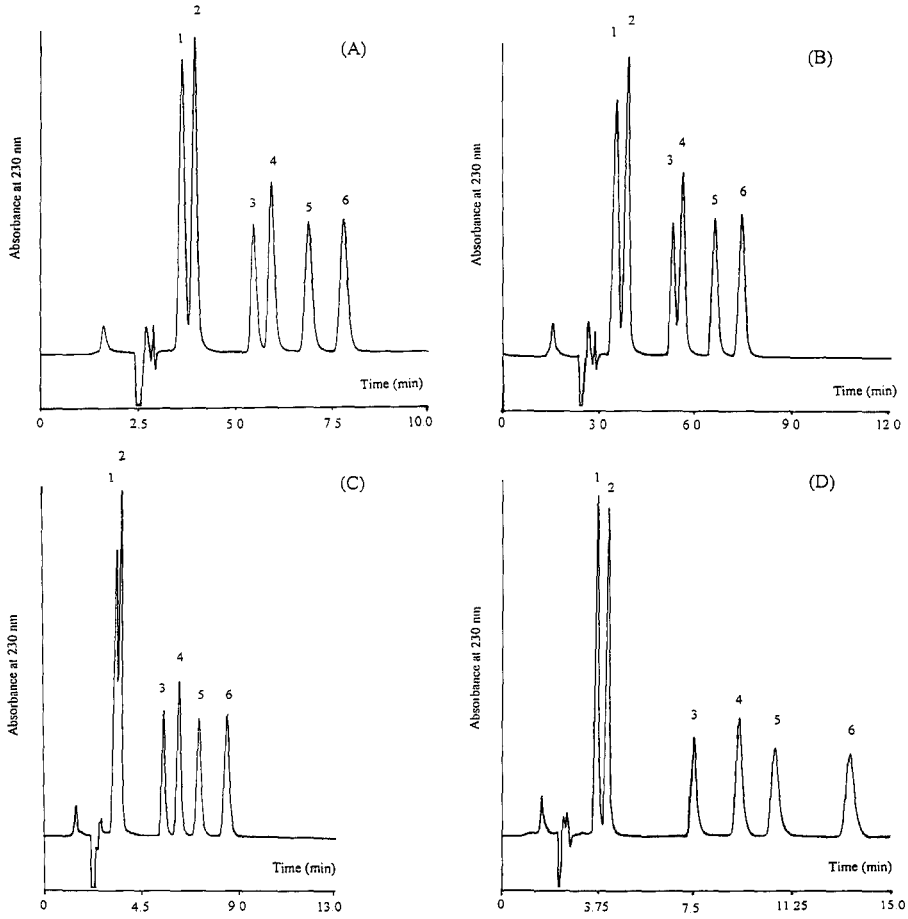

FIGURE 1.

Chromatograms of the steroid mixture in different chromatographic conditions. (A) column : LichroCART RP-8 (250 - 4 mm, 7 mm); mobile phase : methanol-acetonitrile-water (46:32:22, v/v/v). (B) column : LichroCART RP-8 (250 - 4 mm , 7 mm); mobile phase : methanol-acetonitrile-water (40:38:22, v/v/v). (C) column : LichroCART RP-18 (250 - 4 mm, 7 mm); mobile phase : methanol-acetonitrile-water (46:38:16, v/v/v). (D) column : LichroCART RP-18 (250 - 4 mm, 7 mm); mobile phase : methanol-acetonitrile-water (40:38:22, v/v/v). For compound numbers, see Table 1.

TABLE 4. Calculated mean effects (α values) on the retention times of the substances while changing the variables according to TABLE 2

Steroid No.[a]	α_0	α_1	α_2	α_3	α_4	α_5	α_6	α_7
1	3.71	-0.20	-0.29	-0.08	0.06	0.03	0.05	0.01
2	4.09	-0.35	-0.43	-0.15	0.06	0.02	0.05	-0.01
3	6.82	-1.00	-1.21	-1.25	0.24	0.27	0.37	-0.05
4	8.19	-1.49	-1.90	-2.17	0.40	0.53	0.75	-0.13
5	9.40	-1.75	-2.12	-2.34	0.46	0.54	0.73	-0.13
6	11.63	-2.53	-3.03	-3.58	0.73	0.94	1.22	-0.26

[a] The corresponding steroids are listed in Table 1

TABLE 5. Comparison between actual and predicted retention times(t_R)

Steroid No.[c]	RP - 8[a]		RP - 18[b]	
	Actual t_R (min)	Predicted t_R (min)	Actual t_R (min)	Predicted t_R (min)
1	4.49	4.35	4.40	4.40
2	5.35	5.08	5.22	5.19
3	8.98	8.25	11.58	11.33
4	10.68	9.63	15.95	15.64
5	12.83	11.53	18.09	17.66
6	15.82	13.98	24.92	24.22

[a] Mobile Phase : methanol-acetonitrile-water(37:32:31,v/v/v)
[b] Mobile Phase : methanol-acetonitrile-water(37:34:31,v/v/v)
[c] The corresponding steroids are listed in Table 1

Negative α_1, α_2, and α_3 indicated that there was a negative relationship between main effects and t_R. In the eight experiments, the mean dead time was 2.21 min. With the computer simulation, satisfactory HPLC conditions were not found between the +1 and −1 range of the experimental variables, so we extended the searched intervals to between +2 and −2. When X_1, X_2 and X_3 were (−2, −1, +1) and (−2, −0.333, −1), optimum theoretical HPLC conditions were found. These represent the mobile phase composition methanol-acetonitrile-water (37:32:31, v/v/v) with RP-8 column used and the mobile phase composition methanol-

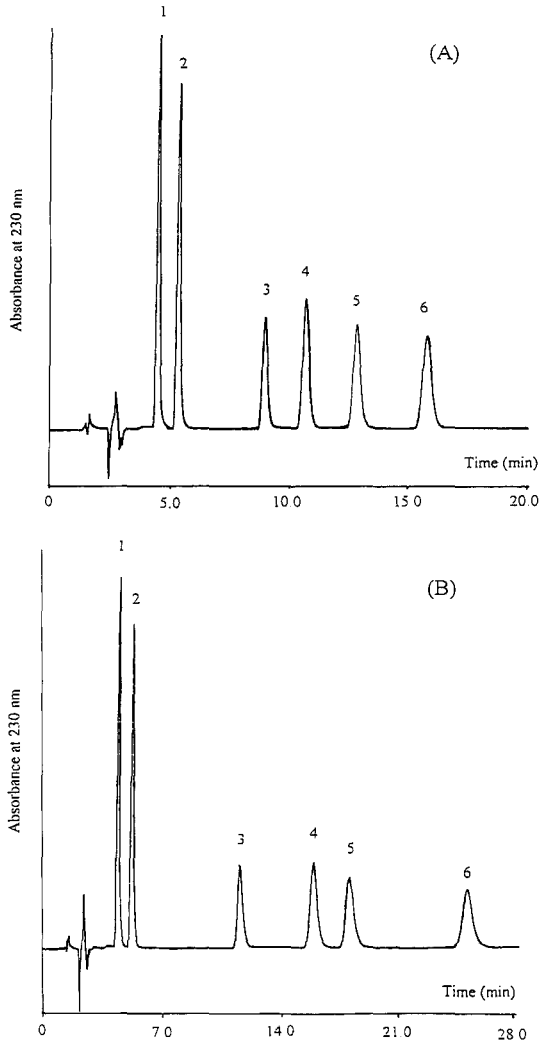

FIGURE 2.
Chromatograms of optimized isocratic separation of a steroid mixture. (A) column : LichroCART RP-8 (250 - 4 mm, 7 mm); mobile phase : methanol-acetonitrile-water (37:32:31, v/v/v). (B) column : LichroCART RP-18 (25 0 - 4 mm., 7 mm); mobile phase : methanol-acetonitrile-water (37:34:29, v/v/v). For compound number, see Table 1.

acetonitrile-water (37:34:29 v/v/v) with RP-18 column used, respectively. On testing these optimum theoretical conditions with the HPLC system, satisfactory separations were obtained. The actual t_R and Rs values were closely corresponding to the predicted values (Table 5 and Fig. 2). With the same resolution of chromatograms, analysis time required was shorter in a RP-8 column and longer in a RP-18 column.

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SIMULTANEOUS DETERMINATION OF 18-OXYGENATED CORTICOSTEROIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

SHINJI KUROSAWA[1], TERUKI YOSHIMURA[2], TAKAO KUROSAWA[2], HITOSHI CHIBA[3], KUNIHIKO KOBAYASHI[3], TAKAO KOIKE[1], AND MASAHIKO TOHMA[2]

[1]Department of Internal Medicine II
Hokkaido University School of Medicine
Sapporo, Hokkaido 060, Japan
[2]Faculty of Pharmaceutical Sciences
Health Sciences University of Hokkaido
Ishikari-Tobetsu, Hokkaido 061-02, Japan
[3]Department of Laboratory Medicine
Hokkaido University School of Medicine
Sapporo, Hokkaido 060, Japan

ABSTRACT

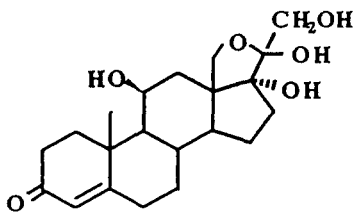
A method for differentially measuring the 18-oxygenated corticosteroids, 18-hydroxycortisol, 18-hydroxycortisone and 18-oxocortisol, in human urine from the patients with primary aldosteronism has been developed by high-performance liquid chromatography with fluorescence detection. The method involves the derivatization of 18-oxygenated corticosteroids into the fluorescent 21-anthroyl esters by the action with 1-anthroyl nitrile. Urine was first extracted with a mixture of ether-dichloromethane, and solid-phase

extraction of the anthroyl derivatives on a Bond Elut CN cartridge column was used as a clean up step before final separation by high-performance liquid chromatography with fluorescence detection. 11,18-Epoxypredonisolone was used as an internal standard. The linearity of the calibration curve for each steroid was from 0.5 pmol to 25 pmol per injection, and the detection limit was 0.1 pmol (SN = 5).

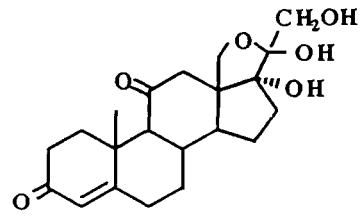
INTRODUCTION

18-Oxygenated corticosteroids, such as 18-hydroxycortisol, 18-oxocortisol and 18-hydroxycortisone (Fig. 1) are often called hybrid steroids since they have the characteristic structure of glucocorticoids and mineralcorticoids (1). Such cortisol derivatives were first reported by Chu and Urick (1) in the urine of patients with primary aldosteronism, and a number of studies have shown that these compounds are effective discriminators for primary aldosteronism (2-5). Although gas chromatography - mass spectrometry (1), radioimmunoassay (4, 6), enzyme immunoassay (7) and high-performance liquid chromatography (5) have been reported for the measurement of 18-hydroxycortisol, a method for the simultaneous determination of the above three 18-oxygenated corticosteroids in human biological fluids has not been established. To clarify the excretion of these steroids in urine, a highly sensitive quantitation method is required because of the low concentration of such steroids in biological fluids.

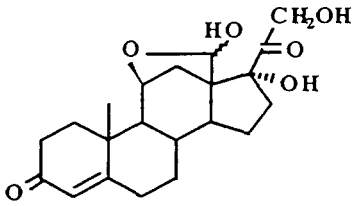
We wish to report here the method developed for the quantitation of 18-oxygenated corticosteroids, and its application to the quantitative determination of them in human urine.



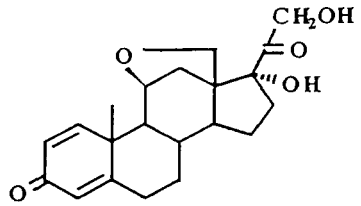
18-hydroxycortisol



18-hydroxycortisone



18-oxocortisol



internal standard
11,18-epoxyprednisolone

FIGURE 1. Molecular structures of steroids.

MATERIALS AND METHODS

Materials

18-Hydroxycortisol, 18-hydroxycortisone and 11,18-epoxyprednisolone (11β,18-epoxy-17α,21-dihydroxypregna-1,4-diene-3,20-dione) were chemically synthesized as reported previously(8). 1-Anthroyl nitrile was prepared as reported by Goto et al.(9) Bond Elut CN cartriges were obtained from Analytichem International (Haber City, CA, U.S.A). All other reagents were of analytical grade. 18-Oxocortisol

was synthesized as follows: The mixture of 18-hydroxycortisol-17α, 21α-acetonide (480 mg), lead tetraacetate (450 mg) and iodine (300 mg) in dry 1, 2-dichloroethane (20 ml) and cyclohexane (20 ml) was irradiated with a 500 W tungsten lamp for 30 min at room temperature. The reaction mixture was filtered and the filtrate was washed with 5 % sodium thiosulfate, then water, and then dried over sodium sulfate. The crude product was subjected to silica-gel column chromatography eluted with chloroform - methanol (30 : 1, v/v) to give 18-oxocortisol actonide (25 %) as a hemiacetal form, mp 215 - 218 °C. The acetonide was hydrolyzed in the usual manner to give 18-oxocortisol. mp 198 - 200 °C. Analysis Calculated for $C_{21}H_{28}O_6$: C, 67.00; H, 7.50. Found : C, 67.16; H, 7.35.

Urine samples

Urine samples (24 h) were collected without preservatives from patients with primary aldosteronism before or after adrenalectomy, and from healthy subjects as a control were collected. All specimens were stored at -25°C until analyzed.

Apparatus and chromatographic conditions

The HPLC apparatus consisted of a LC-6A system equipped with RF-535 spectrofluorometer (Shimadzu, Kyoto, Japan). The chromatographic separation was performed on a μBondasphere phenyl (150 x 3.9 mm i.d., 5 μm, Waters, Milford, MA, USA) column using gradient elution mode at a flow rate of 1.0 ml/min. The column temperature was ambient. The mobile phases

were 10 mM ammonium acetate - methanol (50 : 50, v/v; mobile phase A) and acetonitrile (mobile phase B). The gradient program was as follows; an isocratic elution with 15 % of mobile phase B for 10 min, then a linear gradient to 30 % of mobile phase B over a period of 30 min. The fluorescence intensity of the eluent was monitored with an emission wavelength at 470 nm and an excitation wavelength at 370 nm.

Sample extraction

To a urine sample (100 - 500 µl), an appropriate amount of 11,18-epoxyprednisolone was added as an internal standard, and the whole was extracted twice by shaking for 3 min with 5 ml of diethyl ether - dichloromethane (3 : 2, v/v). After being centrifuged at 800 x g for 10 min, the organic layer (ca. 8 ml) was evaporated to dryness below 40°C under reduced pressure. The residue was subjected to derivatization with 1-anthroyl nitrile.

Derivatization procedure

The sample extract was mixed with 100 µl of 0.4 % 1-anthroyl nitrile in acetonitrile and 100 µl of 0.4 % quinuclidine in acetonitrile (10). The mixture was allowed to stand at room temperature for 10 min, and then the excess reagent was decomposed by the addition of 25 µl of water. After standing at room temperature for 5 min, the mixture was neutralized with 25 µl of 1 % acetic acid, and the solvent was evaporated to dryness below 40°C under reduced pressure. The residue was dissolved in a small amount of benzene and loaded on a Bond Elut CN

cartridge equilibrated with hexane for elimination of interfering peaks on the chromatogram. The cartridge was washed with 6 ml of ethyl acetate - hexane (1 : 15, v/v), and the resultant fluorescent derivatives were eluted with 6 ml of ethyl acetate - hexane (3 : 2, v/v). After evaporation of the solvent under reduced pressure, the residue was dissolved in methanol, and applied to HPLC analysis.

RESULTS AND DISCUSSION

Derivatization of 18-oxygenated corticosteroids with 1-anthroyl nitrile

A highly sensitive derivatization of hydroxysteroids with 1- or 9-anthroyl nitrile for HPLC with fluorescence detection, has been previously developed by Goto et al.(9). It was noted that, in the derivatization of cortisol with both reagents, the primary hydroxyl group at C-21 was quantitatively converted to the corresponding esters, but the 11β-secondary and 17α-tertiary hydroxyl groups underwent no reaction. In order to employ these reagents for fluorescence labeling for the precolumn derivatization of 18-hydroxycortisol, 18-hydroxycortisone and 18-oxocortisol, the reactivities of both reagents for 18-oxygenated corticosteroids were initially examined according to the method described by Goto et al. A marked difference in the reactivity between the two reagents was observed; the reactivity of 9-anthroyl nitrile for the three 18-oxygenated steroids was less than 10 % of that of 1-anthroyl nitrile, despite the modification of

conditions such as reaction time, reaction temperature and kinds of base catalysts. These results may be due to steric interaction between the hydrogens at the 1- and 8- positions of the 9-anthroyl nitrile and the intramolecular hemiacetal system of the 18-oxygenated steroids. Therefore, 1-anthroyl nitrile was selected as the derivatization reagent for the development of a sensitive HPLC method.

The quantitative formation of the anthroyl derivatives of all the steroids with 1-anthroyl nitrile was easily achieved at room temperature within 10 min in the presence of quinuclidine as a catalyst. Unfavorable side reactions and the dehydration of the hydroxyl groups through the derivatization procedure were not observed. All the fluorescent derivatives, the detector responses of which were of the same degree, showed a single peak on the chromatogram, showing that derivatization with 1-anthroyl nitrile occured in the C-21 primary hydroxyl group.

Separation of 1-anthroyl derivatives

Various combinations of column, pH of buffer solution and organic solvent were examined in order to obtain a complete separation of the anthroyl derivatives of the three 18-oxygenated corticostroids and 11,18-epoxyprednisolone. 11,18-Epoxyprednisolone, which is not present in human biological fluids and gaves the fluorescent derivative in the same yield, was used as an internal standard (5). When the C18 columns were tested with mixtures of ammonium acetate and organic solvents such as methanol and

acetonitrile as mobile phase, the peaks due to 18-oxocortisol and 18-hydroxycortisol could not to be resolved successfully. The use of the C8 and phenyl columns gave a good separation of the anthroyl derivatives of the four 18-oxygenated steroids, however, the peak widths obtained on the C8 columns were broader than those obtained on the phenyl columns. Therefore, the phenyl columns were selected for the method. The elution with mixtures containing both methanol and acetonitrile provided more sufficient separation, and sharp and symmetrical peaks, compared with that with methanol or acetonitrile alone as a organic solvent, when combinations of ammonium acetate and organic solvents were tested on phenyl columns. The pH and the concentration of ammonium acetate of mobile phase had little effect on the separation and the peak shape of each steroid. From the results, the best separation of the anthroyl derivatives was achieved on a μBondasphere phenyl column by gradient elution with mixtures of 10 mM ammonium acetate - methanol and acetonitrile within 35 min. A typical chromatogram obtained with authentic specimens of 18-oxygenated corticosteroids and the internal standard is shown in Fig. 2. These derivatives were completely separated from each other without noticeable peak asymmetry. The retention time of the 18-oxygenated corticosteroids increased in the following order : 18-oxocortisol < 18-hydroxycortisol < 18-hydroxycortisone < 11,18-epoxyprednisolone. Reproducible retention times were obtained for all the 18-oxygenated corticosteroids under the present HPLC conditions.

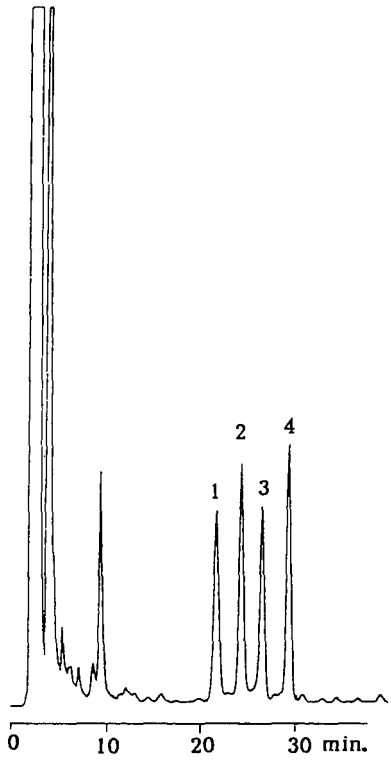


FIGURE 2. Chromatogram of standard 18-oxygenated steroids. 1: 18-oxocortisol 2: 18-hydroxycortisol 3: 18-hydroxycortisone 4: 11,18-epoxyprednisolone (internal standard).

The calibration curves were constructed by plot ting the relative peak area of each 18-oxygenated corticosteroid to the internal standard against the amounts of the corresponding 18-oxygenated corticosteroid. A good linear relationship to each 18-oxygenated steroid was obtained over the range of

0.5 - 25 pmol with the linear correlation coefficients of more than 0.999 for all the 18-oxygenated corticosteroids. The coefficients of variation for the measurement of 10 pmol of each standard 18-oxygenated steroid was less than 3 %. The detection limits of all the 18-oxygenated corticosteroids was estimated to be 0.1 pmol (signal-to-noise ratio = 5). The proposed HPLC method was at least 100 times more sensitive than the reported HPLC method using ultraviolet detection (5).

Determination of 18-oxygenated corticosteroids in human urine

Extraction of 18-oxygenated corticosteroids from urine samples was performed with a mixture of diethyl ether - dichloromethane (3 : 2, v/v) in the usual manner. Maximum and consistent recoveries of all the 18-oxygenated corticosteroids were achieved by extracting twice with 10 ml of the organic solvent. Furthermore, to eliminate interfering peaks appearing on the chromatogram, extraction of the 1-anthroyl derivatives before HPLC analysis was tested by several cartridge columns such as Bond Elut and Sep-Pak series with mixtures of organic solvents. The interferences could be removed by a Bond Elut CN with mixture of ethyl acetate - hexane as an eluent.

The recovery rates through the extraction and derivatization as described in "MATERIALS AND METHODS" were tested by adding known amounts of 18-oxygenated corticosteroids (20 ng and 100 ng) to steroid-free urine (0.5 ml). As listed in Table I, all of the 18-oxygenated corticosteroids were

TABLE 1

Recoveries of 18-Oxygenated Corticosteroids

	added amounts* (ng)	recoveries (%)
18-Oxocortisol	22.3	87.6 ± 1.8**
	111.6	86.3 ± 1.2
18-Hydroxycortisol	20.5	89.1 ± 1.8
	102.7	91.1 ± 1.5
18-Hydroxycortisone	22.2	89.6 ± 1.8
	111.2	91.6 ± 1.9

* added amounts of each standard sample into 0.5 ml of steroid free urine, internal standard (19.5 ng).

** Mean ± S.E.M. (n = 6)

recovered at a rate of more than 86 %, and the within-day and day-to-day coefficients of variation were less than 5 % (n=6) and 7 % (n=5), respectively.

Figures 3a and 3b show typical chromatograms obtained from the urine of a patient with primary aldosteronism and from the same patient after adrenalectomy, respectively. The peaks due to 18-hydroxycortisol, 18-hydroxycortisone and 18-oxocortisol in Fig. 2a were identified, respectively, on the basis of their retention times. The predominant 18-oxygenated corticosteroid was 18-hydroxycortisol as reported previously, accounting for more than 70 % of the total amounts of the three 18-oxygenated corticosteroids. Of particular interest was the

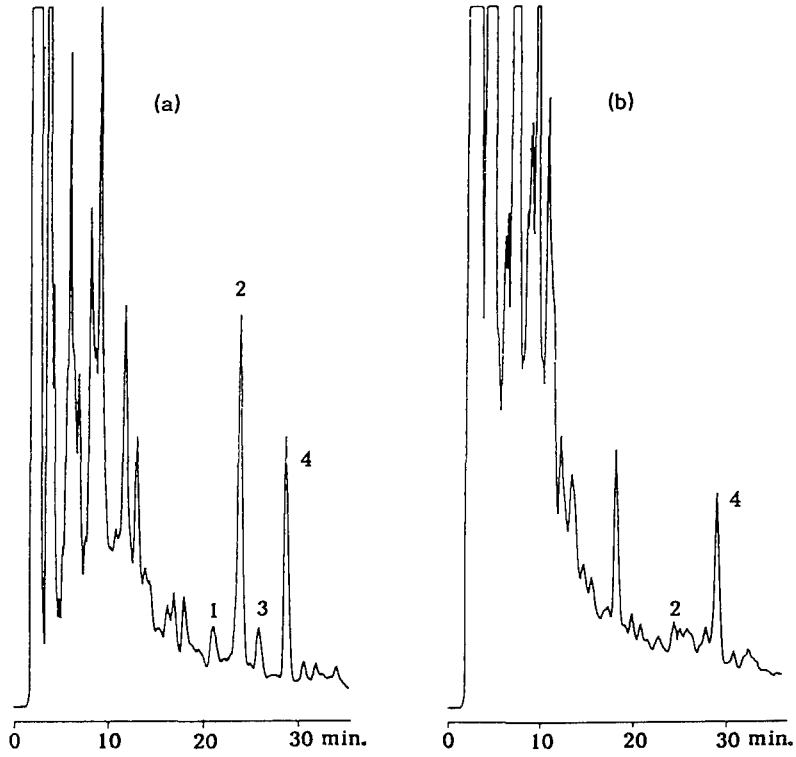


FIGURE 3. Chromatograms of urine samples from a patient with primary aldosteronism, before (a) and after adrenalectomy (b). 1:18-oxocortisol 2:18-hydroxycortisol 3:18-hydroxycortisone 4:internal standard.

identification of 18-hydroxycortisone in the urine of patients with primary aldosteronism in addition to 18-hydroxy and 18-oxocortisol. The concentrations of 18-hydroxycortisol, 18-hydroxycortisone and 18-oxocortisol in the urine of the patients with primary aldosteronism were estimated to be 350-2300, 45-100,

30-75 nmol / l, n=5), respectively. On the other hand, all the 18-oxygenated corticosteroid levels after adrenalectomy showed significant decreases to the level of the control in comparison with that before adrenalectomy, accounting for less than 100 nmol/l for 18-hydroxycortisol,and 4 nmol/l for 18-oxocortisol and 18-hydroxycortisone (Fig. 3b).

This study has provided an HPLC method with fluorescence detection for the simultaneous determination of 18-hydroxycortisol, 18-hydroxy-cortisone and 18-oxocortisol in human urine. Further application of this method is under way to clarify the physiological and pathophysiological roles of these 18-oxygenated corticosteroids in relation to primary aldosteronism.

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STUDY OF PAH'S SEPARATION AND PHASE-SOLUTE INTERACTION BY MICELLAR LIQUID CHROMATOGRAPHY

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

ABSTRACT

The large k' values obtained in micellar liquid chromatography of Brij-35 on C-18 and C-8 columns clearly decrease in the presence of methanol or propanol as organic modifiers, propanol being superior to methanol although resolution is better with the latter. A linear relationship between k,' not log k', and the number of carbons is obtained. The distribution coefficient of the solutes between the stationary and aqueous phases increases, as do the micelle-solute association constants, with the number of aromatic rings for naphthalene, phenanthrene, chrysene and dibenzo(ah)anthracene. Sensitivity is increased with respect to that obtained in the classic isocratic RPLC methanol/water method. In best conditions nine PAH's are resolved to the baseline.

INTRODUCTION

Nowdays it is well accepted that micellar liquid chromatography (MLC) on alkyl-bonded stationary phases has some advantages over reversed phase liquid chromatography (RPLC);

these include the use of smaller amounts of organic solvents, which means less environmental pollution and lower cost as well as higher sensitivity for aromatic compounds by fluorimetric detection. Selectivity can also be increased by controlling additional chromatographic parameters such as the nature of the surfactant as well as its ionic charge and concentration[1-7]. However, there are some drawbacks, including greater broadening of the chromatographic peaks and larger capacity factors, which make analysis time impractically long. This effect is more important in RPLC when the polarity of the analytes decreases; stationary phases based on short hydrocarbonated chains decrease the capacity factors but they also decrease the resolution[7-9].

The behaviour of analytes in MLC arises from more complex interactions than in RPLC, making it difficult to predict the results in given chromatographic conditions. Consequently more data are required to probe further into this field of chromatography.

In this paper we study the behaviour of 13 polycyclic aromatic hydrocarbons (PAH's) in MLC, based on the use of Brij-35, a non ionic surfactant, using two alkyl-bonded stationary phases, C-8 and C-18, and fluorimetric detection. Some relevant thermodynamic data based on equations described in the literature[1-4,10,11] are applied to explain this behaviour. From the analytical point of view the results could also be useful for determining these compounds in environmental studies.

EXPERIMENTAL

Apparatus and material

The chromatographic system consisted of the following components: a high pressure gradient LDC Analytical CM-4000 pump, a Rheodyne 7125 sample injector with a 20 μl loop, a Waters 420 fluorimetric detector with the excitation and emission filters of 254 and 375 nm (long-pass), respectively, and a Milton Roy CI 4100 integrator. The columns were a C-18 Hypersil 5 μm particle size (100x4.0 mm) and a C-8 Hypersil 5 μm particle size (100x4.6 mm). A P-Selecta Precisterm bath was used for thermostating the columns. A P-Selecta Ultrasons bath was used for preparing all the solutions. A Lida nylon membrane filter with 0.45 μm pore size was used to filter the eluents employed to prepare the mobile phase. Vapor pressure osmometry measurements were carried out on a Knauer VPO instrument.

Chemicals

Stock standard methanolic solutions of 13 PAH's from Sigma at concentrations in the range 10^{-3} - 10^{-4} M were prepared by weighing and dissolving the solid products in methanol. More dilute solutions were prepared by dilution with methanol.

An aqueous micellar solution of Brij-35 [polyoxyethylene 23 lauryl ether, C_{12} H_{25} $(OCH_2-CH_2)_{23}$ OH; formula weight = 1199.6; 30% (w/v)] from Sigma, was prepared by dissolving 200

ml in water up to 500 ml to give a final concentration of 0.1 M; critical micellar concentration (c.m.c.) = 10^{-4} M[12]. More dilute solutions were prepared by dilution with water, methanol/water or propanol/water, where appropriate.

Methanol, propanol and acetone (Carlo Erba) of chromatographic grade were used. Water was obtained from a Milli-Q system (Millipore). Before use, all eluents were degassed under vacuum and filtered.

Chromatographic procedure

Micellar mobile phases containing Brij-35 concentration in the range 4.10^{-4}-0.1 M were used; depending on the case, propanol or methanol in the ranges 2-30% and 20-80%, respectively, were used as organic modifiers at flow-rates of 1 and 0.6 ml/min, for C-18 and C-8 columns, respectively.

Stock solutions of the PAH's were used and their concentrations were adjusted to allow detection in the range $4.0.10^{-3}$-120 μg/ml for naphthalene and anthracene, respectively, by the injection of 20 μl of standard samples. For fluorimetric detection, excitation and emission filters of 254 and 375 nm (long-pass), respectively, were used.

Temperatures in the range 30-60ºC and flow-rates in the range 0.4-1 ml/min were tested.

The columns were conditioned by applying the following gradient: methanol for 15 minutes and then water for a further 15 minutes, which changed to the micellar Brij-35 solution

working concentration in 60 minutes at a flow-rate of 1 ml/min; this micellar solution was then maintained for a further 60 minutes. Acetone/water was used to determine the void times.

RESULTS AND DISCUSSION

Conditioning of the stationary phase

It is known that work in MLC requires prior conditioning of the column to achieve reproducible results. It is accepted that the column is modified by adsorption of the surfactant. When using the Brij-35 surfactant, the C-18 and C-8 columns are completely regenerated by passing pure methanol; consequently, the conditioning procedure specified in the experimental section was applied. Reproducibility of the naphthalene, which is the earliest-eluting solute, with a retention time in the range ± 0.1 min was taken as a measure of the stability of the column. Reconditioning of the column was necessary after a month's work, equivalent to 175 hours; in any case elution of the column with methanol for 120 minutes allows it to be used in RPLC without significant changes.

Determination of void volume

Different approaches have been used to determine the void volume in MLC. The uracil method[2,10] was not successful in this case despite using fluorimetric detection. Other methods based

on the change of refraction index, such as the use of water, urea, acetonitrile/water and deuterated water were also unsuccessful[3,7,10]. A new approach based on the use of acetone/water yielded good results, although acetone can be retained somewhat on the stationary phase. Injection of 20 μl of acetone/water 20/80 (v/v) gave a signal similar to the one obtained by injection of 20 μl of naphthalene 5.10^{-4} M; this signal is also based on a refraction index change. This method was also successful for SDS in MLC[5,9]. Under the typical working conditions (flow-rate 1 ml/min, Brij-35 concentration 0.05 M and temperature 60ºC) the void volumes were 0.80 ml and 1.08 ml for the C-18 and C-8 column, respectively. These values scarcely changed over the Brij-35 concentration range of 10^{-2}-10^{-1} M. It should be noted that the calculated bonded phase coverage in μ mol/m^2 was 2.84 and 3.85 for C-18 and C-8 columns, respectively.

Effect of the surfactant concentration on the mobile phase

A micellar mobile phase containing a Brij-35 concentration 4.10^{-4} M, which is close to its c.m.c., gave capacity factors for naphthalene above 61 and 26 on C-18 and C-8 columns, respectively. As shown in Figure 1, retention times (and, consequently, capacity factors) on a C-18 column decrease with increasing Brij-35 concentration in the mobile phase. However, at 0.05 M Brij-35, capacity factors are impractical from an analytical point of view. In these conditions, only five PAHs

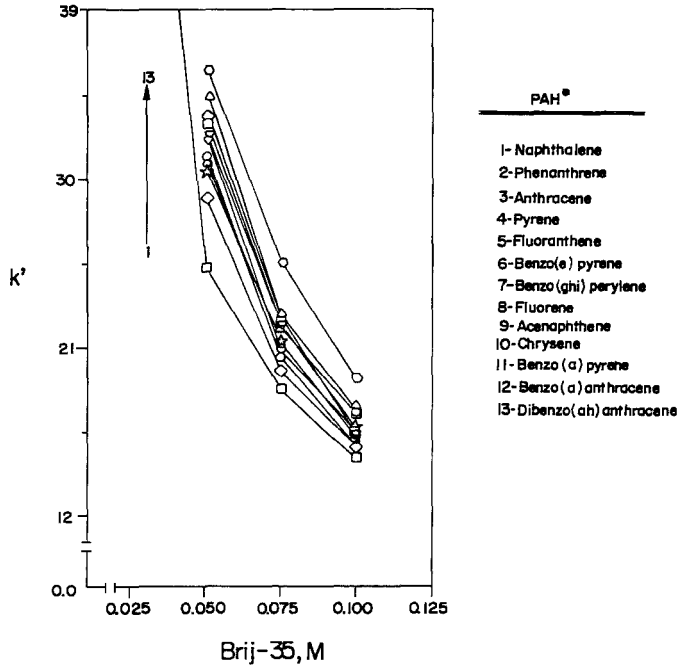


Figure 1: Capacity factors versus micellar concentration of Brij- 35. Flow-rate, 1 ml/min; temperature, 60ºC; detection, 254 nm excitation and 375 nm emission (long-pass); C-18 column. *Elution order in 0.05 M Brij-35.

are resolved to the baseline. Moreover, at 0.1 M Brij-35 only four PAH's are resolved. Similar changes were observed on a C-8 column, where capacity factors changed from the range 12-16.2 to 6.5-9.0 with the same Brij-35 concentration change. On the other hand, the elution order changed with Brij-35 concentration and with the column type (C-18, C-8). These changes are consistent with some thermodynamic parameters determined below.

Effect of the organic modifiers

A common practice to decrease capacity factors is based on addition of organic modifiers, such as methanol, propanol, butanol or acetonitrile[1-9]. An additional beneficial wetting effect is assumed for these modifiers. Methanol and propanol were tested, the elution strength of propanol being higher than that of methanol. Similar analysis times were obtained both for 30% propanol and 50% methanol, resolution being lower with propanol. As shown in Figure 2, capacity factors also decrease with methanol percentages. A change in the chromatographic mechanism for the larger PAH's is apparent in this figure. Capacity factors lower than 10 are obtained for methanol percentages above 60%, which makes the analytical separation practical. Eleven PAH's can be separated with 50% methanol at a flow-rate of 1 ml/min. Similar results were obtained with a C-8 column, changing log k' values from the range 0.7 - 1.0 to (-0.5) - (-0.2) for the change of methanol percentage from 30 to 60%. On the other hand, elution orders are independent of the column type and of the methanol percentages. Thus PAH's can be separated with 50% methanol and flow-rate of 0.6 ml/min.

Determination of the critical micellar concentration

After selection of the percentages of methanol, the critical micellar concentrations (c.m.c.) in these conditions were determined by vapor presure osmometry[13,14]. The results

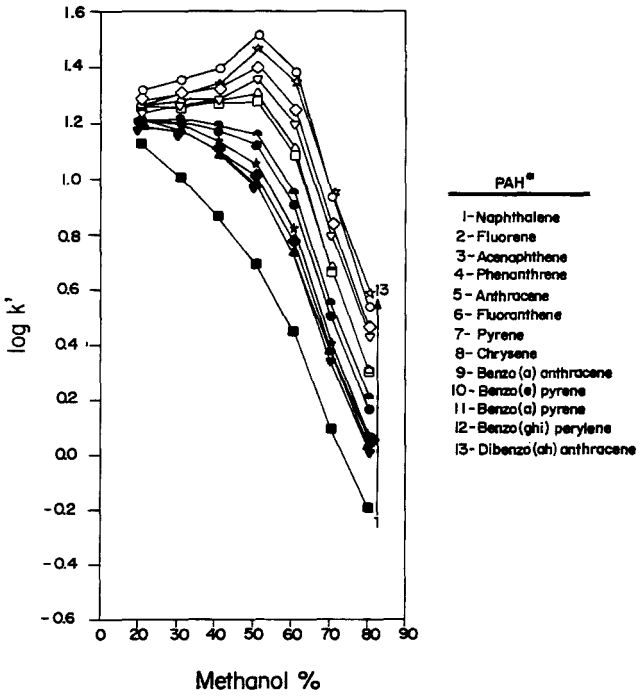


Figure 2: Capacity factors versus methanol concentration. Brij-35 concentration, 0.05 M; Flow-rate, 1 ml/min; temperature, 60ºC; detection, 254 nm excitation and 375 nm emission (long-pass); C-18 column. *Elution order in 50% methanol.

found are shown in Figure 3. Graph number 1 relates to glucose solutions taken as a reference and the graph number 2 to Brij-35 solutions. In both cases we started with a 50/50 v/v methanol/water solution and added glucose or Brij-35.

Graph 1 shows a regular increase of the resistence, ΔR, with glucose concentration; in contrast, the inflection point of graph 2 allows the c.m.c. of Brij-35 to be determined in 50%

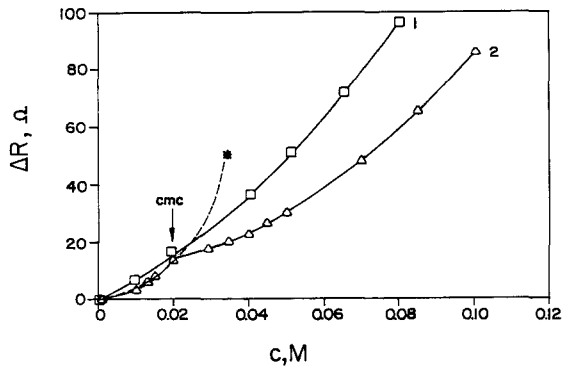


Figure 3: Determination of the critical micellar concentration. Methanol/water, 50/50 (v/v). 1, glucose solution; 2, Brij-35 solution; *Assuming the absence of micelles.

methanol. This c.m.c. value was 0.02 M, which is higher than that obtained in the absence of methanol, 10^{-4} M[12]. Obviously, lower methanol contents and a 0.02 M Brij-35 concentration will always give a micellar system. The existence of micelle structures in the presence of such a high concentration of organic modifiers could be questioned; however, results obtained seem to confirm that micelles exist in these experimental conditions.

Solvent strength

According to the literature[1,10] the solvent strength of the mobile phase decreases in the presence of micelles. We determined the solvent strength of the micellar mobile phases

with different percentages of methanol, using the equation[15] described elsewere:

$$\log k' = - S \theta + \log k_o' \qquad (1)$$

For a particular solute, plotting the logarithm of the capacity factor (k') against the volume fraction (θ) of methanol allows the solvent strength (S) to be determined from the slope. The intercept, log k_o', is the logarithm of the capacity factor in micellar mobile phase without a modifier.

As shown in Table 1, the solvent strength decreases with the molecular size of the PAH. This agrees with results for other compounds and for other micellar mobile phases in the presence of propanol as a modifier[10]. The range of variation is even lower for the C-8 column. Some PAH solvent strength values for a traditional methanol mobile phase on a C-18 column are included in this table for comparison purposes.

Effect of flow-rate

The effect of flow-rate on retention times for a C-18 column is shown in Figure 4. Retention times decrease with the flow-rate, and eleven PAH's can be separated with analysis times below 36 minutes for a flow-rate of 1 ml/min. The large retention times observed for flow-rates below 1 ml/min seem to indicate the important contribution of kinetic factors. Good resolution up to nine PAH's was obtained with the C-18 column.

TABLE 1

Solvent strength of the mobile phase in C-18 and C-8 columns

PAH	S, C-18		S, C-8*
	*	**	
Naphthalene	2.2	3.0	2.2
Fluorene	1.9		2.4
Acenaphthene	2.0		2.3
Phenanthrene	1.9		2.3
Anthracene	1.9		2.3
Fluoranthene	1.7		2.2
Pyrene	1.6	3.3	2.2
Chrysene	1.5		2.1
Benzo(a)anthracene	1.5		2.1
Benzo(e)pyrene	1.2	2.9	1.9
Benzo(a)pyrene	1.2	2.9	1.9
Benzo(ghi)perylene	1.0	2.8	1.7
Dibenzo(ah)anthracene	1.1		1.7

* Mobile phase, 0.05 M Brij-35 in water/methanol solution.
** Mobile phase water/methanol.

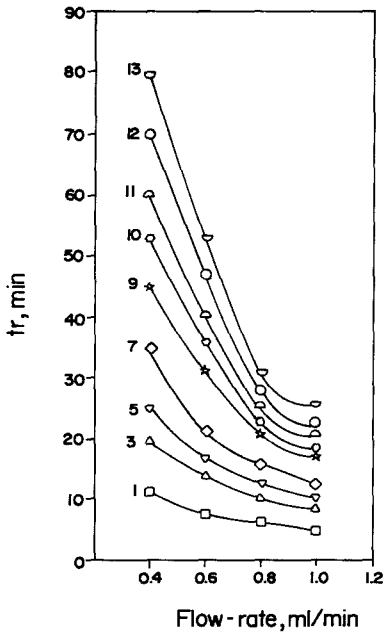


Figure 4: Effect of flow-rate on retention time. Brij-35 concentration, 0.05 M in methanol/water 50%; temperature, 60ºC; detection, 254 nm excitation and 375 nm emission (long-pass); C-18 column. PAH's identified in Figure 2.

The same behaviour was found for the C-8 column; separation of ten PAH's was achieved with a flow-rate of 0.6 ml/min.

Effect of temperature

The effect of temperature on MLC is greater than in RPLC[5,7-9]; the decrease in viscosity of the micellar mobile phase with increasing temperature reduces retention time significantly. The results obtained are shown in Figure 5, for

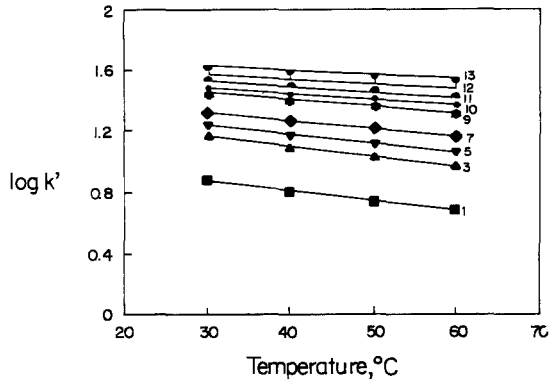


Figure 5: Effect of temperature on capacity factors. Brij-35 concentration, 0.05 M in methanol/water 50%; Flow-rate, 1 ml/min; detection, 254 nm excitation and 375 nm emission (long-pass); C-18 column. PAH's identified in Figure 2.

a C-18 column. The change in k' is higher for the PAH's with smaller molecular size. An increase of 10ºC produces a log k' change of 0.2 units. Again, similar changes were observed for a C-8 column.

Correlation between retention factors and the number of carbons

In RPLC there is a linear relationship between log k' and the number of carbons, Nc, as shown in the following equation[16,10]:

$$\log k' = (\log \alpha)\ Nc + \log \beta \qquad (2)$$

where the slope, log α, is a measure of hydrophobic selectivity which characterizes non-specific interactions; the intercept

indicates the specific interactions between the residue of the molecule and the mobile and stationary phases.

However in MLC a linear relationship between k' and Nc is obtained, as follows[10]:

$$k' = B\ Nc + A \qquad (3)$$

where A and B are the intercept and slope, respectively, of the straight line.

The results obtained for three Brij-35 micellar mobile phases are shown in Figure 6; straight line plots of k' versus Nc were also obtained for different methanol percentages in mobile phases, Figure 7. This confirms the results obtained for other homologous series[1,2,10]. It should be noted that the slope of the graph increases significantly with the presence of methanol, thus increasing resolution. These results are clearly different from those obtained with a 50% methanol/water mobile phase; in this case, only naphthalene is resolved, the capacity factor being 26.5.

Determination of the micelle-solute association constants and the distribution coefficients of the solute.

Several parameters have been proposed[1-3,10,11] in order to explain the above relationship between k' and Nc. The micelle-solute association constants per surfactant monomer (K_2) can be determined from the Armstrong and Nome equation[17]:

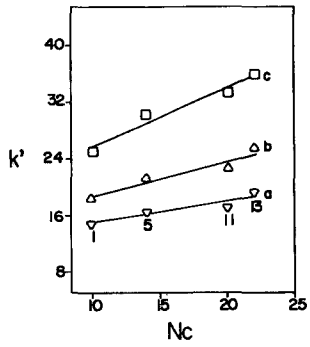


Figure 6: Correlation of capacity factors and the number of carbons. Mobile phase, Brij-35, M, a, 0.10; b, 0.075; c, 0.050. PAH's identified in Figure 2.

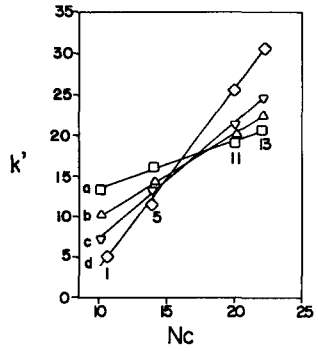


Figure 7: Correlation of capacity factors and the number of carbons. Mobile phase, Brij-35 0.05 M. Methanol, %, a, 20; b, 30; c, 40; d, 50. PAH's identified in Figure 2.

$$V_s/(V_e - V_m) = [V(P_{mw}-1)/P_{sw}]C_m + 1/P_{sw} \; ; \; K_2 = V(P_{mw}-1) \qquad (4)$$

The graph of the ratio of the stationary phase volume (V_s) to the difference between elution volume of the solute (V_e) and the void volume of the column (V_m) versus the micellized surfactant concentration (C_m), which is the surfactant concentration minus the c.m.c., gives rise to straight-line plots with slope K_2/P_{sw}; where P_{sw} is the distribution coefficient of the solute between the stationary and aqueous phases, which can be calculated from the intercept. V is the molar volume of the surfactant. P_{mw} is the distribution coefficient of the solute between the micellar and aqueous phases.

Similarly, the Arunyanart and Cline Love equation can be used for the same purpose[18]:

$$1/k' = [K_2/\Phi(L_s)K_1]\ C_m + 1/\Phi(L_s)K_1 \qquad (5)$$

where Φ is the ratio V_s/V_m, L_s is the concentration of stationary-phase ligate and K_1 the association constant for the solute between the bulk solvent and the stationary phase. In this case K_2 can be determined from the slope of 1/k' versus C_m; K_2 is obtained easily as a ratio between the slope and the intercept.

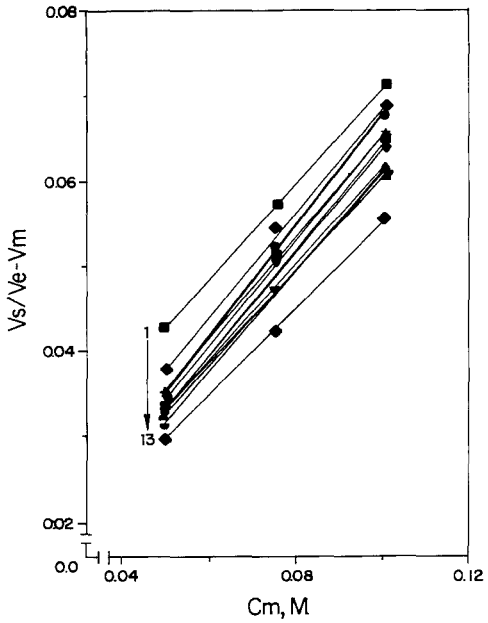


Figure 8: Effect of micellized Brij-35 concentration on the $V_s/(V_e-V_m)$ ratio. PAH's identified in Figure 1.

Figures 8 and 9 respectively show the variations of $V_s/(V_e - V_m)$ and $1/k'$ as a function of the micellized Brij-35 concentration. The results are shown in Table 2.

Related to K_2 is the solute-micelle association constant per micelle (K_s), which can be calculated by multiplying K_2 by the aggregation number, which is 40 for Brij-35[12]. Both the above equations yielded similar results for K_2. There is apparently no correlation between K_2 and molecular size of the solute; in fact the PAH's listed have different structures. However the slopes of the graphs in Figures 8 and 9 are not the

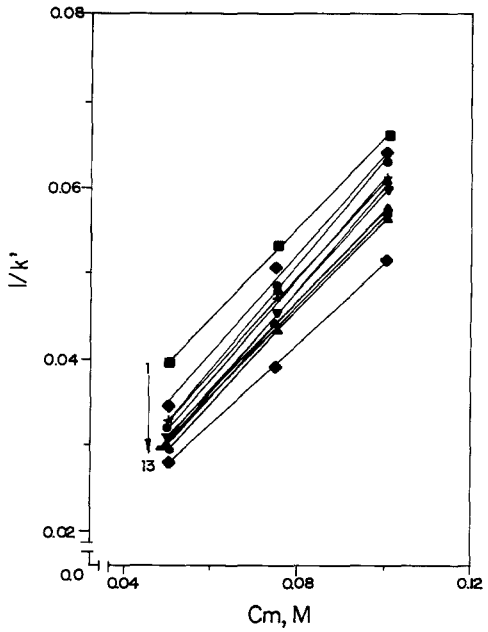


Figure 9: Variation of 1/k' versus the micellized Brij-35 concentration. PAH's identified in Figure 1.

same for all the PAH's studied, indicating that the effect of Brij-35 concentration differs with each PAH, as indicated before. Focusing on PAH's with similar structure, for two, three, four and five aromatic rings (e.g. naphthalene, phenanthrene, chrysene and dibenzo(ah)anthracene), increasing K_2 values were obtained for increasing numbers of rings in the linear ring structure; this agrees with results reported recently for Brij-35 MLC[19]. The size of the micelle cavities must be related to the behaviour of the different PAH's.

Similarly, there is apparently no correlation between the molecular size of all PAH's and the distribution coefficient of

TABLE 2

Binding constant calculations.

	Equation (4)			Equation (5)	
PAH	K_2,*10 (M^{-1})	K_S,*10^3 (M^{-1})	P_{sw}, *10	K_2,*10 (M^{-1})	K_S,*10^3 (M^{-1})
Naphthalene	4.0	1.6	6.9	4.0	1.6
Acenaphthene	10	4.1	19	11	4.2
Phenanthrene	9.3	3.7	15	9.5	3.8
Anthracene	12	4.8	20	12	4.7
Fluoranthene	39	16	59	39	15
Pyrene	32	13	48	31	12
Chrysene	13	5.3	23	13	5.2
Benzo(a)anthracene	33	13	56	31	12
Benzo(e)pyrene	23	9.4	37	26	10
Benzo(a)pyrene	20	7.9	33	20	8.1
Benzo(ghi)perylene	26	10	42	26	10
Dibenzo(ah)anthracene	14	5.7	23	14	5.6

the solute between the stationary and aqueous phases. However, for linear structures, as is the case of the four compounds mentioned above, increasing P_{sw} values have been obtained for increasing ring number. As before, when the straight-line plots

of $V_s/(V_e-V_m)$ or 1/k' versus C_m for two PAH's are parallel, the effect of C_m is similar for them.

Free energy of transfer from water to micellar phase

Another parameter used to show interactions between the solute and the micelle is the free energy of transfer from water to micelle, defined as:

$$\Delta \mu^{o}_{t}(w/m) = -RT \, Ln(55.5 \, K_2) \qquad (6)$$

where R and T are the gas constant and the temperature, respectively[20]. These energies are usually compared with those from octanol to water, which involves mainly hydrophobic interactions described by the following equation:

$$\Delta \mu^{o}_{t}(o/w) = -RT \, Ln(55.5 \, P) \qquad (7)$$

where P is the octanol-water distribution coefficient in molarity units, which can be calculated from

$$\log P = \log P_{o/w} - \log (MW)_o \, d_w/(MW)_w \, d_o \qquad (8)$$

where $(MW)_o$, $(MW)_w$, d_o and d_w are the molecular weight and density of octanol and water, respectively, and $P_{o/w}$, which is a measure of the hydrophobic nature of a solute, is expressed in molar fractions[11,21]. The results are shown in Figure 10. A

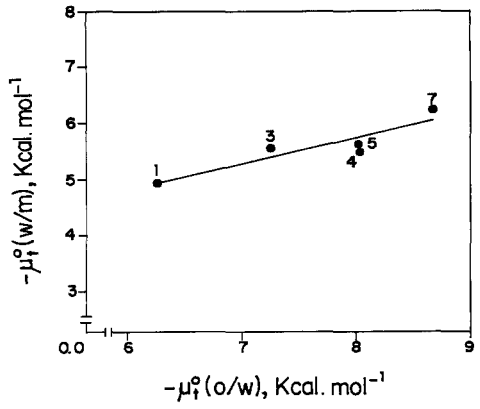


Figure 10: Variation of μ^{o}_{t}(w/m) versus μ^{o}_{t}(o/w). PAH's identified in Figure 2.

straight-line plot of μ^{o}_{t}(w/m) versus μ^{o}_{t}(o/w) was obtained. The variation of the transfer energy for octanol-water is higher than for water-micelle, whose slope is 0.46.

Correlation between binding constants and number of carbons

According to the literature[10], there is a linear relationship between the logarithm of the binding constant of the solute to micelle (log K_2) or the solute distribution coefficient between the stationary and aqueous phases (log P_{sw}) versus the number of carbons in the normal chain length; these relationships are defined by the following equations:

$$\log K_2 = a\ Nc + b \qquad (9)$$

$$\log P_{sw} = a' Nc + b' \quad (10)$$

where a is a measure of the free energy of transfer of additional carbon from the bulk solvent to the micelle, or to the stationary phase (a'); and b represents the interaction between the homologous rest with the micelle or stationary phase (b'). The results are shown in Figures 11 and 12. Clearly, plots of both log K_2 and P_{sw} versus Nc are linear for the compounds studied. The larger binding constant values for the homologous PAH's indicate the stronger interactions of the solutes with the Brij-35 micelles.

Correlation between transfer free energy from water to micelle and the number of carbons

The transfer free energy per mole of solute from water to micelle, $\mu^o_t(w/m)$, is related to the number of carbons, Nc, by the following equation[22]:

$$\Delta \mu^o_t(w/m) = Nc \Delta \mu^o_C + \Delta \mu^o_{Ar} \quad (11)$$

where the slope represents the free energy for each additional carbon and the intercept is the free energy of the rest of the aromatic structure. As shown in Figure 13, most of the PAH's studied fit a linear graph. The slope of the graph, i.e. the increase of the transfer free energy per additional carbon, is about - 90 cal mol^{-1}.

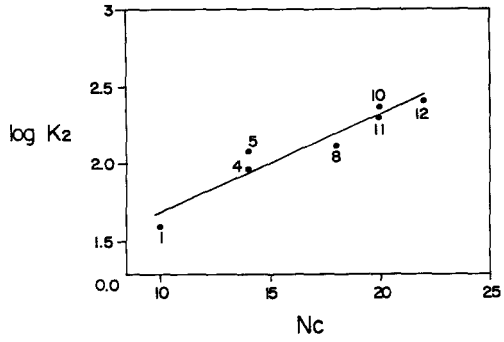


Figure 11: Correlation between binding constants and number of carbons. PAH's identified in Figure 2.

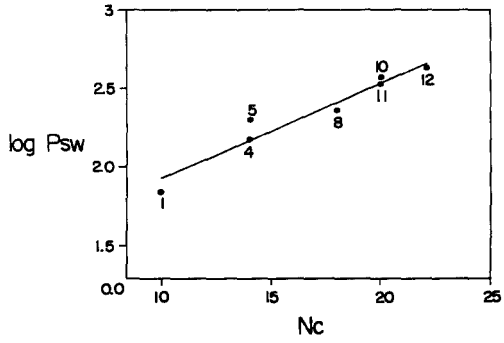


Figure 12: Correlation between distribution coefficients and number of carbons. PAH's identified in Figure 2.

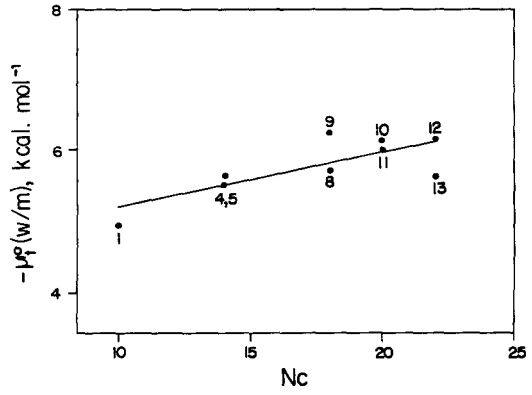


Figure 13: Correlation between transfer energy from water to micelle and the number of carbons. PAH's identified in Figure 2.

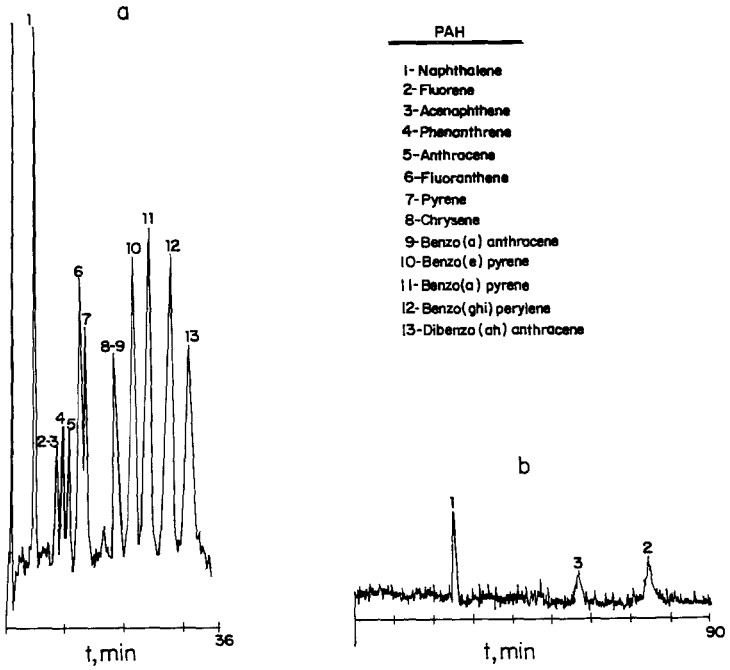
TABLE 3

Optimum experimental conditions

Variable studied	Range	Recommended
Mobile phase micellar concentration	4.10^{-4} - 10^{-1} (M)	5.10^{-2} (M)
Organic modifiers	propanol: 2 - 30% methanol: 20 - 80%	methanol: 50%
Flow-rate	0.4 - 1 (ml/min)	1 (ml/min)
Temperature	30 - 60 (ºC)	60 (ºC)
Stationary phase	C-8 and C-18 columns	C-18

Analytical considerations

The optimum experimental conditions for separating the PAHs studied are summarized in Table 3. In these conditions eleven PAH's can be separated, but only nine PAH's can be resolved to the base-line: naphthalene, fluorene or



Figures 14: Chromatograms of standard PAH's solutions. C-18 column; flow-rate, 1 ml/min; temperature, 60ºC; detection, 254 nm excitation and 375 nm emission (long-pass); mobile phase, 50% methanol/water; a, 0.05 M Brij-35; b, without Brij-35.

acenaphthene, phenanthrene or anthracene, fluoranthene or pyrene, chrysene or benzo(a)anthracene, benzo(e)pyrene, benzo(a)pyrene, benzo(ghi)perylene and dibenzo(ah)anthracene. The chromatogram is shown in Figure 14 a. The chromatogram for 50% methanol/water is also included (Figure 14 b) for comparison; it should be noted that optimum conditions are methanol/water, 85/15 (v/v); up to an analysis time of 90 minutes only naphthalene, acenaphthene and fluorene are

Table 4

Analytical characteristics

PAH	Sensitization factors*	Dynamic range, (μg/ml)	DL**, (ng/ml)	RSD, %***
Naphthalene	1	6.15 - 117	500	2.4
Fluorene	1.1	3.74 - 29.9	250	3.5
Acenaphthene	1.1	3.78 - 432	300	5.5
Phenanthrene	2.7	0.050 - 0.668	4.01	3.4
Anthracene		0.004 - 0.046	0.250	2.6
Fluoranthene	2.5	0.086 - 1.15	10.0	1.8
Pyrene	3.5	0.035 - 0.400	2.02	4.2
Chrysene	1.3	0.093 - 0.748	3.50	2.4
Benzo(a)anthracene	1.3	0.041 - 0.478	1.52	1.7
Benzo(e)pyrene	2.5	0.121 - 1.39	6.51	5.6
Benzo(a)pyrene	2.1	0.026 - 0.297	1.01	1.9
Benzo(ghi)perylene	1.9	0.092 - 1.06	3.50	1.3
Dibenzo(ah)anthracene	1.7	0.272 - 3.12	8.50	2.0

* Defined as the MLC/RPLC areas ratio
** DL = 3 S/N
*** n = 3

resolved, with retention times of 22, 57 and 76 minutes, respectively. The sensitivity of PAH's with fluorimetric detection is significantly higher than that obtained by the classical separation using a mobile phase of methanol/water 85/15, v/v. The sensitization factors defined as the area ratio MLC/RPLC are summarized in Table 4.

Other analytical characteristics are included in this table. The detection limits (DL) have been evaluated as three times the signal-noise ratio (3 S/N); the noise was defined as the peak to peak ratio. The precision, expressed as relative standard deviation (RSD), was determined from three replicates at a concentration level in the middle of the dynamic range.

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COMPARATIVE STUDY ON THE ENANTIOMERIC SEPARATION OF SEVERAL NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON TWO CELLULOSE-BASED CHIRAL STATIONARY PHASES*

A. VAN OVERBEKE[1], W. BAEYENS[1], AND C. DEWAELE[2]

[1]Laboratory of Drug Analysis
Faculty of Pharmaceutical Sciences
University of Ghent
Harelbekestraat 72, B-9000 Ghent, Belgium
[2]Bio-Rad RSL
Begoniastraat 5, B-9810 Nazareth, Belgium

ABSTRACT

Two cellulose-based chiral stationary phases were compared on their feasibility to resolve a representative number of 2-arylpropionic acids : a new experimental phase (Tolylcellulose, EXP B101) manufactured by Bio-Rad RSL and the Chiralcel OJ phase by Daicel. Both columns were tested under normal phase conditions, applying a n-hexane:isopropanol mobile phase. The Bio-Rad column was also assayed under reversed phase conditions using a methanol and perchlorate buffer system. Enantiomeric separation without prior derivatization of most 2-arylpropionic acids was far better on the Chiralcel OJ column than on the Bio-Rad column, the latter performing somewhat better under reversed phase conditions. Prior derivatization of the carboxylic acid group with an amine (naphthylmethylamine, (2-methyl)benzylamine) or an alcohol (benzylalcohol) permitted to separate the tested drugs to different extents on both columns.

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INTRODUCTION

2-Arylpropionic acids (2-APAs) are a group of non steroidal anti inflammatory (NSAI) drugs that are characterised by a chiral carbon atom adjacent to the carboxylic acid moiety. With the exceptions of naproxen and flunoxaprofen, they are marketed as racemic compounds and widely prescribed in relief of acute and chronic rheumatoid arthritis and osteoarthritis.

Numerous chiral stationary phases (CSPs) for the liquid chromatographic discrimination of these frequently used pharmaceuticals are described in literature [1-2]. Various protein columns with excellent selectivity towards underivatised 2-APAs have been developed and commercialised [3-4]. Efficient separations were afforded by immobilised phases of Human and Bovine Serum Albumin [5-6], α_1- Acid Glycoprotein [7-11], Ovomucoïd [12-14] and Avidine [15-16].

The first commercially available "brush type" phases for HPLC, designed by the groups of Pirkle (CSP1, DNB-phenylglycine derivatives) [17-24,53] and Ôi [19-20], permitted a separation of profens only after derivatization of the carboxylic acid moiety, necessary to meet the requirements for stereoselective interaction. Several other versions have been created, still asking for a pre-column derivatization of the 2-APAs with an amine or an alcohol [27-32]. An extensive overview of the numerous phases designed by Pirkle and his group has recently been published [33] and includes the successfully improved Pirkle concept that separates the profen family into their enantiomers as such [34-37].

Interesting results have also been obtained using diverse modified and immobilised cyclodextrins [38-41].

The possibilities offered by different derivatised cellulose columns to discriminate the enantiomers of 2-arylpropionic acids and many other classes of drugs have extensively been examined by many researchers. A variety of derivatised cellulose phases have been synthesised since the successful introduction of the cellulose triacetate phases [38-46]. Pioneering studies in the development of cellulose columns were performed by Okamoto and his group [47-51]. Many of these chiral stationary phases have been commercialised by Daicel (Tokyo, Japan) [52].

Carbamate derivatives and tris(3,5-dimethylphenylcarbamate) cellulose in particular (Chiralcel OD), offer many possibilities for enantioselective interactions and have proved to be efficient in separating most profen enantiomers [53-55]. For some 2-APAs, chiral resolution could only be obtained after derivatization of the carboxylic acid moiety. Among

the ester cellulose derivatives coated on varied silica gel supports, the methylbenzoate CSPs have also found many applications [56-60]. These polymers have likewise proved their enantioselective capabilities configured as pure beads offering a much higher loadability [61-64].

A new experimental cellulose-based CSP, called Tolylcellulose column, manufactured by Bio-Rad RSL (Nazareth, Belgium) contains a similar tris(4-methylbenzoate) cellulose polymer as the Chiralcel OJ phase (Daicel, Tokyo, Japan) and has already been examined on its enantioselectivity towards several NSAI drugs [65-66]. In order to obtain an acceptable chiral separation for most analytes, a derivatization of the carboxylic group was necessary. Due to the fact that the cellulose layer is bound onto the modified silica support rather than being adsorbed as is the case for the Chiralcel OJ column, this CSP was used under reversed phase conditions.

The mobile phase recommended for analyses with the Chiralcel OJ column consists mainly of a mixture of n-hexane and an alcoholic modifier, preferably isopropanol. Pure ethanol however can also be applied and even aqueous acetonitrile has been tested [67]. Its analogue for reversed phase conditions, similar as the Chiralcel OD-R column, has not been commercialised yet.

In this paper the chiral discriminative properties towards ten 2-arylpropionic acids (Fig. 1) on a Chiralcel OJ column were compared with the experimental Tolylcellulose phase using a mobile phase of n-hexane:isopropanol:acetic acid. The same Tolylcellulose column was also used with a methanol:perchlorate buffer system. As only poor or no resolution of the free acids was obtained on this CSP [65-66], several derivatives (amides and esters) were prepared and analysed on both columns.

EXPERIMENTAL

Chemicals

Benzylamine, 1-naphthylmethylamine, 2-methylbenzylamine, 1-ethyl-3-dimethylamino-propyl-carbodiimide, 1-hydroxybenzotriazole, *rac*-2-phenylpropionic acid and *rac*-ketoprofen were purchased from Sigma-Aldrich (Bornem, Belgium); benzylalcohol from UCB (Leuven, Belgium). *Rac*-carprofen was a kind gift of Produits Roche (Brussels,

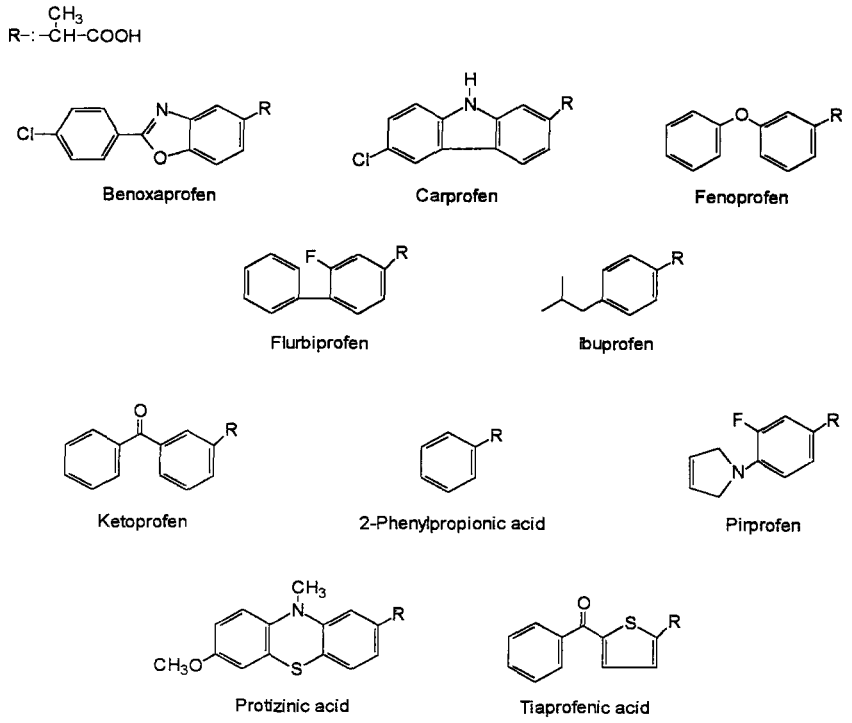

FIGURE 1. Chemical structures of the molecules under investigation

Belgium), *rac*-flurbiprofen of Upjohn Co. (Kalamazoo, MI, USA), *rac*-pirprofen of Ciba-Geigy (Groot-Bijgaarden, Belgium), *rac*-calcium fenoprofen of Eli Lilly Co. (Indianapolis, IN, USA), *rac*-protizinic acid of Rhône-Poulenc Rorer (Brussels, Belgium) and *rac*-tiaprofenic acid of Erfa (Brussels, Belgium). *Rac*-benoxaprofen was obtained from Eli Lilly Co. (Windelsham, UK) before withdrawal from the market and *rac*-ibuprofen from Profarma (Oud-Turnhout, Belgium).

Sodium-perchlorate (Merck, Darmstadt, Germany) perchloric acid 70 % aqueous solution (UCB, Leuven, Belgium), methanol (Labscan, Dublin, Ireland) and dichloro-

methane (UCB, Brussels, Belgium) were all of analytical grade. n-Hexane and isopropanol (HPLC-quality) were from J.T. Baker (The Netherlands). Deionised water was used throughout.

Apparatus

Chromatography was performed with a Varian 9010 SDS pump (Varian Associates Inc., Walnut Creek, CA, USA) using a Rheodyne injector with a 20 µl loop. Detection was achieved at two wavelengths simultaneously (230 and 254 nm) with a Hewlett Packard 1050 DAD (Waldbronn, Germany). Integration of the more intense chromatogram was made with the Hewlett Packard software package (1990). The following parameters were measured:

k'1 : capacity factor of the first eluted enantiomer : $(t_1-t_0) / t_0$.

k'2 : capacity factor of the second eluted enantiomer : $(t_2-t_0) / t_0$.
Tri-*t*-butylbenzene was used to determine the t_0-value under normal phase conditions on both columns [68]. As it was retained on the Tolylcellulose column under reversed phase conditions, the elution time of methanol was used as t_0-value.

α : selectivity factor : k'2 / k'1.

Rs : resolution factor : $Rs = 1.18 (t_2-t_1) / (w_1+w_2)$; w is the width at half-height of the peak based on peak area and height.

Rp : Kaiser's peak separation index : the ratio of mean valley height between two peaks and the mean peak height, which rises to 1 for perfectly separated peaks. This factor is therefore measured of enantiomeric peaks that were not baseline separated.

Chromatographic conditions

The columns under investigation (250 mm x 4.6 mm ID) are both characterised by a tris (4-methylbenzoate) cellulose layer (Fig. 2). In case of the Tolylcellulose column, EXP B101 (Bio-Rad RSL, Nazareth, Belgium) the polymer is covalently bound onto a 10 µm aminopropylsilinazed silica gel with mean pore size of 300 Å; coverage is about 10 %.

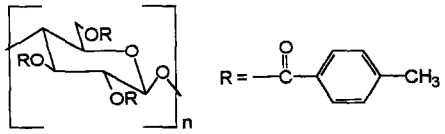

FIGURE 2. Chemical structure of the tris (4-methylbenzoate) cellulose layer

This phase has been compared with a commercially available Chiralcel OJ column (Daicel Chemical Industries, Tokyo, Japan, purchased from J.T. Baker, The Netherlands), adsorbed onto macroporous (1000 Å) 10 μm aminopropylsilinazed silica gel. The mobile phase for normal phase (NP) conditions consisted of n-hexane and isopropanol with 0.5 % (V/V) acetic acid (HAc), applied on both columns. The Tolylcellulose column was also used with methanol and perchlorate buffer 0.1 M pH 2.0 (Reversed phase, RP). The eluents were mixed in varying ratios and ultrasonicated and degassed before they were pumped at a flow rate of 1 $ml.min^{-1}$. For preparation of the buffer solution, 14.05 g sodium perchlorate was dissolved in water and after pH adjustment with a concentrated perchloric acid solution, water was added up to 1 litre. Chromatography was carried out at ambient temperature.

Derivatization procedure

Ester and amide derivatives were formed using 1-ethyl-3-dimethylaminopropyl-carbodiimide (EDC) as coupling agent in combination with 1-hydroxybenzotriazole (HOBT). The amines used were 1-naphthylmethylamine and benzylamine as applied in former studies [28-29] and 2-methylbenzylamine. Esters were formed with benzylalcohol.

Taking the derivatization of ibuprofen with benzylamine as an example, the given recipe was followed

To 1 ml of a solution of ibuprofen (1.0 $mg.ml^{-1}$ dichloromethane) were added HOBT (300 μl of a 1.0 $mg.ml^{-1}$ dichloromethane solution containing 1 % pyridine), EDC (300 μl of a 11 $mg.ml^{-1}$ dichloromethane solution) and benzylamine (300 μl of a 2.6 $mg.ml^{-1}$ dichloromethane solution). The mixture was vortexed and left for about 1.5 h. The

dichloromethane layer was evaporated to dryness under a stream of nitrogen and the residue was taken into 5.0 ml isopropanol (NP) or methanol (RP).

Solutions of other acids and amines or alcohol were prepared relative to their molecular weight. About 1.5 molar amounts of amine or alcohol are used relative to acid. Calcium fenoprofen was converted to its acid form prior to derivatization by an acid extraction.

RESULTS AND DISCUSSION

Although a tris(4-methylbenzoate) cellulose derivative forms the chiral layer of both columns in the present study, the stereoselective properties towards the tested analytes differ tremendously. A major difference between the two applied CSPs is the way the polymeric layer is coated onto its silica support, covalently in case of the Tolylcellulose and via adsorption for the Chiralcel phase. Differences in the solvents used in the synthesising process, washing steps, packing procedures, etc. may certainly be of influence as well on the stereoselective characteristics of the resulting CSP.

Enantiomeric separation of free 2-arylpropionic acids

The necessity to derivatise the carboxylic moiety in order to obtain a chiral discrimination of the 2-APAs on the Tolylcellulose column as observed in former experiments [65-66], was demonstrated not to be required for some analytes on the Chiralcel OJ column. An enantiomeric resolution of several (underivatised) 2-APAs using the Chiralcel OJ column has been described in literature, of ketoprofen and fenoprofen [52], of 2-(10,11-dihydro-10-oxodibenzo-[*b,f*]thiepin-2-yl)propionic acid [69], and of a methylester derivative of flurbiprofen [52]. Of all analytes considered in this paper, only flurbiprofen and 2-phenylpropionic acid could not be resolved on the Chiralcel OJ column. Table 1 summarises the results using n-hexane:isopropanol:HAc (80:20:0.5) as mobile phase (Fig. 3). Upon decrease of the isopropanol portion, resolution increases at the expense of higher capacity factors.

As mentioned before, the chiral discriminative capability for underivatised acids on the Tolylcellulose column turned out to be far worse. The mobile phase, as an essential and dynamic part of the chiral system, modifies the enantioselective properties substantially. Under normal phase conditions, no enantiomeric separation was obtained. The capacity

TABLE 1. Resolution of Underivatised Acids on Chiralcel OJ Column

analyte	k'1	α	Rs	Rp
ibuprofen	0.27	1.14	0.67	0.27
ketoprofen	1.65	1.35	3.04	>1
flurbiprofen	1.16	1.00	0.00	0.00
tiaprofenic acid	2.69	1.06	0.65	0.23
fenoprofen	1.23	1.23	2.02	0.99
2-phenylpropionic acid	1.42	1.00	0.00	0.00
pirprofen	1.04	1.33	2.62	>1
protizinic acid	4.09	1.25	2.31	>1
carprofen	5.85	1.38	3.55	>1
benoxaprofen	2.86	1.13	1.17	0.83

Mobile phase : n-hexane:isopropanol:HAc 80:20:0.05

factors were a multiple fold of those obtained on Chiralcel OJ. Under reversed phase conditions, using methanol:perchlorate buffer 0.1 M, pH 2 mixtures, some of the free acids were only slightly resolved into their enantiomers on Tolylcellulose.

By increasing the buffer fraction of the mobile phase, the acids were retained longer. If a mobile phase with e. g. 40 % buffer was applied, ibuprofen (Rp=0.36) and tiaprofenic acid (Rp=0.38) were enantiomerically resolved as well to a small extent. Piketoprofen, an amide of ketoprofen with 2-amino, 4-methylpyridine (Almirall, Barcelona, Spain) was partially resolved on the Tolylcellulose column under normal phase conditions only (n-hexane:isopropanol 95:5; k'1=6.30, Rp=0.38).

Enantiomeric separation of derivatives of 2-arylpropionic acids

As no acceptable resolution was obtained for the free acids on the Tolylcellulose column, the carboxylic acid group was derivatised to improve possible stereoselective interactions with the CSP. Amides were formed with 1-naphthylmethylamine, 2-methylbenzylamine and benzylamine; esters were formed with benzylalcohol. These four series of derivatives were chromatographed on the Chiralcel OJ column and on the Tolylcellulose column, the latter in NP and RP.

Comments that hold for all ten of the studied acids, cannot easily be provided. It was obvious that if the free acids eluted at higher retention times, their derivatives showed the same relative behaviour. This may indicate that the cyclic moiety on the chiral carbon atom

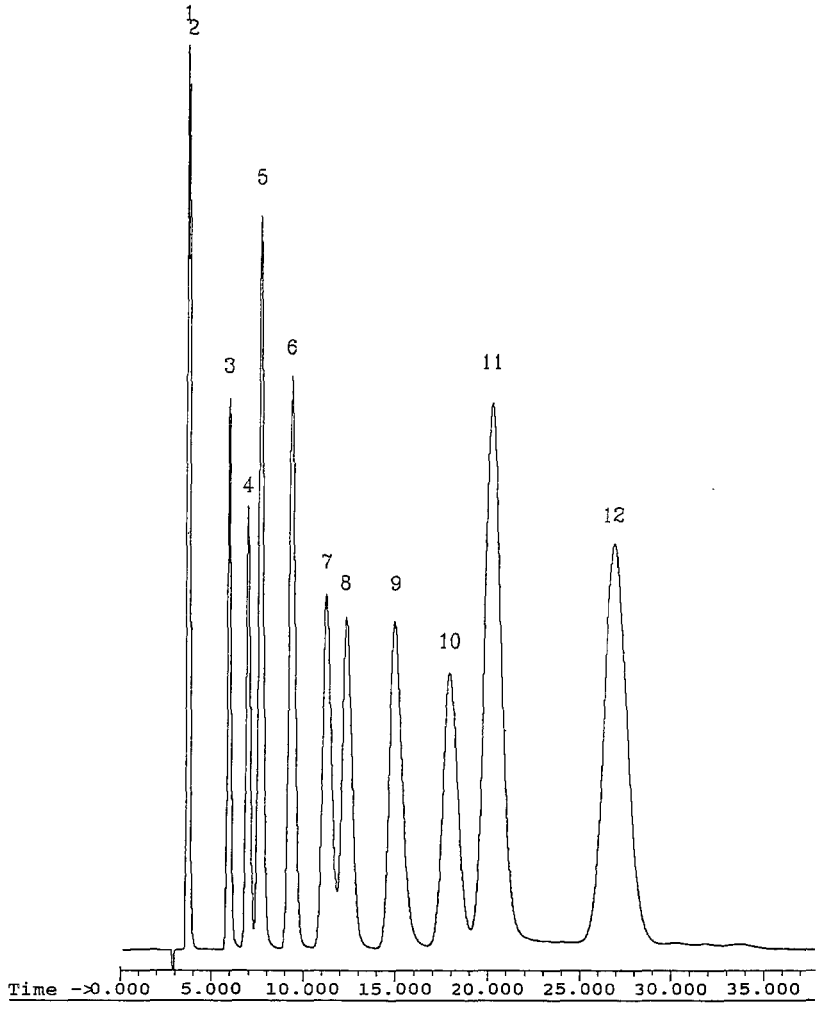

FIGURE 3. Mixture in isopropanol of 0.5 mg.ml^{-1} ibuprofen (1,2), 0.5 mg.ml^{-1} pirprofen (3,4), 0.5 mg.ml^{-1} ketoprofen (5,6), 0.5 mg.ml^{-1} benoxaprofen (7,8), 0.5 mg.ml^{-1} protizinic acid (9,10), 0.3 mg.ml^{-1} carprofen (11,12). Mobile phase: n-hexane:isopropanol:acetic acid 80:20:0.5. Detection wavelength: 230 nm.

TABLE 2. Resolution of Underivatised Acids on Tolylcellulose Column

analyte	k'1	α	Rs	Rp
flurbiprofen	1.24	1.10	0.61	0.25
protizinic acid	3.15	1.05	0.43	0.06
carprofen	1.85	1.07	0.52	0.11
benoxaprofen	2.24	1.10	0.68	0.28

Mobile phase : methanol:perchlorate buffer 0.1 M, pH 2 80:20

interacts, e.g. via inclusion phenomenon, with the stationary phase. On the other hand, a good resolution of the free acid does always not imply good chiral discrimination of the derivatives.

As for the *1-naphthylmethylamine derivatives,* the best results were obtained using the Tolylcellulose column under reversed phase conditions. All the samples under investigation with the exception of the ketoprofen derivative were separated yielding a resolution Rs of at least 0.8.

Under normal phase conditions, better results were obtained for Chiralcel OJ. However, only half of the investigated analytes could be enantiomerically discriminated, the obtained resolutions on the other hand were mostly better than in RP on Tolylcellulose. The bicyclic amides had higher capacity factors than the *(2-methyl)benzylamine derivatives.* Most NSAI drugs showed stereospecific interaction following derivatization with these smaller amines. The addition of a methyl group on benzylamine was thought to improve the stereoselective interactions with the cellulose layer. As a weak electron donating group, the methyl function may increase the electron density of the aromatic ring (promoting $\pi-\pi-$ interactions with the methylbenzoate group of the CSP) and favour hydrogen bonding interaction with the ester group of the CSP, thus contributing to the electron negativity of the carbonyl group or the proton character on the amine moiety. A consistent improvement however of the separations induced by the o-substitution on the aromatic amine moiety was obvious for the Tolylcellulose column only; for the Chiralcel OJ no clear preference could be assigned to either of the series. More recent studies in our lab on other derivatives using the Tolylcellulose phase have shown that a methyl group in *meta-* or *para*-position leads to a decrease in resolution versus *ortho*-substitution.

The ester analogues of the benzylamide derivatives were formed with *benzylalcohol,* having no capabilities for hydrogen bonding interactions. In most cases, better separations were obtained with the amide analogues. The retention times of the ester derivatives were

TABLE 3.a. Under the applied normal phase conditions, *ibuprofen* turned out to be a too small a molecule for its derivatives to be sufficiently retained on either column. Consequently, best chiral separations were noted following derivatization with a naphthylgroup. (ND because of overlapping with blank peaks)

	naphthylMeamide			benzylamide			2-Mebenzylamide			benzylester		
MP	k'1	α	R	k'1	α	R	k'1	α	R	k'1	α	R
COJ	0.84	1.36	0.95*	ND	-	-	ND	-	-	ND	-	-
TNP	2.98	1.22	0.91*	ND	-	-	ND	-	-	ND	-	-
TRP	1.31	1.53	2.36	4.07	1.33	1.95	1.07	1.47	1.97	4.41	1.14	0.86*

TABLE 3.b. *Ketoprofen* enantiomers were better resolved as amide than as ester derivatives. The Tolylcellulose column could not separate the 1-naphthylmethylamide nor the ester derivatives.

	naphthylMeamide			benzylamide			2-Mebenzylamide			benzylester		
MP	k'1	α	R	k'1	α	R	k'1	α	R	k'1	α	R
COJ	8.94	2.15	3.95	2.31	1.86	4.46	1.92	1.43	0.97*	3.52	1.07	0.34*
TNP	2.75	1	0	4.97	1.20	0.98*	4.49	1.34	2.03	2.18	1	0
TRP	3.48	1	0	5.85	1.41	2.30	1.80	1.80	3.01	3.21	1	0

TABLE 3.c. *Flurbiprofen* enantiomers could not be separated as such on the Chiralcel OJ column, but were easily resolved after derivatization. In NP, a better resolution was obtained for ester derivatives; in RP, derivatization into amides was preferred.

	naphthylMeamide			benzylamide			2-Mebenzylamide			benzylester		
MP	k'1	α	R	k'1	α	R	k'1	α	R	k'1	α	R
COJ	3.64	1.46	1.67	2.30	1.33	2.21	2.08	1.37	0.99*	4.34	1.37	3.17
TNP	2.51	1.15	0.39*	4.41	1.17	0.96*	4.21	1.23	0.98*	1.42	1.21	0.98*
TRP	6.89	1.10	0.32*	3.22	1.23	0.98*	4.15	1.45	2.52	9.94	1.13	0.91*

TABLE 3.d. *Tiaprofenic acid* enantiomers were easily separated after derivatization, amides performing generally better than esters.

	naphthylMeamide			benzylamide			2-Mebenzylamide			benzylester		
MP	k'1	α	R	k'1	α	R	k'1	α	R	k'1	α	R
COJ	5.98	1.70	2.72	4.06	1.40	2.71	3.69	1.51	2.60	6.34	1.46	3.78
TNP	4.32	1.41	1.85	3.60	1.20	0.95*	9.81	1.33	2.32	4.31	1.25	2.21
TRP	3.73	1.43	2.27	1.82	1.19	0.87*	2.30	1.43	2.21	4.73	1.17	0.96*

TABLE 3.e. *Fenoprofen* enantiomers were easily separated after derivatization. Under NP conditions ester derivatives were not resolved.

	naphthylMeamide			benzylamide			2-Mebenzylamide			benzylester		
MP	k'1	α	R	k'1	α	R	k'1	α	R	k'1	α	R
COJ	3.74	2.60	4.62*	1.83	1.58	3.78	1.61	1.50	2.51	2.96	1	0
TNP	2.09	1	0	1.39	1.16	0.77*	2.92	1.33	2.28	0.95	1	0
TRP	4.46	1.20	0.91*	1.88	1.33	1.88	2.42	1.88	3.73	4.50	1.12	0.74*

(continued)

TABLE 3.f. *2-Phenylpropionic acid* probably is too small an acid to bring about an efficient interaction with the cellulose polymer. On the Tolylcellulose column, only derivatives of 1-naphthylmethylamine were partially resolved. On the Chiralcel OJ phase, all other derivatives were poorly chirally discriminated.

	naphthylMeamide			benzylamide			2-Mebenzylamide			benzylester		
MP	k'1	α	R	k'1	α	R	k'1	α	R	k'1	α	R
COJ	1.14	1	0	1.25	1.09	0.29*	1.05	1.14	0.47*	2.32	1.08	0.61*
TNP	8.68	1.11	0.74*	2.16	1	0	1.88	1	0	ND	-	-
TRP	1.06	1.78	0.72*	ND	-	-	0.74	1	0	1.73	1	0

TABLE 3.g. *Pirprofen* enantiomers could be separated after derivatization with an amine or an alcohol. Some analyses however suffered from interferences of impurities or degradation products present in the acid samples.

	naphthylMeamide			benzylamide			2-Mebenzylamide			benzylester		
MP	k'1	α	R	k'1	α	R	k'1	α	R	k'1	α	R
COJ	2.44	1.20	0.64*	1.81	1.38	0.98*	1.70	1.35	0.98*	2.75	1.35	3.15
TNP	2.10	1.07	0.07*	1.79	1.11	0.34*	3.75	1.17	0.93*	1.26	1.12	0.66*
TRP	3.73	1.22	0.96*	2.10	1.27	1.43	2.58	1.32	0.95*	5.97	1.16	0.95*

TABLE 3.h. *Protizinic acid* enantiomers were most efficiently separated as free acid on the Chiralcel OJ column. Generally, no chiral discrimination was obtained for amide derivatives, ester derivatives were only partially resolved.

	naphthylMeamide			benzylamide			2-Mebenzylamide			benzylester		
MP	k'1	α	R	k'1	α	R	k'1	α	R	k'1	α	R
COJ	7.45	1	0	8.11	1	0	7.81	1	0	10.9	1.21	0.97*
TNP	6.40	1	0	15.6	1.10	0.53*	5.55	1	0	5.39	1.13	0.77*
TRP	13.0	1.14	0.56*	9.50	1	0	12.5	1	0	22.1	1.07	0.19*

TABLE 3.i. The best separation for *carprofen* was obtained on the Chiralcel OJ column as free acid. Chiral discrimination was generally better for ester than for amide derivatives on the Tolylcellulose phase. (ND: not detected within an analysis time of 2 hours)

	naphthylMeamide			benzylamide			2-Mebenzylamide			benzylester		
MP	k'1	α	R	k'1	α	R	k'1	α	R	k'1	α	R
COJ	ND	-	-	3.31	1.31	0.91*	3.46	1	0	5.63	1	0
TNP	3.67	1	0	10.5	1.05	0.09*	9.44	1.08	0.29*	7.75	1.13	0.90*
TRP	8.17	1.22	0.89*	6.57	1.18	0.87*	9.00	1	0	10.1	1.21	0.99*

TABLE 3.j. *Benoxaprofen* enantiomers could be largely baseline separated after derivatization with an amine. Esters were significantly worse resolved. (ND: not detected within an analysis time of 2 hours)

	naphthylMeamide			benzylamide			2-Mebenzylamide			benzylester		
MP	k'1	α	R	k'1	α	R	k'1	α	R	k'1	α	R
COJ	ND	-	-	5.72	2.66	7.11	4.95	2.64	4.24	8.82	1.12	0.77*
TNP	4.68	2.09	3.08	2.17	1.63	3.03	8.97	2.21	5.06	3.21	1.11	0.63*
TRP	16.1	2.81	7.01	9.03	3.01	7.68	10.5	4.04	8.80	18.3	1	0

1.5 to 2 times higher on the Chiralcel OJ and the RP-Tolylcellulose columns. In the case of Tolylcellulose column in NP, amide and ester derivatives interacted to similar extents, consistently worse than under RP conditions. This may indicate that for the Tolylcellulose phase, hydrogen bonding interactions are favoured using an aqueous mobile phase. This assumption may also be confirmed by former findings that the use of acetonitrile instead of methanol causes a faster elution of the amide derivatives with loss of resolution that could partly be compensated by increasing the buffer portion of the mobile phase [66]. The mobile phase modifies the CSP at both achiral and chiral sites. It functions as a dynamic part of the total chiral system and competes for interaction locations with the analytes.

Table 3 (subdivided for each individual acid) summarises the obtained resolutions for the four series of derivatives of the ten acids under various conditions on both columns. For the resolution R, either Rs or Rp* is given. (COJ stands for the Chiralcel OJ column, TNP for the Tolylcellulose column, both used with n-hexane:isopropanol:acetic acid, TRP for the Tolylcellulose column used with methanol:perchlorate buffer 0.1 M, pH 2). Plate numbers of the enantiomeric peaks eluting at comparable retention times, were of the same order for the Chiralcel OJ and the Tolylcellulose column in RP. Plate numbers were often slightly worse for Tolylcellulose in NP.

This selection of chromatographic results shows that there is a derivative to be formed of each acid that interacts stereoselectivily with tris(4-methylbenzoate)cellulose stationary phases.

CONCLUSIONS

In general, the feasibility of chiral separation of the investigated group of NSAI drugs turned out in favour of the Chiralcel OJ column as more 2-arylpropionic acids could be resolved without prior derivatization. The tested Tolylcellulose phase however has the advantage that it can be used under reversed phase conditions without significant deterioration of the phase over several months of experiments. Derivatization of the carboxylic acid moiety accounts for an improved resolution or an increased absorbance. Further experiments on derivatives with mono-, bi- and tricyclic amines and alcohols are currently under investigation on the Tolylcellulose column with promising results.

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DETERMINATION OF NITRATE AND NITRITE IN TAP WATER AND VEGETABLES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

D. BLANCO[1], L. MARTINEZ[1], J. J. MANGAS[2], E. DAPENA[2], AND D. GUTIERREZ[1]

[1]*Departamento de Química Física y Analítica*
Universidad de Oviedo, 33006
Oviedo, Spain

[2]*Instituto de Experimentación y Promoción Agraria*
Villaviciosa, Asturias, Spain

ABSTRACT

A method is presented for the simultaneous determination of nitrate and nitrite by high performance liquid chromatography (HPLC) with an anion exchange (Partisil SAX) column, a 0.030 M potassium dihydrogen orthophosphate/phosphoric acid buffer of pH 3.5 mobile phase and UV detector. Without using a preconcentration system the detection limits are 0.2 ng for nitrate and 1 ng for nitrite. A suitable extraction procedure has been established for its application to the analysis of tap water, lettuce and apple tree leaves. The reproducibility of the method, calculated as the relative standard deviations in the optimum range, is always less than 2% for nitrates and 5% for nitrites.

INTRODUCTION

The abundant use of chemical fertilizers, animal manure, compost, etc. in agriculture and horticulture has to be considered as the most important reason for the presence of

high concentrations of nitrates in vegetables (1). On one hand, these high nitrogen contents in leaves of apple trees (2) can rise the susceptibility to various parasites such as scab (*Venturia inaequelis* (Cke.) Wint.), canker (*Nectria galligena* Bres.) and european spider red mite (*Panonychus ulmi* Koch.) (3).

On the other hand, it is generally accepted that, except for the possible production of changes in the thyroid, nitrate itself is not toxic. However, nitrate can be reduced to nitrite, most importantly by bacterial action during the storage of nitrate containing food (4) or in the human body (5) during digestion.

Nitrite can then react under certain conditions with secondary and tertiary amines and amides to form the N-nitroso compounds (6), most of which so far tested in laboratory animals have been found to be carcinogenic. Also, nitrite formed by the reduction of nitrate can react with haemoglobin to form methaemoglobin which impairs the capacity of the bloodstream to carry oxygen (7). Problems of this nature have occurred very occasionally in babies.

There are many approaches to nitrate and nitrite determination: spectrophotometric techniques and potentiometry using a nitrate selective electrode are widely used (8-12). These methods, however, do not allow the simultaneous determination of both anions and have limitations in terms of detection limits and interference by a variety of ions. Gas chromatography after derivatization can also be used in nitrate determination (13).

Several authors have reported successful nitrate analysis by ion chromatography (14-16) using an anion-exchange column and conductivity detector. The precision, except for some exceptions (17), is limited.

Anion-exchange high performance liquid chromatography together with an UV detector is also able to determine nitrate and nitrite in one step, without derivatization (18-20).

The objective of the present work was to develop an alternative method for the simultaneous determination of nitrate and nitrite so as to establish a suitable extraction procedure for its application to the analysis of tap-water, lettuce and apple tree leaves.

EXPERIMENTAL

Chemicals

Potassium nitrate and sodium nitrite were purchased from Sigma (St Louis, MO, U.S.A.). Potassium dihydrogen phosphate and all other chemicals used to prepare buffer or samples were analytical reagent grade and supplied by Merck (Darmstad, Germany). The methanol used was HPLC grade and was employed as supplied by Romil (Loughborough, Leics, U.K.). High purity water was produced with a Millipore Milli-Q system (Mildford, MA, U.S.A.).

Equipment

A Hewlett-Packard HPLC system was used, comprising of a HP 1090 pump, a Hewlett Packard 79881A filter photometric detector, a HP 85B personal computer, a HP 3390A integrator and a Rheodyne 7010 injection valve with a 20 µL sample loop.

The stainless steel column used was a Partisil SAX (250x4.6 mm i.d., 10 µm).

An ultrasonic bath, a vibromatic stirrer and a centrifuge (all three from Selecta, Spain) were used.

For pH measurements, a PW 9422 Philips pHmeter equipped with a combined glass-Ag/AgCl electrode was employed.

Chromatographic conditions

The mobile phase was 0.030M potassium dihydrogen orthophosphate/phosphoric acid buffer of pH 3.5 and was

pumped at a flow-rate of 1.5 mL/min. Before being used the solution was vacuum-filtered through a 0.22 µm Nylon filter and degassed with helium.

Detection was performed at 210 nm. The chromatographic experiments were carried out at room temperature (20±2°C).

Standard solutions

Individual stock solutions of nitrate and nitrite were prepared in ultrapurified water to provide a concentration of 100 ppm. For the nitrite solution it was necessary to add 5 mL of NH_4Cl/NH_3 0.2M buffer of pH 9 to avoid its oxidation. These solutions were stored below 4°C.

Standard solutions were prepared by appropiate dilution of the stock solution, always maintaining the basicity required, and filtered through a 0.45 µm membrane (Millex-HV_{13}, Millipore) before being injected into the system.

Sample treatment

Tap-water can be injected directly after removing particulate matter with a Millex filter. For lettuce extracts, the alkaline extraction method described by Sen and Donaldson (21), with some modifications in the reactive concentration, was used: 50 g of several inside and outside leaves of lettuce were washed and cut in pieces less than 1 cm. This sample was homogenized without solvents in a Polytron mixer for 5 minutes. 1 g of a representative sample was then diluted and extracted with 30 mL water and 0.5 mL 10% NaOH. The resulting mixture was allowed to stand for 15 minutes at 50-60°C before adding 4.5 mL NaOH and 6 mL 30% $ZnSO_4$ (Carrez II), the slurry being kept another 15 minutes at 50-60°C. The mixture was cooled to room temperature, centrifuged, washing twice with alkaline water and diluted to 100 mL water. Extracts were filtered before analysis through a 0.22 µm filter. This step is very important for preventing the column from clogging.

For the apple tree leaves, the method described by Schuster and Lee (22), with some modification, was used: 0.5 g of liofilized leaves was diluted and extracted with 10 mL water, 20 mL 2% borax, 5 mL 15% $K_4Fe(CN)_6$ (Carrez I) and 5 mL 30% $ZnSO_4$ (Carrez II). The resulting mixture was heated and centrifuged as above and diluted to 50 mL water. An aliquot was filtered through a 0.22 µm filter and cleaned-up through a solid-phase extraction C_{18} cartridge used to retain several interference compounds. It was then injected into the chromatographic system.

RESULTS

Standards

The relative retention times of the investigated anions are dependent on pH and eluent concentration.

The results obtained (Figure 1.a) show that the capacity factor, k', decreases when the ionic strength increases due to the competitiveness between the ions used to establish the ionic strength and the nitrate and nitrite anions. Figure 1.b shows the variation of the retention with pH. As can be seen, a rise in pH causes an increase in the nitrite capacity factor, because the anion nitrite concentration increases (pK_a = 3.4) with pH.

The optimum mobile phase flow-rate, tested between 0.8 and 1.8 mL/min, was found to be 1.5 mL/min.

An optimal resolution in all cases allows us to choose a concentration of 0.030M KH_2PO_4 and pH 3.5 in order to avoid any overlapping of the nitrite peak with the elution front that presumably could appear in some vegetable samples, without prolonging the analysis time, since both anions are eluted in less that 8 minutes.

Figure 2 shows a chromatogram of a standard containing both ions. With this system, the detection limits for both

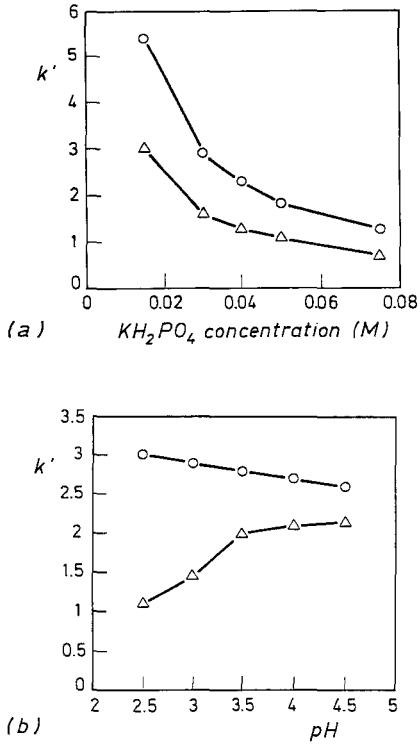

Figure 1:

Capacity factor variation of nitrate and nitrite with: a) ionic strength and b) pH. Column: Partisil SAX (250x4.6 mm i.d., 10 µm). Mobile phase: 0.03M KH_2PO_4/H_3PO_4, pH 3.5. Flow-rate: 1.5 $mL.min^{-1}$. $\lambda_{det.}$ = 210 nm.

ions - based on a signal-to-noise of 3:1 - were 0.2 ng for nitrate and 1 ng for nitrite.

Samples

Examples of the application of the described procedure for the determination of nitrate and nitrite in samples of

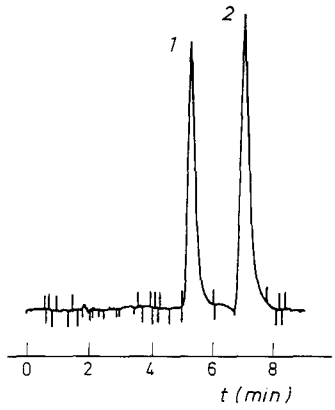

Figure 2:

Chromatogram of a standard solution containing 2 ppm of each anion. Column: Partisil SAX (250x4.6 mm i.d., 10 µm). Mobile phase: 0.03M KH_2PO_4/H_3PO_4, pH 3.5. Flow-rate: 1.5 mL.min^{-1}. $\lambda_{det.}$ = 210 nm. Peaks: 1) NO_2^-, 2) NO_3^-.

tap-water, lettuce and apple tree leaves are shown in Figure 3. The samples peaks were identified by comparing the relative retention times of each one with those of the standard reference anions. Nitrite could not be detected in the analysed lettuce extracts nor the tap-water.

The quantification of nitrate and nitrite was achieved by using the external standard method. Standards were injected and the integrator response factors were computed. These were then stored in the integrator for the computation of unknown concentrations. Injection volumes of 20 µL were employed for all quantitative evaluations and the amount of anions was directly obtained from the data module. The calibration curves constructed from the peak area versus anion concentrations were linear (r = 0.9999) from the determination limit to at least 80 ppm for NO_3^- and 40 ppm for NO_2^-. Recalibration was performed regularly.

To study the accuracy of the method, recovery experiments were performed. Known amount of each anion were

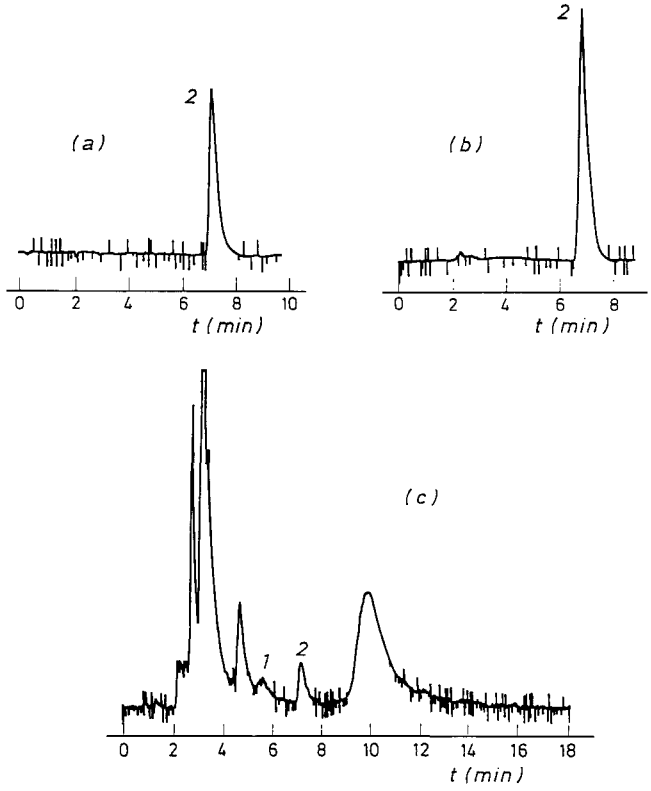

Figure 3:

Typical chromatograms of a) tap-water, b) lettuce extract and c) apple tree leaves extract. Column and chromatographic conditions as in Figure 2. For peak identification see also Figure 2.

added to a variety of samples and the resulting spiked samples were subjected to the entire analytical sequence. Each solute was spiked at three different concentrations and recoveries were calculated based on the difference between the total amount determined in the spiked samples and the amount observed in the non-spiked samples. All analyses were carried out in triplicate. The recoveries obtained for tap-

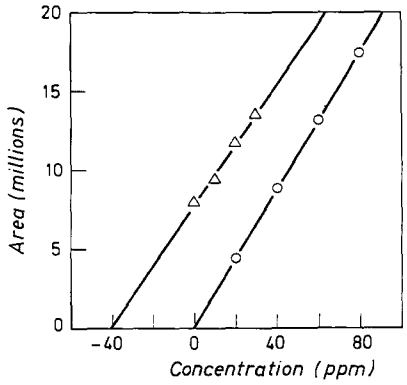

Figure 4:

Calibration graph with standards and the standard additions graph with a lettuce sample for NO_3^-.

TABLE 1

Sample	Nitrate	Nitrite
Tap-water (mg/L)	2.58±0.01	n.d.
Lettuce (mg/Kg fresh weight)	4.100±300	n.d.
Apple tree leaves (mg/Kg fresh weight)	18.2±0.4	8.9±0.4

n.d.: not detected

water samples, which ranged between 98.0 and 100.5%, testify to the accuracy of the proposed method for this type of sample.

In the lettuce extracts and the apple tree leaves samples, the matrix effects were present, as can be seen in Figure 4 and 5 which present the calibration graphs with standards and the standard additions graphs. There are different slopes in the calibration lines, which explain the

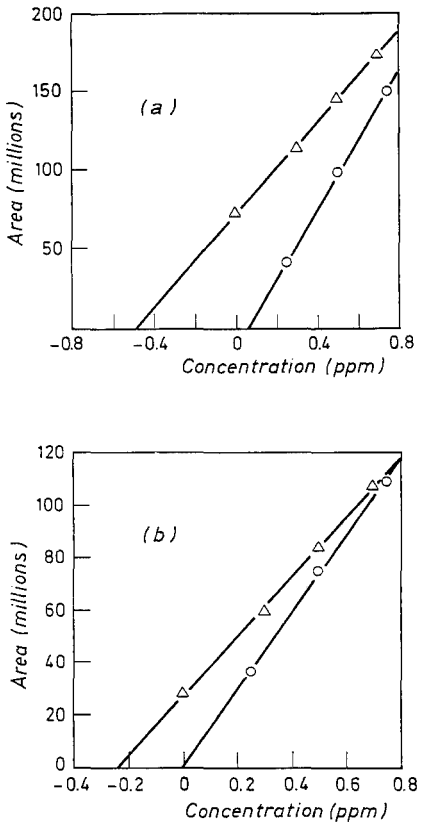

Figure 5:

Calibration graph with standards and standard additions graph with an apple tree leaves sample for a) NO_3^- and b) NO_2^-.

increased variability obtained by the recovery data (69%-87% for nitrate in lettuces and 63%-65% for nitrate and 70%-77% for nitrite in apple tree leaves). In both cases the determination of nitrate and nitrite was carried out by using the standard addition method.

The precision of the method was investigated using three different pool sample and by analysing each one in

triplicate. The variation coeficient was always less than 2% for nitrates and 5% for nitrites.

The results of some determinations are given in Table I. As can be seen, the nitrate values in the lettuce extracts show considerable variations according to their origin.

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SIMULTANEOUS DETERMINATION OF AMPICILLIN AND DICLOXACILLIN IN PHARMACEUTICAL FORMULATIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

KHALID A. AL-RASHOOD
Department of Pharmaceutical Chemistry
College of Pharmacy
King Saud University, P.O. Box 2457
Riyadh 11451, Saudi Arabia

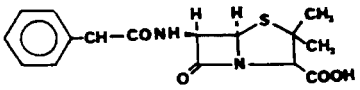
ABSTRACT

A rapid liquid chromatographic procedure for simple determination of each of ampicillin and dicloxacillin in bulk forms, their binary admixtures and in dosage forms is illustrated. The assay method has been adopted for simultaneous quantification of both penicillins in the presence of their degradation products. The best HPLC-resolution could be achieved on a reversed-phase, LiChrospher 100 RP-18 (5 μm), column by using a mobile phase containing acetonitrile + acetic acid (1%, aqueous) (39:61, v/v) isocratically at a rate of 2 ml.min^{-1} with UV-detection at 240 nm. Recovery testing of varying masses of each individual penicillin added to dosage forms was found to be fairly satisfactory.

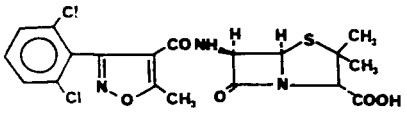
INTRODUCTION

Synergisms due to certain penicillins admixtures have been demonstrated against clinical isolates of some β-lactamase-producing and non-producing bacteria (1,2). Penicillins combinations exhibit more broad spectra and usually prescribed for infections induced by β-lactamase-producing strains. Synergy between ampicillin [I] and dicloxacillin [II] has been evaluated, so; several combinations containing them are now available on drug market.

The British Pharmacopoeia (BP 1988) (3) describes a mercurometric method after hydrolysing the β-lactame ring or complexation with an imidazole-mercury reagent followed by measuring the absorbance at 325 nm for determination of ampicillin in pure form and in



(I)



(II)

pharmaceutical preparations. While, the United States Pharmacopeia (USP 1990) recommends the high performance liquid chromatography and iodometric titrimetry for determination of ampicillin. Dicloxacillin is only official in the USPXXII (USP 1990) (4) which describes an HPLC-procedure for its determination in bulk material and pharmaceutical preparations. The difficulty arises when a simultaneous quantification of both penicillins is required.

Reversed-phase liquid chromatography, RP-HPLC (5) and reversed-phase thin-layer chromatography, RP-TLC (6) have been described only for separation and identification of several penicillins, including ampicillin and dicloxacillin, but not for their quantification. Various HPLC-methods have been reported for the determination of β-lactame antibiotics, individually (7-12). Recently, HPLC-methods with derivative UV-detection (13) and normal-mode UV-detection (14) have been investigated for determination of amoxycillin and dicloxacillin in capsules. A potentiometric procedure for the contemporaneous determination of ampicillin and dicloxacillin as such, in their binary mixtures, and in capsules, has been described (15).

It appears to be of high value to develop rapid and accurate assay methods for simultaneous estimation of ampicillin and dicloxacillin. The versatility of HPLC in antibiotics assay

is well discussed (16). So, the present work describes a simple liquid chromatographic-method for determination of the intact individual antibiotics in their binary admixtures and in some dosage forms. The stability-indicating characteristics, i.e. determination of the intact penicillins in the presence of their degradation products were investigated.

EXPERIMENTAL

Materials

Ampicillin trihydrate-WHO reference, assessed purity 99.4% (BP 1988), water content 12.5%- and dicloxacillin sodium monohydrate - claimed purity 99.5%, water content 3.9%, Gruppo Lepetit S.p.A., Milan-Italy - were utilized without further treatments. Capsules and powders for suspensions containing ampicillin and dicloxacillin were purchased randomly from local pharmacies. The penicillin contents in all investigated preparations were calculated as the free anhydrous bases. The antibiotics and their capsules were kept in a dry cool place in tightly closed moisture-proof containers.

Acetonitrile, HiPerSolv (HPLC-grade), pure glacial acetic acid (AnalaR™), BDH Chemicals, Poole-U.K., and all-glass bidistilled water was used to prepare the mobile phase.

Instrument

Shimadzu LC-10 AD liquid chromatograph was attached to SPD-10A tunable UV-detector, CTO-10A column oven controller, DGU-3A mechanical degasser, and C-R4A Chromatopac data unit, Shimadzu Corp., Analytical Instruments Division, Kyoto-Japan. Fixed loop injector (Rhydone, 20-μl) was utilized to carry the samples onto the column.

Analytical Procedure

Chromatographic conditions

The separation was performed isocratically at a rate of 2 $ml.min^{-1}$ on a reversed-phase [LiChrospher RP-18 (5 μm) Hibar prepacked column (12.5 cm × 4 mm ϕ), E. Merck, Darmstadt-

F.R.G.] at ambient temperature by using a mobile phase consisting of CH_3CN + 1% aq. CH_3COOH (39:61, v/v). The detection wavelength was set at 240 nm (AUFS = 1×10^{-3}).

Calibration graphs

Working standard solutions containing 50-200 $\mu g.ml^{-1}$ of each penicillin were prepared by taking 0.5-2 ml from the stock solutions (1 $mg.ml^{-1}$) of one penicillin in the mobile phase into separate 10-ml volumetric flasks and each was diluted with the mobile phase to the volume. Triplicate injections of each solution were done to get the standard plot of each antibiotic. Linear regression equations were computed for each penicillin:

$$Y = -0.07 + 0.089\ C(amp.)\ (r = 0.998)$$

$$Y = -0.031 + 0.327\ C(diclox)\ (r = 0.999)$$

where; Y; the area count ($\times 10^{-5}$) of the penicillin, C; the concentration ($\mu g.ml^{-1}$), and r; the correlation coefficient.

Alternatively, triplicate injections of the samples were done with similar standard working solutions in order to enable reliable sample/standard matchings. Calculation of drug contents was achieved by adopting the following formula:

$$C\ (mg.capsule^{-1}) = \frac{A_\mu D C(\%)\ W_1}{A_s W_2}$$

where, A_u and A_s are the areas for the ampicillin in sample (unknown) and standard, respectively, D is the dilution factor, W_1 and W_2 are the average weight (mg) of a capsule or 1-g powder for suspension and the taken weight (mg), in order, C(%) is the concentration percent of standard solution in the final dilution.

Recovery Testing

Spicking with equimasses of each individual reference penicillin was undertaken by adding varying aliquots to admixture solutions (25-100 $\mu g.ml^{-1}$) in the mobile phase. To test the degree

of recovery of the added amounts of each drug, triplicate injections of the solution mixtures were carried out. The reproducibility was evaluated by 12 consecutive injections of a standard solution equivalent to full amounts of each component drug based on its theoretical quantities.

RESULTS AND DISCUSSION

On adopting the method of the British Pharmacopoeia (BP-1988) (3) recommended for determination of ampicillin on dosage formulations specimens containing ampicillin and dicloxacillin combination, the reaction products of imidazole-mercury reagent with dicloxacillin show overlapping absorption at the wavelength specified for ampicillin. On the other hand, binary mixtures of both penicillins exhibit considerable UV-band overlap, which makes the direct UV-spectrophotometric determination of one penicillin in the presence of the other remains quite unrealizable.

The potentiometric titrimetry (15), described for simultaneous determination of ampicillin and dicloxacillin in capsules, has been tested for its applicability on degraded penicillin, where poor stability-indication was proved. The method depends on the fact that the amino group of ampicillin and the carboxylate group of dicloxacillin are differentially protonated, but most of the penicillin degradates still contain such functional grouping and behave on protonation likely as the intact penicillin.

Depending on conditions, the degradative pathway and products, i.e. degradates, are widely different. The main penicillins' degradates are penicilloic, penacilloic, penicillenic, penilloic and penillic acids (17). Detection of any penicillin degradation is of special interest in view of the implications of possible degradates in allergic responses to penicillin therapy (17). Analytical methods with significant stability-indicating characteristic are always needed for detection and determination of drug degradation as well as for quantification of the intact drug in presence of its prime and minor degradates.

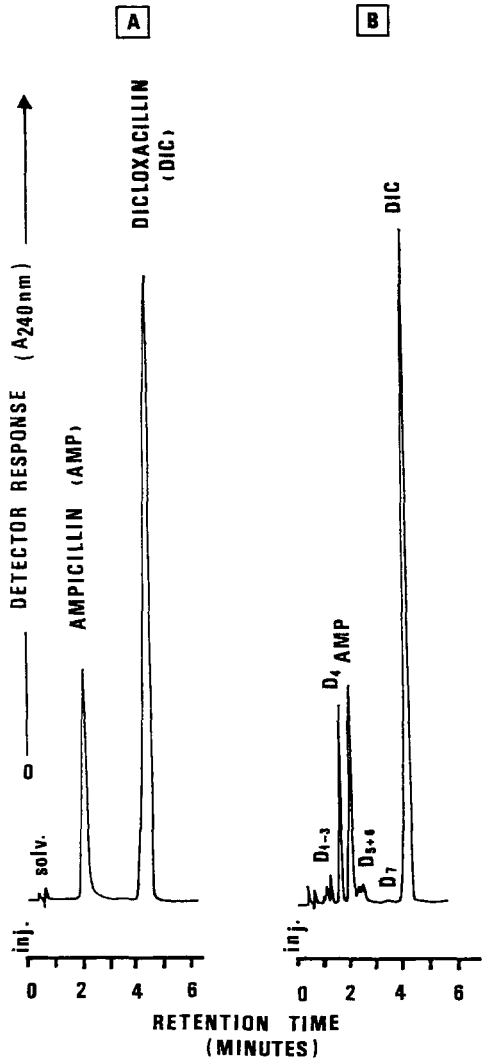


Figure 1: HPLC-Separation of (A) admixture of refrence ampicillin & dicloxacillin, and (B) same sample degraded at room temperature (22°C) for 24 hours; D means degradates.

HPLC has achieved pronounced advantages over some other techniques in being the most stability-indicating. The liquid chromatographic procedure herein investigated thus allows the simultaneous determination of ampicillin and dicloxacillin in admixtures even in the presence of their products of degradation. A reversed-phase column, namely, LiChrospher 100 C18 (5 μm), has revealed validity on utilizing the isocratic elution at 2 ml.min^{-1} rate of flow with a polar mobile phase composed of acetronitrile and 1% aq. acetic acid (39:61, v/v). Symmetric peaks were obtained for both penicillins, the tailing factors, i.e. asymmetry factors, were 1.12 and 1.02 for ampicillin and dicloxacillin, respectively. The relative standard deviation (SDrel.) for twelve replicates was $\leq$ 2.0%. Figure 1 demonstrates the HPLC-resolution of both penicillins before and after degradation (24 hrs at 22°C). All the penicillin degradates $D_{1\text{-}7}$ were resolved quite away from the peaks of the two intact drug substances. Several chemicals, such as clavulanic acid, amoxycillin, ... etc., have been tried to serve as an internal standard but they were eluted either with one of the penicillins or near the retention times of the degradates. Fixed-loop injections as well as standard/sample matching give always consistent results. The mean deviations in case of ampicillin were relatively higher than that of dicloxacillin. This may be attributed to the slightly higher absorption coefficient (ϵ) of dicloxacillin at the specified wavelength for drug detection (240 nm). The selected chromatographic conditions seemed to be the best for indicating the penicillins stability.

Table 1 collects the obtained results of assay, recovery and reproducibility testing of the proposed chromatographic method of analysis. The described method is rapid , so, the two penicillins and all degradates leave the column completely after about 5 minutes. No sample clean-up or extra sample preparation are needed. The described HPLC-method has proved its advantage in the capability of finding the accurate contents of each drug substance in the presence of its degradates. The drug degradation was undertaken in solution either by one-day stay at room temperature (~22°C) or heating for 20 minutes. No justification of the decay products has been

Table 1: HPLC-Analysis (assay and recovery) of ampicillin and dicloxacillin in capsules.

Pharmaceutical Formulations	Analysis*	Penicillin content (%)	
		Ampicillin	Dicloxacillin
Cloxapen-250™ Capsules[a]	A	100.4 ± 1.56 (5)	100.5 ± 0.22 (5)
	R**	99.7 ± 0.50 (4)	99.9 ± 0.38 (6)
Cloxapen-500™ Capsules[a]	A	101.2 ± 2.34 (4)	100.3 ± 0.58 (4)
	R**	100.8 ± 0.50 (4)	100.2 ± 0.86 (4)
Diclopen™ Capsules[b]	A	102.4 ± 0.60 (5)	101.0 ± 0.68 (5)
	R**	101.1 ± 0.83 (4)	100.0 ± 0.46 (4)
Dipenacid™ Suspension[c]	A	99.6 ± 2.10 (6)	99.4 ± 0.94 (6)
	R**	100.4 ± 0.94 (4)	100.1 ± 0.63 (4)

*X ± CV (n); n : the average mean of at least 3 determinations.
A; assay R; recovery
**recovery of added mass (50%) of the named penicillin.
a. Cloxapen™ (250 & 500) capsules are products of each capsule contains equal amount (125 or 250 mg) of each penicillin (anhydrous base).
b. Diclopen™ capsules is a product of Kahira Pharm. & Chem. Ind. Co., Cairo-Egypt.
c. Dipenacid powder for oral suspension, dissoluted 5 ml contain 250 mg of each penicillin, is manufactured by CID Co., Giza-Egypt.

done in this study. Moreover, the recovery testing of the investigated method indicates good accuracy and precision. Excellent reproducibilities have been observed for different replicates of both penicillins as reflected in the relative low coefficient of variations.

It can be concluded that the proposed method for simultaneous quantification of ampicillin and dicloxacillin in their admixtures and/or pharmaceutical preparations is simple, rapid, quite accurate and stability-indicating in addition to its high precision and confidence.

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DETECTION OF BENZYLPENICILLIN IN MILK 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 benzylpenicillin (Penicillin G) in milk is presented. The samples were extracted with acetone, the organic layer then being evaporated to dryness and cleaned-up using C_2 solid phase extraction column. The method is simple and robust.The lower limit of quantification was 4 µg/l and the limit of detection close to 2 ppb. The average recovery was 82%.

INTRODUCTION

Beta-lactam antibiotics, especially benzylpenicillin (BZP), are commonly used in veterinary medicine. Their wide application

represents a potential hazard to consumers due to the persistence of residues in milk. Despite their low toxicity, beta-lactam antibiotics are nevertheless a group of drugs which have been reliably documented to harm human health, residues of BZP in food inducing allergic reactions (1).
A number of sensitive screening tests have been described for the detection of β-lactam antibiotic residues in milk. These include microbiological tests (2), immunoassays (3,4), competitive binding (2, 5, 6) and enzyme inhibition (7). These are all capable of detecting residues at levels of 10 μg/l or less. With the possible exception of immunoassay, none of the screening procedures can differentiate the various β-lactam antibiotics from one another. False positive tests may occur. A capillary gas chromatographic method has been described (8), with a sensitivity of <1 μg/l, which requires lengthy partitioning clean-up and derivatization steps. Several analytical methods for the determination of BZP based on high-performance liquid chromatography have been published (9-14). The methods are, however, time-consuming and either require the use of large quantities of chemical reagents, or do not achieve the required sensitivity.
The purpose of the present study was to develop a rapid, simple and sufficiently sensitive method (15), for the determination of BZP, which required only small quantities of chemical reagents. The fact that many β-lactam antibiotics cannot be partitioned between buffers and organic solvents had to be taken into consideration(10).

MATERIALS AND METHODS

Chemicals and Reagents

Samples of cows milk were used.
All chemicals and solvents were of analytical or HPLC grade.
BZP was supplied by Sigma Co. (St. Louis, MO, USA). Stock

solutions (1mg/ml) and working standards were prepared fresh weekly by dilution with distilled water. The solutions were stored in the refrigerator.
Extraction columns Bond Elut (1cc/100 mg) C_{18}, C_8 and C_2, were purchased from Varian (Harbor City, CA, USA).

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 (12°c) from Messgeräte Werk Lauda, (Lauda Königshafen, Germany), and a LC 235C Diode Array detector (Perkin-Elmer, Norwalk, CT, USA). The detector was operated at 200 nm. The integration was carried out using the software programme Turbochrom 4.0 (Perkin-Elmer), which was operated on a Brick personal computer connected to a BJ-330 printer (Canon).
The analytical column (stainless steel, 25 cm x 4.6 mm. ID) and guard column (stainless steel, 2 cm x 4.6 mm. ID), were packed with 5-µm particles of Supelcosil LC-C_{18} DB (Supelco, Bellefonte, PA, USA), and operated with a constant column temperature of 35°c.
The mobile phase was a mixture of two solutions, A and B (66:34). Solution A was 0.02 M heptanesulphonate-0.01 M $Na_2HPO_4 \cdot 2H_2O$, made by dissolving 4.45 g/l 1-heptane sulphonic acid sodium salt (Supelco) and 1.78 g/l di-sodium hydrogenphosphate-2-hydrate (Ferax, Germany) in c. 750 ml of water. The pH was then adjusted to 2.15 with 5 M phosphoric acid, and the solution made up to volume (1l) with water. Solution B was acetonitrile.
The flow-rate was 0.9 ml/min for 3 min, 0.6 ml/min for 10 min, followed by 2 ml/min for 2 min.The samples were injected at intervals of 17 min. Aliquots of 125 µl were injected onto the column for the determination of BZP.

Sample pretreatment

To 1 ml milk was added 200 µl H_2O (or standard) and 10 ml acetone. The sample was mixed for approx. 10 sec. and then centrifuged for 3 min. (3000 rpm.). The supernatant was transferred to a clean glass-stoppered centrifuge tube. The organic layer was evaporated to c. 600 µl at 45 °c under a stream of nitrogen and then 1 ml hexane was added.The sample was shaken vigorously for 5 sec., and centrifuged for 2 min. The upper layer (hexane) was discarded and the water layer washed again with hexane, and the water was evaporated to dryness. The dry residue was dissolved in 350 µl H_2O-CH_3CN (8 : 2) and the solution loaded onto a conditioned C_2 column.

Clean-up on SPE-column. The column was activated with 1 ml CH_3CN and 2x1 ml water and suctioned to dryness for 5 sec. The extract of milk was loaded into the column and slowly suctioned through (c. -1 in. Hg.). The column was suctioned to dryness for 5sec., and with vacuum -5 in. Hg. washed with 2x50 µl H_2O-CH_3CN (9:1), suctioned to dryness for 5 sec., and then eluted with 4x100 µl H_2O-CH_3CN (8:2) with full vacuum using a VacMaster system (International Sorbent Technology). The collected eluate was centrifuged for 3 min. at 3000 rpm. prior to injection into the HPLC.

Validation of the pretreatment procedure

The precision, recovery and linearity of the pretreatment procedure were determined by analyses of spiked milk samples in the concentration range 4-300 ng/g.
The spiked samples were extracted using the above described procedure. Duplicate samples were used. The recovery rates were determined by comparing results of analysis of the spiked milk samples with those of standard solution. The linearity of the

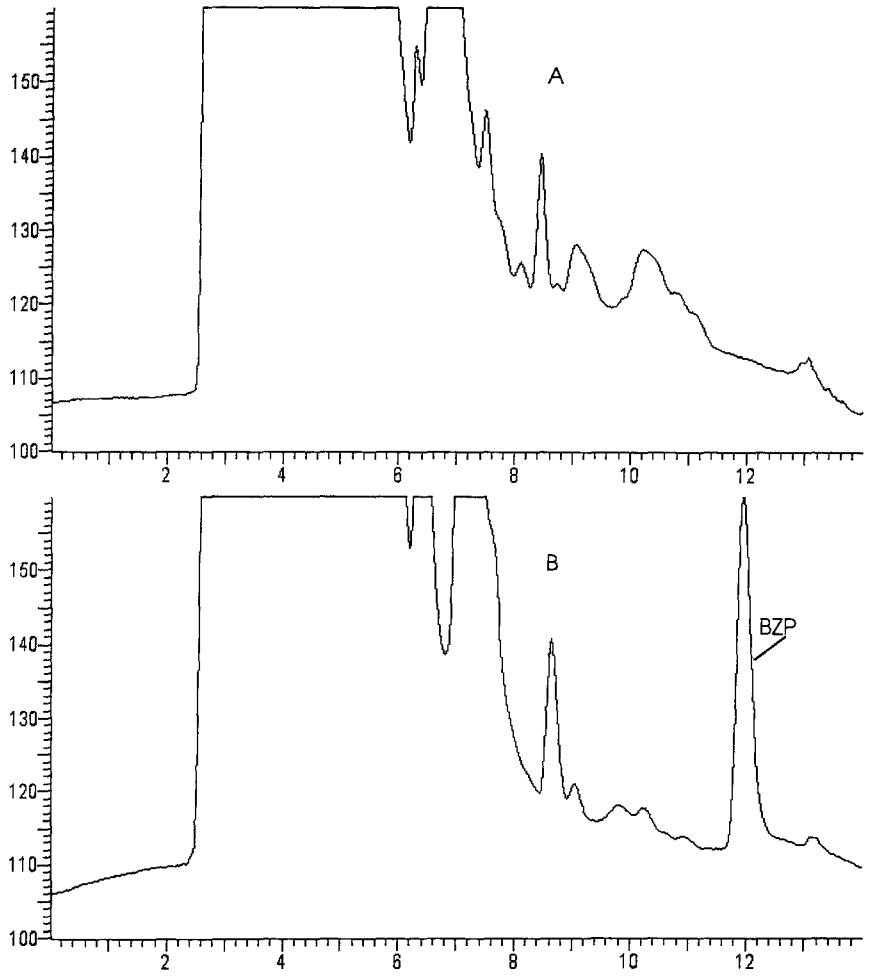

FIGURE 1

Chromatograms of extracts from milk.

A: drug-free milk, **B**: "real" sample of milk contains 200 ng/ml BZP.

TABLE 1

Recovery and repeatability for benzylpenicillin from spiked samples of milk.

Sample	No. of Samples	Amount of drug added (ng/ml)	Recovery % BZP Mean	SD*
Milk (1 ml)	8	10	82	0.8
	8	100	82	0.7

SD*= standard deviation

standard curves for BZP in milk was tested using peak-height measurements.

RESULTS AND DISCUSSION

Chromatograms of extract of blank samples and real samples are shown in Figure 1.
The chromatographic system appeared to be efficient for the determination of BZP in milk, the limit of quantification being 4 ng/ml and the limit of detection close to 2 ng/ml. No interference was seen during analysis, when calibrating the curves, or when performing recovery studies. The extraction procedures were validated, and the results are shown in Table 1. The average recovery for BZP over the concentration range of the standard curve was 82%.

The precision of these recovery studies varied from 0.7 to 0.8% for BZP in milk. The linearity of the standard curves was 0.999 for milk, when using the external standard method of calculation. The results also show that the precision and accuracy of the quantification of BZP are good.
The retention and elution properties of BZP were studied on the bonded-phase extraction columns. The drugs were retained on C_8 and C_2 and only partly retained on the C_{18} column using water-acetonitrile (8:2). C_8 columns seem to retain a little more impurities that C_2 columns. A large amount % CH_3CN in the elution mixture gave more impurities. Moreover c. 15% of BZP remains in the column when using water-acetonitrile (8:2) as elute. A suitable pH in the mobile phase is important if good separation from residues of endogenous compounds is to be obtained.
This study has shown that residues of the antibiotic compound BZP in milk may be determined without being partitioned between buffers and organic solvents. The cost of chemicals and the manual work-up procedures are also reduced compared to previously published methods. An experienced technician can carry out sample clean-up of about 24-27 milk samples per day. The assay shows good precision when using the external standard method. The method is robust, simple, and sufficiently sensitive. The quantification is linear over a wide concentration range. The chromatographic system was specific with regard to BZP.

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QUALITY ASSURANCE OF PETROL BY HPLC

VIVEK R. DHOLE[1]* AND G. K. GHOSAL[2]
[1]*Regional Forensic Science Laboratory*
State of Maharashtra, Ganeshkhind
Pune - 411 007, India
[2]*Petro-Chem. Technology Department*
Laxminarayan Institute of Technology (LIT)
Amravati Road, Nagpur (M.S.), India

ABSTRACT

A simple and rapid reverse phase HPLC method has been developed for the determination of adulteration in petrol (gasoline) with Kerosene. Methanolic solutions of petrol, Kerosene and their synthetic admixtures were analysed on HPLC using C_{18} ODS column and Acetonitrile : Water (8:2) as mobile phase, at the flow rate of 1 ml/min and UV-absorption detection at 285 nm. The UV detector at 285 nm gives specificity to detection, since the alkyl naphthalenes predominantly present in kerosene have strong, characteristic absorption around 285 nm. The amplitudes of the peaks corresponding to these naphthalenes (C-10 - C-12) increased with the added quantum of kerosene linearly. Average values with ± SD of intrachromatographic ratios generated for the peak heights of the naphthalenes Σ(C-10 to C-12) divided by peak height of solvent methanol were tabulated. These ratios could be used to get a linear curve if evaluated graphically against corresponding (P:K) composition for determining extent of adulteration with kerosene, even down to a level of 5% v/v in suspected petrol samples.

INTRODUCTION

Kerosene being a readily available, specially subsidised domestic cooking fuel has a substantially lower price than the commonly used automobile fuel, petrol (motor gasoline) in Indian-sub-continent and many other countries. The large price difference, between those of petrol and kerosene, has induced an expensive economic offence of the petrol adulteration with kerosene. Moreover, use of such adulterated petrol not only reduces the performance but it also causes serious damage to the automobile engines. Hence early detection and estimation of kerosene in adulterated petrol samples is highly important for the law enforcement agencies and also for ensuring the quality and performance of the fuel (petrol) before using in the motor vehicles. For this purpose the suspected petrol samples are often referred to the Forensic Science Laboratories and also to the quality control,testing laboratories of the petroleum oil companies/refineries.

Number of publications have dealt with the quality assurance/detection of adulteration in petrol/diesel by using various physico-chemical and analytical instrumental methods such as, distillation ranges (1-3), flash point (4), octane number (1,2), specific gravity (5), aniline point (5), thin-layer-chromatography (TLC) (6-9),gas-chromatography (GC) (10-12), UV -spectrophotometry (13), semimicro-chromatography (14),

critical-micelle-concentration (CMC) (15), rapid-phase-titration-procedure (16), and high-performance-size exclusion chromatography (17) with varying degree of success.

To supplement detection of adulteration in petrol with kerosene usually done by us by monitoring, distillation ranges (1-3), specific gravity (5), aniline point (5), TLC (6-9) and gas-chromatography amongst the other parameters, the utility of reverse-phase HPLC with a UV absorption detector has been presented here.

EXPERIMENTAL

Materials :-

Number of authentic/standard petrol samples (n=10), belonging to different petroleum companies, viz., Esso, Hindustan Petroleum, Indian Oil Corporation (IOC) and Bharat Petroleum etc. and number of kerosene samples (n=10) were procured from different sources from the market. HPLC grade solvents acetonitrile, methanol and water and AR grade (spectra pure) naphthalene (I), 2-methyl naphthalene (II) and 2,6-dimethyl naphthalene (III) and standard Borosil pipettes, volumetric flasks were used.

Equipment :-

The HPLC system consisted of "Theromo-separation-products" (formerly "Spectra physics") company HPLC Unit, RP-C-18 column

(Lichrospher-100-RP-18, 5 μm, 250 mm length x 4 mm ID), variable wave-length UV absorption detector fitted with Datajet CH-1 integrator.

Chromatographic conditions :-

Mobile phase of isocratic solvent system, comprising of Acetonitrile : Water (8:2) was used. A flow rate of 1 ml/min. at ambient temperature ($30^{o}C$) was maintained throughout the experiment. Detection wavelength was chosen at 285 nm on the UV absorption detector. Integrator conditions were kept at, Attenuation = 4, Chart-speed = 1 cm/min, Threshold = 1, Peakwidth = 0.04.

Standards/sample preparation :-

Number of authentic/standard petrol (P) and kerosene (K) samples (n=10 each) procured as described above were utilised to prepare ten different sets of synthetic admixtures in the range of (P), (9P + 1K), (8P + 2K) (7P + 3K), (6P + 4K), (5P + 5K), (4P + 6K) and (K) separately. Exactly 1% v/v solutions of all these synthetic admixtures were separately prepared with help of suitable standard volumetric flasks and pipetts by using methanol as a solvent. Exactly 1% v/v methanolic solution of suspected petrol sample if any from case work was also prepared.

10 µg/ml of naphthalene (I) and 20 µg/ml each of 2-methyl naphthalene (II) and 2,6 - dimethyl naphthalene (III) were separately prepared as reference markers in methanol.

Method :-

Exactly 10 µl each of the 1% v/v methanolic solutions of fresh petrol, kerosene and their synthetic (P + K) admixtures, reference markers of naphthalenes (I), (II) and (III) and suspected petrol sample, if any, prepared as described above, were separately injected on HPLC unit employing the "Chromatographic conditions", as described above.

RESULTS AND DISCUSSION

Typically characteristic patterns of chromatograms were observed for petrol, kerosene and their synthetic admixtures. The representative chromatograms for (P), (9P + 1K)... to (4P + 6K) and (K) are shown in FIGURES 1 to 8 respectively. Peaks of the standard reference markers of naphthalene (I), 2-methyl-naphthalene (II) and 2, 6-dimethyl-naphthalene (III) were identified at Retention Times (RT) 4.9, 6.2 and 8.0 ± 0.1 min., respectively and solvent methanol (M) showed a small peak at RT = 1.8 ± 0.1 min. in these chromatograms (FIGURES 1 to 8).

Petrol (gasoline) samples chiefly consist of hydrocarbons of paraffinic, cycloparaffinic and aromatic series. Higher boiling

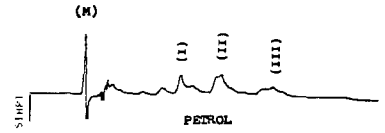

FIGURE - 1* : HPLC Chromatogram of Petrol (1% v/v in methanol).

FIGURE - 2* HPLC chromatogram of synthetic admixture of 9 Petrol + 1 Kerosene (1% v/v in methanol).

FIGURE - 3* : HPLC chromatogram of synthetic admixture of 8 petrol + 2 kerosene (1% v/v in methanol).

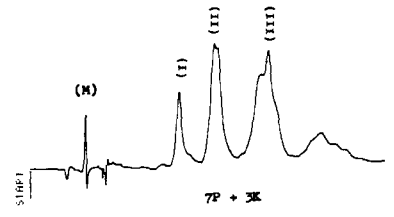

FIGURE - 4* : HPLC chromatogram of synthetic admixture of 7 Petrol + 3 Kerosene (1% v/v in methanol).

* FIGURES 1 to 8 : Quantity of injection = 10 µl each. Symbols (M) (I)(II) and (III) marked in the chromatograms represent the peaks for Methanol, Naphthalene, 2-Methyl-naphthalene and 2,6 - Dimethyl naphthalene respectively. Chromatographic conditions are described in the text.

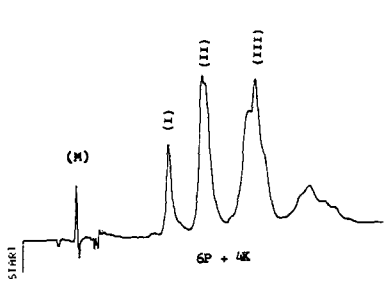

FIGURE - 5* : HPLC chromatogram of synthetic admixture of 6 Petrol + 4 Kerosene (1% v/v in methanol).

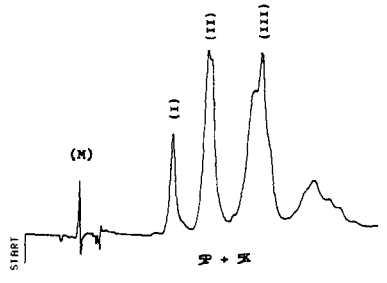

FIGURE - 6* : HPLC Chromatogram of synthetic admixture of 5 Petrol + 5 Kerosene (1% v/v in methanol).

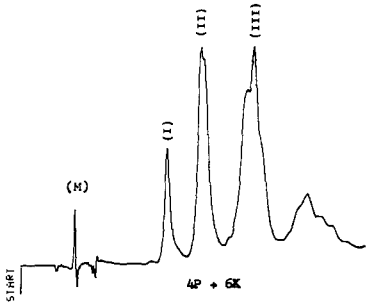

FIGURE - 7* : HPLC chromatogram of synthetic admixture of 4 Petrol + 6 Kerosene (1% v/v in methanol).

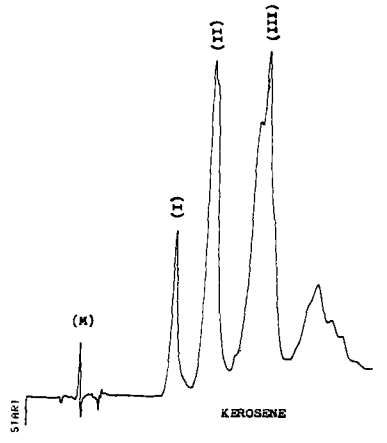

FIGURE - 8* : HPLC chromatogram of kerosene (1% v/v methanol).

petroleum products like kerosene or diesel also comprise of similar classes of hydrocarbons though with more complex molecules and higher carbon numbers of mixed nature (6-9, 13,18). Aromatics present in fresh (unevaporated) petrol are predominantly mononuclear (alkyl benzenes) with relatively low concentrations of dinuclear aromatics (alkyl naphthalenes) (13, 19-22). In contrast kerosenes are richer in dinuclear aromatics (alkyl naphthalenes) (13,22) with substantial proportions of mononuclear aromatics, diphenyls and traces of highly condensed aromatics (13,18). Thus the concentration of total naphthalenes/alkyl naphthalenes would be invariably high in kerosene and diesel than in fresh (unevaporated) petrol.

The above concept was experimentally confirmed by TLC results in our earlier work (6-9), and further by HPLC results in the present work, wherein the peak heights/amplitudes of the naphthalenes (I), (II) and (III) show an increasing trend in the chromatograms of the (P : K) synthetic admixtures as the percentage of kerosene goes on increasing from (P), (9P + 1K) ... to (4P + 6K) and (K) (FIGURES 1 to 8).

Criterion for the selection of wavelength :-

Among the petroleum hydrocarbons absorption of radiations in the ultraviolet region is mainly due to aromatics. Alkyl naphthalenes could be measured by their absorptions at 225 ± 5

nm (23-26), 275 nm (22-24) 285 (13,22-24, 27,28) and 319 nm (13,24). However, petrol (gasoline) generally shows high absorption upto 275 nm (due to predominance of alkyl benzenes) and thereafter shows a rapid tapering off (13), since the absorptivities (log E values) at 285 nm of mononuclear aromatics, i.e., alkyl benzenes (C-8 to C-12) are less than 1.0 (29). However, the absorptivities (log E) are more than 2.5 for diphenyls (C-12 and C-13) and are more than 3.5 for naphthalenes (C-10 to C-13) at 285 nm (29). Hence UV-absorption measurements at 285 nm would be most useful for the determination of total naphthalenes from kerosene in petrol. The criterion for selection of wavelength for UV detection in the present HPLC work is based on the concept discussed above. In fact absorption at 285 nm is the basis of ASTM method D-1840 for the determination of total naphthalenes in jet fuels (27). Normal UV-Spectrophotometric method for the detection of naphthalenes at 285 nm has been presented by other workers also (13). However, it was further established by other workers that the direct, normal UV-spectrophotometric detection of naphthalenes at 285 nm is inapplicable in petroleum (gasoline) sample because of the serious interference from monoaromatics (28). But the HPLC technique because of its powerful chromatographic separation facility if coupled with characteristic UV detection at 285 nm as suggested in the present work could be used as a better alternative approach than the ASTM (27) and other direct UV-spectrophotometric method (13) for determining

total naphthalenes in petroleum products without interference from alkyl benzenes etc.

Intra-chromatographic ratios :-

A close look at the chromatograms for synthetic admixtures from (P), (9P + 1K) ... to (4P + 6K) and (K) (FIGURES 1 to 8) reveals an increasing trend of peak heights, as described above, especially for the peaks corresponding to naphthalenes (I), (II) and (III) at RT = 4.9, 6.2 and 8.0 ± 0.1 min. respectively. However, the peak height for solvent methanol (M) at RT = 1.8 ± 0.1 min. is almost the same in all these chromatograms (FIGURES 1 to 8) since the quantity of injection is exactly same, i.e., 10 ul each for all the (P : K) synthetic admixtures. It was also observed that the peak height for solvent methanol is directly proportional to the quantity of injection. Apart from characteristic chromatographic pattern recognition for different (P:K) compositions (FIGURES 1 to 8) it was further attempted to determine intrachromatographic peak height ratios in the following manner, i.e., $\Sigma((I) + (II) + (III))$ divided by (M). For finding out these ratios, peak heights were utilised rather than peak areas due to the incomplete separation of bands in the 15 min. elution range, utilising optimum chromatographic conditions. Average values with ± SD of these peak height ratios for various (P:K) synthetic admixtures derived from ten different sets are shown in TABLE 1.

TABLE 1 :-

Average (X) with ± SD values (n = 10) of the intrachromatographic ratios of the peak heights for ((Naphthalene (I) + 2 methyl-naphthalene (II) + 2,6-Dimethyl-naphthalene (III)) ÷ Methanol (M), identified in the HPLC chromatograms of various (P:K) synthetic admixtures.

Sr. No.	(P : K) Composition	Average with ± SD* values of peak height ratios. ((I)+(II)+(III)) ÷ (M)	% CV.@
1.	Petrol (P)	1.2632 ± 0.0551	4.362
2.	(9P + 1K)	2.5790 ± 0.0665	2.578
3.	(8P + 2K)	3.7894 ± 0.0731	1.929
4.	(7P + 3K)	5.2631 ± 0.0818	1.554
5.	(6P + 4K)	6.6316 ± 0.0867	1.307
6.	(5P + 5K)	7.8947 ± 0.0848	1.074
7.	(4P + 6K)	9.1578 ± 0.0918	1.002
8.	Kerosene (K)	14.1052 ± 0.0935	0.663

* SD = Standard Deviation @ C.V. = Coefficient of Variation.

Since % C.V. is less than even 5% for all the (P : K) compositions, the results from the present HPLC work (TABLE-1) indicate good precision of assay. The average values of the peak height ratios from TABLE -1 if plotted against corresponding (P : K) composition, i.e., (P), (9P + 1K) ... to (4P + 6K) and (K) etc. give a straight line curve which could be utilised for determining the percentage of kerosene in the suspected petrol

sample by finding out the peak height ratio from its HPLC chromatogram and then with the help of straight line curve graph derived from TABLE-1 as described above one can easily find out the extent of adulteration in the suspected petrol sample.

Thus the proposed HPLC method/approach could be routinely used as a simple, rapid, sensitive (even down to a level of 5% v/v) and reliable complementary method for the determination of adulteration in petrol with kerosene in Forensic Science Laboratories. It could also be used by the quality control/testing laboratories of the petroleum refineries and petrochemical / (PAH) etc. industries in day-to-day work.

ACKNOWLEDGEMENT

The authors are thankful to Mr. S.O. Baisane, Director, Forensic Science Laboratories, State of Maharashtra, Bombay for the encouragement in this work. The authors are also grateful to M/s Chromline Equipment Co., Bombay for providing HPLC instrumental facility for this work.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 18(12), 2489-2493 (1995)

THE BOOK CORNER

CENTRIFUGAL PARTITION CHROMATOGRAPHY, Edited by A.P. Foucault, Chromatographic Science Series, Volume 68, Marcel Dekker, Inc., New York, 432 pages, 1994. Price: $150.00.

Modern countercurrent chromatography (CCC) originates from the pioneering studies of Y. Ito et al., who first constructed, in Japan, an apparatus designed to differentiate particles in suspension or solutes in solution in a solvent system subjected to a centrifugal acceleration field. This first machine opened the way in two main directions: one, pursued by Y. Ito in the United states, is based on a wide variety of countercurrent chromatographic apparatuses most of the recent ones using a variable gravity field produced by a two-axis gyration mechanism and rotary seal-free arrangement for the column; the other, pursued by K. Nunogaki in Japan, is based on the "CPC apparatus" (centrifugal partition chromatographic apparatus) and uses a constant gravity field produced by a single-axis rotation mechanism, and two rotary seal joints for inlet and outlet of the mobile phase.

The historical linkage between countercurrent distribution (Jantzen, Watanabe, Van Dijek, Martin and Synge, Craig and others) and countercurrent chromatography is responsible for the name *countercurrent chromatography* in which a strong gravitational field is used to keep a liquid stationary phase in a steady immobilized state while the mobile phase is pumped through. With technological improvements, the performance of today's instruments is much closer to that of liquid-liquid chromatography using a solid support to hold the liquid stationary phase.

The goal of this volume is to provide a forum for scientists who are already using centrifugal partition chromatographs in their research to share with others their personal knowledge in this specific field of chromatography. This book is devoted exclusively to the CPC apparatus (single-axis rotation mechanism).

CPC and HPLC (high-performance liquid chromatography) are similar in several respects. They share the same fundamental mechanism (partitioning of solutes), the same goal (separation, purification), and the same ancillary equipment (pumps, injectors, detectors).

The book introduces centrifugal partition chromatography (CPC) for any biphasic system - offering in-depth coverage of instrumentation, theory, liquid-liquid partition coefficients, and CPC in organic and inorganic chemistry - and provides over 80 ternary phase

diagrams of three-solvent systems that can be applied to virtually all partitioning, separation, and purification situations.

The book is divided into 12 chapters written by international experts from North American, Europe, and Japan. It examines chromatographic properties, illustrates practical operations, and gives examples of CPC solutions to real experimental problems, highlights the distinction between CPC and high-performance liquid chromatography, explains hydrostatic, hydrodynamic, and overall pressure drops, discusses solvent systems, strategies for solvent selection, and the elution mode in CPC, shows how to design solvent systems for CPC of complex organic mixtures, describes carrier-aided CPC for preparative-scale separations and the use of CPC as a multistage liquid-membrane transport system, and much more.

With nearly 800 references, tables, equations, and figures, *Centrifugal Partition Chromatography* is a good resource for analytical and pharmaceutical chemists and biochemists, separation scientists, pharmacologists, and upper-level undergraduate and graduate students in these disciplines.

Table of Contents:

CARBOHYDRATE ANALYSIS HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ELECTROPHORESIS, Edited by Z. El Rassi, Journal of Chromatography Library Volume 58, Elsevier, Amsterdam, The Netherlands, 668 pages, 1995. Price: $242.75.

This book is an excellent reference for every one interested in carbohydrate analysis. The volume discusses all aspects of carbohydrate analysis from sample preparation to separation and detection in an easy to understand manner. The editor, Dr. Z. El Rassi is a leader in this field and should be commended for a job well done. The authors are leaders in their areas. The book is free of errors and well illustrated and referenced, and is a must for all those interested in carbohydrates and glycoconjugates analysis.

Carbohydrates and glycoconjugates are very important biological species involved in many life processes. Because of the structural diversities and the multilateral importance of carbohydrates, the analytical methodologies used to analyze them continue to evolve. High performance liquid chromatography (HPLC) has been extensively used in the separation and isolation of carbohydrate species. More recently, high performance capillary electrophoresis (HPCE), has been explored. The objective of this book is to provide a comprehensive review of carbohydrate analysis by HPLC and HPCE by covering the separation methods for all classes of carbohydrates including mono- and disaccharides; linear and cyclic oligosaccharides; branched oligosaccharides (e.g., glycans); polysaccharides; glycoconjugates (e.g., glycolipids, glycoproteins); carbohydrates in food and beverage; compositional carbohydrates of polysaccharides; carbohydrates in biomass degradation; etc.

The book is well balanced in terms of its content: covers the fundamental aspects of the various modes of HPLC and HPCE that are currently applied to the analysis of carbohydrates; discusses analytical and preparative separations; describes the principles of detection and quantitative determination of carbohydrates by HPLC and HPCE; reviews sample preparations; and provides an ample amount of important applications.

The book is divided into three major parts. The first part, Chapter 1, reviews enzymatic and chemical methods currently utilized in sample preparation.

The second part deals with Analytical and Preparative Separations, and encompasses a series of 8 chapters. Seven of these chapters, Chapters 2 through 8, describe, in detail, the different HPLC and HPCE systems currently used in analytical separations of carbohydrates and glycoconjugates. An additional chapter, Chapter 9, reviews the various aspects of semi-preparative and preparative HPLC for the isolation of small and large quantities, respectively, of intact and pure carbohydrates and glycoconjugates.

The third part is on The Detection, a topic as important as the separation part. In general, carbohydrates lack chromophores or fluorophores in their structures. This inherent property of carbohydrates causes difficulties in determining these species at low levels. This section contains a series of 8 chapters, Chapters 10 through 17, covering, in detail, the different direct and indirect detection methods that have been introduced for the sensitive detection of carbohydrates. The various detection topics include electrochemical, refractive index, mass spectrometry, light scattering, chiroptical, pre- and post-column derivatization reactions for optical detectors (UV, Vis and fluorescence), post-column enzyme reactors, indirect UV and

fluorescence detection, low wavelength UV and other miscellaneous modes of detection. Each of these chapters discusses the basic principles, advantages and limitations, and applications of the particular detection technique. It is highly recommended.

Table of Contents

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(12), 2495-2496 (1995)

MEETING ANNOUNCEMENTS

PrepTech'96

February 5 - 7, 1996

Sheraton Meadowlands Hotel
East Rutherford, New Jersey

PrepTech'96 will cover a wide range of topics in industrial separation science, including preparative liquid chromatography, membrane separations, electroseparation techniques, extraction and centrifugation. Although the primary focus of the conference will be process-scale liquid chromatography, the following topics of special interest will be included:

* Scale-up strategies

* Process cost & economics

* Reduction of solvent usage & cost

* Regulatory compliance

* New modes of chromatography, e.g., simulated moving bed, continuous displacement, new column packings

* Membrane separations, electrophoresis, dialysis, field-flow fractionation, gas chromatography, supercritical fluid techniques

* Recent case studies of separations of chiral compounds, DNA, plasmids & analogs, new drugs

* Integration of unit processes

* In-place sterilization and cleanup

Process development chemists and engineers are invited to submit abstracts for lecture and poster presentation. The chairman of the technical committee is Dr. Robert Stevenson; abstracts of no more than 350 words should be submitted to him at the following address: Dr. Robert Stevenson, PrepTech'96, 3338 Carlyle Terrace, Lafayette, CA 94549.

7TH INTERNATIONAL SYMPOSIUM ON SUPERCRITICAL FLUID CHROMATOGRAPHY & EXTRACTION

March 31 - April 4, 1996

Westin Hotel
Indianapolis, Indiana, USA

The purpose of this conference is to build upon previous programs to provide a forum for maximum exchange of information on techniques, theory and applications of supercritical fluid chromatography, extraction and fractionation. The program will emphasize recent accomplishments and research results rather than review. It will include lecture presentations featuring leading researchers from academia, government and industry; posters; intensive, yet informal discussion sessions; an instrument exhibit; and an optional tutorial.

An optional tutorial program will precede the symposium for those who are interested in a review of the technologies. Participants will have ample opportunity to meet commercial vendors at the instrument exposition and to view state-of-the-art SFC, SFE and SFF instrumentation and supplies.

A limited number of student travel scholarships will be available to selected students wishing to present a poster. Students may apply by submitting a short letter of nomination from their major professor together with a two-page abstract to the Symposium Manager by December 15, 1995.

Two-page abstracts are required in advance and will be reproduced in a bound volume which will be distributed to all symposium participants

Further details may be obtained from Mrs. Janet Cunningham, Symposium Manager, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(12), 2497-2498 (1995)

MEETING REPORT

PittCon'95
46th Annual Pittsburgh Conference
on Analytical Chemistry & Applied Spectroscopy

March 5 - 10, 1995

New Orleans, Louisiana

PittCon'95 was a very successful conference, both from the point of view of its technical content and its instrument exposition. Attendance was the largest for a Pittsburgh Conference held away from the northeastern part of the United States, with total attendance exceeding 31,000, and an exposition featuring over 1150 companies in nearly 3150 booths.

Registration:		
	Total	31,089
	Exhibitor personnel	15,836
	Conferees	14,908
	Media representatives	345
Technical Program:		
	Poster presentations	473
	Oral presentations	1,355
	Short courses	41
Exposition:		
	Companies	1,163
	Booths	3,144

One of the most exciting activities of the Conference was the presentation, to the Louisiana Children's Museum, of a number of gifts totalling $40,000, including an Earth Awareness Portable Classroom, an inflatable globe measuring 22 feet in diameter, The Science Spectrum (a collection containing "hands-on" activities in science areas such as spectroscopy, magnetism, optics, and a large microscope with slides mounted on a turntable for easy viewing by Museum visitors).

Science Week activities included awards to outstanding high school science teachers and equipment grants. Elementary school teachers attended a workshop on demonstrations to aid them in their teaching activities, while high school teachers built equipment to take back to their schools. More than 300 eight grade students participated in hands-on activities, while more than 1000 high school students attended a lecture-demonstration to learn scientific concepts and their applicability to everyday life.

PittCon'96 will be held on March 3-8, 1996 at McCormick Place in Chicago.

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(12), 2499 (1995)

ANNOUNCEMENT

BASIC PRINCIPLES OF HPLC
and
HPLC SYSTEM TROUBLESHOOTING
One-Day & Two-Day
In-House Training Courses

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

The following topics are covered in depth:

- Introduction to HPLC Theory
- Modes of HPLC Separation
- Developing and Controlling Resolution
- Mobile Phase Selection & Optimization
- Ion-Pairing Principles
- Gradient Elution Techniques
- Calibration & Quantitation
- Logical HPLC Troubleshooting

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

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

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(12), 2501-2506 (1995)

LIQUID CHROMATOGRAPHY CALENDAR

1995

JUNE 11 - 14: 1995 International Symposium and Exhibit on Preparative Chromatography, Washington, DC. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JUNE 13 - 16: Capillary Electrophoresis, Routine Method for the Quality Control of Drugs: Practical Approach (in English); L'Electrophorese Capillaire, Methode de Routine pour le Controle de Qualite des Medicaments: Approche Pratique (in French), Monpellier, France. Contact: Prof. H. Fabre, Lab. de Chimie Analytique, Inst. Europeen des Sciences Pharmaceutiques Industrielles de Montpellier, Ave. Charles Flahault, 34060 Montpellier Cedex 1, France.

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.

JUNE 25 - 28: Method Development in HPLC, Virginia Tech, Blacksburg, Virginia. Contact: Dr. H. McNair, Chem Dept, Virginia Tech, Blacksburg, VA 24061-0212, 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.

JULY 23 - 27: American Society of Pharmacognosy, 36th Annual Meeting, University of Mississippi, Oxford, Miss. Contact: Russell Cooper, Center for Public

Service & Continuing Studies, 14 E.F. Yerby Center, Box 1667, University of Mississippi, University, MS 38677, USA.

JULY 23 - 28: 35th Rocky Mountain Conference on Analytical Chemistry, Hyatt Regency, Denver, Colorado. Contact: Patricia Sulik, Rocky Mt. Instrum. Labs, 456 S. Link Lane, Ft. Collins, CO 80524, USA.

AUGUST 13 - 17: ICFIA'95: International Conference on Flow Injection Analysis, Seattle, Washington. Contact: Prof. G. D. Christian, Dept of Chemistry BG-10, University of Washington, Seattle, WA 98195, USA.

AUGUST 14 - 19: 35th IUPAC Congress, Istanbul, Turkey. Contact: Prof. A. R. Berkem, 35th IUPAC Congress, Halaskargazi Cad. No. 53, D.8, 80230 Istanbul, Turkey.

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

AUGUST 20 - 25: ACS sponsored Symposium on Saponins: Chemistry and Biological Activity, Chicago, Illinois. Contact: G. R. waller, Oklahoma State University, Dept of Chem & Molecular Biology, Stillwater, OK 74078, USA.

SEPTEMBER 4 - 7: 13th International Symposium on Biomedical Applications of Chromatography and Electrophoresis and International Symposium on the Applications of HPLC in Enzyme Chemistry, Prague, Czech Republic. Contact: Prof. Z. Deyl, Institute of Physiology, Videnska 1083, CZ-14220 Prague 4, Czech Republic.

SEPTEMBER 7 - 8: Engineering & Construction Contracting Conference, Phoenician Resort, Scottsdale, Arizona. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, 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.

SEPTEMBER 18 - 21: Safety in Ammonia Plants & Related Facilities, Loews Ventana Canyon, Tucson, Arizona. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

SEPTEMBER 26 - 29: CCPS International Conference on Modelling & Mitigating the Consequences of Accidental Releases of Hazardous Materials, Fairmont Hotel, New Orleans, Louisiana. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

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

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

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

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

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

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

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

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

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

1996

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

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

MARCH 3 - 8: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

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

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

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

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

JUNE 16 - 21: "HPLC '96: Twentieth International Symposium on High Performance Liquid Chromatography," San Francisco Marriott Hotel, San Francisco, California. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

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

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

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

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

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

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

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

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

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

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

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

1997

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

APRIL 14 - 19: Genes and Gen Families in Medical, Agricultural and Biological Research: 9th International Congress on Isozymes, sponsored by the Southwest Foundation for Biomedical Research, Hilton Palacio del Rio, San Antonio, Texas. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

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

1998

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

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

1999

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

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

2000

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

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

2001

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

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

2002

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

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

The **Journal of Liquid Chromatography** will publish, at no charge, announcements of interest to scientists in every issue of the journal. To be listed in Meetings & Symposia, we will need to know:

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Volkswriter 3, 4	Volkswriter Deluxe 2.2
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F. D. Pierce and H. R. Brown
Utah Biomedical Test Laboratory
520 Wakara Way
Salt Lake City, Utah 84108

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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.

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Contents Continued

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