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HIGH PERFORMANCE LIQUID PHASE SEPARATION OF GLYCOSIDES. I. REVERSED PHASE CHROMATOGRAPHY OF CYANOGENIC GLYCOSIDES WITH UV AND PULSED AMPEROMETRIC DETECTION

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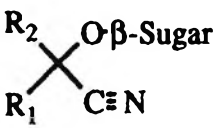
ABSTRACT

High performance liquid chromatography procedures based on reversed phase chromatography (RPC) using microparticulate octadecylsilica columns were introduced for the separation and detection of some representative cyanogenic glycosides and their degradation products. Pulsed amperometric detection (PAD) provided relatively low detection limits (10^{-5}–10^{-7} M) for the cyanogenic glycosides and permitted the detection of those lacking a chromophore in their structures (e.g., linamarin) which could not be detected in the UV even at low wavelength. In addition, the PAD was a more selective method of detection when compared to UV at 200 nm, a wavelength at which the molar absorptivity and in turn the detection sensitivity were relatively high for the chromophoric cyanogenic glycosides. However, and in the presence of acetonitrile in the eluent, the detector response in PAD was linear in concentration range over

2 to 3 orders of magnitude as opposed to 4 to 5 orders of magnitude in the UV. Finally, RPC proved very useful in monitoring the rate of hydrolysis of mandelonitrile to benzaldehyde, which is a degradation product of the cyanogenic glycosides amygdalin and prunasin. This hydrolysis was found to be a first order reaction.

INTRODUCTION

Many plant derived foodstuffs or tissues release HCN by the hydrolysis of secondary products, namely the cyanogenic glycosides and cyanolipids.[1] This ability of releasing HCN is know as cyanogenesis. Hydrolysis generally follows the disruption of cyanophoric tissues either by crushing, mastication or fungal injury, whereby endogenous hydrolases are allowed access to cyanogenic glycosides or cyanolipids, whose rapid catabolism to the lethal metabolite HCN is promoted. Cyanogenic glycosides are naturally occurring toxins that have been determined to be present in more than 2050 different plants distributed throughout 110 different families[2] that may be used as food sources by humans and domestic animals. There are, however, only approximately 26 known cyanogenic glycosides, all of which are derived from α-hydroxynitriles.[1] The general formula for cyanogenic glycosides is as follows



where the sugar moiety is usually a glucose and less often a gentiobiose, vicianose or primeverose. R_1 is either an aliphatic or aromatic group while R_2 is an H in a majority of the cyanogenic glycosides. When the sugar of the glycoside is removed, both HCN and a carbonyl are released.[3] Many of these cyanogenic glycosides are ingested by animals and also are nutritionally useful. Toxicity is the result of the release of HCN upon hydrolysis or enzymatic action on the glycoside.[4]

High performance liquid chromatography (HPLC) has been applied to the isolation and determination of cyanogenic glycosides including the use of hydroxyapatite chromatography,[5] normal-phase chromatography using silica gel columns[6] and reversed phase chromatography using primarily octadecylsilica (C_{18}) columns[7,8] (see Ref. 9 for a discussion) and to a lesser extent octylsilica (C_8) columns.[10,11] In these HPLC systems, UV detectors have been used for the detection of chromophoric cyanogenic glycosides while

refractive index (RI) detectors have been employed for detecting non absorbing cyanogenic glycosides. While UV detectors have provided an adequate detection sensitivity, RI detectors have been known for their low sensitivity and incompatibility with gradient elution. To overcome the difficulties associated with the detection of cyanogenic glycosides that lack strong chromophores in their structures, electrochemical detection has been attempted.[10] However, in that work,[10] a post-column enzymatic reactor was needed for the cleavage of the cyanogenic glycosides to form HCN, which was followed by converting the liberated HCN to CN^- by the addition of NaOH, and the ultimate cleavage product CN^- was then detected electrochemically. Thus, there is a need for a method for determining weak chromophore-containing cyanogenic glycosides that is simple and reproducible.

This report is concerned with the evaluation of pulsed amperometric detection (PAD) in the detection of cyanogenic glycosides, and the results are compared with UV detection. To the best of our knowledge, PAD has not been yet exploited in the determination of cyanogenic glycosides, despite the fact that PAD has been found to be quite useful for the detection of analytes that do not possess a strong chromophore.[12] With PAD, there is no need for post-column modification of the separated analytes; thus, providing an easily managed method of detection. In addition, PAD is a very simple, sensitive, and selective method for the detection of a variety of different analytes, such as carbohydrates, alcohols, alkanolamines, and sulfur-containing compounds.[13-15]

EXPERIMENTAL

Instruments and Columns

The liquid chromatograph was assembled from (i) an LDC Analytical (Riviera Beach, FL, USA) solvent delivery system comprising a ConstaMetric 3500 pump with a gradient programmer, which also controlled a ConstaMetric Model III pump, (ii) a Model 7125 sample injector from Rheodyne (Cotati, CA, USA), (iii) a variable wavelength detector Model SpectroMonitor 3100 from LDC Analytical, (iv) a pulsed amperometric detector (PAD) from Dionex (Sunnyvale, CA, USA), and (v) a Shimadzu (Columbia, MD, USA) integrator Model C-R5A Chromatopac.

For UV monitoring of the column effluent, the detector was set at 200 nm with a response time of 0.50 sec. For pulsed amperometric detection of the solutes, 0.50 M NaOH was added to the column effluent at a flow rate of 0.7–0.8 mL/min. This post-column addition was accomplished via a T-connector

which allowed the mixing of the column effluent with the stream of the NaOH solution that was delivered by a nitrogen pressurized reservoir containing the NaOH solution. The two liquids (i.e., the column effluent and NaOH solution) were further mixed in a post-column reaction coil packed bead before entering the detection cell of the PAD. The pH of the combined streams of column effluent and post-column addition of NaOH yielded a solution whose pH was found to be 12.7.

The PAD is equipped with a gold working electrode, Ag/AgCl reference electrode and a solvent compatible PAD-2 cell. The PAD settings for pulse potentials and duration times were E_1 = 0.00 V, t_1 = 240 ms; E_2 = 0.60 V, t_2 = 60 ms; E_3 = -0.80 V, t_3 = 60 ms.

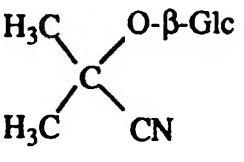
The reversed phase packing consisted of Microsorb-MV C18, 5 µm, 100 Å from Rainin (Woburn, MA, USA), and was supplied in packed columns of dimensions of 250 x 4.6 mm or 150 x 4.6 mm.

The UV spectra of the various cyanogenic glycosides were obtained with a diode array spectrophotometer from Hewlett Packard (Waldbronn, Germany) Model 8452A which was utilized over the spectral range from 190 nm to 400 nm with an integration time of 1.0 sec.

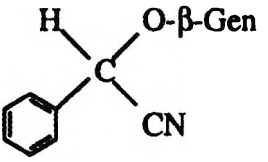
Reagents and Materials

Acetonitrile of HPLC grade as well as high purity UV was obtained from J.T. Baker (Phillipsburg, NJ, USA) and from Baxter Diagnostics Inc. (McGraw Park, IL, USA), respectively. Sodium hydroxide solution (50% w/w) was purchased from Fisher (Pittsburgh, PA, USA), and purified water was prepared with a Barnstead Nanopure ultrapure water system. Benzaldehyde was purchased from Mallinckrodt (St. Louis, MO, USA).

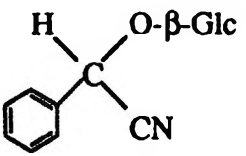
The cyanogenic glycosides linamarin (phaseolunatin), prunasin (D-mandelonitrile-β-D-glucoside) and D-amygdalin (D-mandelonitrile 6-O-β-D-glucosido-β-D-glucoside) from apricot kernels as well as their degradation product DL-mandelonitrile (α-hydroxyphenylacetonitrile) were purchased from Sigma (St. Louis, MO, USA). The structures of the three cyanogenic glycosides and mandelonitrile (the degradation product of prunasin and amygdalin) are shown below.



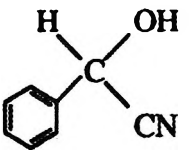
Linamarin



Amygdalin



Prunasin



Mandelonitrile

where Glc = glucose and Gen = gentiobiose. The standard cyanogenic glycoside samples were dissolved in water, while the standard mandelonitrile was dissolved in acetonitrile. Eluents were filtered, sonicated and degassed with ultra pure grade helium.

RESULTS AND DISCUSSION

Two different methods of detection were utilized and compared in this study, namely UV and pulsed amperometric detection (PAD). The solutes were chromatographed by either gradient or isocratic elution.

Figure 1a and b shows typical chromatograms obtained by 10 and 6 min linear gradient elution, respectively, at increasing acetonitrile concentration in the eluent using PAD for sensing the column effluent. As can be seen in Figure 1a and b, the four solutes are fully resolved into narrow peaks. In contrast, Figure 2 shows a typical isocratic separation using a mobile phase of 82:18 water/acetonitrile (v/v). It should be noted that peak No. 4 in Figures 1a and 2 is believed to be the peak of benzaldehyde which was assigned (see below) by studying the degradation of mandelonitrile. Although the same analysis time is achieved in both cases, gradient elution provided higher separation efficiency (compare Figures 1a and 2). In addition, linamarin which is not strongly retained eluted very close to the dead volume where other contaminants of the sample emerged; see Figure 2.

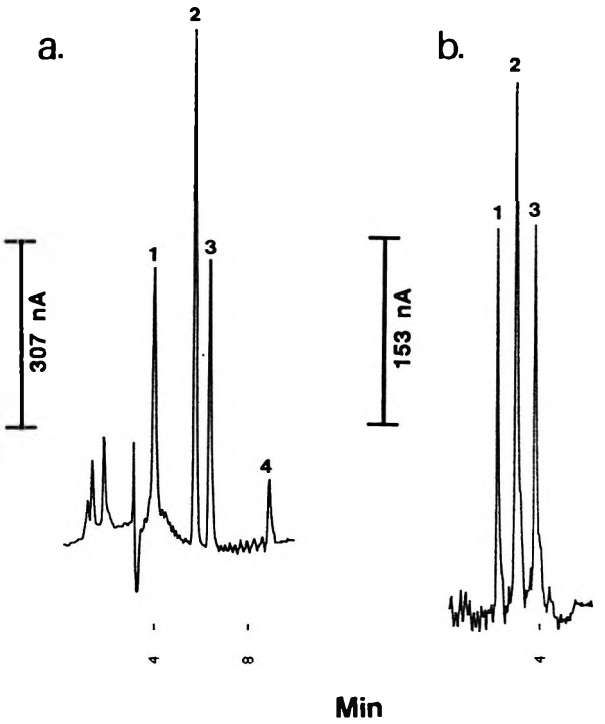


Figure 1. Typical chromatograms of cyanogenic glycosides obtained by gradient elution using PAD. Column, Microsorb MV C18, 15 cm x 4.6 mm i.d. in (a), 25 cm x 4.6 mm i.d. in (b); (a) linear gradient in 10 min from 0.0 to 60% (v/v) acetonitrile in water; (b) linear gradient in 6 min from 18.0 to 60% (v/v) acetonitrile in water; flow rate, 1.5 mL/min.
Peaks: 1, linamarin; 2, amygdalin; 3, prunasin; 4, benzaldehyde.

To select the appropriate wavelength for UV detection, spectral data were obtained for each of the cyanogenic glycosides studied as well as for the degradation products in the range of 190 to 400 nm. With the exception of linamarin, each of the solutes exhibited two distinct absorption maxima, see Table 1. The highest absorption maxima were at 192 nm for both amygdalin and prunasin. The other less pronounced absorption maxima were at 206 nm and 208 nm for amygdalin and prunasin, respectively. While prunasin showed a small maximum at 262 nm, amygdalin did not show a distinct maximum at this wavelength and the molar absorptivity was relatively low, see Table 1.

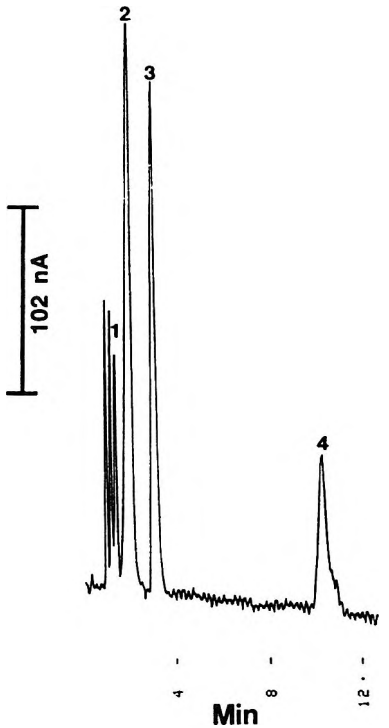


Figure 2. Typical chromatogram of cyanogenic glycosides obtained by isocratic elution using PAD. Column, Microsorb MV C18, 15 cm x 4.6 mm i.d.; mobile phase, water at 18% acetonitrile (v/v); flow rate, 1.5 mL/min. Peaks as in Fig. 1.

The intermediate and degradation products of both amygdalin and prunasin, i.e., mandelonitrile and benzaldehyde, respectively, showed the highest absorption maxima at 196 and less pronounced maxima at 251 nm. Based on these spectral data, a 200 nm wavelength was selected for the detection of the four UV detectable solutes.

Returning to Table 1, it is clear that adding a sugar residue to mandelonitrile to yield prunasin decreased slightly the molar absorptivity and shifted the less pronounced absorption maximum to lower wavelength (i.e., from 251 nm to 208). Adding another glucose moiety to prunasin to yield amygdalin did not result in a significant change in the spectral maxima and molar absorptivity, see Table 1.

Table 1

Absorption Maxima and Molar Absorpitivity

Cyanogenic	λ_1 (nm)/ε_1 ($M^{-1}cm^{-1}$)	λ_2 (nm)/ε_2 ($M^{-1}cm^{-1}$)	λ_3 (nm)/ε_3 ($M^{-1}cm^{-1}$)
Linamarin	200 / 6 420		
Amygdalin	192 / 37 280	206 / 5 700	262 / 716
Prunasin	192 / 35 320	208 / 7 355	262 / 3 807
Mandelonitrile	196 / 42 435	251 / 10 472	
Benzaldehyde	196 / 45 555	251 / 10 041	

As just stated above, only four solutes could be detected at 200 nm, namely amygdalin, prunasin, mandelonitrile and benzaldehyde. Figure 3 illustrates a typical UV chromatogram obtained with a 5 min linear gradient at increasing acetonitrile concentration in the eluent. The starting eluent contained 30% (v/v) acetonitrile to affect the elution and separation of the three solutes with the gradient time selected. This high organic modifier was necessary since increasing gradient steepness (from 3 to 12% v/v acetonitrile/min) by decreasing the gradient time while maintaining the composition of the starting eluent the same (i.e., 0% v/v acetonitrile) did not allow the elution of all analytes. As with the PAD detection, when eluted isocratically, the peaks were broader, and in addition the analysis time was doubled (results not shown).

Returning to Figures 1a, 2 and 3, as mentioned above, the last eluting peak was the degradation product of mandelonitrile, namely benzaldehyde. The rationale behind studying the chromatographic behavior of mandelonitrile resides in the fact that mandelonitrile is a degradation product of prunasin and amygdalin. In fact, in cyanophoric plants, there exists enzymes which are specific for the β-glycosidic linkage.

Generally, when the plant tissue is crushed or otherwise disrupted, the glycoside comes into intimate contact with β-glucosidases which hydrolyze the cyanogenic glycoside producing D-glucose and the respective α-hydroxynitrile (see also **INTRODUCTION**). The latter may dissociate spontaneously, releasing HCN and the corresponding aldehyde or ketone. However, cyanogenic tissues generally possess a second enzyme, an hydroxynitrile lyase, which catalyzes the dissociation of the cyanohydrin.[9]

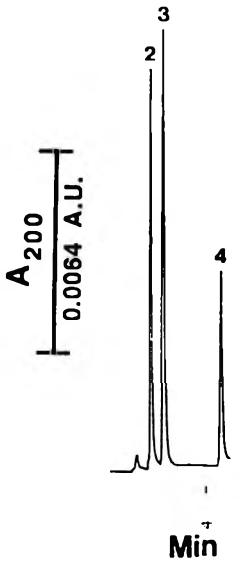
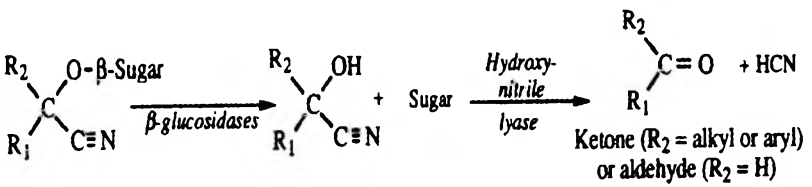


Figure 3. Typical chromatogram separation of cyanogenic glycosides obtained by gradient elution using UV detection at 200 nm. Column, Microsorb MV C18, 25 cm x 4.6 mm i.d.; linear gradient of 5 min from 30.0 to 60.0% (v/v) acetonitrile in water; flow rate, 1.5 mL/min. Peaks as in Fig. 1.



Over time, and as it has been reported in the literature,[10] mandelonitrile breaks down due to hydrolysis to form benzaldehyde and HCN. In fact, whenever the mandelonitrile sample solution was freshly prepared, two peaks were consistently associated with the solution of mandelonitrile. However, over time the apparent ratio of the peaks changed reflecting the appearance of the product and disappearance of the analyte. To determine the time course and conditions required for total breakdown of mandelonitrile, a series of injections was undertaken using isocratic elution with a mobile phase at 27% (v/v) acetonitrile. Figure 4 shows three chromatograms corresponding to the initial

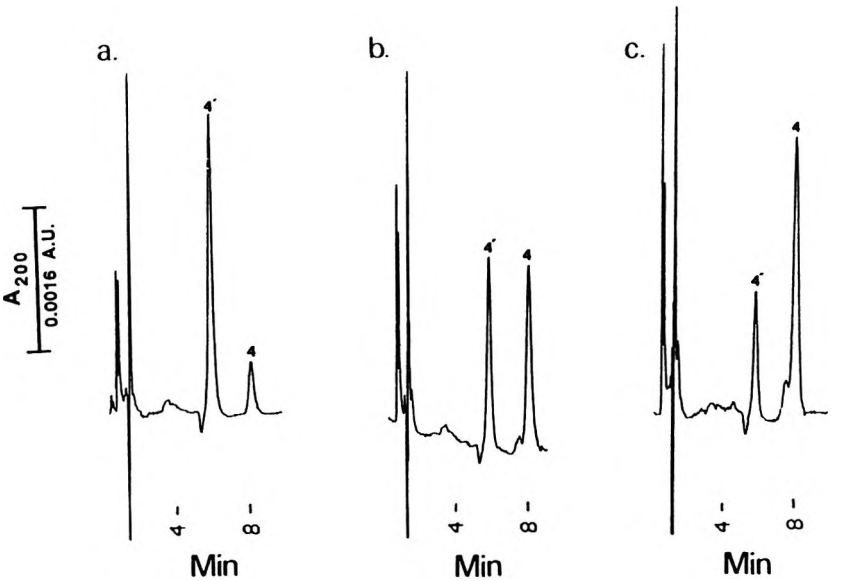


Figure 4. Typical chromatograms for the degradation of mandelonitrile to the product benzaldehyde obtained by isocratic elution using UV detection at 200 nm. (a) Initial preparation; (b) 1 hour after preparation; (c) 3 hours and 30 minutes after preparation. Column, Microsorb MV C18, 15 cm x 4.6 mm i.d.; mobile phase, water at 27% (v/v) acetonitrile; flow rate, 1.5 mL/min. Peaks: 4', mandelonitrile; 4, benzaldehyde.

preparation, 1 hour after preparation, and after 3 hours and 30 min of standing at room temperature. Figure 5a gives the time course of the degradation of mandelonitrile, expressed in terms of peak height ratio (mandelonitrile/benzaldehyde) vs. time. To determine the order of the hydrolysis reaction, Figure 5b shows the dependence of the natural logarithm of the peak height of mandelonitrile, which reflects the natural logarithm of mandelonitrile concentration, on time. The plot is linear indicating a first-order reaction.

Using isocratic elution, the retention of glycosides under investigation showed strong dependence on the percent acetonitrile in the eluent. Plots of logarithmic capacity factor of the solutes under investigation vs. percent acetonitrile (in the percent range from 0 to 25%) were linear with R values greater than 0.993 (results not shown), thus indicating a reversed phase chromatography behavior.

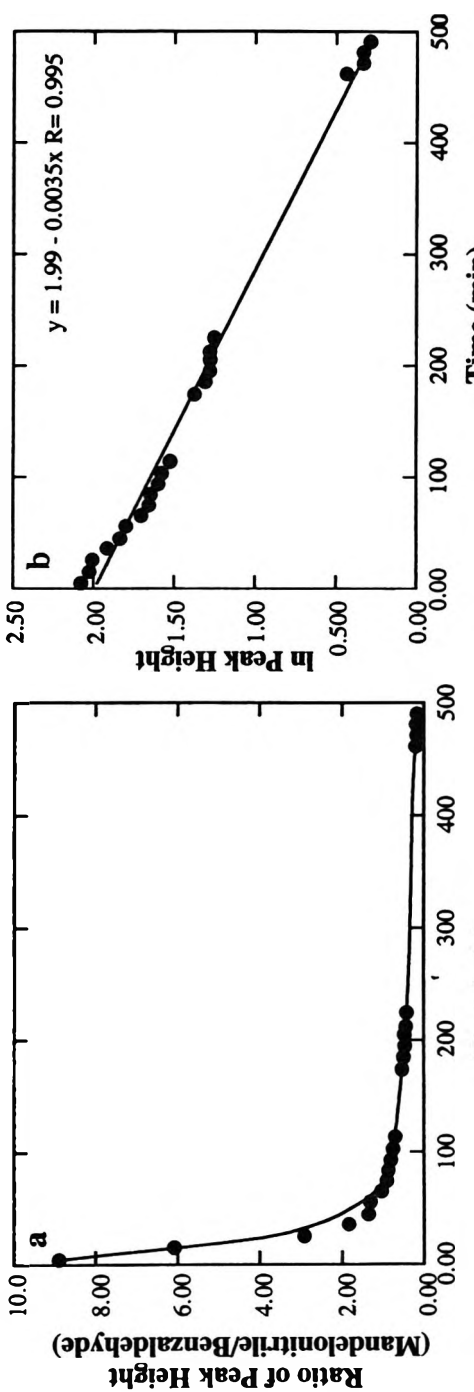


Figure 5. (a) Plot of the ratio of mandelonitrile/benzaldehyde peak height *versus* time; (b) plot of logarithmic peak height of mandelonitrile *versus* time. Conditions as in Fig. 4.

It should be noted that with PAD detection, as the percent acetonitrile in the eluent was increased, baseline noise increased. It has been reported that acetonitrile can interfere with the detector response by adsorbing to the surface of the noble-metal electrode.[16] Therefore, detection is inhibited as the percent acetonitrile in the eluent is increased, thus lowering the detection efficiency of PAD. Peak area *versus* the percent acetonitrile in the eluent was tested in PAD using amygdalin as a representative cyanogenic glycoside. As the percent acetonitrile in the eluent was increased, the peak area of amygdalin was unaffected up to 15% (v/v) acetonitrile. At percent acetonitrile ≥ 18% (v/v) in the eluent, peak area was reduced (results not shown).

Detection limits were determined with both the 15 cm and 25 cm columns under gradient and isocratic elution. The limits of detection were determined at a signal-to-noise ratio of ca. 3. It should be noted that with PAD, as the percent acetonitrile in the eluent was increased, it was necessary to decrease the setting of sensitivity (i.e., increase attenuation) to reduce baseline noise. Table 2 shows the limits of detection found for the solutes using both UV and PAD detection methods based on a 20 μL injection. In gradient elution, using a 10 min linear gradient (from 0 to 60% acetonitrile) provided the lowest limit of detection for the solutes with PAD, and similar limits of detection were found for the 10 min and 20 min linear gradient with the UV detector. In gradient elution, PAD as well as UV detection required a lower setting for sensitivity (i.e., higher attenuation) in order to avoid a noisy baseline. An elution at 15% acetonitrile (v/v) provided the lowest limit of detection for isocratic analysis. The limit of detection in gradient elution was lower than in isocratic elution making gradient elution a more sensitive choice. In comparing UV and PAD, both detection methods provided similar results concerning the limits of detection for two solutes, namely amygdalin and prunasin. As expected, benzaldehyde, which is the degradation product of mandelonitrile, was detected at a lower level using UV detection than in PAD. This was the result of a weak response which is usually obtained for aldehydes using PAD.[15] The pulsed amperometric detector, however, was able to detect the presence of linamarin whereas UV detection did not; therefore, a combination of PAD and UV detection would provide the best method for the determination of the four solutes.

The linear dynamic range for the detector response was determined in both UV and PAD detection. The analytes amygdalin and prunasin were chromatographed isocratically with 15% (v/v) acetonitrile while linamarin was analyzed with a mobile phase at 6% (v/v) acetonitrile. Mandelonitrile was not tested for linear response due to its degradation to benzaldehyde. Using PAD, linamarin responded linearly in the concentration range of $1.25 \times 10^{-6} - 0.0075$ M ($R = 0.994$). For the analyte amygdalin and using UV detection the

Table 2

Limit of Detection (LOD) Obtained with UV and PAD in Terms of Molar Concentration

	LOD (mol/L) UV		LOD (mol/L) PAD	
Solute	**Isocratic**	**Gradient**	**Isoctatic**	**Gradient**
Linamarin	-----	-----	2.50×10^{-7}[a]	-----
	-----	-----	1.25×10^{-6}[b]	-----
	-----	-----	-----	1.25×10^{-6}[c]
	-----	-----	-----	2.50×10^{-7}[d]
	-----	-----	-----	9.83×10^{-6}[e]
Amygdalin	1.26×10^{-7}[f]	-----	7.58×10^{-7}[f]	-----
	4.40×10^{-7}[g]	-----	-----	-----
	-----	5.70×10^{-7}[h]	-----	1.00×10^{-6}[c]
	-----	6.83×10^{-7}[I]	-----	8.80×10^{-8}[d]
	-----	-----	-----	8.80×10^{-7}[e]
	1.00×10^{-7}[k]	-----	-----	-----
Prunasin	1.35×10^{-7}[f]	-----	1.35×10^{-6}[f]	-----
	4.68×10^{-7}[g]	-----	-----	-----
	-----	6.08×10^{-7}[h]	-----	1.35×10^{-6}[c]
	-----	7.28×10^{-7}[I]	-----	9.90×10^{-8}[d]
	-----	1.50×10^{-6}[j]	-----	9.90×10^{-6}[e]
	7.30×10^{-8}[k]	-----	-----	-----
Benzaldehyde	4.98×10^{-7}[g]	-----	1.25×10^{-5}[g]	----
	-----	1.53×10^{-7}[h]	-----	9.90×10^{-6}[c]
	-----	7.73×10^{-7}[I]	-----	9.90×10^{-7}[d]
	-----	1.50×10^{-6}[j]	-----	9.90×10^{-6}[e]

[a] Isocratic elution at 0% acetonitrile with a 15 cm column; [b] isocratic elution at 6% acetonitrile with a 15 cm column; [c] 20 min linear gradient with a 15 cm column; [d] 10 min linear gradient with a 15 cm column; [e] 6 min linear gradient with a 15 cm column; [f] isocratic elution at 12 % acetonitrile with a 15 cm column; [g] isocratic elution at 18% acetonitrile with a 15 cm column; [h] 20 min linear gradient with a 25 cm column; [I] 10 min linear gradient with a 25 cm column; [J] 6 min linear gradient with a 25 cm column; [k] isocratic elution at 15% acetonitrile with a 15 cm column. In [c, d, h and I] the gradient was from 0 to 60% acetonitrile while in [e and J], the gradient was from 18 to 60% acetonitrile.

response was linear (R = 0.995) in the concentration range of 1.00 x 10^{-7} – 0.007 M, while the detector response in PAD remained linear from 1.40 x 10^{-5} – 0.00138 M (R = 0.992). At 15% acetonitrile, the baseline noise with PAD was relatively high, reason for which the limit of detection is now about 1.40 x 10^{-5} M as opposed to 7.58 x 10^{-7} M at 12% acetonitrile in the eluent, see Table 2. In UV detection, prunasin maintained a range of linearity from 7.30 x 10^{-8} – 0.0080 M (R = 1.000), and in PAD the response was linear from 1.00 x 10^{-5} – 0.0083 M (R = 0.999). As with amygdalin, the baseline noise was relatively high at 15% acetonitrile permitting a limit of detection of only 1.00 x 10^{-5} M. In all cases, response was linear to the limits of detection for all of the analytes sampled.

In conclusion, the use of PAD and UV detection coupled with reversed phase chromatography provides a sensitive and efficient method for the determination and separation of cyanogenic glycosides. Under optimal conditions, linear detector response holds over a wide range of concentration for the solutes, and limits of detection are in the low micromolar to high nanomolar range (10^{-6} - 10^{-8} M).

ACKNOWLEDGMENTS

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SELECTIVITY IN MICELLAR LIQUID CHROMATOGRAPHY: SURFACTANT BONDED PHASE INTERACTIONS. I. C-18

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ABSTRACT

Micellar liquid chromatography and solid state ^{13}C NMR spectroscopy have been used to study the interactions of three ionic surfactants with the C_{18} alkyl bonded phase. The three surfactants, sodium dodecylsulfate (SDS), cetyltrimethyl ammonium bromide (CTAB), and dodecyltrimethylammonium bromide (DTAB), are commonly used in micellar RPLC. Surfactant adsorption is found to produce distinct changes in the selectivity of the stationary phase. Specifically, the differing nature of the surfactant monomer-bonded phase association is largely responsible for the observed differences in selectivity between SDS, CTAB, and DTAB micellar RPLC. For SDS, the association leads to the formation of an anionic hydrophilic surface layer on C_{18} which would explain the superior resolution

achieved by SDS for hydrophilic compounds. For CTAB or DTAB adsorbed on C_{18}, the nitrogen head group is probably incorporated (at least partially) in the bonded phase due to hydrophobic interactions between the methyl nitrogens and the C_{18} alkyl bonded phase. Chemical models depicting the structure of the surfactant coated C_{18} stationary phase are proposed from the NMR data, and these models are in good agreement with retention data obtained for these micellar RPLC systems.

INTRODUCTION

In reversed phase liquid chromatography (RPLC), a hydro-organic solvent mixture is commonly used as the mobile phase. However, aqueous micellar solutions, i.e., solutions containing surfactant at a concentration above the critical micelle concentration, have been shown to possess properties analogous to those of conventional mobile phases in RPLC. This unusual variation of the RPLC experiment is known as miceliar liquid chromatography (MLC).

Armstrong and Henry[1] first demonstrated that micelles can be used in place of traditional organic modifiers, such as methanol or acetonitrile, in RPLC. Micelles, which are dynamic assemblies of surfactant molecules, can organize and compartmentalize solutes at various sites within the surfactant assembly. The actual location of the solute in the assembly is dictated by the nature of the solute and the surfactant system employed.[2-3] Each solubilization site, i.e. microenvironment, in the micelle is unique, and its properties (e.g., polarity, fluidity, and acidity) are distinctly different from those of the bulk solvent.

Retention in MLC has been shown to be correlated to surfactant type and to the concentration of surfactant in the mobile phase.[4-6] Solute retention in MLC generally decreases with increasing surfactant (i.e., micelle) concentration, but the rate of decrease can vary considerably from one organic solute to the next. Equations relating the capacity factor (k') to the concentration of micelles in the mobile phase have been formulated by Armstrong and Nome[7] and Cline-Love and Arunyanart.[8] These equations which are based on a three-way partition model have been verified experimentally[9-11] for a large number of organic compounds.

Many of the advantages offered by micellar mobile phases, e.g., enhanced luminescence detection, simultaneous separation of charged and neutral compounds, and the ability to directly inject biologicals onto the column without prior sample work-up, is due to the unique ability of micelles to

organize and compartmentalize solutes at the molecular level. The ability of micelles to selectively solubilize and interact with solute molecules is believed to be the basis of separation in MLC.[12] However, surfactant molecules are readily adsorbed on hydrocarbonaceous stationary phases. The architecture assumed by adsorbed surfactant molecules on conventional RPLC stationary phases has been postulated to vary from hemi-micellar or admicellar to mono-, bi-. Since many properties of HPLC stationary phases are altered by the process of surfactant adsorption, the modification of the bonded stationary phase by adsorbed surfactant molecules can have profound implications with regard to retention and selectivity in MLC.

Micellar liquid chromatography and solid state ^{13}C NMR spectroscopy have been used to study the interactions of three ionic surfactants with the C_{18} alkyl bonded phase. The three surfactants, sodium dodecylsulfate (SDS), cetyltrimethylammonium bromide (CTAB), and dodecyltrimethylammonium bromide (DTAB), are commonly used in micellar RPLC. Surfactant adsorption is found to produce distinct changes in the selectivity of the stationary phase. Specifically, the differing nature of the surfactant monomer-bonded phase association is largely responsible for the observed differences in selectivity between SDS, CTAB, and DTAB micellar RPLC. For SDS, the association leads to the formation of an anionic hydrophilic surface layer on C_{18} which would explain the superior resolution achieved by SDS for hydrophilic compounds. For CTAB or DTAB adsorbed on C_{18}, the nitrogen head group is probably incorporated (at least partially) in the bonded phase because of hydrophobic interactions between the methyl nitrogens and the alkyl bonded phase. Chemical models depicting the structure of the surfactant coated C_{18} stationary phase are proposed from the NMR data, and these models are in good agreement with retention data obtained for these different micellar RPLC systems.

EXPERIMENTAL

Chemicals

The six vanillin compounds (see Figure 1) which constituted the hydrophilic test mixture used to characterize the surfactant coated stationary phases were obtained from Aldrich and were used as received. Stock solutions of the various test solutes were prepared in methanol and then diluted to the appropriate working concentration (550 μg/mL) using 50% methanol in water. The surfactants, SDS, CTAB, and DTAB, were obtained from BDH Chemicals (99% purity) and were purified prior to use by first dissolving them in ethanol

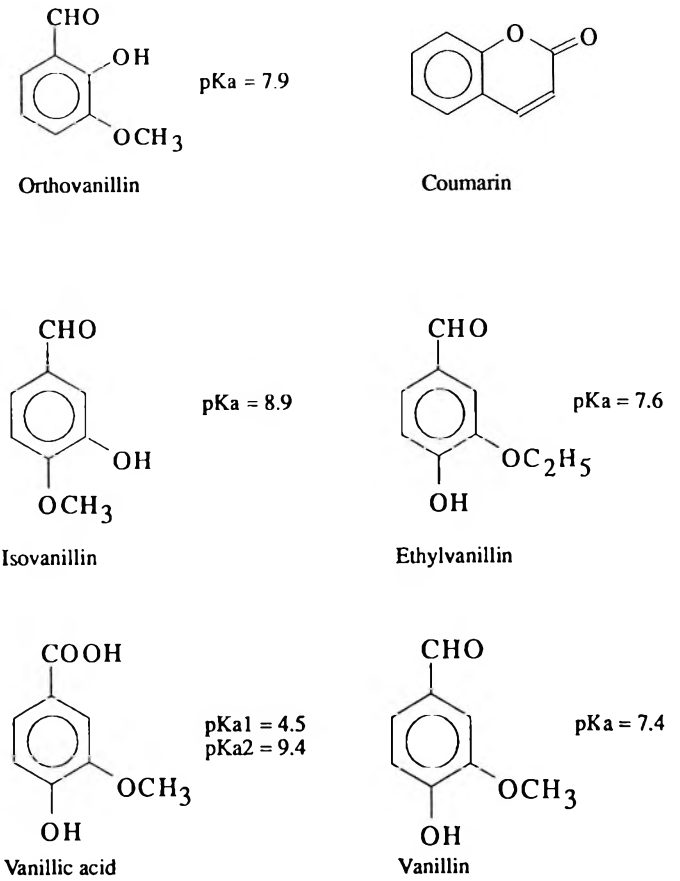

Figure 1. The vanillin compounds. The pKa values are from reference 13.

followed by addition of charcoal to the solution. After the charcoal was separated from the mother liquor by filtration, the surfactant was recrystallized from the ethanol and dried in an oven at 65° C. Micellar solutions were prepared from the recrystallized surfactants using HPLC grade distilled water. (Methanol-water mobile phases were also prepared using HPLC grade solvents.) All mobile phase solutions were filtered twice with a 0.45 μm Nylon membrane filter (Rainin Instruments, Woburn, MA) to remove particulate matter. Prior to use, the solutions were degassed and their pH adjusted to 3 with hydrochloric acid to prevent ionization of polar solutes in the mobile phase solutions.[13]

High Performance Liquid Chromatographic (HPLC) Measurements

All HPLC measurements were made using either a Perkin Elmer TriDet HPLC or a Rainin 81-20 M analytical HPLC system. The analytical column was Apex I C-18 (5-μm, 10cm x 4.6 mm i.d.). The columns were purchased from Jones Chromatography (Golden, CO). The analytical column was water-jacketed and temperature controlled. Separate columns were used for each surfactant because of strong and irreversible adsorption of ionic surfactants on the stationary phase of the C_{18} bonded phase. The dead volume of each column which was determined by injecting different solutions such as methanol-water, or water onto the Apex I column was approximately 1.0 mL and was used for all k' calculations. The k' values determined in this study were averages of at least triplicate determinations, and deviations in individual k' values were never greater than 5%. All k' measurements were made at a flow rate of 1.0 mL/min and were measured at 25°C for SDS and DTAB and 30°C for CTAB. (Since the Kraft point of CTAB is 23°C, it was necessary to perform the CTAB studies at a higher temperature.)

Estimation of Critical Partitioning Parameters in Micellar RPLC

Solute-stationary phase and solute-micelle binding constants were determined for the vanillin compounds using an equation developed by Cline-Love and Arunyanart[8]

$$1/k' = [M]K_2/\theta[L_s]K_1 + 1/\theta[L_s]K_1 \qquad (1)$$

where [M] is the concentration of surfactant, K_2 is the solute-micelle binding constant per monomer of surfactant, θ is the chromatographic phase ratio. $[L_s]$ is the concentration of ligate on the stationary phase, and K_1 is the solute-stationary phase binding constant. A plot of 1/k' vs [M] should yield a straight line, and in fact excellent linearity was observed for all six compounds using SDS, CTAB or DTAB.

Solid State NMR Measurements

Adsorption of SDS, DTAB, and CTAB on C_{18} chemically derivitized silica was investigated using cross polarization/magic angle spinning ^{13}C NMR with high-power proton decoupling (CP/MAS ^{13}C NMR). All NMR experiments were performed at 50 MHz on a Bruker/IBM WP-200 SY Spectrometer equipped with an IBM solids control accessory and a Doty-type solid-state probe that was software controlled which permitted automatic

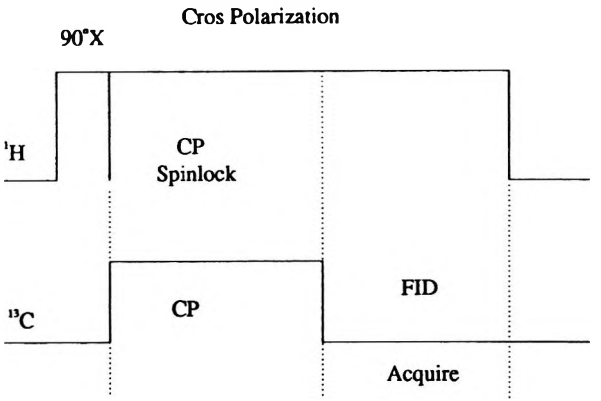

Figure 2. Pulse sequence for the determination of T_{CH}.

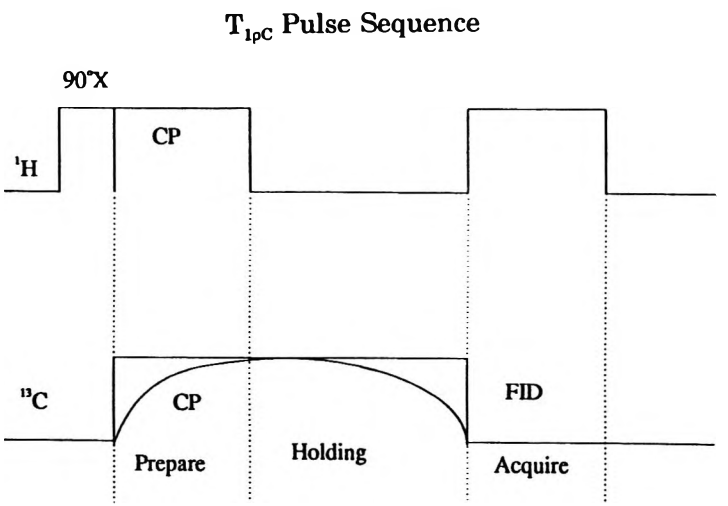

Figure 3. Pulse sequence for the determination of T_{1YC}.

variation of all pulse parameters. The magic angle spinning probe used was a double-tuned, single-coil design with a bullet type rotor which held a sample volume of 0.75 cm^3. Two different pulse sequences were used in these NMR experiments (see Figures 2 and 3). However, each pulse sequence was performed with a constant 3-s recycle time. The ^{13}C spectra collected were

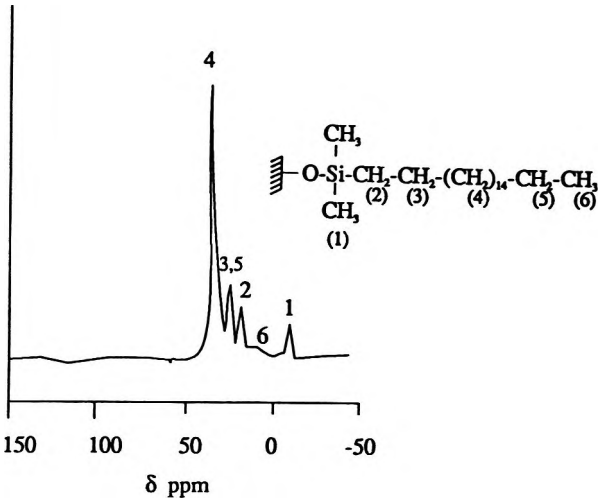

Figure 4. ^{13}C CP/MAS NMR spectrum and chemical shift assignments for C_{18}. A thousand pulses were used to generate the spectrum.

externally referenced to para-di-t-butyl benzene. All chemical shift values were expressed as parts per million down-field from tetramethylsilane. The ^{13}C data were collected in 2 Kbytes of memory, exponentially multiplied prior to Fourier transformation, and zero-filled to 8 kilobytes.

Sample Preparation

To prepare a sample for solid-state NMR, 0.5 g of 5μm C_{18} reversed phase material was equilibrated with 10 mL of 0.05 M aqueous CTAB, DTAB, or SDS solution. The equilibration period for the stationary phase material and surfactant was at least 24 h.

During equilibration, a wrist action shaker was periodically used to agitate the samples. After equilibration, each sample was vacuum filtered onto a 0.45 μm Nylon 66 membrane filter and vacuum dried at 35°C for 2 days prior to being packed into the rotor of the solid-state probe.

X-ray Diffraction Studies

Low angle X-ray diffraction spectra were obtained for pure SDS, CTAB, and DTAB with a Siemens Crystalloflex 4, with a Tennelec detector system, PSD 100. The path length was 50 cm, and the sample was suspended in a 0.5 mm capillary with 0.01 mm wall thickness. The spectra were run at room temperature at 40 kV and 30 mA.

RESULTS & DISCUSSION

Solid state NMR was employed in this study because surfactant molecules not in contact with the bonded phase can be readily differentiated from surfactant molecules that are intercalated or in direct contact with the bonded phase. However, solid state NMR cannot sample the stationary phase under chromatographic conditions. Even though solid state NMR measurements can provide information about the structural environment of the surfactant coated stationary phase, no direct information on how the stationary phase interacts with the solute is provided by this NMR technique. One approach for the direct measurement of these interactions is the use of retention probes which can be selected to emphasize specific physical or chemical interactions of the solute with the mobile or stationary phase. Because our objective was to study hydrophilic interactions in MLC, not hydrophobic interactions (which is the usual practice), we chose a set of six vanillin compounds to serve as retention probes. NMR and micellar RPLC retention data obtained for a C_{18} bonded phase are summarized and discussed below for three ionic surfactants: SDS, CTAB, and DTAB.

NMR

Solid state ^{13}C NMR spectra are shown in Figures 4, 5, 6, and 7 for the following materials: (1) C_{18}, (2) pure SDS, (3) SDS adsorbed on C_{18}, (4) pure CTAB, (5) CTAB adsorbed on C_{18}, (6) DTAB, and (7) DTAB adsorbed on C_{18}. Chemical shift assignments were made on the basis of previously published literature reports[14-16] on related materials and the observed CP/MAS spectra of pure SDS, CTAB, and DTAB. For pure SDS (see Figure 5a) in order of increasing frequency from left to right, the lowest field line at 68 ppm is assigned to the methylene carbon nuclei alpha to the sulfate head group (i.e., the alpha carbon), the highest field line at 12 ppm is assigned to the terminal methyl group. There is a second peak for the alpha carbon at 50 ppm which only appears after adsorption of SDS on C_{18} (see Figure 5b). This peak represents surfactant monomer in contact with the bonded phase. We attribute

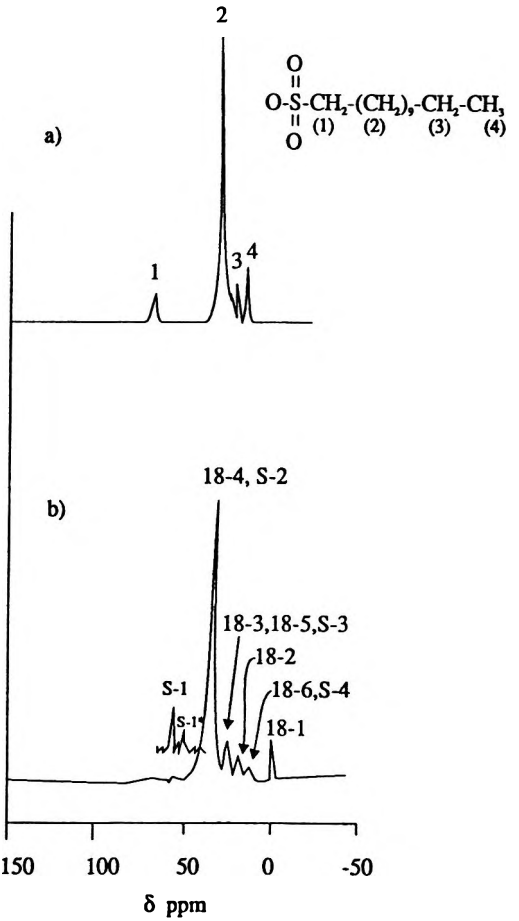

Figure 5. ^{13}C CP/MAS NMR spectrum and chemical shift assignments for (a) pure SDS, and (b) SDS adsorbed on C_{18}. Bonded phase resonances are indicated by labels starting with numerals (i.e., 18-), while surfactant resonances are indicated by labels beginning with letters (S=SDS).

the change in the chemical shift value of the alpha carbon to a change in the chemical environment of this nuclei. This conclusion is reinforced by X-ray diffraction studies performed in our laboratory which show that pure SDS has an ordered structure with an interlayer spacing of 37.3 A which is in good agreement with the calculated length of a close-packed SDS structure,[17] whereas a less ordered arrangement is implied by the accepted model[18-19] for

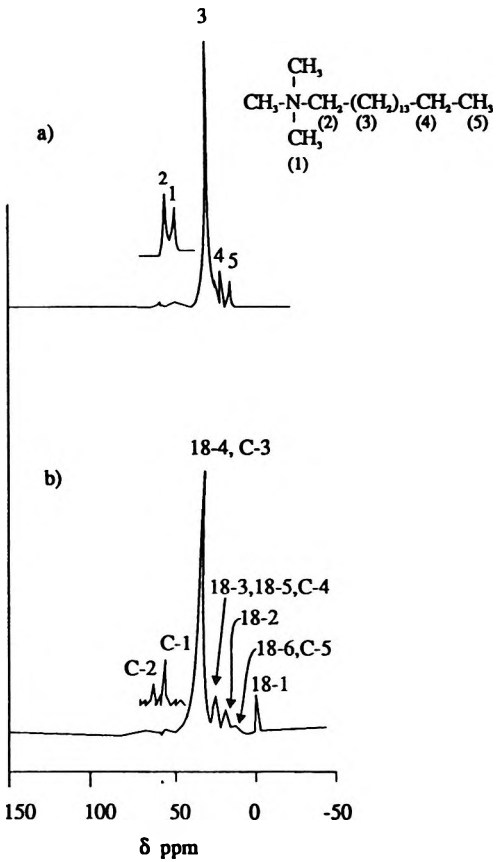

Figure 6. ^{13}C CP/MAS NMR spectrum and chemical shift assignments for (a) pure CTAB, and (b) CTAB adsorbed on C_{18}. Bonded phase resonances are indicated by labels starting with numerals (i.e., 18-), while surfactant resonances are indicated by labels beginning with letters (C=CTAB).

surfactant adsorption at a buried interface. Because of differences in ordering between these two phases (solid vs adsorbed SDS), a change in the chemical shift value of the alpha carbon is not unexpected and constitutes direct evidence for wetting of the C_{18} bonded phase by the surfactant monomer.

For both pure and adsorbed CTAB (see Figure 6) and DTAB (see Figure 7), the peak at the most down-field position (62 ppm) is due to the methylene carbon alpha to the ammonium head group (i.e., the α-carbon), while the peak

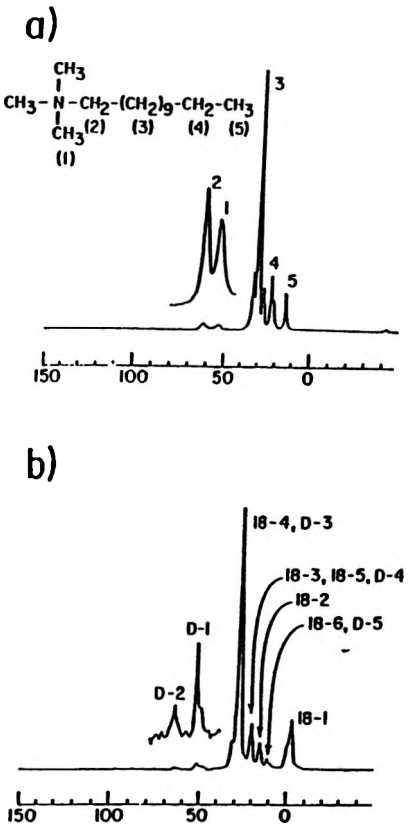

Figure 7. ^{13}C CP/MAS NMR spectrum and chemical shift assignments for (a) pure DTAB, and (b) DTAB adsorbed on C_{18}. Bonded phase resonances are indicated by labels starting with numerals (i.e., 18-), while surfactant resonances are indicated by labels beginning with letters (D=DTAB).

at 54 ppm is due to the N-methyl carbon. There is no change in the chemical shift value of the α-carbon after adsorption of CTAB or DTAB onto the bonded phase, and this is consistent with low angle X-ray diffraction data which shows both pure CTAB and DTAB to be amorphous solids. Hence, we should not observe any change in the chemical shift value of the CTAB or DTAB α-carbon because of the similarity in ordering of these two phases (solid vs adsorbed surfactant).

Table 1

Relaxation Parameters of the α-Carbon Nuclei*

Surfactant	T_{CH} (ms)	$T_{1\rho C}$ (ms)
CTAB (62 ppm)	0.03 (± 0.003)	14.5 (± 0.58)
CTAB on C_{18} (62 ppm)	0.54 (± 0.22)	24.2 (± 1.58)
DTAB (62 ppm)	0.03 (± 0.002)	25.7 (± 0.5)
DTAB on C_{18} (62 ppm)	0.30 (± 0.11)	20.8 (± 5.2)**
SDS (68 ppm)	0.13 (±0.03)	203.4 (± 0.24)
SDS on C_{18} (68 ppm)	0.12 (± 0.03)	21.3 (± 0.68)
SDS on C_{18} (50 ppm)	1.23 (± 0.00)	Dispersion Pattern

* The uncertainty in T_{CH} and $T_{1\rho C}$ was determined from the statistical parameters of the least squares fitting.
**At short holding times, i.e., less than 2 milliseconds.

From an examination of the NMR spectra, it is evident that the methylene carbon nucleus alpha to the head group of the surfactant can serve as a probe to study the sorptive behavior of SDS, CTAB, and DTAB on C_{18}. Resonances from the other surfactant nuclei are obscured by resonances from the bonded phase, preventing their use as probes. In other words, there is significant peak overlap in the 0 to 50 ppm region - one simply cannot distinguish aliphatic surfactant resonances from other resonances due to the bonded alkyl phase. Although the ^{13}C nucleus of the N-methyl group of CTAB and DTAB is not obscured by other surfactant or bonded phase resonances, the N-methyl group is not a good probe of molecular motion because of the rapid rotation of the methyl groups which can partially decouple the carbon and hydrogen nuclei.

It is also important to note that in Figures 5, 6, and 7, the relatively small peaks available to us as probes for this work (e.g., S-1, C-2, and D-2) appear to be inconsequential. In reality, this is not true. There is plenty of signal available to accurately measure peak intensity, with signal to noise (S/N) ratios of 100 or greater. The favorable S/N ratio of these peaks is simply obscured by the intensity of the "larger" peaks.

Table 1 lists cross polarization time constants (T_{CH}) for the alpha carbon of SDS, CTAB, and DTAB before and after surfactant adsorption onto the bonded phase. Since cross polarization is most efficient for static and near static C-H dipolar interactions, it can be related to the mobility of the nuclei

under investigation. For CTAB and DTAB, a significant increase in T_{CH} is observed after surfactant adsorption onto the bonded phase which indicates that the polar head group of the surfactant is more mobile after adsorption than in the pure solid form. This observation is consistent with a model of the CTAB or DTAB head group in intimate contact with the semi-rigid fluid like alkyl bonded phase. Hence, the decrease in the polarization transfer rate (i.e., the increase in T_{CH}) is significant because it constitutes direct evidence for wetting of the bonded phase by the surfactant monomer.

For adsorbed SDS, we observe two resonances for the α-carbon - one at 68 ppm and the other at 50 ppm. The 68 ppm resonance is very similar to the α-carbon peak of pure SDS as evidenced by the similar T_{CH} values (see Table 1), which suggests this peak represents SDS not in direct contact with the C_{18} bonded phase. However, the 50 ppm resonance behaves differently - the carbon magnetization build-up is not as rapid, and the T_{CH} value of the 50 ppm peak is substantively larger, suggesting that an increase in the mobility of the α-carbon nuclei has occurred. Hence, the 50 ppm peak probably corresponds to SDS in direct contact with the bonded hydrocarbon chains. During the cross-polarization experiments, $T_{1\rho H}$ (which represents ^{13}C magnetization relaxation through ^{1}H magnetization) was also determined for both pure and adsorbed surfactant. However, we could not relate this relaxational parameter to changes in motional behavior of the samples under investigation due to the problem of maintaining the Hartman-Hahn match at long contact times which is a concern since $T_{1\rho H}$ is determined from the falling portion of the variable contact time plot. Furthermore, the observed $T_{1\rho H}$ is an average over all the protons in the sample as a result of spin diffusion. Hence, a simple and direct interpretation of $T_{1\rho H}$ in terms of the various types of motion of carbon nuclei is not possible.

Clearly, spin diffusion can complicate the analysis of carbon relaxation behavior which is the reason why $T_{1\rho C}$ was used in the present study as an indicator of carbon relaxation behavior. Unlike $T_{1\rho H}$, spin diffusion is not a serious problem because the low natural abundance of ^{13}C ensures a physical separation within the solid and hence a slow spin diffusion rate.

Figures 8, 9, and 10 show the results from several variable holding time ($T_{1\rho C}$) experiments for SDS, CTAB, and DTAB. The holding time data were plotted in familiar semilog fashion. Information about the relaxation and motional behavior of the nuclei can be obtained from these plots. A linear decay plot suggests homogeneous relaxation behavior, whereas a nonlinear decay plot indicates a distribution of relaxation times for the nucleus. From the reciprocal of the slope of the semilog decay curve, $T_{1\rho C}$ can be obtained.

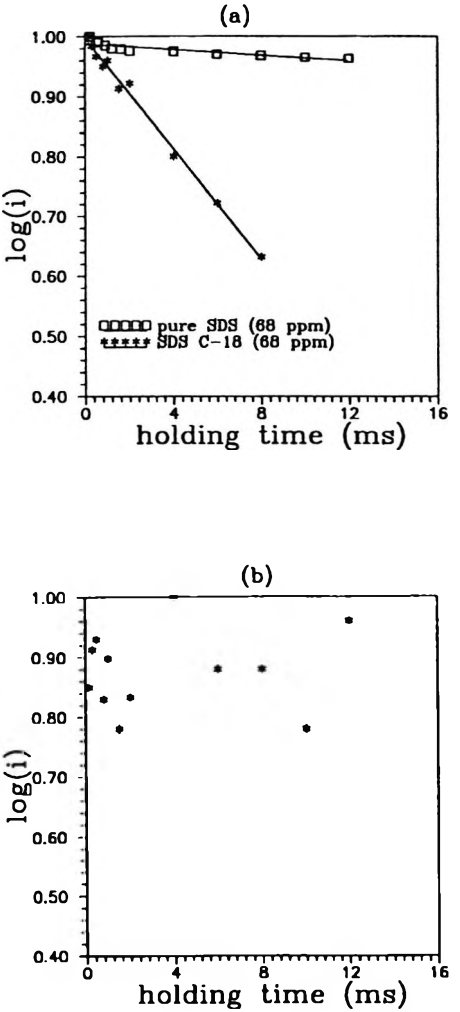

Figure 8. A plot of log intensity versus holding time for the alpha carbon of pure and adsorbed SDS: (a) 68 ppm resonance, and (b) 55 ppm resonance.

The semilog decay curves for the 68 ppm peak of solid and adsorbed SDS (see Figure 8) are linear, which suggests homogeneous relaxation behavior. Taken together, T_{CH} and $T_{1\rho C}$ data (see Table 1) suggest that the carbon atom associated with the 68 ppm peak in the NMR spectrum of SDS adsorbed on C_{18} is as rigid as the α-carbon nuclei of pure SDS. In other words, the 68 ppm peak represents solid SDS. On the other hand, the 50 ppm peak, which is

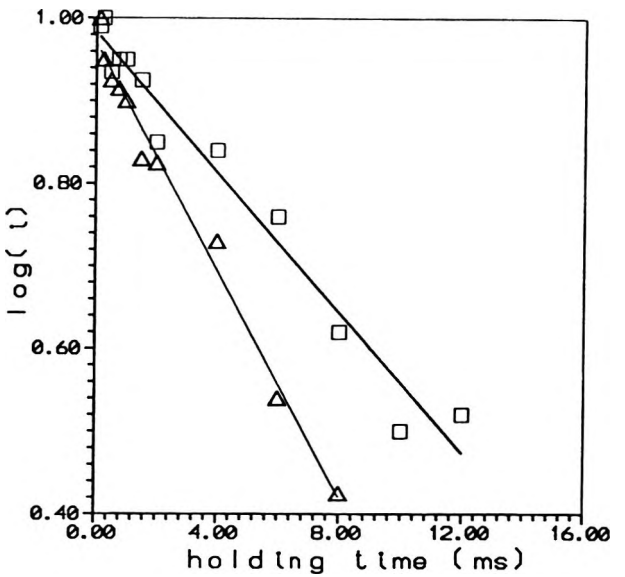

Figure 9. A plot of log intensity versus holding time for the alpha carbon of pure and adsorbed CTAB. (triangles = CTAB; squares = CTAB adsorbed on C_{18})

only observed after adsorption of SDS onto C_{18} and C_8, does not exhibit homogeneous relaxation behavior. We attribute the so-called dispersion pattern in the decay plot to the sulfate head group of SDS which is very mobile. The sulfate head group is not in direct contact with the bonded phase and will have many different orientations available to it. Because the α-carbon will possess a unique relaxation time for each orientation available to the sulfate head group, it is not surprising that a dispersion pattern is observed for the α-carbon nuclei which is in direct contact with the fluid-like bonded phase.

The decay curves shown for CTAB (see Figure 9) are also linear which indicates that the α-methylene carbon atom of both adsorbed and solid CTAB exhibits homogeneous relaxation behavior. The value of $T_{1\rho C}$ for the α-methylene carbon atom of adsorbed CTAB is greater than $T_{1\rho C}$ for pure CTAB, suggesting that an increase in the mobility of the carbon nuclei has occurred after adsorption of CTAB onto the C_{18} bonded phase (see Table 1). These conclusions are reinforced by the variable contact time data previously

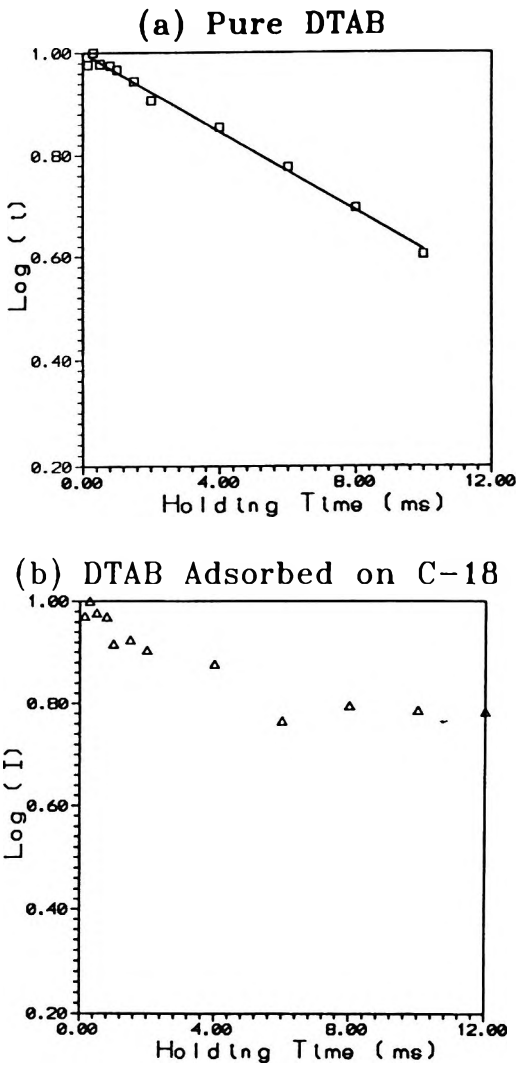

Figure 10. A plot of log intensity versus holding time for the alpha carbon of pure and adsorbed DTAB.

discussed, where T_{CH} of the CTAB α-carbon nuclei increased after adsorption of CTAB onto the bonded phase. Taken together, changes in T_{CH} and $T_{1\rho C}$ values of the α-carbon nuclei indicate that the polar head group of CTAB is in intimate contact with the fluid-like bonded phase.

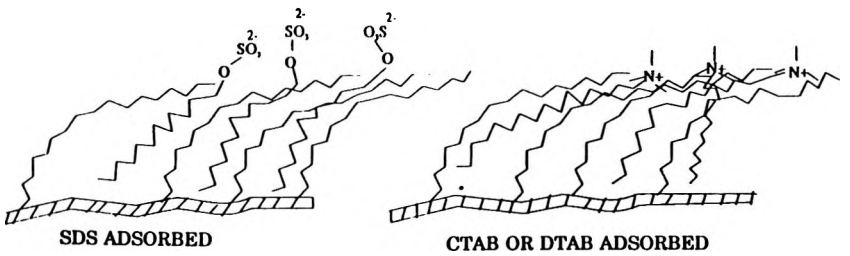

Figure 11. Model depicting the structure of SDS, DTAB, and CTAB-modified C_{18}.

Semilog decay curves for solid and adsorbed DTAB are shown in Figure 10. DTAB is similar to CTAB: T_{CH} and $T_{1\rho C}$ data follow the same trend. However, there are differences in the relaxation behavior of the α-methylene carbon atom of DTAB and CTAB. $T_{1\rho C}$ plots for adsorbed DTAB are not linear at long holding times, and the value of $T_{1\rho C}$ for the α-methylene carbon nuclei of adsorbed DTAB is not greater than $T_{1\rho C}$ for pure DTAB at short holding times. Since the only difference between DTAB and CTAB is hydrocarbon chain length, this factor is evidently important, influencing the adsorptive behavior of these amphiphiles on alkyl bonded phases.

We interpret the observed changes in α-carbon mobility upon adsorption of surfactant on the bonded phase as resulting from two entirely different forms of surfactant monomer association with the bonded phase. In the case of SDS, the hydrophobic alkyl tail and the alpha carbon of the adsorbed surfactant are associated with the C_{18} bonded phase, with the polar head group oriented away from the bonded phase surface. This orientation would prevent the establishment of a double layer structure at the stationary phase-mobile phase interface, i.e., the formation of hemi- or admicelles. On the other hand, the head group of CTAB and DTAB is oriented closer to the silica surface due to hydrophobic interactions between the N-methyl groups and the bonded phase. Evidently, CTAB is incorporated at least partly in the bonded phase giving rise to a modified bulk phase that is significantly denser. Figure 11 depicts the proposed model developed from the NMR data which summarizes SDS, CTAB, and DTAB adsorption on the C_{18} alkyl bonded phase at concentrations above the cmc of the surfactant.

Berthod and coworkers[20-21] have measured adsorption isotherms for SDS and CTAB on C_{18} bonded phase columns. The presence of large amounts of sodium chloride (ca. 0.20 M) in the micellar mobile phase increased markedly the amount of SDS adsorbed on C_{18} which is consistent with the proposed model for SDS adsorption since an increase in the ionic strength of the mobile

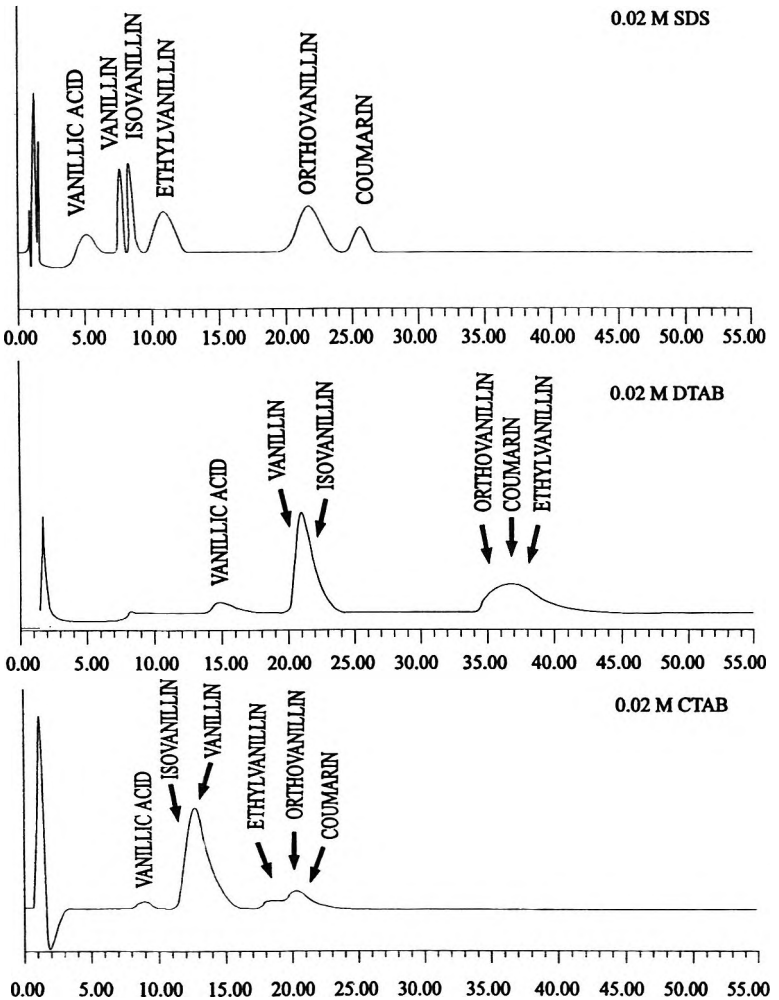

Figure 12. Separation of the vanillin test mixture on Apex I C-18 with a Perkin Elmer TriDet HPLC using the following mobile phases: 0.02 M SDS, 0.02 M DTAB, and 0.02 M CTAB. Flow rate was 1.0 mL/min, and the pH of each mobile phase was 3.0.

phase would diminish electrostatic repulsion between the sulfate head groups of the adsorbed surfactant molecules. However, the presence of a large amount of sodium chloride in the micellar mobile phase did not affect the total amount of CTAB adsorbed on C_{18} which is consistent with the proposed model for CTAB

adsorption since the N-alkyl head groups of adsorbed CTAB are already partly obstructed by the C_{18} alkyl bonded phase; hence, increasing the ionic strength of the mobile phase would not be expected to have much of an effect on reducing electrostatic repulsion between the N-alkyl head groups of adsorbed CTAB.

Retention Data

The conclusions regarding surfactant modification of C_{18} alkyl bonded phases can also be used to explain observed differences in selectivity between SDS, CTAB, and DTAB micellar RPLC. Adsorption of SDS on C_{18} in the manner described, with the sulfate head group projecting away from the bonded phase surface, would lead to the formation of a hydrophilic layer and would explain the superior resolution achieved by SDS for the vanillin compounds (see Figure 12) which probably undergo some form of selective hydrogen bonding interaction with this layer.

The hydrophilic layer formed on the stationary phase would also affect the penetration depth of the vanillin compounds into the bonded phase because of strong hydrogen bonding interactions between these compounds and the layer. The expected result would be a decrease in hydrophobic interactions between the vanillin compounds and the C_{18} stationary phase which would explain why the retention time of the vanillin compounds is greater for 0.02 M CTAB or 0.02 M DTAB than 0.02 M SDS. (The 0.02 M DTAB and 0.02 M SDS micellar solutions contain approximately the same number of micelles since DTAB and SDS have similar cmc's, whereas the CTAB solution contains significantly more micelles because its cmc is an order of magnitude lower than the cmc of SDS.) The proposed model for SDS adsorption can also explain the observation made by Yarmchuk and Cline-Love[6] that acidic solutes, such as phenols, have larger k' values when DTAB is used as the surfactant instead of SDS in micellar RPLC, whereas nonproton donor solutes, e.g., benzene, or nitrobenzene, possess similar k' values for DTAB and SDS.

The type of association between SDS and the bonded phase can also explain why the correlation coefficient for Log P and Log K_1 of SDS is so small (see Table 2), whereas incorporation of CTAB or DTAB in the manner described (see Figure 11) would ensure that much of the hydrophobic character of the modified bulk phase is retained which would explain why the correlation coefficient for Log P and Log K_1 of DTAB or CTAB (see Table 2) is so much larger.

Table 2

Solute Hydrophobicity as Represented by the [1,2]Log of the Octanol/Water Partition Coefficient (Log P) versus Log K_w or Log K_1 for the Vanillin Compounds on C_{18}

Compounds	Log P	[3]Log $K_{1(SDS)}$	[4] Log $K_{1(DTAB)}$	[5]Log $K_{1(CTAB)}$
Isovanillin	0.97	1.02	1.34	1.24
Vanillin	1.21	0.98	1.35	1.24
Orthovanillin	1.37	1.49	1.73	1.42
Coumarin	1.39	1.62	1.60	1.47
Vanillic Acid	1.43	0.62	1.34	1.23
Ethylvamllin	1.88	1.24	1.66	1.56

[1]Log P is a well known index of hydrophobicity.
[2]Log P values were obtained from the CLOGP Program, Medicinal Chemistry Project, Pomona College, Claremont, CA.
[3]The correlation coefficient for Log P and Log $K_{1(SDS)}$ is 0.188.
[4]The correlation Coefficient for Log P and Log $K_{1(DTAB)}$ is 0.600.
[5]The correlation coefficient for Log P and Log $K_{1(CTAB)}$ is 0.758.

Table 3

Sodium Dodecylsulfate

Compound*,**	$\Theta[L_s]K_1$	K_2
Vanillic Acid	4.2 ± 0.2	28.9 ± 2.1
Vanillin	9.6 ± 0.9	39.5 ± 6.1
Isovanillin	10.5 ± 1.0	37.6 ± 4.8
Ethylvanillin	17.5 ± 1.2	45.7 ± 3.7
Orthovanillin	31.2 ± 2.0	41.3 ± 3.0
Coumarin	41.7 ± 3.1	59.0 ± 4.6

* Compounds are listed in their order of elution from Apex I C-18. Concentration of SDS in the mobile phase varied from 0.01 to 0.10 M.
**Uncertainties in $\Theta[L_s]K_1$ and K_2 were determined from the statistical parameters of the least square fitting and from propagation of error.

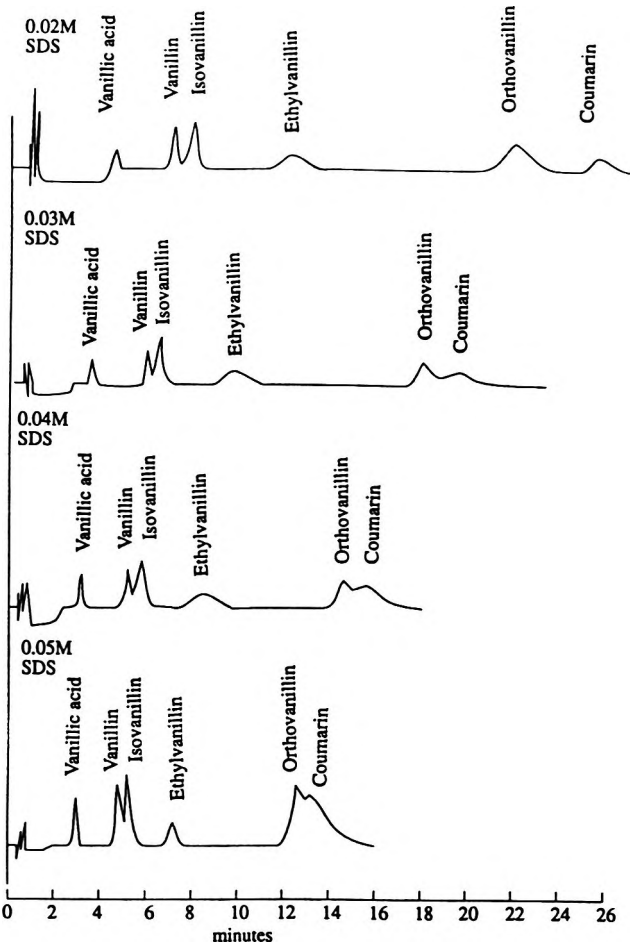

Figure 13. Separation of the vanillin test mixture on Apex I C-18 with SDS micellar solutions of differing surfactant concentration. Flow rate was 1.0 mL/min, and the pH of each mobile phase was 3.0.

Although the association between the surfactant and C_{18} bonded phase plays an important role in defining the selectivity of the separation process, micelle-solute interactions also play a role. For example, SDS micelles interact more strongly with vanillin than isovanillin, as evidenced by the larger K_2 value for vanillin (see Table 3), and this interaction is responsible, at least in part, for the 6 σ or baseline resolution achieved for these two isomers. Nevertheless, the separation of the hydrophilic test mixture is better at lower

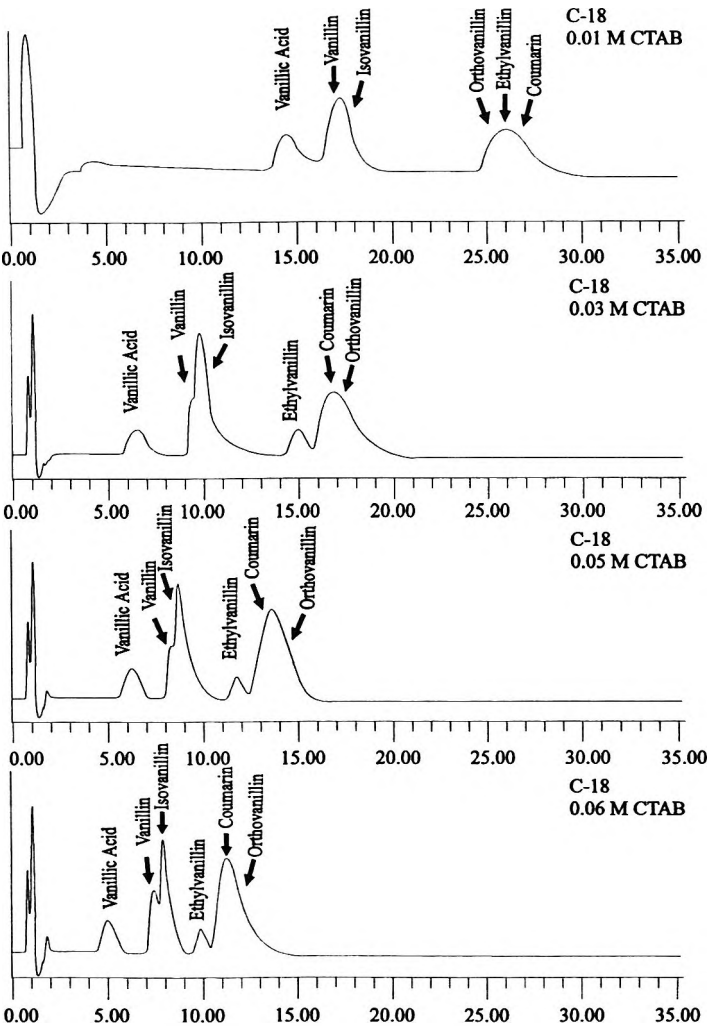

Figure 14. Separation of the vanillin test mixture on Apex I C-18 with CTAB micellar solutions of differing surfactant concentration. Flow rate was 1.0 mL/min, and the pH of each mobile phase was 3.0.

SDS concentrations (see Figure 13) which suggests that solute-stationary phase interactions are largely responsible for the separation. For CTAB and DTAB, the separation of the hydrophilic test mixture is better at higher surfactant

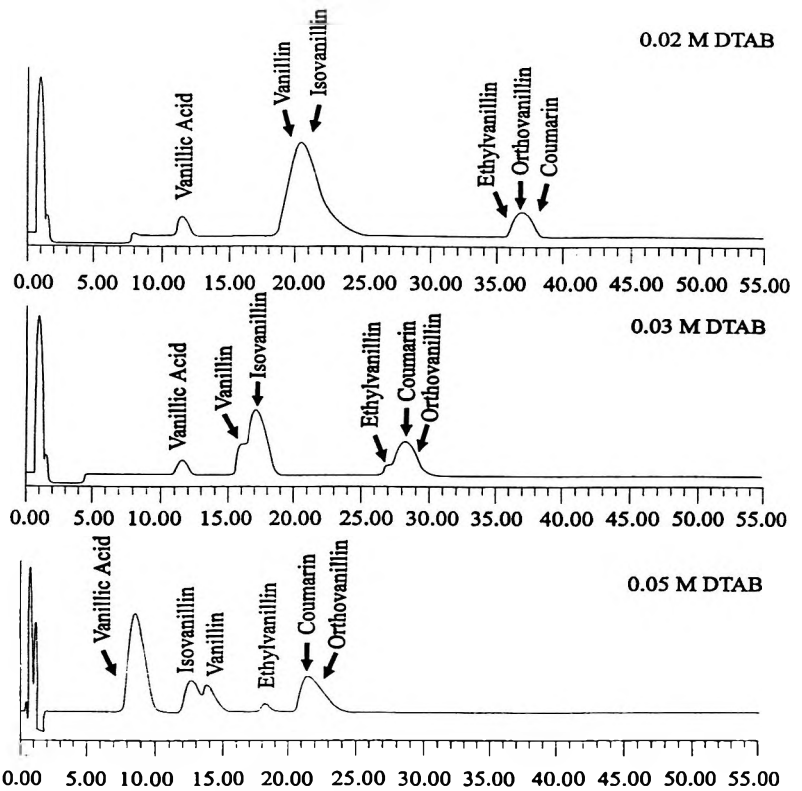

Figure 15. Separation of the vanillin test mixture on Apex I C-18 with DTAB micellar solutions of differing surfactant concentration. Flow rate was 1.0 mL/min, and the pH of each mobile phase was 3.0.

concentration (see Figures 14 and 15), which implies that micelle solute interactions are beneficial for the separation of the vanillin compounds on C_{18} when either CTAB or DTAB is used as the surfactant (see Tables 4 & 5).

This result is not surprising since the selectivity of CTAB and DTAB aggregates towards phenols is well known[22] and is probably the result of a secondary equilibrium process involving the transfer of a proton from the phenol to a water molecule in the Stern region of the micelle.

Table 4

Cetyltrimethylammonium Bromide

Compound*,**	$\Theta[L_s]K_1$	K_2
Vanillic Acid	16.9 ± 1.4	58.6 ± 5.2
Vanillin	17.2 ± 1.2	32.7 ± 2.7
Isovanillin	17.4 ± 1.2	29.7 ± 2.7
Ethylvamillin	36.0 ± 2.6	58.8 ± 4.5
Coumarin	29.5 ± 3.5	37.3 ± 5.3
Orthovanillin	26.5 ± 1.4	31.7 ± 2.3

* Compounds are listed in their order of elution from Apex I C_{18}.
Concentration of CTAB in the mobile phase varied from 0.006 to 0.15M.
**Uncertainties in $\Theta[L_s]K_1$ and K_2 were determined from the statistical parameters of the least squares fitting and from propogation of error.

Table 5

Dodecyltrimethylammonium Bromide

Compound*,**	$\Theta[L_s]K_1$	K_2
Vanillic Acid	21.9 ± 1.7	52.7 ± 5.8
Vanillin	22.8 ± 1.4	31.6 ± 2.7
Isovanillin	24.0 ± 1.6	29.7 ± 2.7
Ethylvanillin	46.1 ± 2.4	48.2 ± 3.1
Coumarin	40.7 ± 3.1	31.8 ± 3.4
Orthovanillin	53.8 ± 5.2	38.5 ± 6.3

* Compounds are listed in their order of elution from Apex I C-18.
Concentration of DTAB in the mobile phase varied from 0.01 to 0.14M.
**Uncertainties in $\Theta[L_s]K_1$ and K_2 were determined from the statistical parameters of the least squares fitting and from propogation of error.

CONCLUSION

Surfactant-bonded phase interactions in MLC are very important. A fundamental understanding of these interactions is crucial for developing separations with greater selectivity in MLC. Hence, finding the appropriate combination of surfactant and stationary phase is crucial in micelle mediated separations. Perhaps, some of the reported differences in selectivity between MLC and RPLC with hydro-organic mobile phases are due in some measure to the modification of the stationary phase by adsorbed surfactant.[23]

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SELECTIVITY IN MICELLAR LIQUID CHROMATOGRAPHY: SURFACTANT BONDED PHASE INTERACTIONS. II. C-8 AND CYANOPROPYL

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ABSTRACT

Micellar liquid chromatography and solid state ^{13}C NMR spectroscopy have been used to study the interactions of three ionic surfactants with C_8 and cyanopropyl bonded phase columns. The three surfactants, sodium dodecylsulfate (SDS), cetyltrimethyl ammonium bromide (CTAB), and dodecyltrimethylammonium bromide (DTAB), are commonly used in micellar RPLC. Surfactant adsorption is found to produce distinct changes in the selectivity of the stationary phase. Specifically, the differing nature of the surfactant-bonded phase association is largely responsible for the observed differences in selectivity between SDS, CTAB, and DTAB micellar RPLC. For SDS, the association leads to the formation of an anionic hydrophilic surface layer on C_8 (as well as on C_{18}) which would

explain the superior resolution achieved by SDS for hydrophilic compounds. For CTAB, small surfactant aggregates form within the C_8 stationary phase, which would explain the differences in the observed selectivity of CTAB mediated separations on C_{18} and C_8 alkyl bonded phases. The observed differences in the selectivity of DTAB and CTAB modified C_8 alkyl bonded phase columns towards hydrophilic aromatic compounds are probably due to the differing nature of the CTAB and DTAB C_8 bonded phase association, which suggests that hydrocarbon chain length is an important factor influencing the adsorptive behavior of these amphiphiles on hydrophilic silica surfaces. The unusual behavior of cyanopropyl bonded phase columns in SDS or CTAB micellar RPLC can be attributed to strong interactions between the polar head group of the surfactant and the cyano group of the polar bonded phase. Chemical models depicting the structure of the surfactant coated C_8 and cyanopropyl stationary phase are proposed from the NMR data, and these models are in good agreement with retention data obtained for these micellar RPLC systems.

INTRODUCTION

In the preceding paper,[1] it was reported that differences in selectivity between SDS, CTAB, and DTAB mediated micellar reversed phase liquid chromatography (RPLC) with C_{18} alkyl bonded phases can be attributed to the differing nature of SDS-, CTAB-, and DTAB-bonded phase association. For SDS, the hydrophobic alkyl tail of the surfactant appears to associate with the C_{18} phase, with the polar head group projecting away from the bonded alkyl phase surface. Incorporation of SDS into the C_{18} alkyl bonded phase in the manner described would lead to the formation of a hydrophilic layer which would explain the superior resolution achieved by SDS for hydrophilic compounds in micellar RPLC. For CTAB or DTAB, the nitrogen head group appears to orient closer to the silica surface due to hydrophobic interactions between the N-methyl groups and the C_{18} alkyl bonded phase. Evidently, CTAB and DTAB surfactant monomers are incorporated partially or wholly into the C_{18} bonded phase, giving rise to a modified bulk phase that is significantly denser. These results suggest that an understanding of surfactant-bonded phase interactions is crucial for developing selective separations in micellar liquid chromatography.

In this paper, the issue of selectivity in micellar liquid chromatography (MLC) as it relates to surfactant-bonded phase interactions is re-examined. MLC and solid state ^{13}C NMR spectroscopy are used to study the interactions of three ionic surfactants with C_8 and cyanopropyl bonded phase columns. The three surfactants, sodium dodecylsulfate (SDS), cetyltrimethyl ammonium bromide (CTAB), and dodecyltrimethylammonium bromide (DTAB), are commonly used in micellar RPLC. Surfactant adsorption is found to produce distinct changes in the selectivity of the stationary phase. Specifically, the differing nature of the surfactant-bonded phase association is largely responsible for the observed differences in selectivity between SDS, CTAB, and DTAB micellar RPLC. For SDS, the association leads to the formation of an anionic hydrophilic surface layer on C_8 (as well as on C_{18}) which would explain the superior resolution achieved by SDS for hydrophilic compounds. For CTAB, small surfactant aggregates form within the C_8 stationary phase, which would explain the differences in the observed selectivity of CTAB mediated separations on C_{18} and C_8 alkyl bonded phases. The observed differences in the selectivity of DTAB and CTAB modified C_8 alkyl bonded phase columns towards hydrophilic aromatic compounds are probably due to the differing nature of the CTAB and DTAB C_8 bonded phase association, which suggests that hydrocarbon chain length is an important factor influencing the adsorptive behavior of these amphiphiles on hydrophilic silica surfaces. The unusual behavior of cyanopropyl bonded phase columns in SDS or CTAB micellar RPLC can be attributed to strong interactions between the polar head group of the surfactant and the cyano group of the polar bonded phase. Chemical models depicting the structure of the surfactant coated C_8 and cyanopropyl stationary phase are proposed from the NMR data, and these models are in good agreement with retention data obtained for these micellar RPLC systems.

EXPERIMENTAL

Chemical

The six vanillin compounds (see Figure 1) which constituted the hydrophilic test mixture used to characterize the surfactant coated stationary phases were obtained from Aldrich and were used as received. Stock solutions of the various test solutes were prepared in methanol and then diluted to the appropriate working concentration (550 μg/mL) using 50% methanol in water. The surfactants, SDS, CTAB, and DTAB, were obtained from BDH Chemicals (99% purity) and were purified prior to use by first dissolving them in ethanol followed by addition of charcoal to the solution. After the charcoal was separated from the mother liquor by filtration, the surfactant was recrystallized

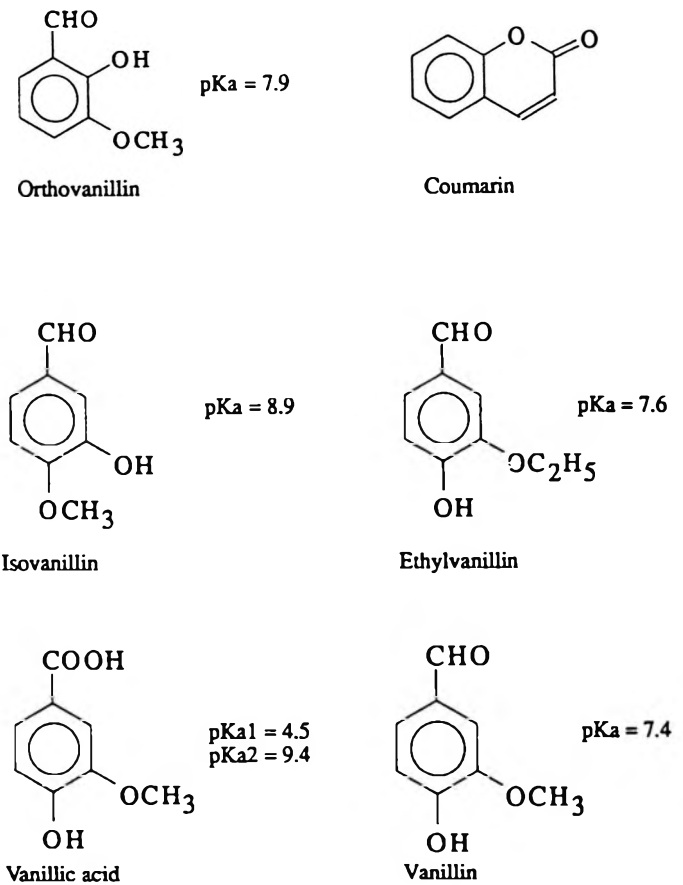

Figure 1. The vanillin compounds. The pKa values are from reference 13.

from the ethanol and dried in an oven at 65°C. Micellar solutions were prepared from the recrystallized surfactants using HPLC grade distilled water. (Methanol-water mobile phases were also prepared using HPLC grade solvents.)

All mobile phase solutions were filtered twice with a 0.45 μm Nylon membrane filter (Rainin Instruments, Woburn, MA) to remove particulate matter. Prior to use, the solutions were degassed and their pH adjusted to 3 with hydrochloric acid to prevent ionization of polar solutes in the mobile phase solutions.[2]

High Performance Liquid Chromatographic (HPLC) Measurements

All HPLC measurements were made using either a Perkin Elmer TriDet HPLC or a Rainin 81-20 M analytical HPLC system. The analytical column was either an Apex I C-8, or an Apex I cyanopropyl (5-μm, 10cm x 4.6 mm i.d.). The columns were purchased from Jones Chromatography (Golden, CO) and were made from the same 5 μm silica support. The analytical column was water-jacketed and temperature controlled. Separate columns were used for each surfactant (as well as the methanol water mobile phase) because of strong and irreversible adsorption of ionic surfactants on the stationary phase of the C_8 and cyanopropyl bonded phase columns. The dead volume of each column which was determined by injecting different solutions such as methanol-water, or water onto the Apex I column was approximately 1.0 mL and was used for all k' calculations. The k' values determined in this study were averages of at least triplicate determinations, and deviations in individual k' values were never greater than 5%. All k' measurements were made at a flow rate of 1.0 mL/min and were measured at 25°C for SDS and DTAB and 30°C for CTAB. (Since the Kraft point of CTAB is 23°C, it was necessary to perform the CTAB studies at a higher temperature.)

Estimation of Critical Partitioning Parameters in Micellar RPLC

Solute-stationary phase and solute-micelle binding constants were determined for the vanillin compounds using an equation developed by Cline-Love and Arunyanart[3]

$$1/k' = [M]K_2/\theta[L_s]K_1 + 1/\theta[L_s]K_1 \qquad (1)$$

where [M] is the concentration of surfactant, K_2 is the solute-micelle binding constant per monomer of surfactant, θ is the chromatographic phase ratio. $[L_s]$ is the concentration of ligate on the stationary phase, and K_1 is the solute-stationary phase binding constant. A plot of 1/k' vs [M] should yield a straight line, and in fact excellent linearity was observed for all six compounds using SDS, CTAB or DTAB.

Solid State NMR Measurements

Adsorption of SDS, DTAB, and CTAB on C_8 and cyanopropyl chemically derivitized silicas was investigated using cross polarization/magic angle spinning ^{13}C NMR with high-power proton decoupling (CP/MAS ^{13}C NMR).

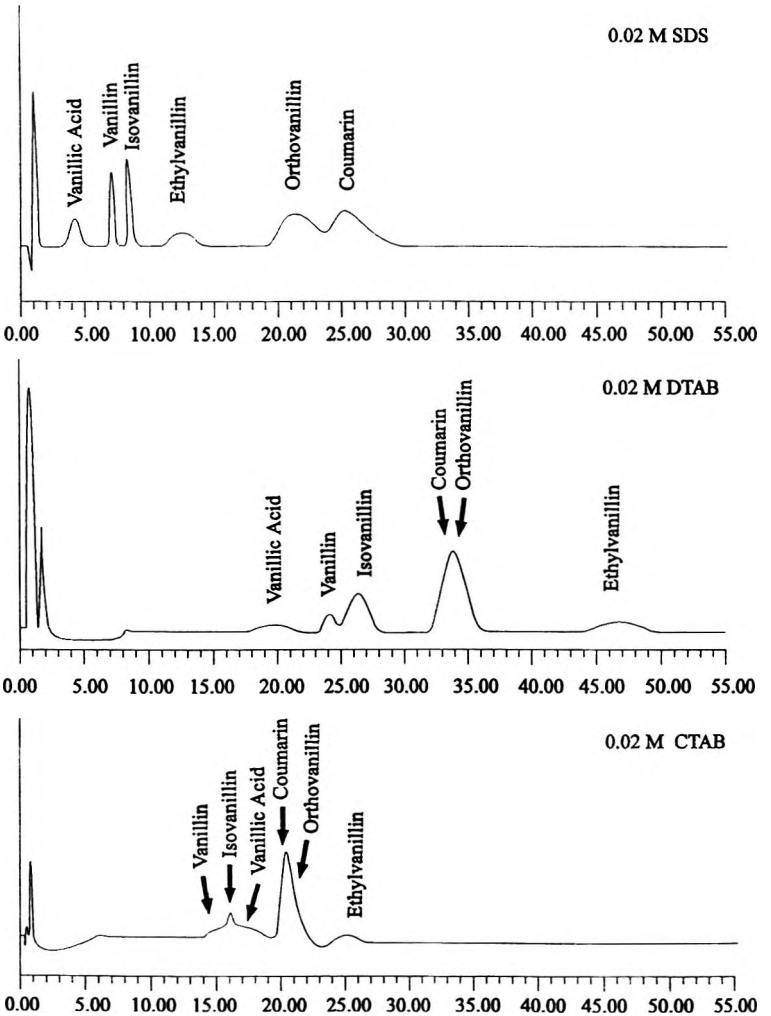

Figure 2. Separation of the vanillin test mixture on Apex I C-8 using a Perkin-Elmer TriDet HPLC with the following mobile phases: (a) 0.02 M SDS: (b) 0.02 M DTAB: and (c) 0.02 M CTAB. Flow rate was 1.0 mL/min, and the pH of each mobile phase was 3.0.

All NMR experiments were performed at 50 MHz on a Bruker/IBM WP-200 SY Spectrometer equipped with an IBM solids control accessory and a Doty-type solid-state probe that was software controlled which permitted

Table 1

Sodium Dodecylsulfate*

Compound	**$\Theta[L_s]K_1$	K_2
Vanillic Acid	4.4 ± 0.4	21.2 ± 2.9
Vanillin	12.5 ± 1.6	41.1 ± 8.1
Isovanillin	14.2 ± 2.0	37.9 ± 7.8
Ethylvanillin	24.1 ± 0.6	58.9 ± 1.6
Orthovanillin	40.5 ± 1.6	66.6 ± 2.9
Coumarin	49.8 ± 1.2	86.0 ± 2.4

* Compounds are listed in their order of elution from Apex I C-18. Concentraiton of SDS in the mobile phase varied from 0.01 to 0.14M.
**Uncertainties in $\Theta[L_s]K_1$ and K_2 were determined from the statistical parameters of the least squares fitting and from propagation of error.

automatic variation of all pulse parameters. The magic angle spinning probe used was a double-tuned, single-coil design with a bullet type rotor which held a sample volume of 0.75 cm^3. Two different pulse sequences[1] were used in these NMR experiments. However, each pulse sequence was performed with a constant 3-s recycle time. The ^{13}C spectra collected were externally referenced to para-di-t-butyl benzene. All chemical shift values were expressed as parts per million downfield from tetramethylsilane. The ^{13}C data were collected in 2 Kbytes of memory, exponentially multiplied prior to Fourier transformation, and zero-filled to 8 kilobytes.

Sample Preparation

To prepare a sample for solid-state NMR, 0.5 g of 5μm C_8 or cyanopropyl reversed phase material was equilibrated with 10 mL of 0.05 M aqueous CTAB, DTAB, or SDS solution. The equilibration period for the stationary phase material and surfactant was at least 24 h. During equilibration, a wrist action shaker was periodically used to agitate the samples. After equilibration, each sample was vacuum filtered onto a 0.45 μm Nylon 66 membrane filter and vacuum dried at 35 C for 2 days prior to being packed into the rotor of the solid-state probe.

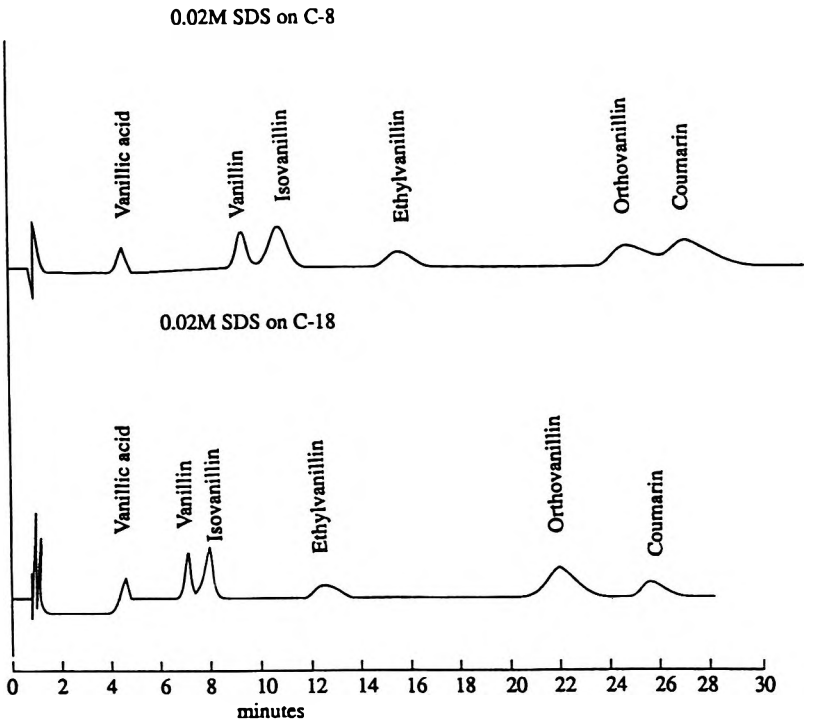

Figure 3. Chromatograms of the test mixture on a C-18 and C-8 Apex I column with a 0.02 M SDS mobile phase. Flow rate was 1.0 mL/min, and the pH of the mobile phase was 3.0.

RESULTS & DISCUSSION

C_8

Figure 2 shows the separation of the vanillin test mixture with the same three mobile phases used in the Apex I C-18 study (see preceding paper). Several things are apparent from an examination of this data. First, the test mixture is completely separated by the 0.02 M SDS micellar mobile phase. As with C_{18}, elution order clocks K_1 suggesting that solute-stationary phase interactions again play a decisive role in the SDS micellar RPLC separation process (see Table 1). Because of the similarity in the micellar RPLC data (see Figure 3), we must conclude that SDS probably forms a similar association with C_8 and C_{18} alkyl bonded phases.

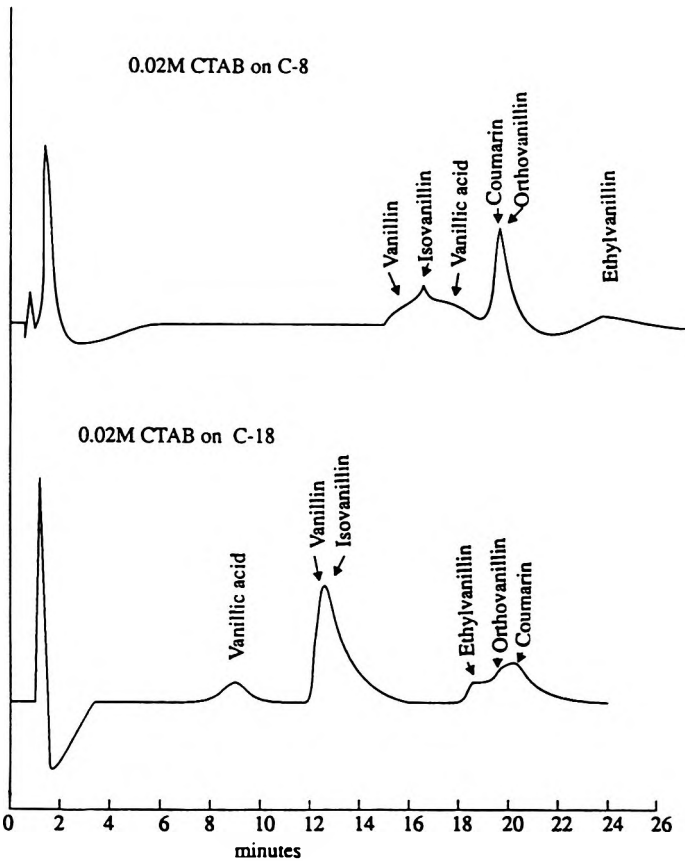

Figure 4. Chromatograms of the test mixture on a C-18 and C-8 Apex I column with 0.02 M CTAB mobile phase. Flow rate was 1.0 mL/min, and the pH of the mobile phase was 3.0.

Second, there is a degradation in the separation of the vanillin test mixture and a change in the elution order when an Apex I C-8 column is used, in lieu of an Apex I C-18 column, with the 0.02 M CTAB mobile phase (see Figure 4). The retention time of the vanillin compounds is also longer on C_8 then on C_{18}. On the basis of these effects, longer retention times, reversals in elution order, and a decrease in resolution, we must conclude that differences in the observed selectivity of CTAB mediated separations on C_{18} and C_8 alkyl bonded phases are due to the differing nature of the CTAB C_{18} and C_8 alkyl bonded phase association. In all likelihood, small surfactant aggregates form

Table 2

Cetyltrimethylammonium Bromide*

Compound**	$\Theta[L_s]K_1$***	K_2
Vanillin	20.4 ± 0.8	46.4 ± 2.3
Isovanillin	21.2 ± 3.2	41.3 ± 7.5
Vanillic Acid	37.9 ± 7.2	126 ± 24
Coumarin	25.1 ± 0.6	44.1 ± 1.3
Orthovanillin	27.3 ± 2.2	36.5 ± 3.6
Ethylvanillin	38.6 ± 1.5	69.1 ± 2.9

* Compounds are listed in their order of elution from Apex I C-8. Concentration of CTAB in the mobile phase varied from 0.006 to 0.15M.
**The correlation between $\Theta[L_s]K_1$ and elution order for the vanillin compounds is greater on C-8 than on C-18. (If vanillic acid is removed from the table, the correlation between $\Theta[L_s]K_1$ and elution order is very high.)
***Uncertainties in $\Theta[L_s]$ determined from the parameters of the least squares fitting and from propagation of error.

within the C_8 stationary phase and are responsible for the longer retention times and reversals in elution order. These aggregates are probably similar in nature to surfactant clusters that form in the presence of water soluble polymers, e.g., polyethylene-oxide in aqueous media.[4]

It has been shown that CTAB aggregates exhibit strong selectivity toward phenols and other aromatic compounds containing acidic functional groups. This selectivity has been attributed to a secondary chemical equilibrium process involving a transfer of a proton from an ionogenic solute to water molecules in the Stern region of the surfactant aggregate.[5] A decrease of 0.5 to 3.0 in the pKa value of a dissociable amphiphile can occur upon incorporation of the guest molecule into a cationic micelle. Clearly, the aforementioned acid-base effect can explain the strong interaction of vanillic acid with the CTAB modified C_8 stationary phase (see Table 2). The existence of surfactant aggregates within the stationary phase would also explain the reversals in elution order, the loss of resolution, and the longer retention times (or greater affinity of the vanillin compounds for the surfactant modified C_8 stationary phase). Since elution order clocks K_1 (see Table 2) which was not the case

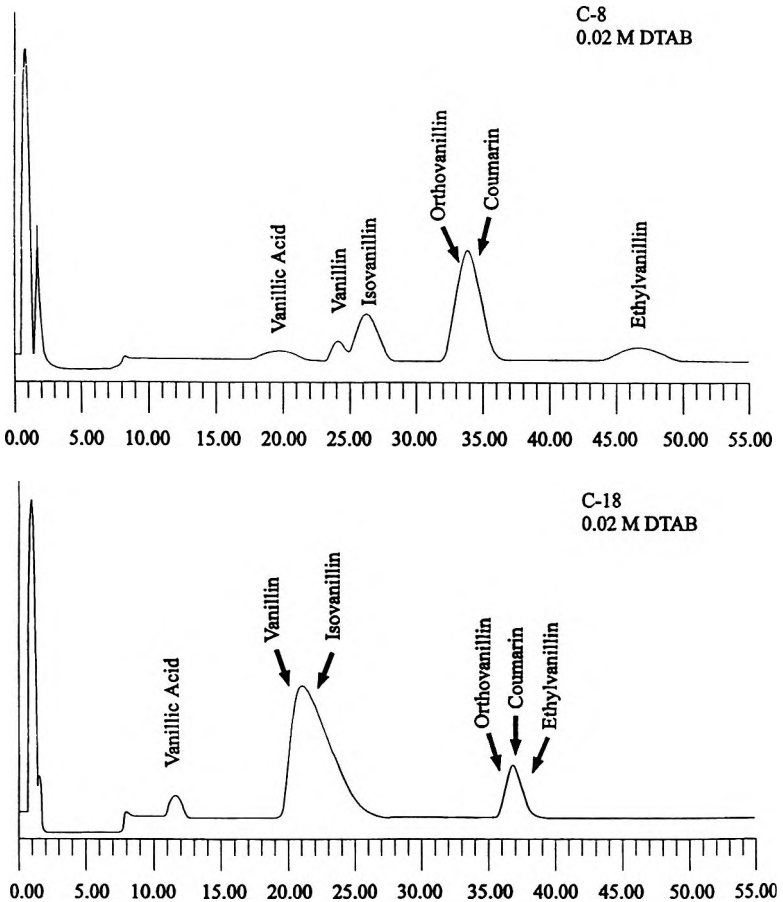

Figure 5. Chromatograms of the test mixture on a C-18 and C-8 Apex I column with a 0.02 M DTAB mobile phase. Flow rate was 1.0 mL/min, and the pH of the mobile phase was 3.0.

when a C_{18} column was used (see Table 4 of preceding paper), we must conclude that solute-stationary phase interactions play a more important role in the separation of the polar test mixture on C_8 than on C_{18} when CTAB micellar mobile phases are utilized.

Third, the separation of the vanillin test mixture is better when an Apex I C-8 column is used, in lieu of an Apex I C-18 column, with the 0.02 M DTAB micellar mobile phase (see Figure 5) which is the opposite to what is observed

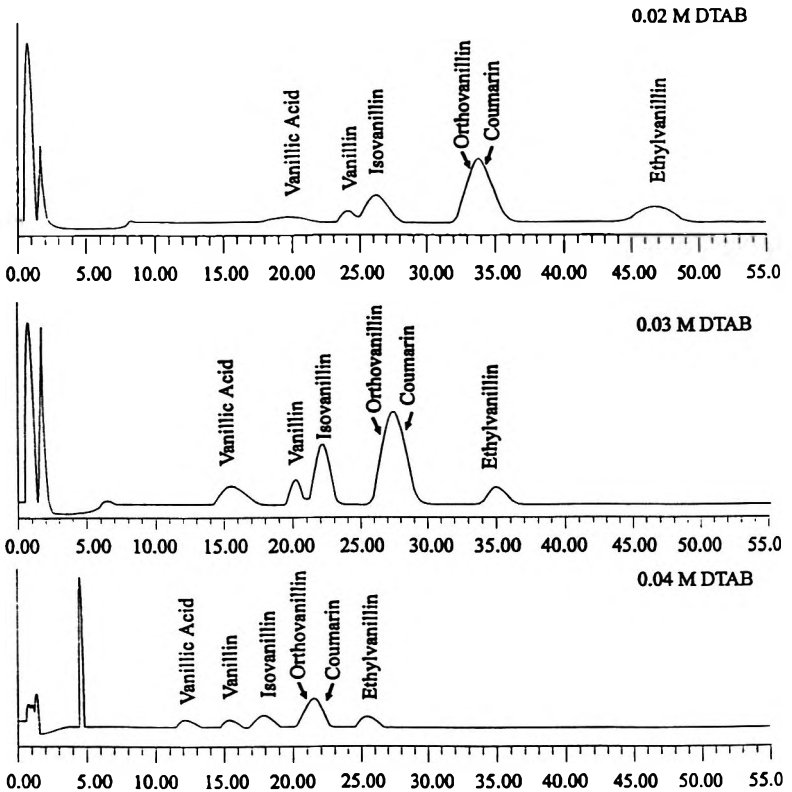

Figure 6. Separation of the vanillin test mixture on Apex I C-8 with DTAB micellar solutions of differing surfactant concentration. Flow rate was 1.0 mL/min, and the pH of each mobile phase was 3.0.

with CTAB (see Figure 4). As with C_{18}, the separation of the test mixture is better at higher DTAB concentrations, but the improvement in the separation of the test mixture with increasing DTAB concentration is far more dramatic with C_8 (see Figures 15 of the preceding paper and Figure 6 of this study). When a C_8 column is used, elution order clocks K_1 (see Table 3) which was not the case when a C_{18} column was used (see preceding paper). Finally, changes occur in elution order when a C_8 column is used; the retention time of most of the vanillin compounds is also longer on C_8. On the basis of these three effects, longer retention times, reversals in elution order, and improved resolution, we must conclude that differences in the observed selectivity of DTAB mediated separations on C_{18} and C_8 columns are due to the differing nature of the DTAB

Table 3

Dodecyltrimethylammonium Bromide*

Compound	$\Theta[L_s]K_1$**	K_2
Vanillic Acid	14.9 ± 2.1	32.8 ± 4.1
Vanillin	19.1 ± 1.1	31.8 ± 1.5
Isovanillin	21.1 ± 1.6	24.1 ± 1.6
Orthovanillin	23.1 ± 2.2	26.5 ± 4.3
Coumarin	27.4 ± 3.1	30.6 ± 2.3
Ethylvanillin	40.1 ± 2.4	44.6 ± 3.5

* Compounds are listed in their order of elution from Apex I C-18. Concentration of DTAB in the mobile phase varied from 0.01 to 0.14M.
**Uncertainties in $\Theta[L_s]K_1$ and K_2 were determined from the statistical parameters of the least squares fitting and from propagation of error.

C_{18} and C_8 alkyl bonded phase association. Furthermore, DTAB and CTAB do not form the same type of association with the C_8 alkyl bonded phase as evidenced by differences in the separation of the vanillin test mixture on C_8 with these two surfactants. Apparently, hydrocarbon chain-length is an important factor, influencing the adsorptive behavior of these amphiphiles on hydrophobic silica surfaces. (CTAB and DTAB micelles interact in much the same manner with aromatics so they cannot be the source of the observed differences in selectivity exhibited by these two surfactants towards the vanillin compounds.)

Because of differences in silanol activity between C_{18} and C_8 alkyl bonded phases, the possibility that silanol groups could be responsible for observed differences in selectivity must also be considered. If the silanol groups were responsible for the differing selectivities, then there would be marked differences in the retention behavior of the vanillin compounds on Jones Apex I C-18 and C-8 columns with a hydro-organic mobile phase. However, the vanillin compounds on C_{18} and C_8 columns exhibit similar retention behavior (see Figure 7) with a 20% methanol in water mobile phase, as well as with 30%, 40%, and 50% methanol in water solvent mixtures. Hence, it is the differing nature of the CTAB and DTAB monomer C_{18} and C_8 bonded phase association that is responsible for differences in the observed selectivity.

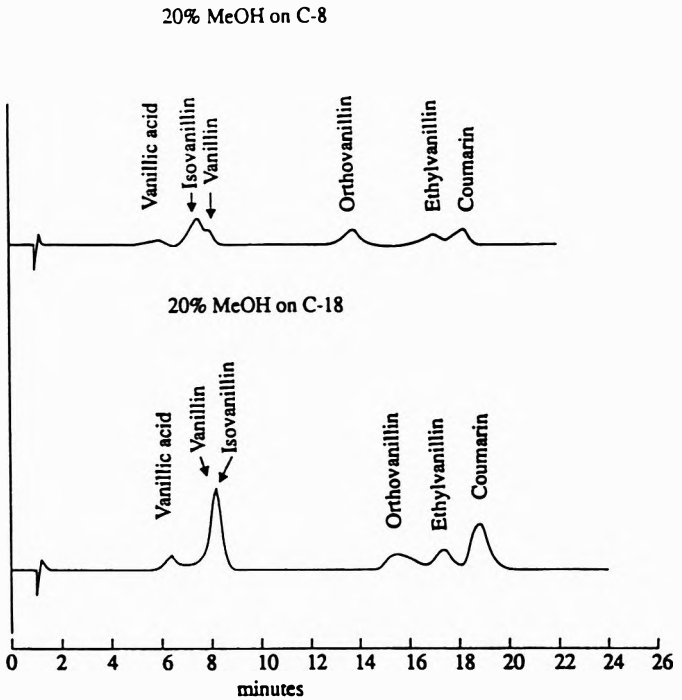

Figure 7. Chromatograms of the vanillin test mixture on a C-18 and C-8 Apex I column with a 20% methanol in water mobile phase. Flow rate was 1.0 mL/min, and the pH of the mobile phase was 3.0.

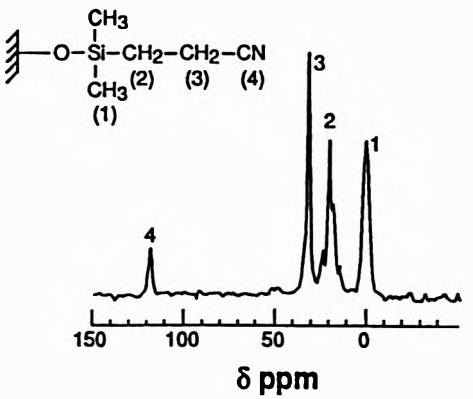

Figure 8. ^{13}C CP/MAS NMR spectrum and chemical shift assignments for the cyanopropyl bonded phase.

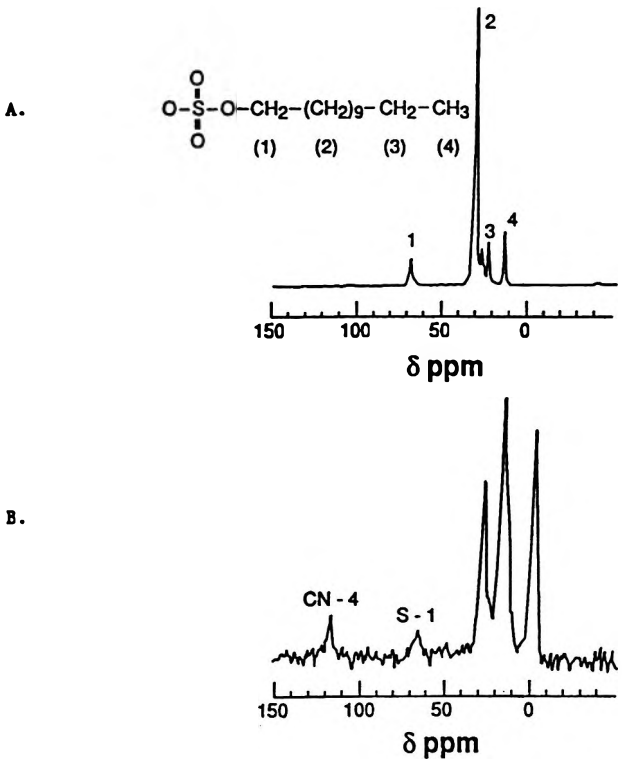

Figure 9. ^{13}C CP/MAS NMR spectrum and chemical shift assignments for (a) SDS, and (b) SDS adsorbed on cyanopropyl. Bonded phase resonances are indicated by labels starting with CN-, while surfactant resonances are indicated by labels beginning with S (S=SDS).

Cyanopropyl

Figures 8-10 show solid state ^{13}C NMR spectra for the following materials: cyanopropyl, SDS adsorbed on cyanopropyl, and CTAB adsorbed on cyanopropyl. Also included in each figure is a structural model that shows tentative chemical shift assignments. From an examination of the NMR spectra, it is evident that the α-carbon nuclei of CTAB (65 ppm) and SDS (68 ppm) can be used as probes to study changes in molecular motion for surfactant molecules adsorbed onto or in the cyano bonded phase. Resonances associated with the other surfactant nuclei cannot be used as probes since these nuclei are obscured by resonances from the cyano bonded phase or are simply not suitable as quantitative probes of molecular motion due to their rapid rotation.

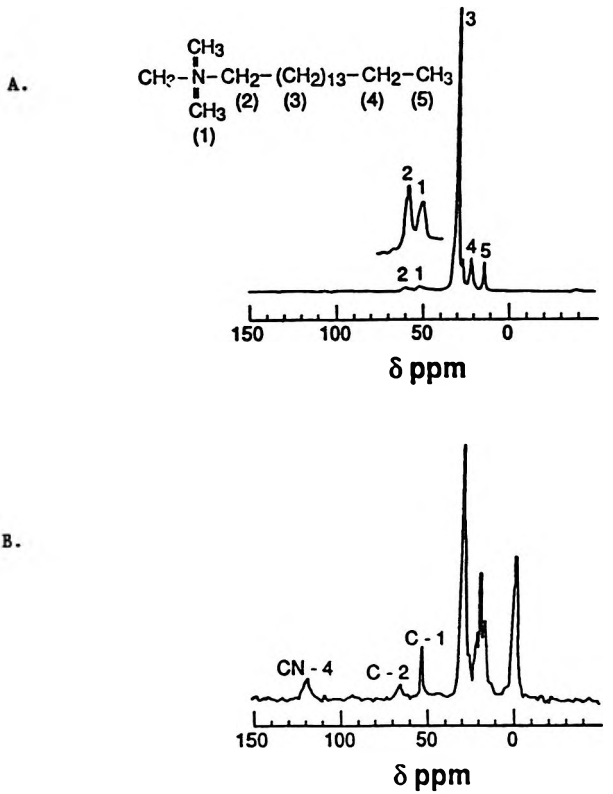

Figure 10. ^{13}C CP/MAS NMR spectrum and chemical shift assignments for (a) CTAB, and (b) CTAB adsorbed on cyanopropyl. Bonded phase resopnances are indicated by labels starting with CN-, while surfactant resonances are indicated by labels beginning with C (C=CTAB).

Interestingly enough, the cyano group of the polar bonded phase (120 ppm) can be used as a probe to study changes in behavior of the bonded phase ligands which can occur as a result of surfactant adsorption, so direct observation of the bonded phase itself is possible.

Table 4 lists cross polarization time constants for the α-methylene carbon atom of SDS and CTAB before and after adsorption of surfactant on the bonded phase. For either CTAB or SDS, there is a significant increase in T_{CH} after

Table 4

***Relaxation Parameters of α-Carbon Nuclei in Pure and Adsorbed Surfactants**

Surfactant	T_{CH} (ms)	$T_{1\rho C}$ (ms)
CTAB	0.03 ± 0.003	14.5 ± 0.58
CTAB ON Cyanopropyl	0.25 ± 0.05	7.41 ± 1.09
SDS	0.13 ± 0.03	203.4 ± 0.42
SDS ON Cyanopropyl	0.40 ± 0.03	2.19 ± 0.31**

* Uncertainties in T_{CH} and $T_{1\rho C}$ were determined from the statistical parameters of the least squares fitting.
**Computed at short holding times.

Table 5

Relaxation Parameters of the Cyano Carbon Nuclei Before and After Surfactant Adsorption[1]

Surfactant	T_{CH} (ms)	$T_{1\rho C}$ (ms)
CN	2.92 ± 0.00	Dispersion Pattern
CTAB ON Cyanopropyl	1.17 ± 0.20	42.3 ± 5.93
SDS ON Cyanopropyl	1.4 ± 0.31	8.4 ± 1.0[2]
		143 ± 50[3]

[1]Uncertainties in T_{CH} and $T_{1\rho C}$ were determined from statistical parameters of the least squares fitting.
[2]Short holding times. [3]Long holding times.

adsorption of surfactant onto the polar bonded phase which indicates that the polar head group of the surfactant is more mobile after adsorption than in the pure solid form. Hence, the 68 ppm and 65 ppm resonances probably represent surfactant monomer in direct contact with the polar bonded phase.

Table 5 lists T_{CH} values for the cyano carbon of the polar bonded phase before and after surfactant adsorption. The decrease in T_{CH} as a result of SDS or CTAB adsorption suggests that an increase has occurred in the polarization

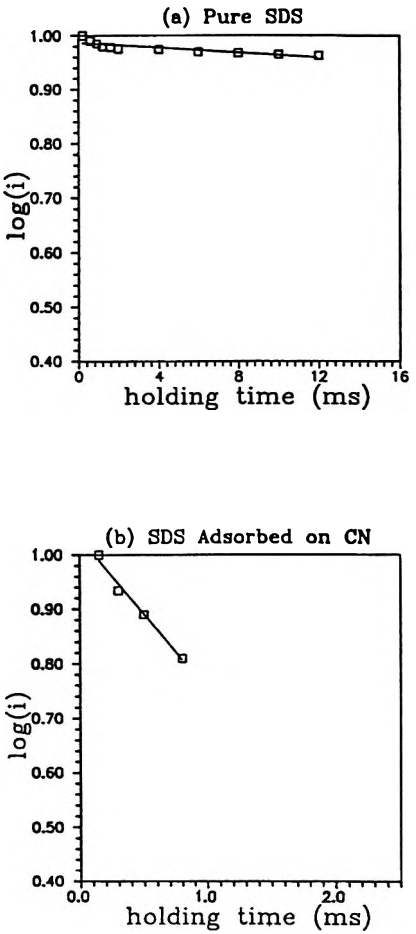

Figure 11. A plot of log intensity versus holding time for the alpha carbon of pure and adsorbed SDS.

transfer rate. Because the cyano functional group carbon atom possesses no direct bonded hydrogen atoms, the enrichment of the hydrogen environment of the cyano phase as a result of CTAB or SDS adsorption is, in all likelihood, responsible for the observed increase in the polarization transfer rate between nonbonded hydrogen atoms and the cyano carbon of the stationary phase. Nevertheless, the decrease in T_{CH} is significant because it is direct evidence for wetting of the cyanopropyl bonded phase by SDS or CTAB.

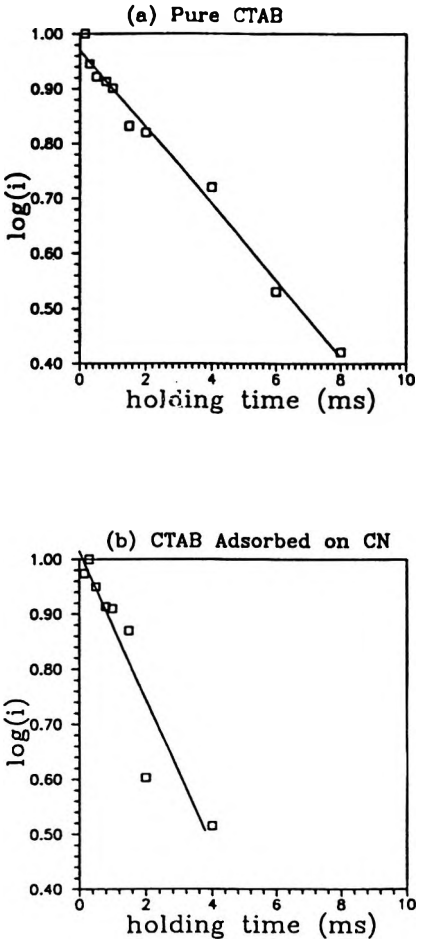

Figure 12. A plot of log intensity versus holding time for the alpha carbon of pure and adsorbed CTAB.

If SDS or CTAB were only physisorbed on the surface, there would be no direct contact between the cyano functional group of the bonded phase and the hydrocarbon chain of the surfactant because the surfactant would be in a different phase.

Figures 11 and 12 show the results from several variable holding time experiments for CTAB and SDS. The linear decay curves indicate that the α-methylene carbon atom of both solid and adsorbed SDS and CTAB exhibit

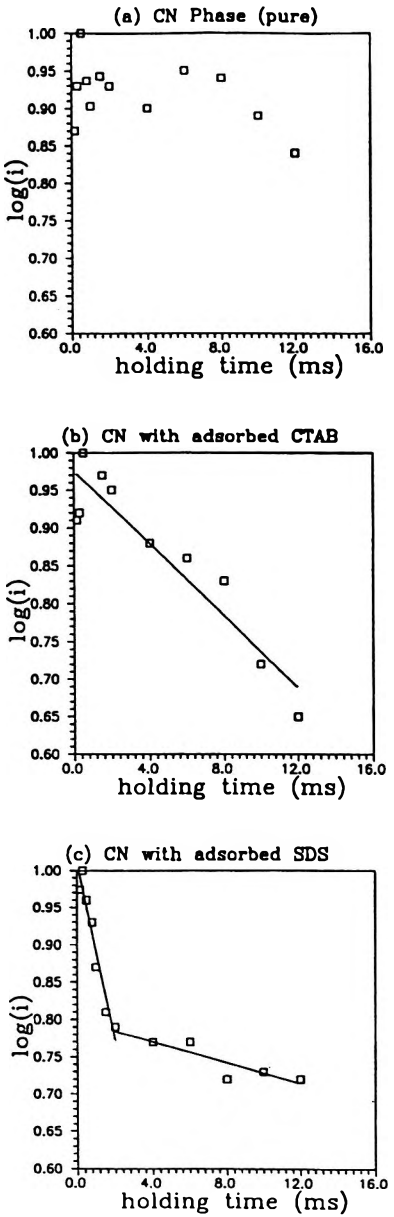

Figure 13. A plot of log intensity versus holding time for the cyano carbon of (a) pure bonded phase, (b) cyano carbon of the bonded phase with adsorbed SDS, and (c) cyano carbon of the bonded phase with adsorbed CTAB.

homogenous relaxation behavior. (For adsorbed SDS, only data at short holding times are shown due to low S/N at holding times greater than 1 ms.) $T_{1\rho C}$ for the alpha methylene carbon atom of solid CTAB and SDS is greater than $T_{1\rho C}$ for adsorbed CTAB and SDS (see Table 4), which is surprising since the bonded phase constitutes a more liquid-like environment than crystalline surfactant. In other words, one would expect $T_{1\rho C}$ for the adsorbed surfactant to be larger than $T_{1\rho C}$ for the solid surfactant, which in fact is what was observed in our study on surfactant adsorption on C_{18} and C_8 alkyl bonded phases.[6] Since this is not the situation with cyanopropyl, the orientation of the alpha carbon nuclei of adsorbed SDS and CTAB must be different on cyanopropyl than on C_{18} or C_8. We believe this difference is due to the strong association between the head group of the surfactant and the cyano group of the polar bonded phase. In other words, the α-carbon nuclei of the adsorbed surfactant is not experiencing random motion; it is not accessing all of the orientations available to it with respect to the magnetic field because of the strong association between the cyano group of the bonded phase and the polar head group of the surfactant, which would explain the ten-fold and two-fold decrease in the value of $T_{1\rho C}$ for the alpha carbon nuclei of adsorbed SDS and CTAB.

Figure 13 shows semilog decay curves for the cyano group carbon atom before and after incorporation of SDS and CTAB into the polar bonded phase. The dispersion pattern obtained for the decay curve of the cyano group of the pure stationary phase material can be rationalized on the basis of chemical considerations. Cyano groups are known to interact with residual silanols.[7,8] The fact that some residual silanols will and some will not interact with cyano groups and to the varying degrees they do would be expected to yield a dispersion pattern.

The linear and bilinear decay curves obtained for the cyano group carbon of CTAB- and SDS-modified cyanopropyl suggest that a decrease in the number of relaxation states available to the cyano functional group carbon atom as a result of SDS and CTAB adsorption onto the polar bonded phase has occurred. We attribute the decrease in the number of relaxation states of the cyano group carbon to the strong association between the polar head group of the surfactant and the cyano group of the polar bonded phase. The sulfate head group of SDS and the N-alkyl head group of CTAB probably interact with the cyano functionality of the polar bonded phase through some form of electrostatic interaction.

Our conclusions regarding modification of the cyanopropyl bonded phase by SDS or CTAB adsorption can explain the unusual behavior exhibited by cyanopropyl bonded phase columns in micellar RPLC. For example, the

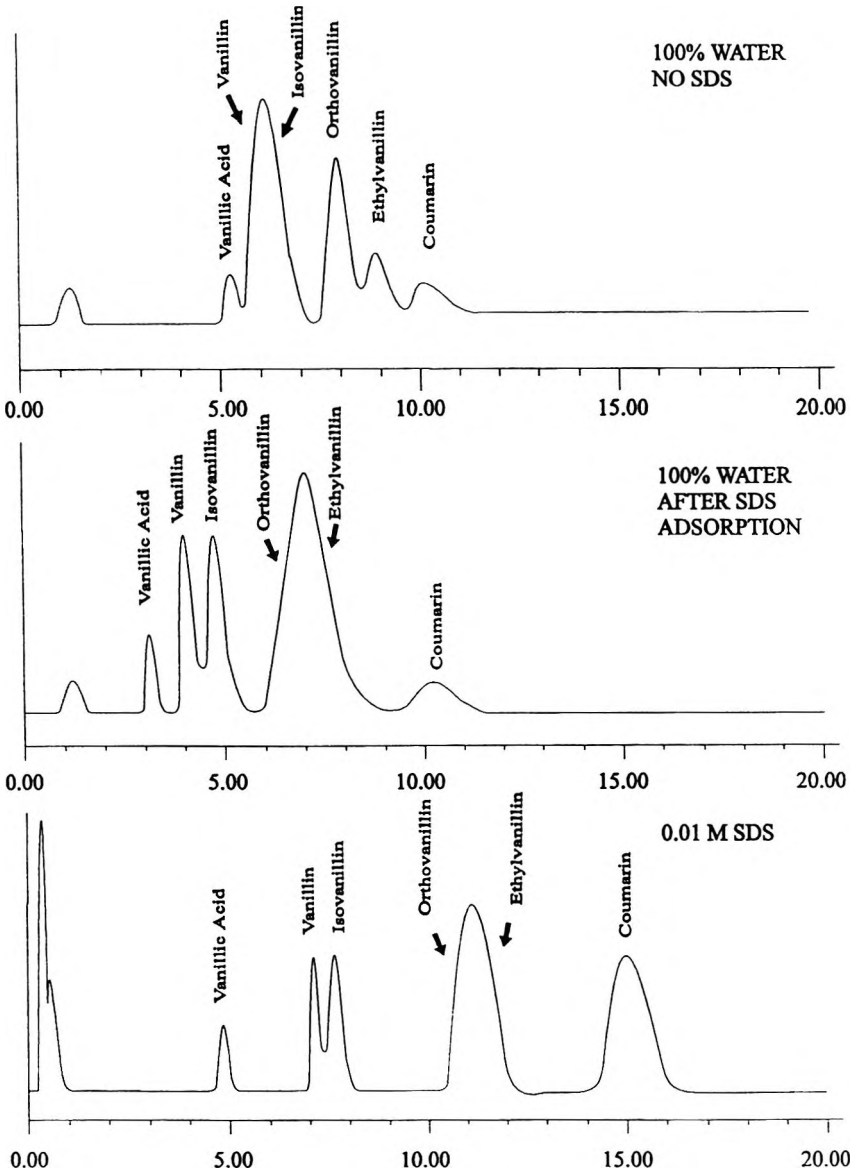

Figure 14. Chromatograms of the vanillin test mixture on a cyanopropyl column before, during, and after separations involving SDS micellar mobile phases. The pH of each mobile phase was 3.0 and the flow rate was 1.0 mL/min.

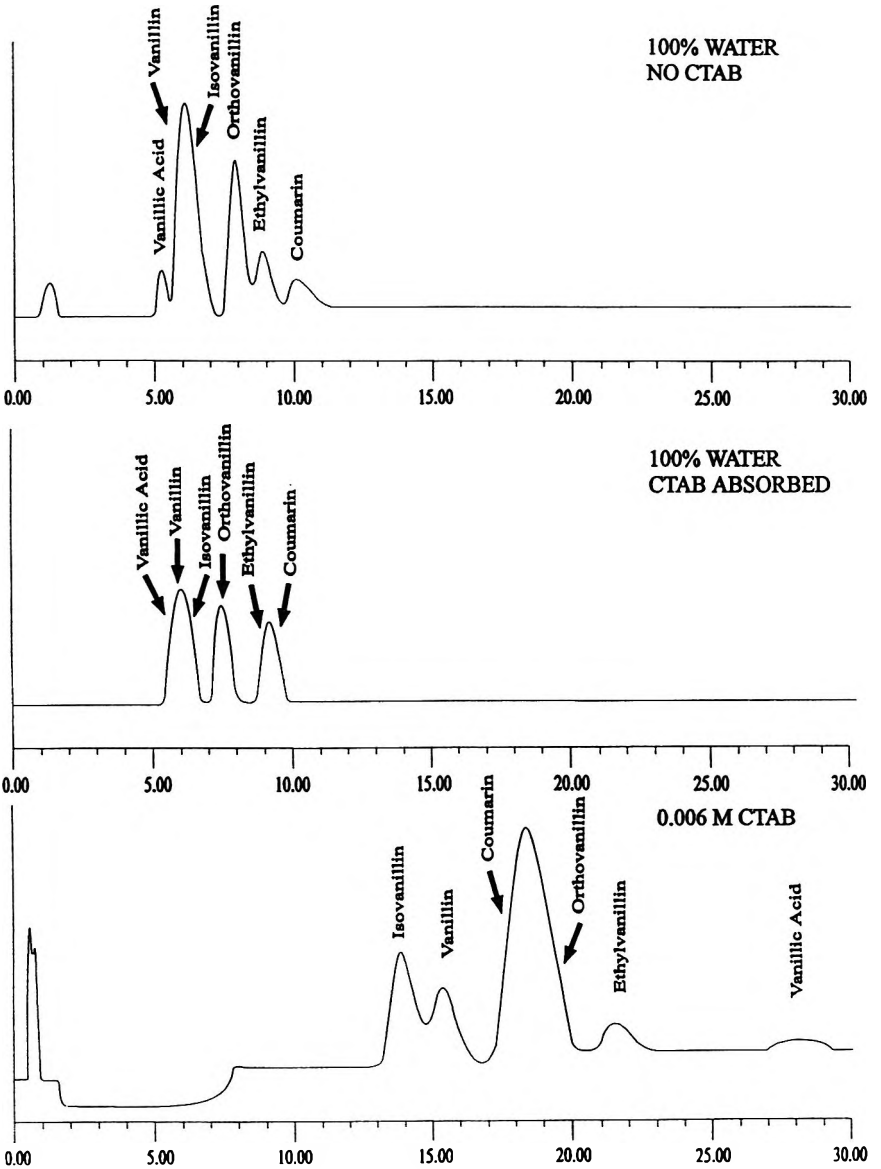

Figure 15. Chromatograms of the vanillin test mixture on a cyanopropyl column before, during, and after separations involving micellar mobile phases. The pH of each mobile phase was 3.0, and the flow rate was 1.0 mL/min.

retention time of some ionogenic compounds on cyanopropyl bonded phase columns actually increases with increasing micelle concentration which is opposite of what is considered to be normal retention behavior in MLC. This effect which is known as antibinding behavior occurs with compounds that have the same charge as the surfactant and is a direct result of a compound being driven into the stationary phase as the micelle content of the mobile phase is increased because the compound is excluded, not only from the micelle, but from the double layer that surrounds the micelle. Because the charged head group of the adsorbed surfactant monomer is "tied up" by the cyano functionality of the bonded phase, there is little free electrostatic charge to prevent migration of this ionized solute into the bonded phase. By comparison, antibinding behavior does not occur on C_{18} or C_8 columns because of the higher surface charge of the CTAB and SDS modified alkyl bonded phase, which is the result of the sulfate and N-alkyl head groups not being as strongly associated with the C_{18} and C_8 alkyl bonded phase.

The model for surfactant adsorption developed from the NMR data, however, cannot explain the S-type adsorption isotherms obtained for SDS or CTAB on cyanopropyl columns[9] which can be interpreted as due to cooperative adsorption. Nor can the model explain the longer retention time of the vanillin compounds when either CTAB or SDS micellar solutions are used as mobile phases in lieu of a purely aqueous mobile phase (see Figures 14 and 15). The latter result is surprising in view of the greater solvent strength of CTAB and SDS micellar mobile phases which suggests that solute stationary phase interactions are also greater when these micellar solutions are used as mobile phases instead of a purely aqueous mobile phase. Perhaps, small surfactant aggregates form within the cyanopropyl bonded stationary phase when SDS or CTAB micellar solutions are used as mobile phases. These aggregates could be responsible for the increase in the retention time of the vanillin compounds and could also explain the S-type adsorption isotherms for CTAB and SDS on cyanopropyl.

CONCLUSION

The results of an in-depth study on the effects of surfactant chain length and charge-type on selectivity in MLC has been presented. A hydrophilic test mixture has been separated on a variety of bonded phase columns using either an aqueous anionic SDS or cationic CTAB or DTAB micellar solution as the mobile phase. To explain the differences in selectivity as a function of surfactant charge type and chain length, it was necessary to determine the relevant partition/binding constants, the extent of surfactant adsorption on the stationary phase, and the molecular orientation of the adsorbed surfactant

monomer in the bonded phase. Our conclusions supported by solid state NMR data indicate that different surfactant molecular orientations on the stationary phase can lead to significant selectivity differences in MLC.

Previously published work by Cline-Love and Berthod[10-11] has stated or implied that one of the disadvantages with the use of micellar mobile phases was that the surfactant coating of the stationary phase rendered them all similar in terms of their polarity and thus masked the columns inherent selectivity. That is, regardless of the stationary phase or surfactant charge type, very similar selectivity would be observed. This assumed, of course, that all surfactants regardless of their charge type or chain length sorbed with similar orientations on different stationary phase materials. Hence, the significance of the present study is that it dispels those previous conclusions/speculations and demonstrates that surfactant-charge type and chain length can influence selectivity. In other words, finding the appropriate combination of surfactant and stationary phase is crucial in micelle mediated RPLC, which is the reason why users should be encouraged to experiment with more than one charge-type micellar system.

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SIZE-EXCLUSION CHROMATOGRAPHY OF LOW MOLECULAR WEIGHT POLYMERS

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ABSTRACT

To overcome the difficulty of the determination of the molecular-weight characteristics by size exclusion chromatography in the low-molecular-weight region ($M < 10^4$), the Sadron-Rempp relationship was used both for the construction of dependence of the universal calibration parameter on elution volume and for the tranformation of chromatographic data to molecular characteristics. Numerical values of constants of the above relation between intrinsic viscosity and molecular weight, which were calculated for some polymer-eluent systems on the basis of literature data, are given.

INTRODUCTION

Size exclusion chromatography (SEC) is suitable for the characterization of low-molecular-weight polymers since the product of intrinsic viscosity and molecular weights $[\eta]M$ determines their hydrodynamic retention.[1-4] Direct determination of molecular weight M is mostly excluded because of insufficient sensitivity of light scattering to short macromolecules. Therefore a calibration of the separation system is not avoidable. The concept of "universal calibration", i.e. the dependence of the parameter $[\eta]M$ on elution volume V,

makes possible characterization of any polymer,[1] for which a set of molecularly uniform standards is not available. For this purpose the relations between $[\eta]$ and M both for calibrants and for polymers under study are needed. The frequently used Mark-Houwink-Sakurada equation is not appropriate for most polymer-good solvent systems if $M<10^4$. For this range of molecular weights, other two-parameter equations were suggested.[5-7] The Dondos-Benoit equation was already used for interpretation of SEC data.[6,8-11] In this paper a more general three-parameter equation suggested by Sadron and Rempp[12] was applied

$$[\eta] = A_1 + A_2 M^a \qquad (1)$$

to demonstrate the determination of molecular weight distribution of small polymers by SEC.

MATERIALS AND METHODS

Polymers

Polystyrene (PS), poly(oxyethylene) (POE), polyisobutylene (PIB), and polybutadiene (PB), were purchased from Chrompack and Polymer Laboratories, Serva and Fluka, Polymers Standard Service, and Polyscience, respectively. Poly(1-decene), poly(1-decene), poly(methyl methacrylate) were prepared by ionic polymerization.

Methods of Measurement

High-speed SEC was performed using an HP 1084 B (Hewlett Packard, U.S.A.) liquid chomatograph with differential refractometric detection (Knauer 2025/50, FRG) and with columns (7.5/600 mm) packed with PL-GEL 10 μm 500 Å and 100 Á (Polymer Laboratories, GB). Tetrahydrofuran (THF, flow rate 1 cm^3 min^{-1}) was the mobile phase. Under chosen separation conditions the axial dispersion was neglected.

Osmotic measurements (VPO) were performed with a Hitachi Perkin-Elmer vapour pressure apparatus M 115 in chloroform at 37°C (cf. 13).

Viscosity of solutions of some polymers in THF was measured at 25°C with an Ubbelohde capillary viscometer adapted for gradual dilution. Viscometric data were evaluated by Heller's extrapolation method.

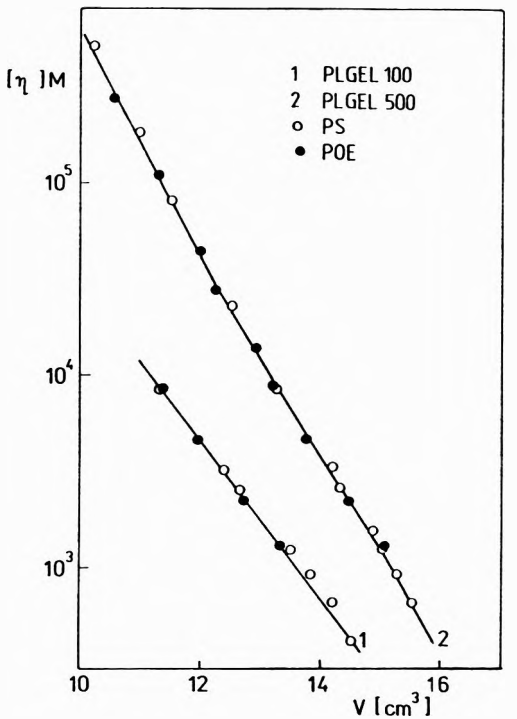

Figure 1. Dependence of universal calibration parameter $[\eta]M$ on elution volume V for two separation systems

RESULTS AND DISSCUSSION

PS and POE standards were subjected to SEC to obtain their elution volumes V. An experimental "universal calibration" (Fig. 1)

$$\log ([\eta]M) = f(V) \qquad (2)$$

was constructed using osmometrically checked molecular weights and intrinsic viscosities calculated according to eq.(1). Parameters of this equation determined on the basis of literature data are summarized in Table 1. (In addition to A_1, A_2, and a for PS and POE in THF, constants for calibrants in other solvents widely used as SEC eluents are listed).

Table 1

The Parameters* of Sandron-Rempp Equation[1] for Various Polymer-Eluent Systems Determined at 25°C in the Molecular-Weight Range (0.3-30)x10^3

Polymer	Eluent	A_1	A_2 x10^2	a	Ref.
PS	Tetrahydrofuran	0.73	2.22	0.65	21, 23
PS	Toluene	0.94	1.33	0.70	22, 23
PS	Chloroform	0.28	4.52	0.59	6, 22
POE	Tetrahydrofuran	0.50	4.75	0.65	24
POE	Dioxane	0.05	4.00	0.70	12, 25
POE	Dimethylformamaide	1.03	5.93	0.63	25
POE	Chloroform	-1.10	18.80	0.55	6, 26
POE	Methanol	0.51	7.74	0.60	12, 25
POE	Water	1.47	4.84	0.66	12, 25, 26
PMMA	Tetrahydrofuran	0.91	3.19	0.60	10, 14
PIB	Tetrahydrofuran	---	5.00	0.60	16
PB	Tetrahydrofuran	---	4.87	0.68	17, 18

* The values of $[\eta]$ atr in cm^3g^{-1}.

Poly(methyl Methacrylate)

For $M<10^4$ the double logarithmic plot of $[\eta]$ against M for PMMA in good solvents[14] deviates upwards. This behaviour of PMMA in THF can be described well by eq.(1) with respective constants given in Table 1. To transform the chromatographic data to molecular weight distribution (MWD), eqs (1) and (2) were combined

$$f(V) = \log (A_1 M + A_2 M^{(a+1)}) \qquad (3)$$

Using Newton's method of successive approximation the values of M along the chromatogram were calculated from eq.(3). Then the calculated M_i and w_i (weight fraction of polymer at V_i) appeared in summations giving weight-average and number-average molecular weight as well as intrinsic viscosity,

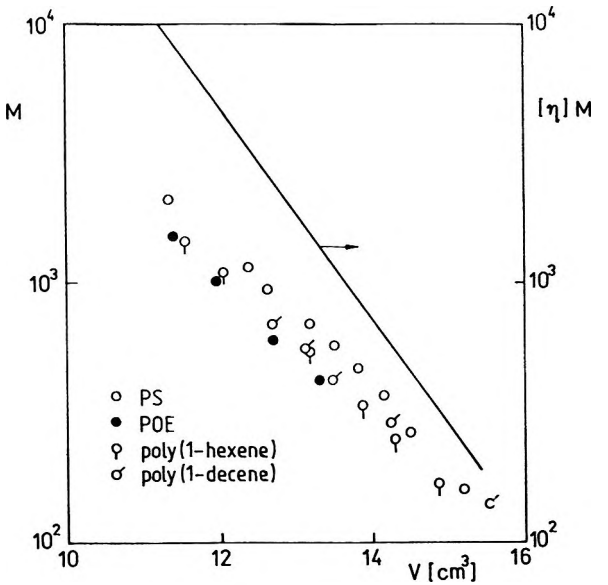

Figure 2. Plot of log $[\eta]$ vs. log M. The lines represent Mark-Houwink-Sakurada relations in the high-molecular-weight region.

Table 2

Comparison of Molecular Weights Obtained by Different Methods for PMMA and PIB Samples

Polymer	VPO	SEC			
		SR eq.		MHS eq.	
	M_n	M_n	M_W/M_n	M_n	$M_W M_n$
PMMA	2700	2780	1.50	3930	1.35
PMMA	3400	3550	1.77	4790	1.57
PMMA	4600	4620	2.46	6130	2.05
PIB	550	---	---	596	1.12
PIB	1500	---	---	1610	1.24

$M_W = \Sigma w_i M_i$, $M_n = (\Sigma w_i / M_i)^{-1}$ and $[\eta] = \Sigma w_i f(V_i) M_i^{-1}$. Characteristics of three PMMA samples obtained by the described way are compared in Table 2 with those calculated using the MHS relation derived for high-molecular-

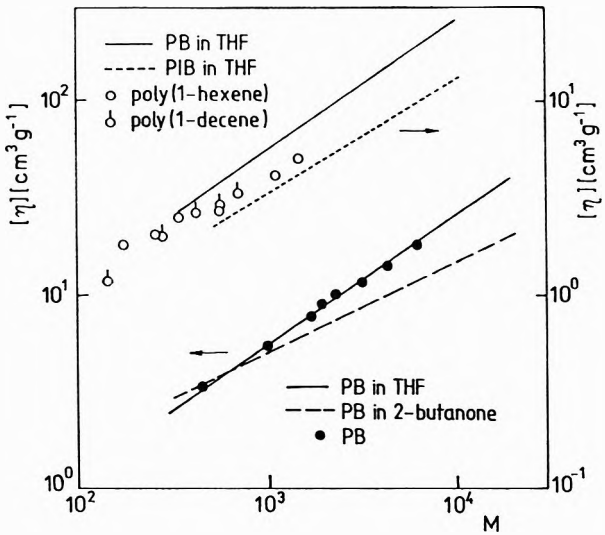

Figure 3. Elution behaviour of different low-molecular-weight polymers in separation system PLGEL 100.

weight region.[15] M_n increassed up to 30-40%, i.e. the nonuniformity became lower (Table 2), when not taking into account specific hydrodynamic behaviour of low PMMA's.

Polyisobutylene and Polybutadiene

For the system PIB-THF the range of validity of the MHS relation[16] can be extended to very low molecular weights, since reliable molecular weight parameters with admissible errors were obtained as shown in Table 2. The same conclusion was drawn from evaluation of the SEC data of PB in THF.[17,18] As this statement contradicts the literature data,[9] the evidence is given in Figure 2. The experimental points with coordinates $[\eta]$ and M follow the MHS dependence[17] satisfactorily.

Poly(1-alkene)'s

Only little is known about hydrodynamic behaviour of these oligomers/polymers in solution. Using the "universal calibration" concept, it

was possible to obtain missing data. The $[\eta]$ value was calculated from the $[\eta]M$ value corresponding to the elution volume in the maximum of a chromatographic curve[19] as demonstrated in Figure 3. Individual low oligomers in multimodal chromatograms were identified by reverse phase liquid chromatography[20] and 75 eV impact mass spectrometry. Other necessary M_n values were measured by osmometry. In Figure 2 the dependences of $[\eta]$ on M for poly(1-hexene) and poly(1-decene) in THF are compared with those for PIB and PB.

CONCLUSION

It was shown that disregarding specific hydrodynamic behaviour of polymers in low-molecular-weight region ($M<10^4$) becomes the cause of possible errors in molecular characterization by SEC. This phenomenon must be taken into account both in construction of universal dependence (eq.1) using molecularly uniform standards and in recalculation of SEC data to MWD by means of the "universal calibration" principle. On the other hand, SEC in combination with an independent measuring method (e.g. VPO) can be used for estimation of hydrodynamic behaviour of short polymer molecules.

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HALLUCINOGENIC SPECIES IN AMANITA MUSCARIA. DETERMINATION OF MUSCIMOL AND IBOTENIC ACID BY ION-INTERACTION HPLC

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ABSTRACT

Ibotenic acid and muscimol are considered the principal components responsible of the hallucinogenic properties of *Amanita muscaria*. In this paper a sensitive ion-interaction HPLC method is presented which permits the simultaneous determination of ibotenic acid and muscimol. A C_{18} reverse phase is the stationary phase and the mobile phase is an aqueous solution of 5.0 mM octylammonium o-phosphate. A spectrophotometric comparative detection performed at 230 and 254 nm is employed. Detection limits are of 18 μg/L for ibotenic acid and of 30 μg/L for muscimol.

Different sample pretreatment processes were compared. The highest recovery yields together with the minimum matrix effect were obtained by a procedure which only consists in the sonication of the fresh sample and its squeezing. The extract obtained is then diluted as required and filtered (0.2 μm) before injection. This preparation offers the further advantage to maximally preserve the native composition.

The method was applied in the analysis of *Amanita muscaria* and permitted to evaluate the content of muscimol and ibotenic acid in heads and stems of the mushrooms. The same analysis performed for samples of *Amanita citrina* confirmed the absence in this species of both ibotenic acid and muscimol.

INTRODUCTION

Amanita muscaria is generally more known for its toxical properties than for the psychotropic ones and the literature methods concerning the analysis of *Amanita* mostly regard identification and determination of its toxic components, such as muscarine, amatoxins and phallotoxins.[1-5]

It is on the other hand reported[6,7] that in North Europe countries, and especially in Siberia, *Amanita muscaria* hallucinogenic properties were known since 4.000 b.C. The "Rig-Veda", which is considered the first politeist text, mentions *Amanita* as the "God plant" because of its exceptional properties to transmit prodigious powers.[8] The species responsible for the psychotropic effects, which have been now identified are: ibotenic acid (amino-(3-hydroxy-5-isoxazolyl)acetic acid) and its decarboxylation product muscimol (3-hydroxy-5-aminomethylisoxazole).[8-10] Their structures are presented in Fig.1.

These compounds are receiving great interest in psychiatric medicine because of their potential ability to be employed as an alternative to synthetic drugs or to be used as possible models for the development and preparation of new drugs devoted to the treatment of psycho-diseases.[9] Analytical methods are therefore required for extraction, purification and determination of these compounds in *Amanita* mushrooms.

Only two determination methods are reported in literature. One makes use of a microbore column and UV detection after reaction with ninhydrin in presence of potassium cyanide.[9] The other (direct source[10] is not easily available) is a HPLC method, which makes use of an amino-bonded stationary phase; the preparation of the sample requires two SPE separation processes respectively performed with Dowex 1-X8 resin (acetate form) for the extraction of ibotenic acid and with alumina-silica gel for muscimol. Detection limits are respectively 18 and 10 mg/L of ibotenic acid and muscimol.

This paper presents a sensitive HPLC ion-interaction method for the simultaneous separation and determination of muscimol and ibotenic acid in the extract which can be easily obtained by squeezing the fresh sample of

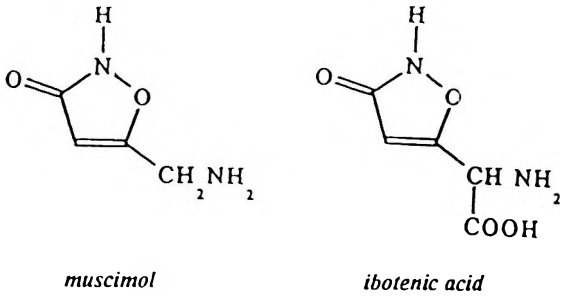

Figure 1. Chemical structures of the analytes.

Amanita muscaria. No other pretreatment is required (a part a suitable dilution and 0.22 μm filtration) and this feature is of particular importance because the native composition is, as far as possible, preserved and conditions for the taking place of decarboxylation reactions (which transform ibotenic acid into muscimol) are prevented. Measurements are also performed for samples of *Amanita citrina*, which is reported not to contain psychotropic compounds.[8]

EXPERIMENTAL

Chemicals and Reagents

Ultrapure water from Millipore MilliQ (Milford, MA) system was used for the preparation of solutions. Ibotenic acid and muscimol are Sigma Chemical Co. reagents. Pentylamine, hexylamine, heptylamine, octylamine, nonylamine, ortho-phosphoric acid and HPLC-grade acetonitrile were Fluka (Buchs, Switzerland) chemicals. All other reagents were C.Erba (Milano, Italy).

Apparatus

The chromatographic analyses were performed with a Merck-Hitachi (Tokyo, Japan) Lichrograph chromatograph Model L- 6200 equipped with a two channel D-2500 chromato-integrator interfaced with a UV-Vis detector L-4200.

The absorbance spectra A/λ were recorded by a Hitachi (Tokyo, Japan) mod. 150-20 spectrophotometer. The analyses were performed at two different wavelenghts, namely 230 nm, which corresponds to the maximum absorbance for the two analytes, and 254 nm, at which absorptivity coefficient for ibotenic acid is much lower than at 230 nm. The choice of the latter wavelenght was due to: a) a lower matrix interference effect as concerns muscimol determination and b) to confirm, under different conditions, the analyte identification and determination.

For pH measurement a Metrohm (Herisau, Switzerland) 654 pH-meter provided with a combined glass-calomel electrode was employed.

Chromatographic Conditions

A Phase Separations (Desidee, CLWYD, UK) Spherisorb S5 ODS-2 (5 μm, 250 x 4.6 mm) cartridge was used together with a guard pre-column Merck (Darmstadt, Germany) Lichrospher RP-18 (5 μm).

The mobile phases were aqueous solutions of the ion-interaction reagents. The use of orthophosphate and salicylate of different alkylamine (namely pentylamine, hexylamine, heptylamine, nonylamine and decylamine) at different pH values (3.0, 6.4 and 8.0) was compared. The solutions were prepared by adding to the amount of the amine (weighted to prepare the desidered molar concentration), o-phosphoric acid or salicylic acid up to the desidered pH value.

The chromatographic system was conditioned by passing the eluent through the column until a stable baseline signal was obtained; a minimum of 1 hour was necessary at flow-rate of 1.0 mL/min. When in use, overnight the flow-rate of the mobile phase was maintained at 0.1 mL/min. After use or anyway after three days of use, the column was washed and a new mobile phase freshly prepared.

The washing steps were performed with water (1.0 mL/min for 30 min), a 50/50 v/v water/acetonitrile mixture (1.0 mL/min for one hour), and finally acetonitrile (0.7 mL/min for 20 min). This procedure is necessary, especially when working with real matrices, to remove highly retained lipophilic matter which doesn't elute in aqueous mobile phase, but could affect column modification. Retention intra-day repeatibility was always within 3% for the same eluent preparation and inter-day reproducibility for different preparations was always within 6%.

Our results fit the model according to which the ion-interaction reagent contained in the mobile phase is bound onto the surface of the stationary phase through adsorption and electrostatic forces, giving rise to an electrical double layer. The interaction properties of the original reverse phase packing material are therefore modified and the modified surface is able to simultaneously retain cationic and anionic species.[11-14]

Sample Preparation

The mushrooms investigated were collected in October in Piedmont (North Italy) and analyzed just within three days after sampling. Alternatively the mushrooms can be frozen soon after picking and cleaning and preserved until the analysis, when they are defrosted and treated as described for the fresh ones.

Different extraction procedures were experimented and compared. The aim was to obtain the maximum recovery yield, taking into account that conditions for the decarboxilation reactions to take place (which transform ibotenic acid into muscimol) must be avoided as much as possible.

The extraction procedures experimented were:

a) solid phase extraction (SPE) employing an anionic-exchange column (QMA Waters) for ibotenic acid and a SPE-C18 for muscimol.

b) liquid-liquid extraction, employing and comparing different extracting solvents mixtures, namely ethanol-water, methanol-water, ethanol-methanol-water in different volume ratios.

c) liquid-liquid extraction, after removal of fats by Soxhlet extractions, respectively performed with chloroform, diethylether and hexane.

d) pounding, sonication and water extraction.

Solid phase extraction (method a) and liquid extraction (method b) led to very low selectivity for the studied analytes and to a consequently heavy matrix effect in the chromatographic analysis. Soxhlet fat extraction also led to a partial extraction of the analytes. The procedure d) was therefore applied for the pretreatment of the sample, also taking into account that the conditions at minimum affect the speciation and at maximum avoid the contamination of the sample. The procedure is described in detail, as follows.

Procedure Followed

The mushrooms were cleaned and the heads and stems were separately pounded in a mortar. After 15 min of sonication treatment, the juice was separated from the pulp and resulted to be about 90% of the fresh weight. In order to improve the recovery of the analytes the pulp was washed twice with ultrapure water. The juice was diluted with ultrapure water and filtered through a 0.22 μm nylon membrane before the injection in the HPLC apparatus. The optimum final dilution prior to the HPLC analysis was 1/1300 v/v.

RESULTS AND DISCUSSION

As mentioned, no example can be found in literature of the simultaneous separation of muscimol and ibotenic acid.

By considering the molecular structure of the analytes (see Fig.1) and their different hydrophilicities, the ion-interaction chromatography seemed to be particularly suitable for their separation.

On the basis of previous results,[11-13] it was pointed out that the good applicability of the method greatly depends on its versatility and the dependance of retention on many variables which must be optimized. Our aim was the development of a method which offers the maximum sensitivity for the analytes, together with the minimum interference effect by the several other components of the matrix.

Method Development

The ion-interaction chromatographic method was optimized as concerns the pH of the mobile phase and the ion-interaction reagent chemical properties. The use of different pH values of the mobile phase showed no significant influence, as concerns both retention and sensitivity, in the pH range between 3.0 and 8.0; a pH value of 6.4 was chosen as more similar to the natural tissue.

As concerns the choice of the ion-interaction reagent, the comparison of salicylate and o-phosphate as counter anions showed that better sensitivities could be gained with o-phosphate. The use of alkylammonium o-phosphate

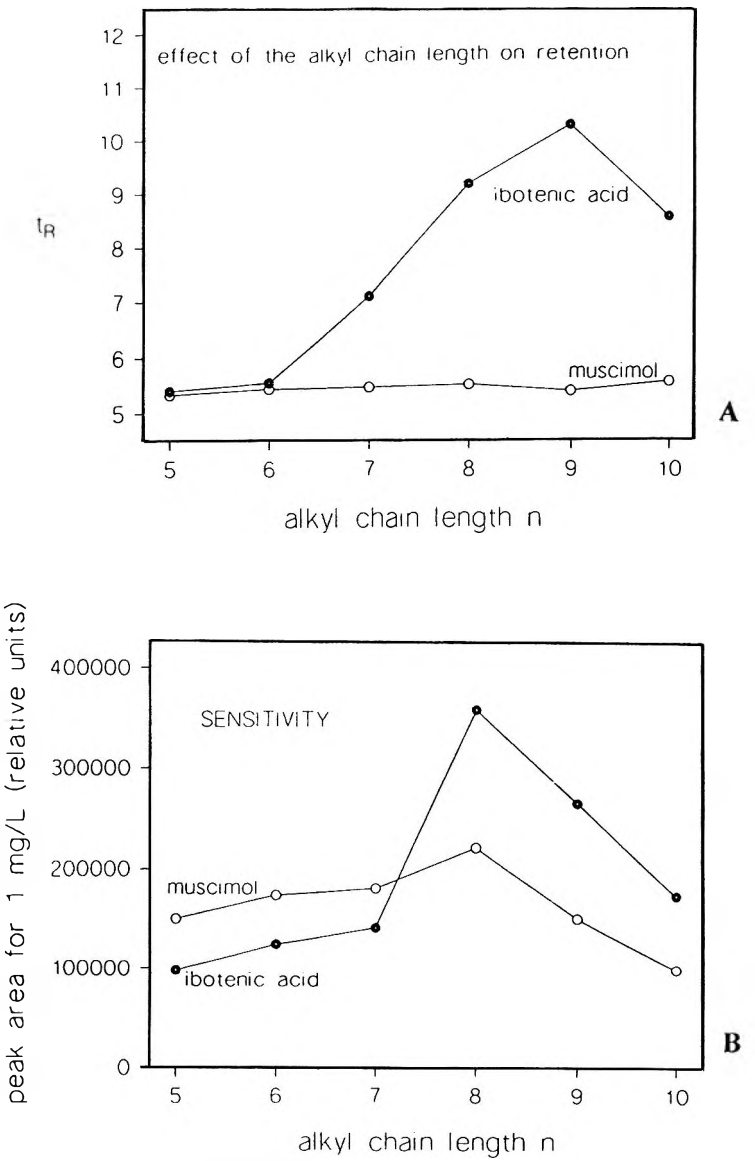

Figure 2. Plot of resolution (A) and of sensitivity (B) vs carbon chain lenght of the ion interaction reagent (aqueous 5.0 x 10^{-3} mol/L alkylammonium orthophosphate, pH 6.4) for the two hallucinogenic compounds.

salts with the alkyl chain lenght *n* varying between 5 (pentylamine) and 10 (decylamine) was then compared. Fig.2A shows that the effect of *n* on retention is very relevant for ibotenic acid, in agreement with its more hydrophilic properties, while it can be considered as negligible for muscimol.

As a consequence, also the resolution between the two analytes is greatly affected by the alkyl chain length. Resolution R_s shows its maximum value for n=9 (R_s=3.04) and is also reasonably high for n=8 (R_s=2.28).

On the other hand, previous studies[14] have shown that the alkyl chain length of the ion-interaction reagent can also affect analyte sensitivity. The behaviour is also followed by the analytes investigated here (Fig.2B): for both analytes the best sensitivity is achieved for n=8. These results suggested the use, as mobile phase, of a 5.0 mM octylammonium o-phosphate aqueous solution at pH=6.4.

As concerns UV detection, this was performed both at 230 and 254 nm, at which wavelengths the two analytes show significantly different absorptivity values: the comparative detection at two different wavelenghts can be helpfully employed to confirm the identification and the quantitation or to better overcome matrix interference effects.

Fig.3 shows a typical separation obtained under the optimized chromatographic conditions, with detection at λ=230 nm, of a mixture of muscimol (0.150 mg/L) and ibotenic acid (0.100 mg/L).

The calibration curves built for ibotenic acid in the concentration range between 0.05 and 1.00 mg/L, and for muscimol in the concentration range between 0.10 and 3.00 mg/L, showed a good correlation between peak area and concentration: the plots could be fitted by straight lines with correlation coefficients always higher than 0.995. From the sensitivity data and for a signal/ noise ratio = 3, detection levels as low as 30 μg/L and 18 μg/L were respectively evaluated for muscimol and ibotenic acid.

Amanita Muscaria and *Amanita Citrina* Analysis

The method was then applied on the analysis of the two extracts respectively obtained, as described in the experimental section, from heads and stems of *Amanita muscaria* and *Amanita citrina*.

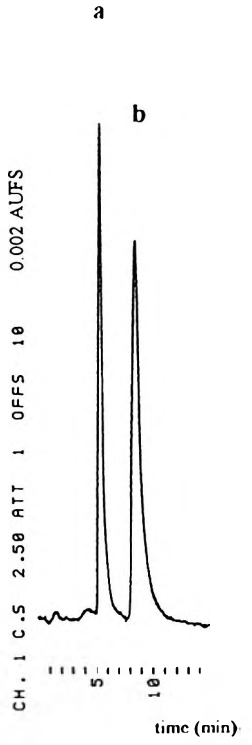

Figure 3. Chromatogram of the standard solution containing 0.150 mg/L of muscimol (a) and 0.100 mg/L of ibotenic acid (b). Experimental conditions. Phase Separation S5 ODS-2 250x4.6 mm column. Mobile phase: aqueous 5.0×10^{-3} mol/L octylammonium o-phosphate, pH 6.4. Flow-rate: 1.0 mL/min. Spectrophotometric detection at 230 nm.

The ion-interaction chromatographic method developed here shows advantages of selectivity towards more lipophilic components present in the matrix. This is likely due to the characteristics of the method which utilizes an aqueous mobile phase and is based on the capability of the analytes to form ion-pairs.

As expected, in agreement with botanic information[8] *Amanita citrina* did not show the presence, at least at the detection limit, of both ibotenic acid and muscimol.

As concerns *Amanita muscaria*, Fig. 4 shows, as a comparison, typical chromatograms respectively recorded at 230 nm and 254 nm for the head extract. In agreement with molar absorptivity values, sensitivity for muscimol

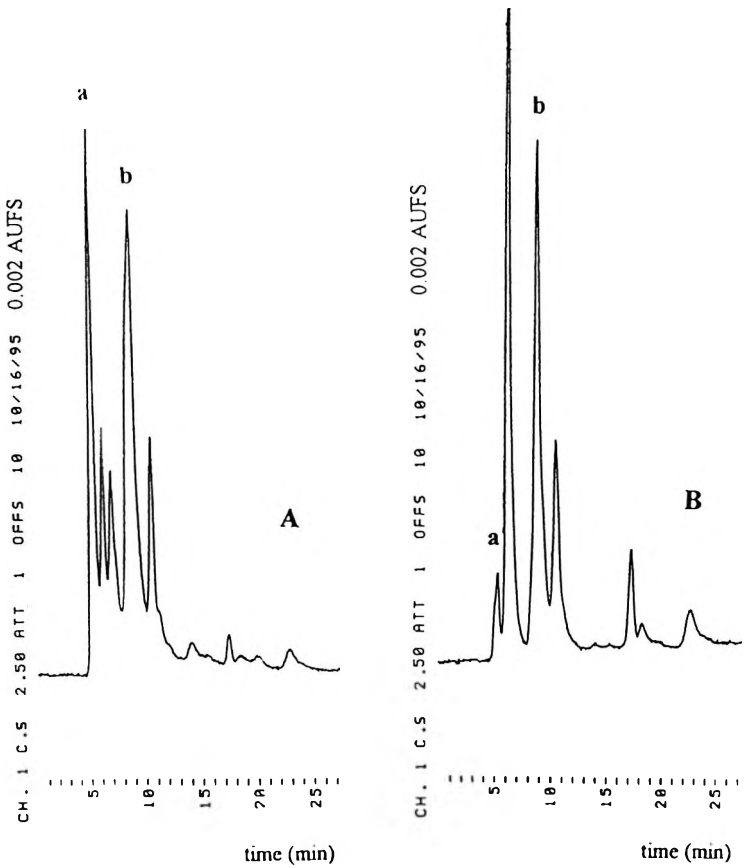

Figure 4. Chromatogram of the diluted (1/1300 w/w) juice from *A. muscaria* heads at 230 (A) and 254 nm (B). Experimental conditions as in Figure 3. a: muscimol; b: ibotenic acid.

is much lower at 254 than at 230 nm, but the interference-freedom is much higher. No significant difference can instead be observed at the two wavelenghts for ibotenic acid.

Quantitation of the two analytes was performed by a standard addition method taking into consideration the chromatographic response in terms of both peak area and peak height at the two wavelenghts. On average, the quantitative results always agree within 15%. As expected, the amount found

for the two compounds is always higher in heads than in stems. The mean values, referred to fresh mushroom, was found to be: for muscimol 0.38±0.03 g/Kg in heads and 0.08±0.01 g/Kg in stems; for ibotenic acid 0.99±0.01 g/Kg in heads and 0.23±0.03 g/Kg in stems.

The only available literature data[9] report for heads of fresh mushroom amounts of respectively 41.8 mg/Kg of muscimol and 86.6 mg/Kg of ibotenic acid.

The higher content we found might be ascribed, besides the different geographical origin of the samples, to the treatment processes performed prior to the analysis, which in the method here proposed at maximum preserves native composition. No particular pretreatment, extraction process or derivatization reaction is in fact required, a part from the squeezing of the mushroom and the subsequent dilution and filtration of the extract. This extraction process and the absence of contaminating reagents can be of practical interest for subsequent use of the extract.

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DETERMINATION OF FOLPET, PROCYMIDONE, AND TRIAZOPHOS IN GROUNDWATER BY HPLC USING PARTIAL LEAST SQUARES AND PRINCIPAL COMPONENT REGRESSION

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ABSTRACT

Three multivariate calibration methods, partial least squares (PLS-1 and PLS-2) and principal component regression (PCR), full spectrum calibration methods, were applied to the simultaneous determination of the three pesticides folpet, procymidone and triazophos, in mixtures, by high performance liquid chromatography with photodiode array detection (HPLC-DAD). The effects of several preprocessing techniques are discussed in order to optimize the calibration matrices by the PLS and PCR methods. The use of mean-centering and smoothing function chromatograms allows better prediction of the samples. The average recoveries from the different mixtures assayed ranged between 79.6 % and 114.0 %. No advantages were found for the prior differentiation step.

The results obtained by the application of the different chemometric approaches are discussed and compared. The methods were applied with satisfactory results in the determination of folpet, procymidone and triazophos in groundwater at ppb levels, having previously employed a solid-phase extraction with C_{18} cartridges.

INTRODUCTION

Pesticides are of major importance in modern agriculture. Their application over a number of decades has led to the development of multirresidue analysis methods by HPLC among others, for their detection and control in the sustracts where they are applied. The development of reliable methods to cover a broad spectrum of relevant substances, in one analytical run, for systematic environmental analysis is an important field of research. The analysis of pesticides in environmental samples by HPLC techniques requires the elution of a wide variety of analytes under conditions as optimum as possible. One way to reach this objective is to apply solvent programming or gradient elution techniques.[1-4]

HPLC using DAD provides an opportunity for chromatographers to explore all wavelengths in the UV-vis, improving the selectivity of HPLC and allowing the identification of compounds not only from their retention behaviour but also from their UV spectral properties. The use of DAD allows choice of the monitoring wavelengths which maximize the instrumental sensitivity.[5-7]

In previous papers we studied the optimization of the separation, isolation and determination of nine pesticides by a new sequential procedure for the automated location of the mobile phase composition optimum[8] and the proposed method was extended to the determination of 21 pesticides in water samples.[9,10] However, it is difficult to avoid the overlapping of peaks in the analysis of complex mixtures owing to similar retention times obtained, either for different analytes or for analytes and interferences.

In this situation, where the overlapping signals do not permit the analysis of all analytes in a single chromatographic run, it is possible to modify the multirresidue method, or apply chemometric techniques in order to extract useful information from the overlapped region[3,4,11-13] The first solution is not the most adequate due to the great cost involved in developing a new method.

Therefore, the second solution can be selected. Moreover, the advent of multi-dimensional data systems used in conjunction with modern computer technology has allowed the development of new experimental procedures for the characterization of unresolved chromatographic peaks.[14-19]

Direct calibration methods, such as multiple linear regression[20] or Kalman filter[21-23] have also been applied to HPLC data. Direct calibration methods assume that chromatograms are available for all chemical species existing in the mixture. The major advantage of these methods is their simplicity to resolve linear analytical systems that obey the Beer-Lambert law. If some species interact, indirect calibration methods present a better alternative than direct methods, since we can design and measure the response of a training set of mixtures with known concentrations (standards), and thus accomodate moderate effects of nonadditivity, which are normally not accounted for with direct methods.

In the present paper, PLS and PCR multivariate calibration methods were applied to resolve highly overlapped chromatographic peaks. Both are examples of indirect calibration methods, i.e. they do not require individual chromatograms for each analyte and interferent to be known in advance, but all expected phenomena must be spanned in the calibration set. They offer full spectrum advantages.

Each method requires a calibration step where the relationship between the chromatograms and the component concentrations is deduced from a set of reference samples by means of a least-squares procedure, followed by a prediction step in which the results of the calibration are used to determine the component concentrations from the sample chromatogram.

Biased regression methods such as PLS and PCR are based on the regression of chemical concentrations on latent variables or factors. PLS differs from PCR in that it uses the concentration data from the training set and the chromatographic data in modelling, whereas PCR only uses the chromatographic data. Hence PLS can reduce the influence of dominant but irrelevant factors, and in some cases yields models of lower dimensionality, in order to achieve better correlations with concentrations during prediction.

PLS also has the advantage of being able to model a number of analytes simultaneously, the so-called PLS-2 approach. These chemometric techniques have been discussed in more detail elsewhere.[24,25]

Table 1

Retention Times of Each Pesticide in the Multirresidue Method

Peak No.	Pesticide	Retention Time (min)
1	metomyl	2.3
2	dimethoate	3.1
3	aldicarb	4.4
4	diclorvos	5.6
5	carbofuran	6.2
6	atrazine	7.3
7	diuron	8.6
8	dichloran	9.9
9	methiocarb	11.2
10	folpet	13.1
11	procymidone	13.4
12	triazophos	13.7
13	iprodione	13.9
14	vinclozolin	14.7
15	chlorfenvinphos	14.9
16	chlorpyrifos methyl	16.4
17	endosulfan sulftate	16.7
18	tetradifon	17.8
19	β-endosulfan	18.0
20	α-endosulfan	18.4
21	chlorpyrifos ethyl	18.7
22	carbophenothion	19.4

This paper describes the development of a combined HPLC-DAD system and direct data treatment using PLS and PCR for simultaneous multi-analyte determination of the components of a mixture of folpet, procymidone and triazophos.

The procedures were applied in order to determine these pesticides in groundwater at ppb levels after a solid-phase extraction (SPE) with C_{18} cartridges.

EXPERIMENTAL

Instrumentation

A Waters (Milford, MA, USA) Model 990 liquid chromatographic system, equipped with a Model 600E constant flow pump, a Rheodyne six-port injection valve with a 20 μL sample loop and a Model 990 photodiode array detector, was used. The detector was interfaced with an Olivetti PCS-386 personal computer using a Waters Model 991 software and a Waters Model 990 plotter. The absorbance (A), wavelength (λ), and time (t) were digitized using the Waters Model 991 software, which allows representation and storage of absorption spectra obtained at the same time. An IBM 486-DX microcomputer, provided with a Grams/386 software package and PLSplus V2.1G,[26] was used for treatment of data. A conversion program written in Array-Basic was used with the object of transferring the files obtained with the Waters Model 991 software to an ASCII XY format, which allows the manipulation of these files with the Grams/386 software.

HPLC separations were carried out using a Hypersil Shandon Green Env. 3 x 150 mm (5 μm particle size) C_{18} column.

Chemicals and Solvents

HPLC grade solvents were used. The pesticide standards (pestanal quality) summarized in Table 1 were obtained from Riedel-de Haën (Seelze, Germany). Solid standards were dissolved in acetonitrile (AcN) and stored at 4°C in the dark, where they were stable for several months. Working solutions were prepared daily by appropriate dilution with AcN. Mobile phases were degassed with helium during and before use. Distilled water was obtained from a Millipore water purification Milli-Q system. All solvents and samples were filtered through Millipore membrane filters before injection into the column. Prepacked Sep-Pak C_{18} cartridges containing 360 mg of C_{18} chemically bonded silica (Waters) were used.

HPLC Operating Conditions

Flow rate: 1 mL. min^{-1}; chart speed: 0.5 cm. min^{-1}; detector sensitivity: 0.02 a.u.f.s. ; column at room temperature; wavelength: 210 nm.

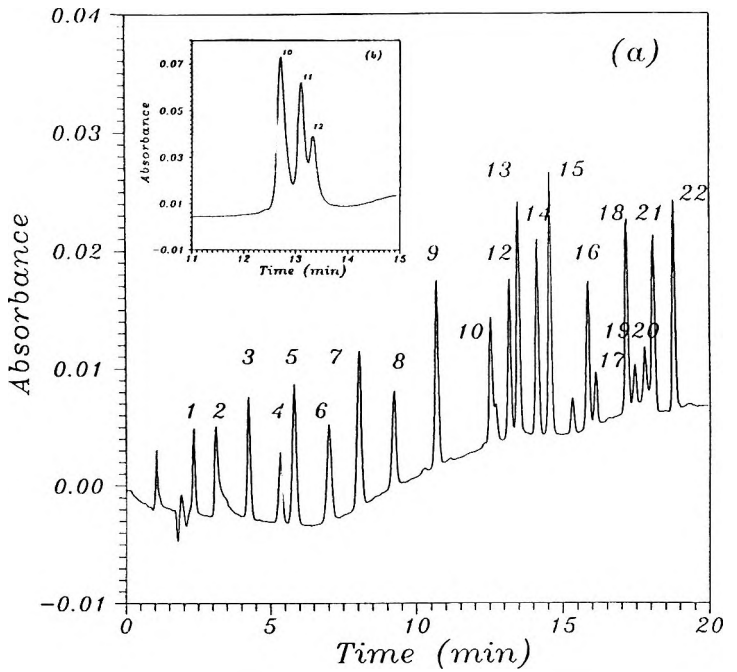

Figure 1. a) Chromatogram obtained by injection of 20 µl of pesticide standard solution with a 20 min gradient, (2 µg. mL^{-1} of each pesticide at 210 nm). Numbers above the peaks correspond with those given in Table 1. b) Chromatogram with a new analyte, procymidone (peak number 11), is observed with 20 min gradient (9 µg. mL^{-1} of folpet, 4 µg. mL^{-1} of procymidone and 6 µg. mL^{-1} of triazophos).

Solvent Programming

The solvent program was as follows: Initially 2 min isocratic with 56 % Water, 27 % AcN, 17 % MeOH, 20 min linear gradient to 5 % Water, 5 % MeOH, 90 % AcN. An additional period of 10 minutes of gradient program was sufficient to return the system to the initial conditions for subsequent analysis runs.

Procedure for Analysis of Mixtures of Folpet, Procymidone and Triazophos

A calibration matrix for folpet, procymidone and triazophos using a fifteen sample set in the range 0-10 µg. mL^{-1} was performed. Volumes of 20 µL were injected into the chromatographic system and the chromatographic

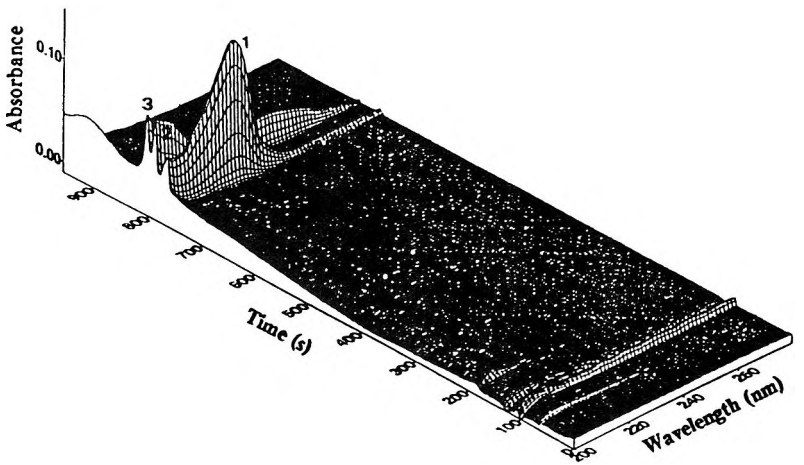

Figure 2. Three-dimensional plot of absorbance, wavelength and time for (1) folpet, (2) procymidone and (3) triazophos at concentrations of 9 μg. mL^{-1} for folpet, 4 μg. mL^{-1} for procymidone and 6 μg. mL^{-1} for triazophos.

separations were performed on a C_{18} column with the solvent programming described above. A mean-centering and smoothing pretreatments of data were applied. The optimized calibration matrices, in the chromatographic region between 11.0 and 14.5 min, calculated by application of PLS and PCR methods, were used to determine folpet, procymidone and triazophos in the prediction set.

Procedure for the Determination of Folpet, Procymidone and Triazophos in Groundwater

The 360 mg C_{18} Sep-Pak cartridges were conditioned with 5 mL of AcN followed by 5 mL of ultrapure water without allowing the cartridges to dry out. 400 mL water samples previously filtered through a 0.4 μm filter were passed at a flow rate of 8-10 mL. min^{-1} through PTFE tubes fitted with the conditioned cartridges; the cartridges were then sucked dry for 5 minutes.

The sample was eluted with 1 mL of AcN and 20 μl was injected into the system. Finally, folpet, procymidone and triazophos were determined as described above.

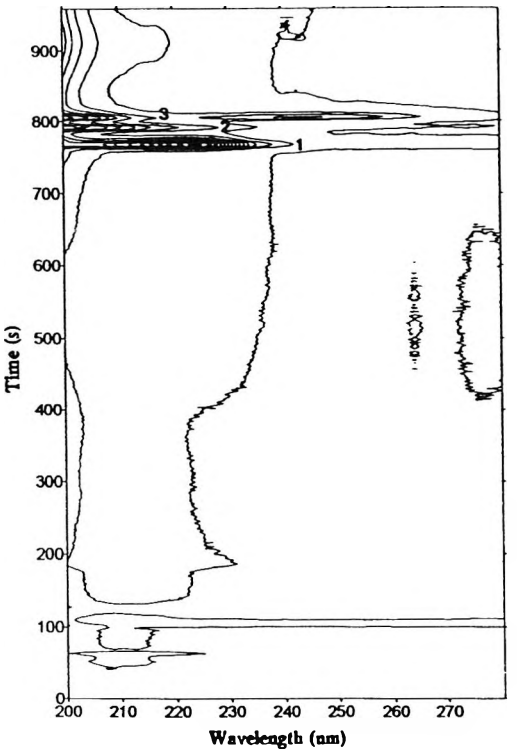

Figure 3. Contour plot of (1) folpet, (2) procymidone and (3) triazophos at concentrations of 9, 4 and 6 μg. mL^{-1}, respectively.

RESULTS AND DISCUSSION

Figure 1(a) shows a chromatogram corresponding to 21 pesticides selected for their agricultural interest. The mixture contains organochlorines, triazines, organophosphorus compounds, carbamates and ureic and imidic derivatives with greatly differing polarities. The composition of the mobile phase was optimized by an automated sequential procedure.[9,10] However, overlapping of peaks occurs if the number of analytes increases. Figure 1(b) shows a chromatogram containing a new analyte, procymidone (peak 11), and overlapping between the peaks of folpet, procymidone and triazophos can be observed. The optimum detection wavelength for a single-channel detector is 210 nm. The R_s values are 0.9 for folpet & procymidone and 0.7 for procymidone and triazophos. Table 1 summarizes retention times of each pesticide.

The DAD allows the collection of full spectral data at rates of up to several scans per second. With the data it is possible to construct three-dimensional plots of absorbance, wavelength and time. Moreover, these plots can be manipulated to allow the data to be viewed from different angles, including from the end of the chromatogram towards the beginning. Such plots depict an incomplete separation of the folpet, procymidone and triazophos when the optimized chromatographic method is used (Figure 2) and it is difficult to extract quantitative data from them. A potentially more informative way of presenting the chromatograms is to use the cartographic technique of a contour plot, a map of signal intensity in the wavelength-time domain (Figure 3). From this plot it is easier to see the incomplete resolution of folpet, procymidone and triazophos. Because of the highly overlapping peaks, conventional measures of the different analytical signals (area or height of chromatographic peaks) can not be realized. With the aim of resolving the ternary mixture, several different chemometric approaches were evaluated.

Calibration

A training set of fifteen samples (C1-C15) was taken; the concentrations are given in Table 2.

The optimum dimensionality of the PCR and PLS methods was selected as that which has the fewest number of factors such that the PRESS (prediction error sum of squares) is not significantly greater than the PRESS from the model that yields a minimum PRESS. The F statistic was used to carry out the significance determination. Empirically it was determined that an F-ratio probability of 0.75 is a good choice.[24]

For each number of factors, "f", an appropriate value of PRESS is obtained. Thus, the PRESS is defined as

$$\text{PRESS (f)} = \sum_{i=1}^{N} \sum_{j=1}^{M} (\hat{x}_i(f) - x_i)^2 \qquad (1)$$

where N is the number of samples, M is the number of analytes, x_i is the true concentration of sample i and $\hat{x}_i(f)$ is the predicted concentration of sample i using a model with f factors. The PRESS was calculated in all cases using a cross-validation method, leaving out one sample at a time, in order to model

Table 2

Concentration Data for the Calibration Set

Standard	Folpet ($\mu g.\ mL^{-1}$)	Procymidone ($\mu g.\ mL^{-1}$)	Triazophos ($\mu g.\ mL^{-1}$)
C1	3	3	5
C2	5	5	6
C3	2	2	10
C4	10	3	9
C5	5	2	6
C6	4	6	8
C7	2	4	8
C8	4	8	6
C9	3	2	10
C10	6	8	8
C11	8	8	8
C12	5	5	5
C13	0	1	1
C14	1	0	1
C15	1	1	0

the system without overfitting the concentration data;[26,27] thus the concentration of the sample left out was predicted using the N-1 model for all N samples. The prediction ability of the methods for each analyte is expressed in terms of the root mean square difference (RMSD):

$$RMSD(f) = \left(\frac{\sum_{i=1}^{N} (xi - xi(f))^2}{N} \right)^{0.5} \qquad (2)$$

and the square of the correlation coefficient (r^2), which is an indication of the quality of fit of all the data to a straight line:

$$r^2(f) = \frac{\sum_{i=1}^{N} \left(\hat{x}_i(f) - \bar{x}\right)^2}{\sum_{i=1}^{N} \left(x_i - \bar{x}\right)^2} \qquad (3)$$

where $\bar{x}$ is the mean of the true concentrations in the prediction set. Often, softwares compute PRESS (0) and RMSD (0), i. e. the PRESS or RMSD value calculated with $\hat{x}_i(0)$, which is defined as the average analyte concentration in the set of all calibration samples when the ith sample is left out. Therefore, RMSD (0) provides an indication of how well we would predict the average analyte concentration in the training set rather than instrumental measurements.

In the process of PLS-1 modelling, the covariance between the chromatographic scores and a single analyte is maximized. This often leads to the loadings of the first PLS-1 factor approximating the pure component chromatogram of the analyte under examination. The PLS-2, however, maximizes the covariance between the spectral scores and a linear combination of a number of variables. In the present study three variables are considered.

Although PCR and PLS are linear methods, in a real spectroscopic or chromatographic application there may be sources of non-linearities, e.g., chemical interactions or non-linear responses in the detector at certain wavelengths. If non-linearities are present, they may be modelled by the inclusion of extra latent variables (factors) in the regression model[25,28] and this could explain the need of the four factors to describe a three-component system. Nevertheless, some non-linearities may be corrected by external methods (transformation of the data, limiting the span of the regression model) while there are non-linearities that are not compensated. To solve this problem, different algorithms of non-linear expansions of PLS regression have been described[29-31] in addition to a method based on local modelling in PCR.[32-34]

Preprocessing

Different methods for the pretreatment of data, as mean-centering, smoothing and differentiation were applied, the aim of which was to eliminate effects of variations in instrumental conditions, background effects. These methods have been discussed in more detail elsewhere.[35] Smoothing and differentiation were done by the convolution algorithm of Savitzky and Golay.[36]

Table 3

Effect of Several Preprocessing Techniques on the Relative Prediction Errors of PLS-2 Model

Pre-processing Technique	Folpet		Procymidone		Triazophos	
	r^2	RMSD[a]	r^2	RMSD[a]	r^2	RMSD[a]
None	0.9962	0.19 (7)	0.9901	0.19 (7)	0.9865	0.17 (7)
Mean-centering (MC)	0.9966	0.13 (6)	0.9937	0.18 (6)	0.9866	0.17 (6)
MC + smoothing	0.9976	0.11 (7)	0.9937	0.18 (7)	0.9968	0.13 (7)
MC + ^{1}D	0.9614	0.44 (6)	0.9695	0.40 (6)	0.7733	0.60 (6)

[a] The number of factors is given in parentheses.

Table 4

Concentration Data for the Prediction Set

Test N°	Folpet ($\mu g.\ mL^{-1}$)	Procymidone ($\mu g.\ mL^{-1}$)	Triazophos ($\mu g.\ mL^{-1}$)
T1	4	2	7
T2	3	1	4
T3	4	1	7
T4	10	10	10
T5	8	8	8
T6	8	6	7

The effect of these preprocessing techniques on the r^2 and RMSD of the calibration matrix for PLS-2 is shown in Table 3. Mean-centering had a beneficial effect on this data set, because it reduces the PLS-2 model dimensionality and RMSD values for folpet and procymidone. Moreover, r^2 values are higher for folpet and procymidone. Also, it can be seen that the first derivative had a detrimental effect on the r^2 and RMSD of this data set.

Table 5

Recoveries of Folpet, Procymidone and Triazophos in the Prediction Set

Test N°	Recovery (%)[a]								
	Folpet			Procymidone			Triazophos		
	PLS-1	PLS-2	PCR	PLS-1	PLS-2	PCR	PLS-1	PLS-2	PCR
T1	86.5 (3.9)	86.5 (3.5)	86.5 (4.3)	88.8 (4.4)	89.9 (4.1)	85.9 (4.3)	80.5 (5.8)	81.9 (5.5)	79.6 (6.2)
T2	107.3 (4.1)	107.3 (4.1)	107.3 (4.5)	94.0 (3.9)	94.0 (3.8)	94.0 (3.9)	112.8 (4.8)	112.7 (4.6)	112.9 (4.8)
T3	98.3 (4.0)	98.5 (4.5)	98.8 (4.3)	114.0 (4.2)	113.0 (3.8)	113.0 (4.1)	100.4 (5.1)	100.4 (5.5)	100.4 (5.4)
T4	101.7 (4.2)	101.7 (4.3)	101.7 (4.0)	103.6 (3.7)	103.6 (4.0)	103.7 (3.6)	89.3 (5.2)	89.3 (5.5)	82.0 (5.6)
T5	81.0 (5.0)	82.2 (4.7)	82.5 (5.0)	104.5 (5.3)	104.5 (5.7)	104.8 (5.2)	91.8 (5.3)	92.3 (5.1)	92.0 (5.3)
T6	103.0 (5.4)	102.6 (5.1)	102.5 (5.5)	101.2 (4.7)	101.2 (4.6)	101.0 (4.7)	90.9 (5.6)	90.4 (5.1)	90.6 (5.2)

[a] The results are averages of three determinations, with RSDs in parentheses.

Contradictory results about the convenience of applying differentiation techniques prior to the use of multicomponent calibration methods can be found in the literature. Jones et al.[37] applied factor-analysis multicomponent methods to the analysis of a binary mixture, by using several luminiscence analytical signals. They found that for the determination of an analyte, the best choice is the use of the synchronous spectral data whereas for the other analyte, the use of second derivative synchronous spectra was the best choice.

MacLaurin et al.[38] and Durán-Merás et al.[39] applied several multivariate calibration methods to UV-vis spectra to resolve ternary mixtures. They did a comparative study of applying methods based on the use of absorbance and first and second derivative spectral data. Both groups found no significant differences in the predictions from the absorbance and first derivative data with PLS and PCR. The second derivative data yielded much less precise

predictions which can be attributed to the poorer signal-to-noise ratio of the second derivative signal compared with that of the direct absorbance signal. However, derivative techniques have proven to be useful in the resolution of simple binary mixtures and/or turbid background samples.[40-44]

On the other hand, the smoothing had a beneficial effect on the r^2 (>0.99 in all cases) and RMSD of this data set. The PCR and PLS-1 models were also built using the preprocessed data and, as expected, resulted in dimensionality, r^2 and RMSD values very similar to those for PLS-2, in agreement with other authors.[38,39,45]

Selection of the Region for the Analysis

We selected the chromatogram region between 660 and 870 s, which involves working with 210 experimental points (the chromatograms are digitalized every 1 s). This region was taken into account because it is the zone with the maximum analytical information from the mixture components of interest. Moreover, the shorter region is selected because by reducing the size of the regions used the amount of memory and time necessary to perform all the calibration calculations is reduced.

Calibration Design and Prediction

The proposed PLS-1, PLS-2 and PCR methods, applied to the chromatograms (using a fifteen-sample training set) with mean-centering and smoothing pretreatments, allowed the resolution of synthetic mixtures containing between 0 and 10 $\mu g.\ mL^{-1}$ each of folpet, procymidone and triazophos. In Table 4 the composition of the mixtures studied are shown while the results obtained by these strategies are summarized in Table 5. It can be observed that the results obtained by all approaches are good and they do not differ significantly among each other, being in agreement with findings by other researchers.[37-39,45]

Simultaneous Determination of Folpet, Procymidone and Triazophos in Groundwaters

The isolation of the pesticides from groundwater was tested by solid-phase extraction (SPE) with C_{18} cartridges. Samples of 400 mL of groundwater, spiked with 7.5 $\mu g.\ L^{-1}$ of folpet, procymidone and triazophos, were passed through Sep-Pak C_{18} disposable cartridges at flow rates of 8-10 $mL.\ min^{-1}$. It

Table 6

Recoveries in the Preconcentration of 7.5 μg. L^{-1} of Folpet, Procymidone and Triazophos from Groundwater

Method	Recovery (%)		
	Folpet	Procymidone	Tirazophos
PLS-1	103.7 (7.4)	96.3 (5.1)	94.7 (7.9)
PLS-2	103.7 (7.4)	98.2 (5.4)	93.4 (7.7)
PCR	98.6 (8.3)	96.3 (5.1)	91.8 (7.9)

The results are averages of three determinations, with RSDs in parentheses.

was found (Table 6) that all the compounds were removed effectively from their aqueous solutions using SPE in all instances, with recoveries ranging from 91.8 to 103.7 %. The results obtained by the PLS-1, PLS-2 and PCR methods were similar.

The proposed method was applied to the determination of pesticide levels in groundwaters of Almeria (Spain) and the chromatograms obtained showed no peaks for the studied pesticides.

CONCLUSIONS

The PLS-1, PLS-2 and PCR methods were successfully applied to the simultaneous determination of folpet, procymidone and triazophos, without a prior separation step, by HPLC. The effect of some preprocessing techniques and the r^2 and RMSD values of the calibration matrix were similar for the PLS-1, PLS-2 and PCR calibration methods. Mean-centering and smoothing (5-points) of the chromatogram to realize the calibration was found to be advantageous, whereas first derivative had a detrimental effect. The results obtained for PLS-1, PLS-2 and PCR were similar.

C_{18} cartridges have shown to be a good adsorbent for SPE of the analytes from groundwater. The method was applied to the determination of folpet, procymidone and triazophos in groundwater samples with satisfactory results.

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ERRATUM

PICOGRAM DETERMINATION OF AN AVERMECTIN ANALOG IN DOG PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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In the above paper, which was published in Journal of Liquid Chromatography and Related Technologies, **20(3)**, 443-458 (1997), an author was inadvertently omitted from the list of authors. The author list should read:

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PICOGRAM DETERMINATION OF AN AVERMECTIN ANALOG IN DOG PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION†

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ABSTRACT

A sensitive and automated method for the determination of a new avermectin analog (MK-324) in dog plasma has been developed and validated. The drug and internal standard were extracted from plasma via solid-phase extraction using an automated Gilson ASPEC XL system. The extracts were evaporated to dryness and reconstituted in a mixture of N,N-diethylmethylamine and acetonitrile. Automated pre-column derivatization of the reconstituted extracts using trifluoroacetic anhydride was performed prior to injection onto the HPLC system. The fluorescent derivatives of the drug and internal standard were chromatographed using a Zorbax RX-C18 column and a mobile phase composed of acetonitrile, water and tetrahydrofuran [6:1:1 (v/v/v)]. The assay was validated in the concentration range of 100 - 5,000 pg/mL. Its applicability, inter-day performance and specificity were demonstrated by analyzing plasma samples from six studies with MK-324.

INTRODUCTION

The avermectins are a group of closely related 16-membered macrocyclic lactones.[1] Two of the members of this family of compounds, ivermectin[2] and abamectin,[2] were introduced as an antiparasitic drug and an agricultural pesticide, respectively. Their worldwide acceptance in the health care of animals and crop protection has made them major commercial successes. MK-324, a new avermectin, is a mixture of two homologous compounds, 22,23-dihydro-13-O-[(2-methoxyethoxy)methyl]avermectin-B_{1a}-aglycone (major component, >90%) (I, Fig. 1) and -B_{1b}-aglycone (minor component, <10%) (II). These two compounds differ by a single methylene group. MK-324 is a substituted ivermectin aglycone.[3,4]

To support studies designed to determine the potency of MK-324, there was a need for the determination of the drug in plasma at pg/mL concentrations to support a number of MK-324 studies in dogs. In addition, a large number of plasma samples needed to be analyzed and automation of some of the sample preparation steps was considered highly advantageous. The use of laboratory automation accessories was previously shown to be a viable alternative to manual sample preparation.[5,6] In order to determine plasma concentrations of MK-324 at pg/mL concentrations, a highly sensitive, automated, and validated assay was required and was developed. The assay was based on the determination of the major component of MK-324 (I, Fig. 1), and involved three steps:

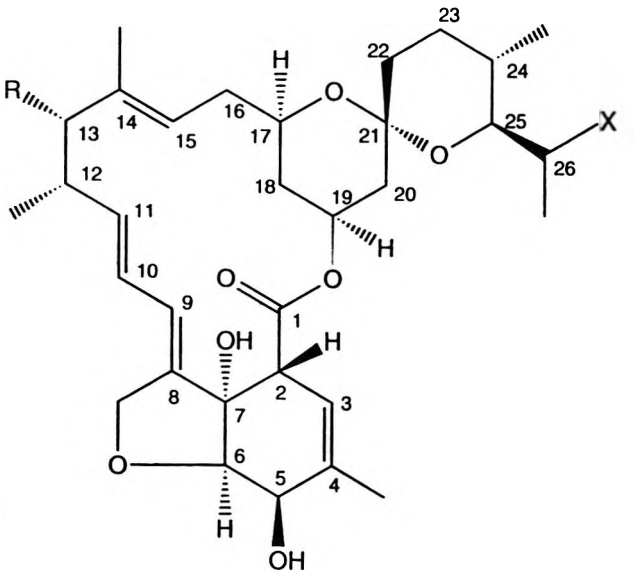
> (1) isolation of I from dog plasma; (2) derivatization to form a strongly fluorescent derivative; and (3) HPLC separation and fluorescence detection of the resulting derivative.

The detailed description of the assay methodology and its application to the analyses of dog plasma samples after dosing with MK-324 is the subject of this paper.

EXPERIMENTAL

Chemicals and Reagents

MK-324 (Fig. 1) and its analogue (IS, Fig. 1), used as an internal standard, were synthesized at the Merck Research Laboratories (Rahway, NJ, USA). Dog control plasma was available from the Department of Drug Metabolism I of Merck Research Laboratories (West Point, PA, USA).



I, R = $CH_3OCH_2CH_2OCH_2O$-
X=CH_2CH_2-
II, R=$CH_3OCH_2CH_2OCH_2O$-
X=CH_3-
IS, R = $CH_3COOCHC(CH_3)CH_2OCH_2O$-
X=CH_3CH_2-

Figure 1. Chemical Structures of MK-324 and Internal Standard IS.

Acetonitrile, chloroform, hexane, and methanol were all HPLC OPTIMA grade, and tetrahydrofuran (certified grade) were all purchased from Fisher Scientific (Springfield, NJ, USA). Trifluoroacetic anhydride (TFAA) and N,N-diethylmethylamine (DEMA, >99%) were purchased from Aldrich (Milwaukee, WI, USA). De-ionized water(18 MΩ conductivity) was obtained through a Milli-Q-filtering system (Millipore, Milford, MA, USA).

Solid-phase extraction (SPE) C_{18} cartridges (3 mL, 200 mg) originated from J. T. Baker (Phillipsburg, NJ, USA).

Instrumentation

A Gilson ASPEC™ XL (Automatic Sample Preparation with Extraction Column) system (Fairview Heights, IL, USA) was used for automated sample preparation and solid-phase extraction. A Zymark TurboVap™ evaporator

(Hopkinton, MA, USA) was utilized for evaporating the chloroform eluent to dryness at 45°C. The HPLC system included a Perkin-Elmer Series 250 pump and ISS-200 programmable autosampler with 150-µL sample loop (Norwalk, CT, USA). A Zorbax RX-C18 analytical column (25 cm x 4.6 mm, 5 µm) (Chadds Ford, PA, USA) protected with a cyano guard column (1cm x 2mm, 5 µm) and a column inlet-filter purchased from Keystone (Bellefonte, PA, USA) were utilized. A Shimadzu RF-551 fluorescence detector (Kyoto, Japan) was employed as an HPLC detector. A Maxi Mix II vortex-mixer from Thermolyne (Dubuque, IA, USA) was also utilized.

Chromatographic Conditions

The mobile phase consisted of a mixture of acetonitrile, tetrahydrofuran and water (6:1:1, v/v/v, pre-mixed), and was delivered isocratically at a flow rate of 1.2 mL/min. The fluorescence detector was set at an excitation wavelength of 365 nm and an emission wavelength of 475 nm with the sensitivity setting at "high" and the gain setting at "2."

A Perkin-Elmer-Nelson ACCESS*CHROM laboratory data system was used for data acquisition, peak integration and quantification.

Preparation of Standards and Quality Control Samples

Stock solutions of MK-324 and IS at concentrations of 0.2 mg/mL were prepared in acetonitrile, and all subsequent dilutions were made using acetonitrile. Working solutions of MK-324 were prepared at concentrations of 50, 20, 10, 5, 2, 1, and 0.25 ng/mL and the internal standard was prepared at a concentration of 20 ng/mL.

An additional stock standard, independent from those used for preparing working standards, was used to make quality-control (QC) samples. QC samples were prepared by pooling control dog plasma and spiking them with the separately prepared stock standard solution of MK-324.

The low quality control (LQC) sample was prepared by diluting the high quality control (HQC) sample with control plasma in the ratio of 1:20 (v/v). The QC samples were divided into 1.25 mL aliquots in glass vials and stored at -15°C until they were analyzed.

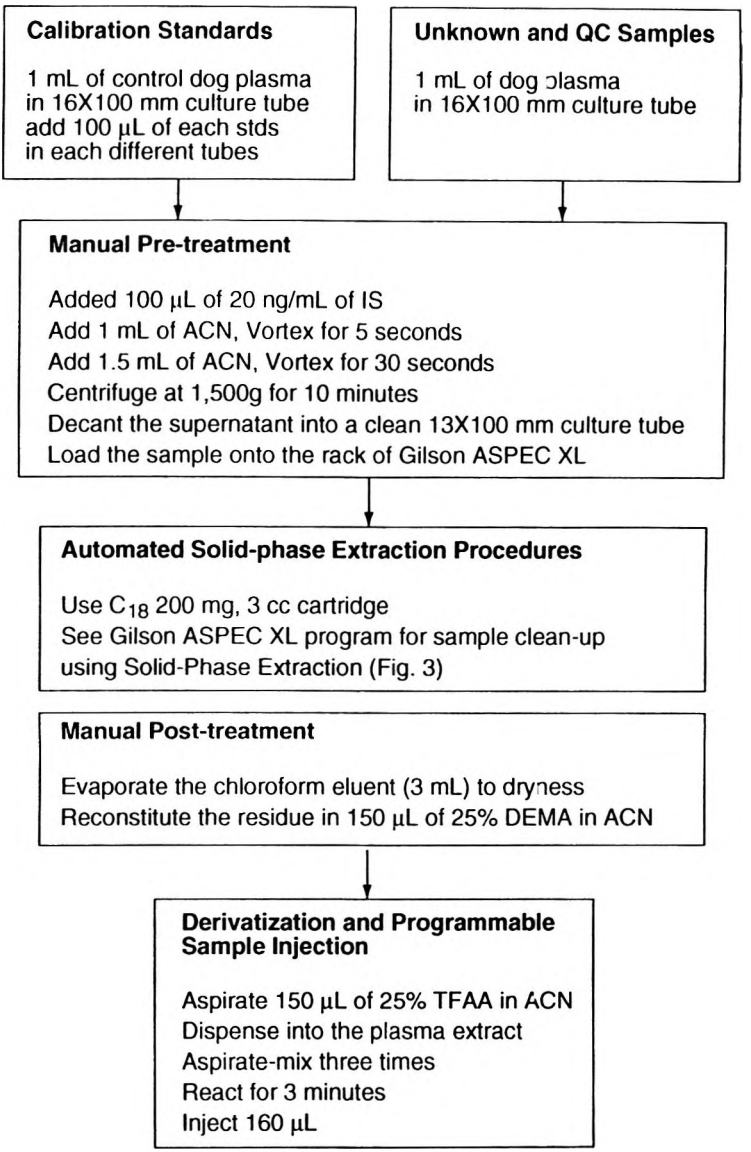


Figure 2. Flow Chart of Sample Preparation Procedure.

A six-point-calibration curve was constructed daily by spiking 1 mL of control plasma with 100 µL of the appropriate working standard and 100 µL of IS working standard solution (20 ng/mL). In addition, three sets of HQC and

LQC plasma samples were analyzed daily, immediately after the analyses of standard line samples, in the middle of the analysis and, later, after the completion of analyses of dog plasma samples.

Sample Preparation

Dog plasma samples were thawed to room temperature and vortexed vigorously for 5 seconds on a vortex-mixer. Fifty to 1,000 µL of dog plasma diluted with 950-0 µL of control make-up plasma (total final volume equal to 1 mL for each sample) and 100 µL of IS (20 ng/mL) were pipetted into a disposable culture glass tube prior to protein precipitation. After the samples were vortex-mixed and centrifuged, the supernatants were transferred to glass tubes and placed onto a Gilson ASPEC™ XL system. The flow chart of sample preparation procedures using Gilson ASPEC™ XL system is presented in Figure 2. The SPE steps which were programmed on a Gilson system are listed below:

Manually put all of the acetonitrile supernatant into culture tube

1. Begin loop
2. Condition SPE cartridge with 6 mL of methanol
3. Condition SPE cartridge with 3 mL of chloroform
4. Condition SPE cartridge with 3 mL of methanol
5. Condition SPE cartridge with 4.5 mL of water
6. Add 2 mL of water into culture tube
7. Mix by aspirate and dispense twice
8. Load 6 mL of the diluted supernatant onto SPE cartridge*
9. Wash SPE cartridge with 3 mL of water
10. Move arm from SPE cartridge to drain position
11. Switch LP valve (valve on)+
12. Wait 0.5 min
13. Switch LP valve (valve off)
14. Wait 0.1 min
15. Switch LP valve (valve on)
16. Move arm to SPE cartridge, stay on for 3 min
17. Switch LP valve (valve off)
18. Wash SPE cartridge with 5.0 mL of hexane
19. Elute sample with 3.0 mL of chloroform
20. End loop

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* draw 0.5 mL of excess volume to build up air gap in needle
+ LP = low pressure

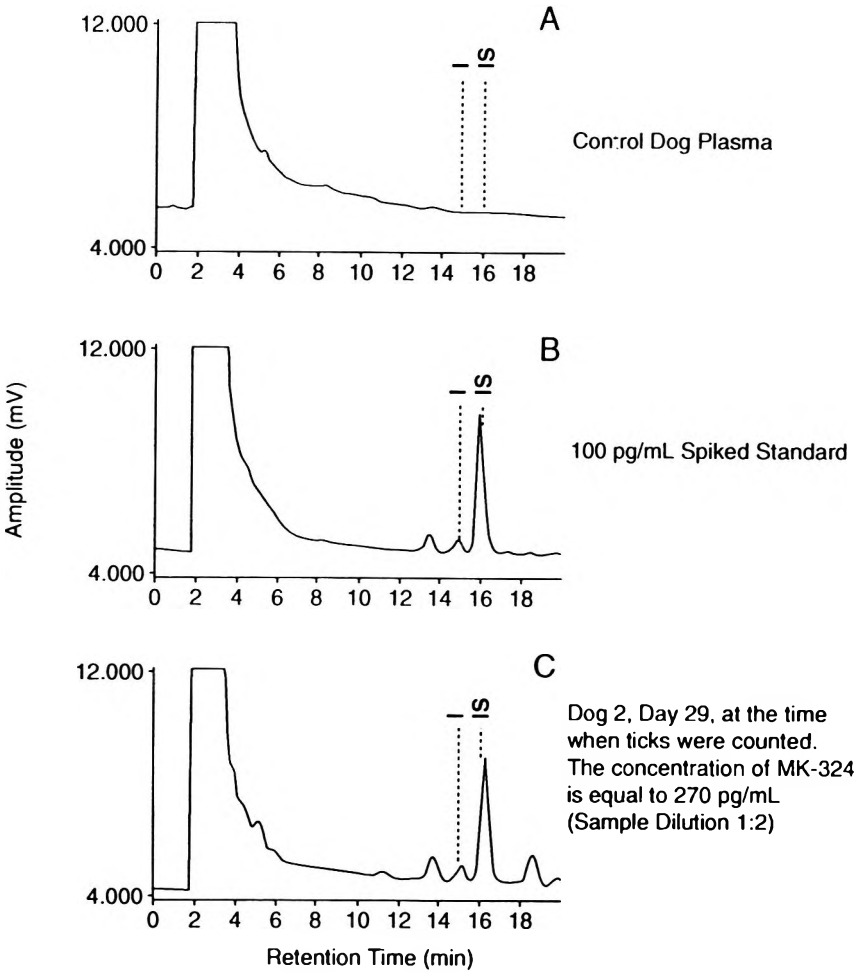

Figure 3. Representative chromatograms of MK-324 and internal standard IS in dog plasma (1 mL). (A) Control plasma, (B) Control plasma (1 mL) spiked with 100 pg of MK-324 and 2 ng of IS, (C) Dog 2, Day 29, the concentration of MK-324 was equal to 270 pg/mL. (sample dilution was 1:2).

After the automated SPE sample clean-up steps were completed, the eluents from SPE cartridges were evaporated to dryness, dissolved manually in 150 µL of 25% DEMA in acetonitrile and derivatized with 150 µL of 25% TFAA in acetonitrile using automated pre-column derivatization capability of the autosampler. After derivatization, 160 µL of the reaction mixture was injected directly onto the HPLC system.

Precision, Linearity, Accuracy, Sensitivity, and Specificity

The precision of the method was determined by replicate analyses (n=5) of dog plasma containing MK-324 at all concentrations utilized for constructing the calibration curves. The linearity of each standard curve was confirmed by plotting the peak-height ratio of I to IS versus concentration of MK-324.

The unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by weighted (1/y) linear regression of the standard line. The standard curve was prepared and assayed daily with quality control and unknown samples.

The accuracy of the method was established by preparing quality control samples in control dog plasma at low and high concentrations on the standard line. These samples were frozen, stored with post-dose dog plasma samples and assayed daily with dog plasma samples. Samples with concentrations exceeding the linear calibration range were diluted with control plasma and re-assayed. The accuracy of the method was expressed by:

(mean observed concentration)/(spiked concentration) x 100%.

The limit of quantification (LOQ) was defined as the lowest concentration on the standard line for which acceptable accuracy (±10 % of the nominal values) and precision (expressed as the coefficient of variation, C.V. ≤ 10 %) were obtained. Assay specificity was assessed by running blank control and pre-dose plasma samples from different dogs.

Peak heights of I and IS were measured and automatically processed using a PE Nelson ACCESS*CHROM laboratory data system. Concentrations of MK-324 in subject plasma samples were calculated from the daily constructed standard curves.

RESULTS

Assay Sensitivity and Precision

Typical chromatograms of control dog plasma, dog plasma spiked with MK-324 and IS (100 pg/mL and 2 ng/mL, respectively) and dog #2 plasma sample are shown in Figure 3. The total analysis time was 20 minutes. The specificity of the assay was demonstrated by the lack of endogenous

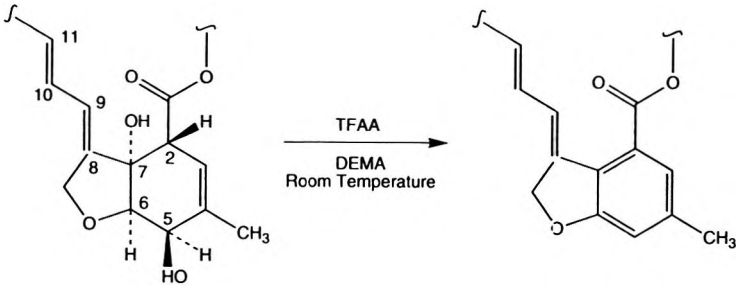

Figure 4. Chemical Derivatization Reaction of MK-324 in the Presence of TFAA.

Table 1

Intra-Day Accuracy and Precision Data for Determination of MK-324 in Dog Plasma

Nominal Conc. (pg/mL)	Conc'n of MK-324 (pg/mL) 1	2	3	4	5	Mean	Accuracy[a]	Precision[b]
100	100.5	91.1	103.6	91.0	96.8	96.6	96.6	5.8
200	197.7	214.8	194.0	192.7	208.7	201.6	100.8	4.8
500	487.9	493.6	483.6	510.1	497.2	494.5	98.9	2.1
1000	1031.8	1011.4	1021.1	982.5	995.3	1008.4	100.8	2.0
2000	2010.7	2030.2	2012.3	1942.6	2013.5	2001.9	100.1	1.7
5000	4972.9	4902.6	4983.5	4821.3	4984.1	4932.9	98.7	1.4

[a] Expressed as [(mean calculated conc.)/(nominal conc.) X 100.
[b] Expressed as coefficient of variation (C.V. %).

interferences observed at the retention times of these compounds in any of the control and pre-dose plasma samples from dogs participating in trials. The assay has been validated in the concentration range of 100 to 5,000 pg/mL in plasma. The typical equation describing the standard line was Y = 0.000852 X - 0.0187 and the average correlation coefficient (r) was 0.9997. The limit of quantification (LOQ) of MK-324 was 100 pg/mL.

The precision of the assay was assessed by calculating the intra-day C.V.'s for calibration standards and QC samples. The precision (Tables 1 & 2), at all concentrations used for constructing the calibr'n curve. was < 10%.

Table 2

Intra-Day Reproducibility of Quality Control Samples during Analysis of MK-324 in Dog Plasma

Replicate	LQC[a] 125 pg/mL	HQC[b] 2500 pg/mL
1	121.4	2543.3
2	123.4	2505.2
3	113.8	2564.7
4	117.3	2570.8
5	123.9	2440.4
Mean	120.0	2524.9
S.D.[c]	4.3	53.8
C.V. %[d]	3.6	2.1

[a] LQC = low quality-control standard.
[b] HQC = high quality-control standard.
[c] Standard Deviation.
[d] Coefficient of Variation (C.V. %).

The inter-day accuracy and precision data are presented in Table 3. The accuracy data for QC samples are also summarized in Tables 2 and 3. The data in Table 3 also indicate that MK-324 was stable in plasma during storage at -20°C for at least one year.

Application

The applicability of the method was demonstrated by analyzing dog plasma samples originating from six different studies with MK-324. As an example, the plasma concentration *vs.* time data after topical and oral administrations of MK-324 in one of these studies are presented in Table 4.

DISCUSSION

The method for the determination of MK-324 in dog plasma included three steps: (1) automated solid-phase extraction (SPE) of MK-324 from dog plasma using a Gilson ASPEC system; (2) automated pre-column derivatization to

Table 3

Inter-Day Precision and Accuracy of Determination of MK-324 in Plasma

Nominal Conc. (pg/mL)	Conc'n Found (pg/mL)		Precision[a] (%)	Accuracy[b] (%)
	Mean[c]	S.D.[c,d]		
Standard				
100	95.8	2.5	2.6	95.8
200	199.8	7.3	3.7	99.9
500	500.5	17.2	3.4	100.1
1000	1049.2	43.2	4.1	104.9
2000	2008.0	36.2	1.8	100.4
5000	4945.7	47.5	1.0	98.9
Quality Control[e]				
125	124.2	3.52	2.84	99.3
2500	2530.1	94.1	3.72	101.2

[a] Coefficient of Variation (n=8).
[b] Expressed as [(Mean calculated conc.)/(nominal conc.) X 100.
[c] n=8.
[d] Standard Deviation.
[e] Three sets of quality control samples at each concentration were analyzed daily with the study samples on thirteen different days and over a period of six months.

form a strongly fluorescent derivative using a Perkin-Elmer programmable autosampler; and (3) HPLC separation and fluorescence detection of the derivatives without further samples clean-up after derivatization. Extensive studies were performed toward the design of extraction conditions of MK-324 from plasma, optimization of the derivatization reaction and HPLC separation of derivatized 1 and IS from endogenous plasma impurities. The results of these studies are described below.

Extraction from Plasma

Several different approaches for isolating of MK-324 from plasma were studied. Direct liquid-liquid extraction at different pH's using various organic solvents gave low and variable recoveries and produced extracts containing

Table 4

Plasma Concentrations (ng/mL) of MK-324 in Dogs Following Different Treatments with MK-324

Pen #	Predose	2 Hr	4 Hr	8 Hr	24 Hr	Day 5	Day 15	Day 29
				Group 1 - Topical				
2	ND[a]	4.88	2.84	1.09	5.43	2.62	0.74	0.27
10	ND	2.32	3.20	1.30	3.53	4.01	2.14	0.44
18	ND	3.53	1.76	0.75	6.19	3.03	1.04	0.21
24	ND	0.37	0.78	2.59	3.99	4.47	1.00	0.40
30	ND	NQ[b]	0.19	0.51	1.11	0.85	0.46	0.14
				Group 2 - Oral				
6	ND	236.60	862.02	NS[c]	15.53	2.29	0.13	NQ
14	ND	373.57	129.53	46.64	16.05	2.87	0.20	NQ
16	ND	196.50	73.47	31.77	15.89	1.82	NQ	NQ
20	ND	299.31	96.07	35.38	12.82	2.77	0.31	NQ
26	ND	414.26	139.78	34.40	12.26	2.73	0.22	NQ

[a] Not detected.
[b] Below limit of quantification.
[c] No sample.

several major interferences. Another approach was based on precipitation of plasma proteins with acetonitrile, followed by the removal and evaporation of the supernatant to dryness and derivatization of the residue. Although the derivatization reaction was effective, variation in the degree of dryness, presence of insoluble substances, and irreproducibility of derivatization prevented this direct method from being rugged and reliable enough for routine application. Therefore, isolation of MK-324 from plasma was attempted using solid-phase extraction. When applied from water or plasma, MK-324 was retained to varying degrees on a variety of SPE cartridges including Si, C-8, and C-18, but the recovery was irreproducible and was dependent on loading speeds and types of cartridges employed. To improve recovery, plasma samples were pre-treated with acetonitrile to denature plasma proteins prior to extraction using SPE. The careful control of sample loading speed and flow-rate of solvents during cartridge elution were found to be critical in achieving high recovery and reproducibility. Since the SPE steps were not only difficult to control manually but were also time consuming, repetitive, and tedious, it was decided to automate the SPE steps prior to HPLC analysis. After precipitation of proteins from a series of plasma samples with acetonitrile and

centrifugation, the supernatants were placed on the ASPEC™ XL system which was programmed (Fig. 2) to add water, load the mixed solution onto a C-18 SPE column and wash the column with water followed by hexane. The drug and IS were eluted with chloroform and, after evaporation of the eluent to dryness off line, the residue was derivatized with DEMA/TFAA in acetonitrile. The use of an automated system for sample clean-up provided a method that was both robust and efficient.

Chemical Derivatization

Due to the presence of the conjugated-diene chromophore in the ivermectin analogs, the ultraviolet (UV) absorption spectra of these molecules exhibit a strong absorption band with the maximum at around 245 nm and a molar absorption coefficient (ε) of approximately 30,000 M^{-1} cm^{-1}. The presence of this absorption band has been used as the basis for the development of several methods based on HPLC with UV detection.[7-11] However, these methods gave the limits of detection (LOD) of 1-2 ng/mL which was far above the LOQ required to support our studies with MK-324.

In order to increase assay sensitivity and specificity, the molecule of I was converted to a highly fluorescent analog using an acid anhydride. This dehydration reaction (Fig. 4), previously described and documented for a number of other avermectin analogs,[12-14] was extensively studied here to establish the best reaction conditions for derivatization of I at pg/mL concentrations of the analyte and compatible with automated pre-column derivatization. Use of acetic anhydride for derivatization[12] required high temperature (100°C) and an extended (one hour) reaction time for the reaction to be completed. Instead of acetic anhydride, the derivatization of I was performed in the presence of TFAA as described earlier for ivermectin.[15] The relative yield and the stability of the derivatized products was also investigated. The effect of presence of various tertiary amines as nucleophilic catalysts for acylation[13,16,17] on the yield and kinetics of reaction of I was evaluated.

Both DEMA and dimethylethylamine were found to improve the yield of the derivatized products. However, interferences in the blank prohibited the use of the latter base for the derivatization. By using DEMA as a base, the reaction of I and IS with TFAA produced stable products within a relatively short period of time (three minutes) which was desired for automated pre-column derivatization. The derivatives formed emitted fluorescence at 475 nm when excited at 365 nm.

Automated Pre-column Derivatization and Chromatography

Automation of the derivatization step was necessary to eliminate the need for careful control of the reaction kinetics and to eliminate the problem associated with the potential chemical instability of the reaction products with high concentration of TFAA and DEMA. Instead of a typical batch pretreatment of many samples and subsequent analysis of these samples over an extended period of time (for example for 20 hours between the first and last sample injection), each sample was derivatized at precisely the same time prior to injection. This was accomplished utilizing the *Derivatization and Automix* capability of the autosampler. After SPE extraction, samples were reconstituted manually in acetonitrile (150 μL) containing 25% of DEMA, and placed in autosampler vials. I and IS were stable in these solutions for up to five days. The autosampler was then programmed to add 25% of TFAA in acetonitrile (150 μL) and, after three minutes, the mixture after derivatization was injected directly onto the HPLC system. While the chromatographic run (20 minutes) was performed on the derivatized sample, the next sample was derivatized in exactly the same manner as the previous one and injected. The derivatives of I and IS were separated from each other and any interfering peaks including the homolog II and IS using a Zorbax RX-C18 column protected with a cyano guard column, and a mobile phase composed of acetonitrile, water and tetrahydrofuran. The cyano guard column acted as a pre-column concentrator, leading to peak compression and a decrease in the baseline noise. The assay was specific, with the LOQ of 100 pg/mL.

In conclusion, a highly sensitive, automated HPLC method for the determination of MK-324 in dog plasma, utilizing a Gilson ASPEC™ XL system for sample preparation and a Perkin-Elmer programmable autosampler for automated pre-column derivatization, has been developed. This method has been validated in the concentration range of 100-5,000 pg/mL. The linearity of the method in the extended concentration range of 25-5,000 pg/mL, ($r=0.9997$) has also been demonstrated. This method is rugged, reliable and was used routinely for the analyses of more than 1,000 dog plasma samples from six different studies with MK-324.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ANTI-TUBERCULOSIS DRUGS IN HUMAN BODY FLUIDS

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ABSTRACT

A rapid, simple and sensitive high performance liquid chromatographic (HPLC) assay for the quantification of pyrazinamidum (PZA), rimifon (INH) and rifapentine (RFT) in human serum, urine and cerebrospinal fluid (CSF) has been developed. After acidification and removal of protein, liquid samples were injected into the HPLC system directly. Separation was achieved using a μBondapak phenyl reversed phase column for RFT and ion-pair chromatography on Nova-pak C_{18} for INH and PZA. The retention times of PZA and INH on the -C_{18} column were 2.66 and 5.33 min, respectively. Retention time of RFT was 9.10 min. on the -phenyl column. The PZA, INH and RFT standard plots were highly linear ($r>0.99$) over the concentration range of 0.1 to 10 μg/mL, 0.1 to 10 μg/mL and 0.5 to 10 μg/mL, respectively. PZA mean recovery was 96.5%±4.2, INH 97.4%±3.7 and RFT 92.1%±6.2. The method was applied for the control of the drug doses on treating tuberculous meningitis and for investigating whether RFT passes through human hematoencephalic barrier.

INTRODUCTION

Pyrazinamidum (PZA), Rimifon (INH), Rifampicin (RFP) and p-aminosalycilic acid (PAS), etc. have been used as anti-tuberculosis drugs for many years.[1,2] PAS has been regarded as a necessary drug for tuberculous meningitis treatment. Although other drugs such as RFP, PZA, EMB, etc. have been recently used and the PAS intravenous (i.v.) treatment in long-term is very troublesome, expensive and has side-effects, the PAS has not been abolished. But PAS is only a bacterium inhibitor and not a sterilization drug. Therefore, PAS should may be theoretically abolished. Rifapentine (RFT) is a new antibiotic. It has been used to treat pulmonary tuberculosis, etc., and has many merits such as long-term treating activity, high anti-bacterium activity, etc. In our work, RFT was used as the main treatment drug, with PZA and INH as auxiliary drugs to treat tuberculous meningitis. Although PZA was abandoned, the treatment was very effective.

To understand the actions of the drugs, the concentrations of the drugs in serum, urine and cerebrospinal fluid (CSF) must be investigated. In view of their wide clinical use, different analytical methods have been developed for the quantification of the drugs in blood, plasma, urine and other body fluids. These methods include spectrophotometry,[3,4] high performance liquid chromatography (HPLC),[5,6] gas chromatography,[7] etc. But analysis of RFT in CSF has not been reported.

In this report, we describe the analysis of INH, PZA and RFT in serum, urine and CSF by HPLC. By monitoring the drug levels, doses can be controlled. Also investigated was whether RFT passes through the hematoencephalic barrier.

EXPERIMENTAL

Chemicals

All the reagents used were of analytical grade; organic solvents were of high purity grade for HPLC; water was Milli-Q (Millipore-Waters) deionized. Standard reagents of anti-tuberculosis drugs were purchased from Merck and the Institute of Medicine Identification, Chinese Academy of Preventive Medicine. The paired ion chromatographic (PIC) reagent used was 1-heptane sulfonic acid (PIC-B7, Millipore-Waters); its UV cutoff wavelength was 200 nm.

Apparatus

The Waters Liquid Chromatography System (Waters Associates. Milford. MA. U.S.A.) used consists of a solvent deliver pump (Model 590). a manual injector (Model U6K). and a UV-VIS programmable detector (Model 490) operated at 230 nm. and a Baseline 810 chromatographic station.

Chromatographic Conditions

The column used for the RFT analysis was μBondapak phenyl packing (300mm x 3.9mm I.D.. 5μm particle size) purchased from Waters Division (U.S.A). The eluent was methanol:water (35:60) at a flow rate of 1.0 mL/min.

The column used for the INH and PZA analysis was Nova-pak C_{18} packing (150mm x 3.9mm I.D.. 5μm particle size) (Waters). The eluent was 5mM PIC-B7 aqueous solution:methanol (90:10) at a flow rate of 1.0 mL/min.

Standards Preparation

Stock aqueous solutions (1.0mg/mL) of INH and PZA were prepared. respectively. Five mixed standards of INH and PZA were prepared by pipetting 10. 20. 40. 80. 100 μL of INH and PZA stock solutions into separated 10mL volumetric flasks and diluting to volume with water. The concentrations of standard series were: INH and PZA 1. 2. 4. 8. 10 ppm. Stock solution (1.0mg/mL) of RFT was prepared with methanol. Five working standards of the drug were prepared by pipetting 10. 20. 40. 80. 100 μL of RFT stock solution into separated 10 mL volumetric flasks and diluting to volume with water. The concentrations of standard series was 1. 2. 4. 8. 10 ppm.

Sample Treatment

A dose of INH was administered to tuberculous meningitis patient via intravenous injection and PZA. RFT orally in a gelatin capsule. Serum. urine and CSF samples were collected at the interval of 10 hrs after administration. An aliquot of 1 mL serum was mixed with 1 mL 10% H_3PO_4 for 15min. followed by extracting in ultrasonator for about 15mir.. 1 mL extract was then mixed with 1 mL methanol and centrifuged at 3000 G for about 15 min. The supernatant was filtered through a 0.45 μm filter. The filtrate was injected into the HPLC system.

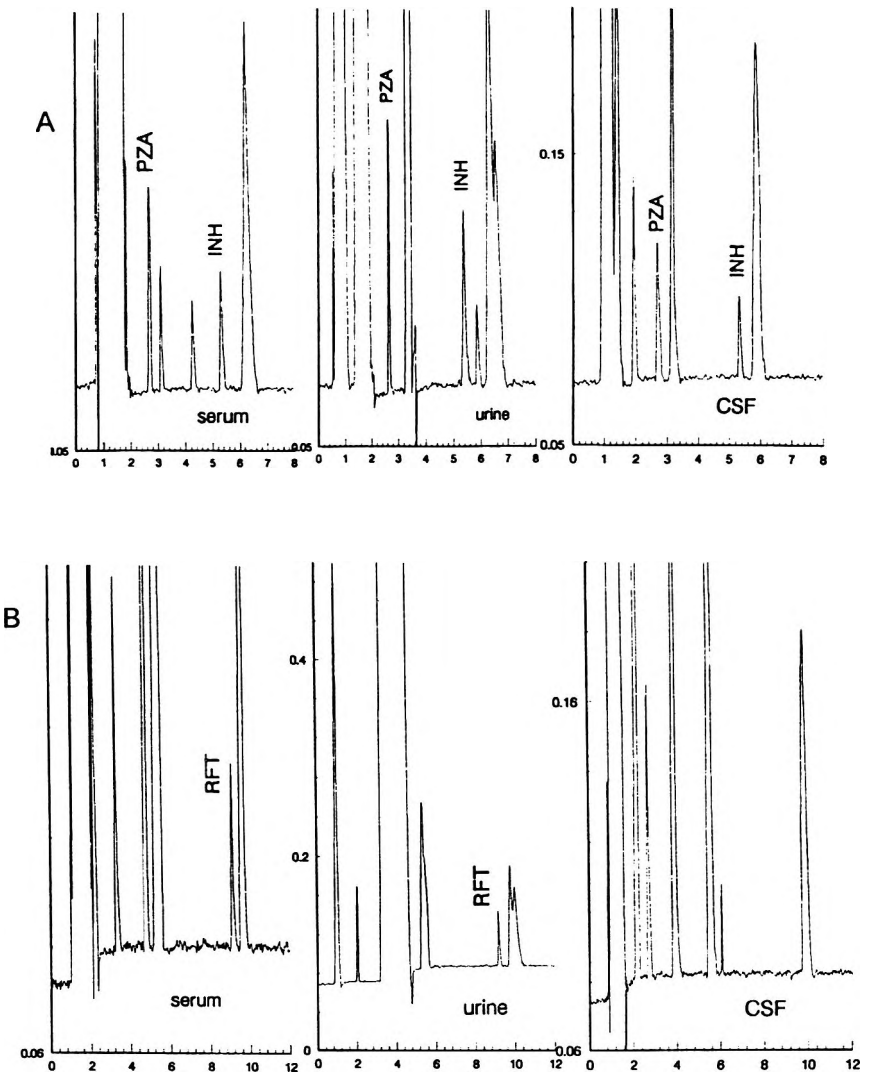

Figure 1. Chromatograms of sample analyses. (A) Serum, urine and CSF for PZA and INH analysis; (B) Serum, urine and CSF for RFT analysis.

An aliquot of 0.2 mL CSF was mixed with 0.2 mL 10% H_3PO_4 for 15min., followed by extracting in an ultrasonator, then filtered through 0.45 μm filter. The filtrate was injected into the HPLC.

An aliquot of 1 mL urine was mixed with 1 mL 10% H_3PO_4 for 15min. followed by extracting in an ultrasonator for about 0.5 hr. The extract was then filtered through a 0.45 μm filter. The filtrate was injected into the HPLC.

RESULTS AND DISCUSSION

Chromatographic Specificity and Sensitivity

Typical chromatograms of (a) INH and PZA analysis of serum, urine and CSF (b) RFT analysis of serum, urine and CSF after administration are shown in Figure 1. The specificity of the method was demonstrated by the lack of interference at the retention times of INH (5.33 min.) and PZA (2.66 min.) on the -C_{18} column and RFT (9.10 min.) on the -phenyl column. The sensitivity of the assay, defined as the minimum concentration that can be quantitated with a statistically acceptable coefficient of variation (10%) in the peak area was 40 ng/mL for INH (CV=7.7%), 45 ng/mL for PZA (CV=9.3%) and 85 ng/mL for RFT (CV=9.6%) (see Table 1). The minimum detectable amounts, defined as the amount, in nanograms, that gives a peak height of the drugs equal to twice the background noise at the most sensitive instrument setting used in the study (0.01 AUFS, time constant: 2.5, injection volume: 50 μL) were 4.0 ng, 4.5 ng and 8.5 ng for INH, PZA and RFT, respectively.

Selection of the Separation Conditions

A mobile phase consisting of water:methanol (35:60) on the reverse phase phenyl column gave the optimum resolution of RFT and other interference components in the samples. The ratio of water with methanol in the mobile phase drastically affected the retention times of the drug: the retention time decreased with increasing percentage of methanol. But, under this condition, INH and PZA could not be separated, and the separation could not be improved by changing the percentage of MeOH (see Figure 2). However, INH and PZA could be separated completely by ion-pair chromatography with PIC-B6 on the Nova-pak C_{18} column, and other components in samples did not interfere with the drugs. But RFT could not be analyzed under this condition. When the ratio of methanol with water was less than 10:90, elution of RFT was very difficult (retention time >18min.), but when the ratio was increased to 15:85, INH and RFT could not be separated. And when the ratio of methanol with water was maintained within 10:90 - 15:85, other components in the samples interfered with the drug.

Table 1

Sensitivity of the Assay (μV•sec)

Repetition	Response of INH (40 ng/mL)	Response of PZA (45 ng/mL)	Response of RFT (85 ng/mL)
1	7432	6821	9627
2	7931	6014	10550
3	8724	5432	10897
4	7855	5243	12843
5	7128	5127	10021
6	8127	6354	9427
7	7043	6742	11456
8	7444	5934	10729
9	8247	5733	9742
10	8931	6217	11737
Mean:	7886	5962	10703
Response (C.V. %):	7.7	9.3	9.6

Additivity standard in blank serum (Injection 50 μL).

Sample Treatment and Recovery of the Drugs

In general, the drug analyses have been completed using a multi-step extraction procedure with different solvents (ethyl acetate, acetonitrile, n-heptane) followed by HPLC.[8,9] The procedures were complex. The drugs exist in body in combined states with other bio-components. Determination of drugs originalating in the body must employ acidification decomposition to free the drugs from combined state. In this paper, 10% H_3PO_4 was used to free the drugs. To investigate effectiveness of treatment, different concentrations of H_3PO_4 and treatment times were tested. The results are shown in Table 2. The results tended to be too low when the hydrolytic decomposition time was less than 10 min.

Absolute recoveries of the three drugs from serum, urine and CSF were determined with samples of known amounts standard addition. Mean recoveries (n=5) of INH, PZA and RFT were, in all cases, not less than 93%, 92% and 85%, respectively.

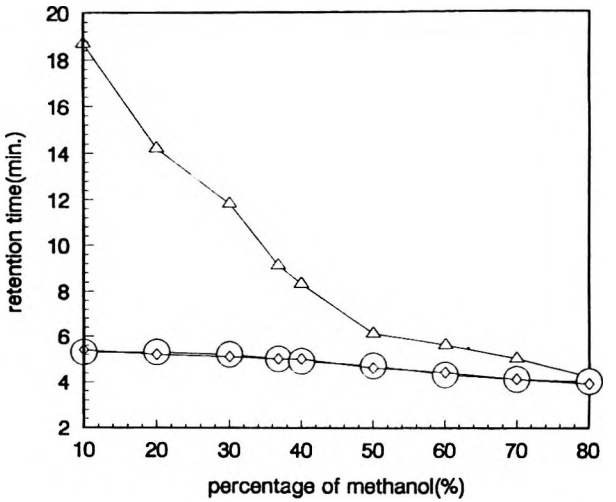

Figure 2. Relationship of drugs' retention time with percentage of mobile phase on phenyl column. —Δ— RFT; ◊ — INH; —○— PZA.

Table 2

Effectiveness of Different Treating Time and H_3PO_4 Conc. (Serum)

		INH (ppm)	PZA (ppm)	RFT (ppm)
Time (min) (with 10% H_3PO_4)	2	4.3	3.1	4.6
	5	5.7	5.2	4.8
	10	6.4	5.4	5.3
	20	6.4	5.4	5.4
	30	6.3	5.3	5.3
	45	6.5	5.4	5.3
H_3PO_4 Conc. (%) (with 30 min)	1	5.1	4.7	5.0
	2	5.3	5.0	5.3
	5	6.2	5.1	5.2
	10	6.4	5.4	5.3
	20	6.3	5.3	5.4
	30	6.4	5.4	5.3

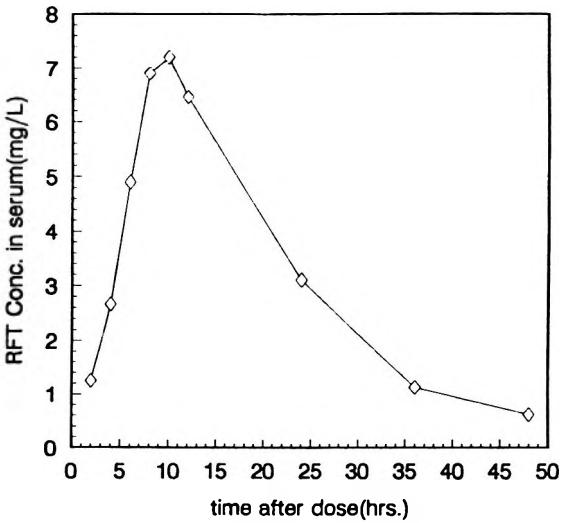

Figure 3. Serum time-concentration profile of RFT to a patient via oral route.

Table 3

Assay Reproducibility (Serum)

Repetition	INH (ppm)	PZA (ppm)	RFT (ppm)
1	13.2	7.4	6.1
2	13.0	7.2	6.4
3	13.2	7.4	6.1
4	13.1	7.3	6.2
5	13.3	7.2	6.6
6	13.6	7.6	6.3
7	13.7	7.1	6.0
8	13.2	7.3	6.3
9	13.4	7.5	6.0
10	13.5	7.6	6.5
Mean	13.32	7.36	6.25
C.V. (%)	1.60	2.20	3.14

Table 4

Sample Analysis Results

Patient No.		INH (ppm)	PZA (ppm)	RFT (ppm)
1	Serum	7.8	8.4	6.5
	Urine	11.3	9.2	7.6
	CSF	5.6	4.1	<0.15
2	Serum	11.2	7.5	8.6
	Urine	12.4	7.8	9.2
	CSF	7.3	3.2	<0.15
3	Serum	9.7	5.6	5.4
	Urine	13.1	7.2	5.2
	CSF	5.2	4.3	<0.15
4	Serum	7.4	8.6	7.2
	Urine	7.7	8.9	9.3
	CSF	4.3	5.3	<0.15
5	Serum	12.8	6.8	8.2
	Urine	14.5	8.3	9.7
	CSF	7.3	4.1	<0.15

Linearity of the Calibration Plots

Least-squares analysis of the calibration curves gave excellent linear responses within the tested concentration range of INH (1-10 ppm), PZA (1-10 ppm) and RFT (1-10 ppm). The correlation coefficients were greater than 0.99.

Reproducibility of the Assay

The intra-day reproducibility of the assay was evaluated by comparing the analysis of the same samples in the same day (Table 3). The Coefficients of variation (CV%) of less than 3.5. indicate excellent intra-day reproducibility.

Analysis Results of Samples and Investigation of RFT Passage through the Human Blood-Brain Barrier

Table 4 shows the analysis results of the three drugs in difference samples. From the results. RFT concentration in CSF is low when the concentration is high in serum. Figure 3 shows the serum concentration-time profile of RFT of a patient via oral route. The peak concentration in serum is at about 7-10 hr. after administration. But, at the time of peak concentration in serum, the concentration in CSF is still less than 0.5 ppm. This indicates that it is difficult for RFT to pass through human blood-brain barrier.

CONCLUSIONS

The HPLC method described herein has sufficient sensitivity to determine INH, PZA and RFT in human serum, urine and CSF following oral and iv bolus dose in the usual therapeutic range. The method is simple, rapid, accurate, and reproducible. Because the drugs have side-effects for humans, this method can be applied to control doses of the drugs to maintain the highest anti-bacterium activity and the lowest concentrations levels of the drugs.

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HPLC ANALYSIS AND VALIDATION OF 5-FLUOROURACIL AND ITS METABOLITES IN RAT PLASMA

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ABSTRACT

An isocratic, reverse phase HPLC method, for the simultaneous determination of 5-fluorouracil (5-Fu), 5-fluoro-5,6-dihydrouracil (FuDH), and 5-fluorouridine (Furd), in rat plasma has been developed. The method utilizes a Spherisorb® column and a mobile phase composition of 100 μM phosphate buffer at pH 3.0. The extraction procedure from the plasma samples has been optimized to give maximum recovery of the analytes. The method has been validated as per USP guidelines in terms of linearity, accuracy and precision. The limit of quantitation for 5-Fu, FuDH, and Furd was found to be 0.2 μg/mL, 0.3 μg/mL and 0.8 μg/mL respectively.

INTRODUCTION

5-Fluorouracil (5-Fu) is an antimetabolite used in the treatment of carcinomas of the gastrointestinal tract and breast.[1,2] The biochemical importance of 5-Fu and its nucleosides has been demonstrated by Heidelberger.[3] Analysis of 5-Fu and its metabolites in biological fluid is, therefore, an imperative parameter not only in pharmacokinetic and pharmacodynamic studies but also during the development of conventional as well as targeted dosage forms of the drug. Reverse phase,[4,5] reverse phase ion-pair[6-8] and normal phase[9] HPLC systems were described for the analysis of 5-Fu. However, few workers have reported the simultaneous analysis of 5-Fu and its metabolites.

The present investigation deals with the development of an HPLC assay procedure for the simultaneous analysis of 5-Fu and its metabolites, 5-fluoro-5,6-dihydrouracil (FuDH) and 5-fluorouridine (Furd), in rat plasma. Optimization of any extraction procedure, in an analytical method that involves quantitation of a number of analytes, is an important step. As 5-Fu and its metabolites were required to be extracted simultaneously, an optimized procedure that would give maximum recovery of the compounds of interest became an obvious necessity in the present study. In order to achieve maximum recovery of the analytes, an ideal extraction procedure was developed by investigating the choice of a suitable extractant system, pH of the redissolving buffer solution and nature of the pH adjusting system required to minimize the co-extraction of plasma matrix components. Precision, accuracy and linearity were the validation parameters addressed for the proposed method and were in accordance with the requirements of Current Good Manufacturing Practice (cGMP) regulations [21 CFR 211.1949a)].[10] Both, Category I and II assays, defined by USP XXII, have been adhered to for compendial compatibility.

MATERIALS AND METHODS

Chemicals and Reagents

5-Fluorouracil (5-Fu), 5-fluoro-5,6-dihydrouracil (FuDH), 5-fluorouridine (Furd) and 5-fluorocytosine (5-Fc) were purchased from Sigma (St. Louis, MO, USA). Ammonium dihydrogen phosphate, HPLC grade water, n-propanol and ether were obtained from Loba Chemie (Bombay, India). All chemicals and reagents were either analytical or spectroanalytical grade.

Apparatus and Chromatographic Conditions

The chromatographic set-up used was a Perkin Elmer Series 410 quaternary solvent delivery system, Rheodyne Model 7125 injector and a Perkin Elmer Model LC-135 diode array UV-Vis detector. Data was recorded and processed on a Perkin Elmer Omega Data System.

Separation was performed on a Spherisorb® ODS 5 μ column (Phase Separation Inc., Norwak, CT, USA, 250 mm x 4.6 mm) using a 100 μM phosphate buffer, pH 3.0, as the mobile phase. The flow rate was kept constant at 1 mL/min and the volume of injection was 20 μL. The wavelength of spectrophotometric detection was 210 nm.

Experimental Animals and Intravenous Administration

Male Wistar rats (200 - 250 g), that were fed a balanced diet and, *ad libitum*, were used during the study. Each experimental station was done in quintuplicate. Rats were dosed with 15 mg per kg body weight through the right femoral vein. Blood samples were withdrawn at regular intervals of time and the concentration of 5-Fu and its metabolites recorded.

To prepare a standard working curve with spiked amounts of 5-Fu and its metabolites, the plasma of untreated rats was used. Plasma was collected in tubes containing sodium heparin, centrifuged at 3000 x g for 15 min and frozen at -20°C until further analysis.

Sample Extraction

Studies were conducted to optimize the extraction of 5-Fu and its metabolites from spiked samples of plasma. Co-solvent systems, as extractant, have been reported e.g. ethyl acetate:methanol,[11] aceton:trile:water[12] and light n-propanol:ether.[13] Preliminary investigations indicated that n-propanol:ether mixture gave good recoveries for 5-Fu. Therefore to optimize the ratio of co-solvents, recoveries of 100 μg each, of 5-Fu, FuDH and Furd, in 1 mL of plasma were determined by extracting with 15 mL of n-propanol:ether mixture (in the ratios of 10:90 through 90:10 v/v) and compared to that of an aqueous standard solution of the same. After ascertaining the optimum ratio of n-propanol:ether mixture, the following procedure was carried out.

Pooled plasma containing spiked amounts of 100 µg/mL each of 5-Fu and its metabolites was mixed with propanol:ether in an appropriate ratio and sonicated for 5 min at 4°C. The solution was then centrifuged at 3000 x g for 15 min at 4°C. The organic phase was separated and evaporated to dryness under a stream of nitrogen. The residue was redissolved in 1 mL of 50 µM ammonium dihydrogen phosphate and adjusted to varying pH values between 2 to 10. The solution was vortexed for 10 min at ambient temperature and filtered through 0.45 µm filter (Millipore® HV4, Millipore, France) prior to filling in autosampler vials.

A parallel study was carried out to study the effect of various pH adjusting systems in minimizing the interference of analyte peaks with that of co-eluents from the plasma matrix. The pH adjusting systems used were 0.1 mL of 1N sulfuric acid or 0.1 mL of 1N sodium hydroxide or 0.1 mL of 100 µM Tris buffer. These solutions were added, separately, to spiked samples of plasma containing 5-Fu and its metabolites, prior to carrying out the extraction procedure discussed above.

Linearity Studies

Two sets of working standard solutions were prepared: one in de-ionized water and the other in pooled plasma. The standard solutions in plasma were prepared by spiking the biological fluid with known concentrations of 5-Fu, FuDH and Furd in separate volumetric flasks. The concentration range for 5-Fu and its metabolites was adjusted between 2 - 200 µg/mL in de-ionized water and 10 - 100 µg/mL in plasma. 5-Fc was used as an internal standard at a concentration of 100 µg/mL. Acceptance criteria of r^2 not less than 0.98 was followed.

Precision and Accuracy Studies

Precision and accuracy of the assay was determined by making replicate injections of known amounts of 5-Fu and its metabolites and assessing the per cent co-efficient of variation (% C.V.) and analytical recovery respectively. A coefficient of variation not exceeding 4% was considered to be an acceptable one.[14]

Plasma samples were spiked with a standard solution of 50 µg/mL each of 5-Fu, FuDH and Furd in separate tubes. Ten replicate injections of these were made on the same day to assess the with-in day precision and accuracy.

To assess the between-day variability, a working standard series of 50 µg/mL each of 5-Fu, FuDH and Furd in plasma was analyzed for 10 replicate injections on three consecutive days. 5-Fc in a concentration of 50 µg/mL was used as an internal standard.

Specificity Studies

The purpose of this study was to ascertain if 5-Fu and its metabolites could be analyzed simultaneously in the presence of other plasma matrix components. This was done by spiking the plasma sample with a standard solution containing a mixture of 50 µg/mL each of 5-Fu and its metabolites. 5-Fc in a concentration of 50 µg/mL was used as an internal standard.

RESULTS AND DISCUSSION

Analysis of sample solutions was done in quintuplicate unless otherwise mentioned. All statistical evaluations were done using Student's t-test (double-sided) at $p < 0.05$ level of significance.

The variables that affected the extraction yield were n-propanol:ether ratio, pH of redissolving buffer solution and nature of pH adjusting system.

The extractant ratio of n-propanol:ether was varied from 10:90 though 90:10 v/v. It was observed that a high ratio of n-propanol resulted in extraction of plasma components as well. Therefore, the peaks for analytes could not be well resolved and the % recovery could not be determined. A high ether ratio minimized interference of plasma components with that of 5-Fu and its metabolites but decreased the % recovery. The optimum ratio of n-propanol:ether mixture, observed for 5- Fu, Furd and FuDH, was found to be 80:20, 85:15 and 80:20 respectively.

When the pH of the redissolving buffer solution was varied from 2 to 10, the % recoveries for 5- Fu, Furd and FuDH decreased gradually upto pH 6.0. After this pH value, the recoveries were not found to alter significantly.

Figure 1 shows the variation of % recovery of 5-Fu as a function of pH of redissolving buffer solution. Therefore, the pH of redissolving buffer solution was kept constant at 2.0 for maximum recovery of 5-Fu and its metabolites.

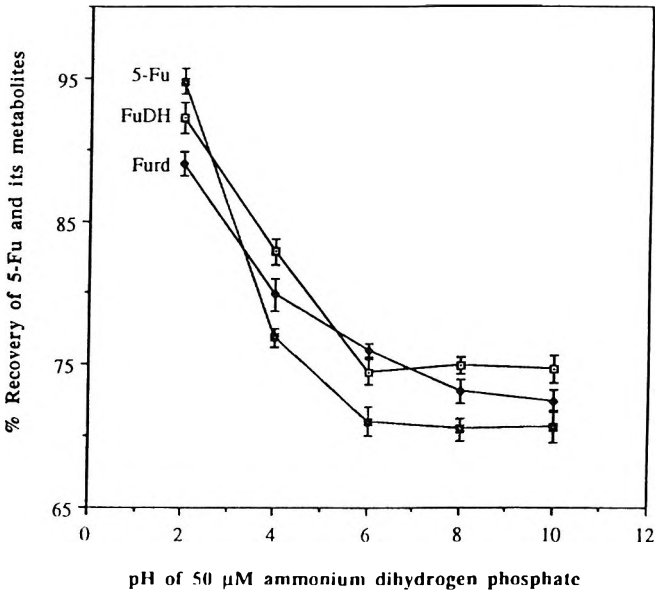

Figure 1. Percent recovery of 5-fluorouracil as a function of pH of 50 µM ammonium dihydrogen phosphate.

Amongst the various pH adjusting systems investigated to minimize the interference of plasma co-eluents with that of analytes, 0.1 mL of 1N sulfuric acid was found to give clear chromatograms. The analysis of samples treated with sodium hydroxide and Tris-buffer gave substantial interference between the analytes and co-eluents of the plasma matrix.

In the light of the above experiments, the extraction procedure established for the simultaneous analysis of 5-Fu and its metabolites requires a n-propanol:ether ratio of 80:20. After the sonication and centrifugation steps as discussed previously, the organic phase was evaporated. The residue was redissolved in 1 mL of 50 µM ammonium dihydrogen phosphate at a pH of 2.0, adjusted using 0.1 mL of 1N sulfuric acid.

A linear relationship was observed between the peak area ratio of the 5-Fu to that of the internal standard. The mathematical expression satisfying the linear regression equation are given in equations (1) and (2).

Table 1

Precision and Accuracy of 5-Fu, FuDH and Furd in Plasma

	5-Fu	FuDH	Furd
Within-day			
Mean concentration (μg/mL)	49.1	48.3	48.1
Standard deviation	0.5	0.7	1.1
% C.V.	1.0	1.4	2.3
% Recovery	98.2	96.7	96.2
Between-day			
Mean concentration (μg/mL)	49.6	48.1	48.5
Standard deviation	0.6	1.0	1.0
% C.V.	1.2	2.0	2.0
% Recovery	99.1	96.2	97.0

$y = 0.047\ x + 0.013,\ r^2 = 0.99$ in de-ionized water (1)

$y = 0.046\ x + 0.033,\ r^2 = 0.99$ in plasma (2)

The limit of quantitation for 5-Fu, FuDH and Furd in plasma was found to be 0.2 μg/mL, 0.3 μg/mL and 0.8 μg/mL respectively.

The with-in day coefficient of variation for 5-Fu, FuDH and Furd was found to be 1.0%, 1.4% and 2.3% respectively. The between-day coefficient of variation for 5-Fu, FuDH and Furd was found to be 1.2%, 2.0% and 2.0% respectively. The accuracy of the assay for 5-Fu and its metabolites was between 96.2% and 99.1%. The results are presented in Table 1.

The method was also found to be specific, as 5-Fu and its metabolites could be analyzed simultaneously with a good degree of precision and accuracy. The retention times of 5-Fc, FuDH, 5-Fu, and Furd were 3.8 min, 4.8 min, 5.5 min and 12.6 min respectively as shown in Figure 2.

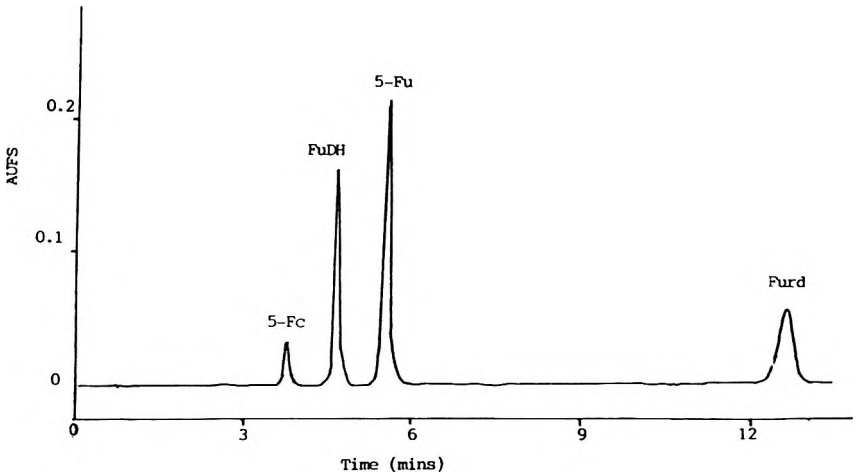

Figure 2. Representative HPLC chromatogram of 5-fluorouracil and its metabolites, 50 μg/mL each.

Table 2

Concentration of 5-Fu and FuDH in Plasma Samples After Intravenous Administration

Time (min)	Concentration in μg/mL	
	5-Fu	**FuDH**
1	89.4 ± 6.1	2.9 ± 1.3
10	25.8 ± 5.4	3.1 ± 1.0
20	29.6 ± 3.3	4.1 ± 0.8
30	12 ± 3.2	4.3 ± 0.9
40	11.1 ± 2.7	5.8 ± 0.5
50	08.5 ± 2.1	4.2 ± 1.1
60	07.6 ± 1.8	3.2 ± 1.0
120	03.1 ± 0.4	2.5 ± 0.9
180		2.2 ± 1.0

± indicates standard deviaton values for a mean of 5 determinations.

Upon intravenous administration of 15 mg of 5-Fu per kg body weight of rats, the concentration of the active drug reached its peak within 1 min. The results are presented in Table 2. FuDH could also be quantitated at this time period of 1 min. Afterwards, 5-Fu concentration gradually decreased over a period of time and could not be detected after 2 h. The concentration of FuDH increased initially upto 5.84% but after 40 min started decreasing. Surprisingly, no traces of Furd could be detected throughout this experiment.

CONCLUSION

The present study demonstrates the importance of an optimized extraction procedure for maximum recovery of 5-Fu and its metabolites. Evaluation of parameters that are necessary for achieving maximum recovery of 5-Fu and its metabolites have been investigated and optimized. The proposed method also confirms the suitability of this assay procedure for the analysis of 5-Fu and its metabolites in plasma samples with a good degree of precision and accuracy. The method has been validated as per compendial standards and can be routinely used for the estimation of 5-fluorouracil and its metabolites during pharmacokinetic experiments using plasma as the biological fluid.

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ANALYSIS AND PHARMACOKINETICS OF APOMORPHINE IN RAT BRAIN BY MICRODIALYSIS COUPLED WITH MICROBORE HPLC ELECTROCHEMICAL DETECTION

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ABSTRACT

The feasibility of an on-line microdialysis coupled with a sensitive microbore high performance liquid chromatography with electrochemical detection (HPLC-ED) system for the direct analysis of apomorphine was investigated. A microdialysis probe was inserted into the right striatum of male Sprague-Dawley rats, which had been administered apomorphine (10 mg/kg, i.v.).

Brain dialysates were automatically injected into a cyano microbore column with an electrochemical detector through an on-line injector. Samples were eluted with a mobile phase containing 0.1 M monosodium dihydrogen phosphate-methanol (84:16, v/v, pH 3.0 adjusted with orthophosphoric acid) at a flow rate of 0.05 mL/min. A monophasic phenomenon with a elimination phase was observed from the brain apomorphine concentration-time curve. The results indicate that the brain pharmacokinetics of apomorphine appears to conform to a one-compartment model.

INTRODUCTION

Apomorphine is well known for its dopaminergic effects.[1-2] However, it exhibits dual effects, where a low dose causes a decrease in dopamine neurotransmission and function by the stimulation of inhibitory pre-synaptic D1 receptor.[3] A larger dose causes direct stimulation of post-synaptic D2 receptor, resulting in an increase in the functional activity of dopamine.[4]

For the determination of apomorphine, a number of analytical methods have been developed including gas chromatographic,[5] fluorometric[6] and mass fragmentographic.[7] Recently, high performance liquid chromatography (HPLC) coupled with ultraviolet,[8,9] fluorometric[10] and electrochemical detection (ED)[11,12] have been reported. HPLC-ED is the most sensitive method.

Currently, microbore columns instead of conventional columns coupled with ED have advantages of very high sensitivity and a relatively small sample introduction.

For the sample processing, apomorphine is a very labile compound in solution. In order to warrant the stability of apomorphine, we used an automatic on-line injection method to inject dialysate directly.[13]

Recent advances in brain microdialysis techniques have enabled the direct measurement of various neurotransmitters in the brain, but pharmacokinetic investigations of psychotropic drugs by this method are limited.[14,15] Since drug monitoring is crucial for the rational therapeutic use of drugs, the feasibility of employing the brain microdialysis method for pharmacokinetic studies is of particular importance.

Of interest in elucidating the central disposition of apomorphine in the brain, a precise and sensitive method using an on-line microdialysis system coupled with microbore HPLC-ED was developed to measure apomorphine in brain dialysates. In addition, the central pharmacokinetics of apomorphine in rat brains was also investigated.

MATERIALS AND METHODS

Materials and Reagents

Apomorphine was purchased from Research Biochemical International (RBI, Natick, MA, USA). Methanol and orthophosphoric acid were obtained from E. Merck (Darmstadt, Germany). Triple de-ionized water (Millipore Corp., Bedford, MA, USA) was used for all preparations.

Chromatography

The HPLC-ED system consisted of a chromatographic pump (BAS, PM-80, Bioanalytical System, West Lafayette, IN, USA) at flow-rate 0.05 mL/min for apomorphine analysis using a cyano microbore column (BAS, SepStik CN-5μ, 150 x 1 mm i.d., particle size 5 μm) in series after an on-line injector (Fig. 1). The mobile phase consisted of 0.1 M monosodium dihydrogen orthophosphate-methanol (84:16, v/v, pH 3.0 adjusted with orthophosphoric acid). The mixture was filtered with a 0.22 μm Millipore membrane and degassed by helium. The injection volume was configured with a 10 μL sample loop on an on-line injector (CMA-160, CMA/Microdialysis AB, Stockholm, Sweden). Apomorphine was measured using an electrochemical detector (BAS 4C). The potential for the glassy carbon working electrode was set at + 0.8 V with respect to a Ag/AgCl reference electrode. The output from the electrochemical detector was recorded using Waters Millennium 2020 software.[16-18]

Microdialysis

Adult, male Sprague-Dawley rats (250-320 g) were initially anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The rat was cannulated with a PE-50 tube at the right femoral vein for drug administration. After the femoral vein cannulation, the rat was placed in a Kopf stereotaxic frame and its body temperature was maintained at 37°C with a heating pad. A microdialysis probe

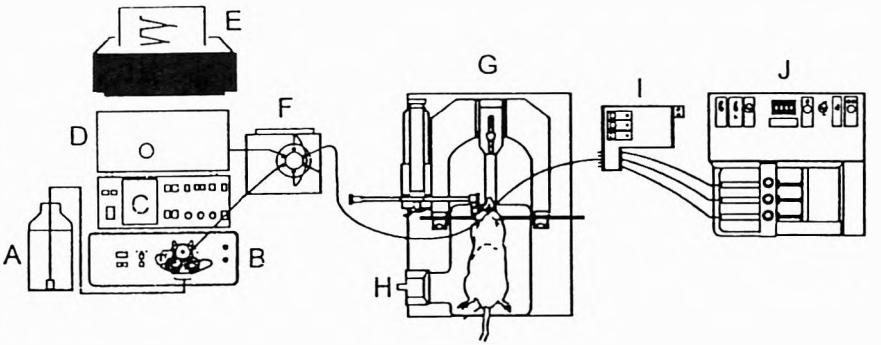

Figure 1. The schematic diagram of an on-line microdialysis coupled with the microbore HPLC-ED system. A: mobile phase; B: chromatography pump; C: electrochemical detector; D: column cabinet; E: data system; F: on-line injector; G: stereotaxic device; H: heating pad; I: solvent selector; J: microinjection pump

(CMA-12; CMA/Microdialysis AB) with a tip length of 4 mm and an outer diameter of 0.5 mm was implanted into the right striatum with its tip located at AP 0.4 mm, ML -3.0 mm, DV -7.0 mm, from the bregma and dura surface, respectively.[19] The probe was perfused with Ringer solution (147 mM Na^+, 4.0 mM K^+, 2.2 mM Ca^{++}) at a flow-rate of 1 μL/min, by a microinjection pump (CMA-100). The outflow from the dialysis probe was connected to an on-line injector (CMA-160) and HPLC-ED.[13] The samples were automatically injected every 10 min for 160 min after drug administration.

Recovery

The recovery of the dialysis probe for apomorphine is the ratio of its concentration in the dialysate, i.e. the outlet from the probe (C_{out}) to its concentration of apomorphine in the medium surrounding the probe (C_m).

$$\text{The recovery}_{\text{in vitro}} = C_{out} / C_m$$

Pharmacokinetic Analysis

Calibration curves were constructed based on the analysis by HPLC-ED of various concentrations of apomorphine (0.5-100 ng/mL) and were used to determine the concentrations of apomorphine in rat brain dialysates. Following a 2-h period for stabilization, dialysates were automatically injected every 10

min for 160 min after drug administration (10 mg/kg, i.v.). The volume of i.v. apomorphine (10 mg/mL) solution administered was 1 mL/kg. After the administration of the drug, the catheter was then immediately flushed with 0.5 mL normal saline.

All brain dialysate concentration-time data were processed by the computer program "PCNONLIN" (SCI Software Inc. Lexington, KY, USA), with reciprocal concentration weights (1/C) for the calculation of pharmacokinetic parameters.

The data were compared with pharmacokinetic models (one- vs two-compartment) according to the criteria of Akaike's information criterion (AIC)[20] and Schwartz criterion (SC).[21] with minimum AIC and SC values being regarded as the best representation of the concentration-time course data. The following equation applies into a one-compartment pharmacokinetic model:

$$C = Ae^{-\alpha t} \quad (1)$$

In equation 1, A is the concentration (C) intercepts and α is disposition rate constant for the disposition phase. The elimination phase half-life ($t_{1/2}$) of apomorphine in brain dialysate were defined as $0.693/\alpha$.

The noncompartmental method for calculating disposition parameters of apomorphine in the brain is based on the theory of statistical moments.[22] The area under the concentration-time curve (AUC) of a plot from time zero to infinity is often referred to the area under the moment curve (AUMC).[22] The ratio of AUMC to AUC for apomorphine in the brain is a measure of its mean residence time (MRT).[23]

RESULTS

Under the conditions described above, the retention times of apomorphine was found to be 6.5 min (Fig. 2). Figure 2(A) shows a standard sample of apomorphine (10 ng/mL). Figure 2 (B) shows a chromatogram of a blank brain dialysate. No discernible peaks were observed within the time frame in which apomorphine was detected. Figure 2(C) shows a chromatogram of a dialysate sample containing apomorphine (27.92 ng/mL) obtained from brain microdialysis 40 min after apomorphine (10 mg/kg, i.v.) administration. The in vitro recovery of apomorphine of the microdialysis probe based on a 10 ng/mL standard, was 32%.

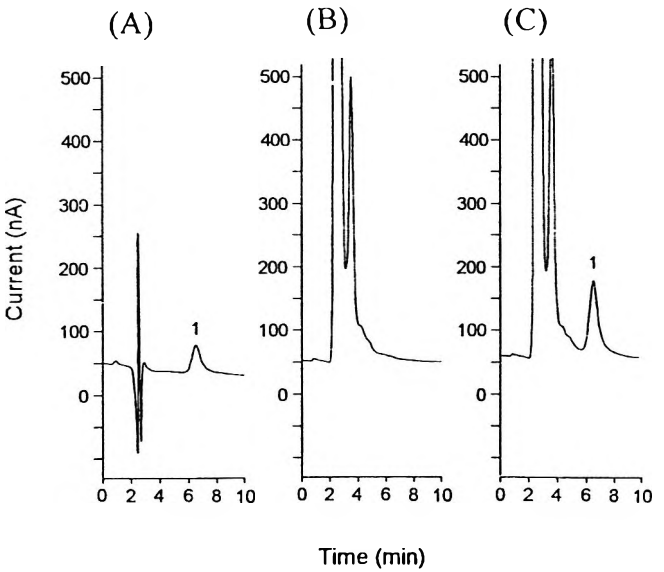

Figure 2. Typical chromatograms of (A) a standard sample containing 10 ng/mL apomorphine, (B) a blank brain dialysate, and (C) a dialysate sample containing apomorphine (27.92 ng/mL) collected from a rat after apomorphine (10 mg/kg, i.v.) administration. 1: apomorphine.

The limit of quantitation is defined as the lowest concentration on the standard curves which can be measured with acceptable accuracy and precision. The limit of quantitation was 0.5 ng/mL for apomorphine. However, the detection limits for apomorphine, at a signal-to-noise ratio of 3, was 0.1 ng/mL.

A one-compartment open model in rat brain with individual animal data after apomorphine i.v. administration was proposed by the computer program "PCNONLIN". Analysis of data in Fig. 3 yields equation 2.

$$C = 55.53e^{-0.021t} \quad (2)$$

The brain pharmacokinetic parameters, as calculated by PCNONLIN program and derived from these data, are shown in Table 1.

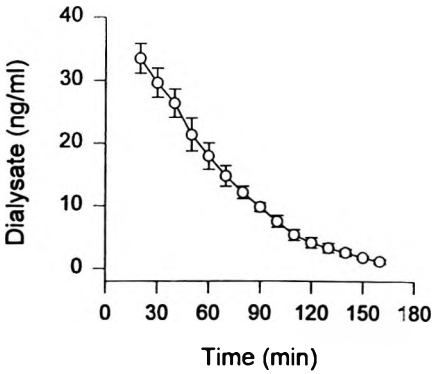

Figure 3. The brain dialysate concentration-time curve after apomorphine (10 mg/kg, i.v.) administration.

Table 1

Brain Pharmacokinetic Parameters of Apomorphine (10 Mg/Kg, I.V.) in Rats

Parameters	Estimate
A, ng/mL	55.53 ± 6.03
α, 1/min	0.021 ± 0.002
AUC, ng min/mL	2666 ± 201
$t_{1/2}$, min	33.92 ± 2.37
AUMC, μg min^2/mL	129.97 ± 11.86
MRT, min	48.94 ± 3.41

Data are expressed as mean±SEM (n=5).

DISCUSSION

Compared to conventional HPLC systems, microbore columns decrease band broadening of analytes so that sharper peaks are obtained. Furthermore, the slow flow-rates in a microbore HPLC-ED system provides a smoother base-line to achieve lower detection limits.[16,17] It also prolongs the time of analytes contact with the working electrode and results in higher coulometric yields.[16,24] Hence, microbore HPLC-ED systems can enhance detection sensitivity and achieve optimum detection limits. Furthermore, the microbore HPLC-ED

system requires only small quantities of samples which is compatible to microdialysis sampling methods. In addition, the on-line analysis improves analytical reproducibility and obviates the need of preservatives in the samples and other tedious manual procedures.

In the present study, a microbore HPLC-ED system was applied to the determination of apomorphine in rat striatal dialysates from an on-line microdialysis system in rats receiving an i.v. administration of apomorphine. The limit of quantification and the detection limit of apomorphine were 10 ng/mL and 1 ng/mL, respectively. A monophasic phenomenon with a first-order elimination rate constant for apomorphine was observed from the brain dialysate concentration-time curve. The results indicate that the brain pharmacokinetics of apomorphine appears to conform to an one-compartment model.

Noncompartmental methods for calculating disposition parameters of apomorphine in brain dialysate are based on the theory of statistical moments.[22] After administration of apomorphine (10 mg/kg, i.v.), MRT and $t_{1/2}$ were 48.94 and 33.92 min, respectively. MRT is a function of both distribution and elimination. Elimination half-life ($t_{1/2}$) is the time required to eliminate 50% of the dose, whereas MRT_{iv} is the time required to eliminate 63.2% of the dose.[22] The disposition of apomorphine in rat brain might process from blood through the blood-brain-barrier. Our results suggest that the disposition of apomorphine in brain exhibit one disposition phase.

In conclusion, the present results recommend that the brain microdialysis method may be applicable to further pharmacokinetic studies of psychotropic or neurotropic agents in the brain.

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1999

MARCH 7 - 12: PittCon '99, Orlando, Florida. Contact: PittCon '99. Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA

MARCH 21 - 25: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington. DC 20036-4899, USA.

AUGUST 22 - 26: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

OCTOBER 8 - 13: 51st ACS Southeast Regional Meeting, Knoxville, Tennessee. Contact: C. Feigerle, Chem Dept, University of Tennessee. Knoxville, TN 37996, USA. Tel: (615) 974-2129; Email: reglmtgs@acs.org.

2000

MARCH 5 - 10: PittCon 2000, Chicago, Illinois. Contact: PittCon 2000. Suite 332, 300 Penn Center Blvd., Pittsburgh. PA 15235-5503, USA.

MARCH 26 - 30: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

AUGUST 20 - 24: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2001

APRIL 1 - 5: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 19 - 23: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2002

APRIL 7 - 11: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 12: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

2003

MARCH 23 - 27: 225th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 11: 226th ACS National Meeting, New York City. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2004

MARCH 28 - APRIL 1: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

AUGUST 22 - 26: 228th ACS National Meeting, Philadelphia, Pennsylvania. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

2005

MARCH 13 - 17: 229th ACS National Meeting, San Diego, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 28 - SEPTEMBER 1: 230th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

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1. 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.

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