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DIRECT CHIRAL HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATION OF O,O-DIETHYL-(p-METHYL-BENZENE-SULFONAMIDO)- ARYL (ALKYL)-METHYLPHOSPHONATE

R. Y. Gao,[1] G. S. Yang,[2] H. X. Shen,[2]
R. Y. Chen,[1] Q. Dai[1], Q. S. Wang*[1]

[1]National Laboratory of Elemento-Organic Chemistry
[2]Department of Chemistry
Nankai university
Tianjin, 300071
People's Republic of China

ABSTRACT

The enantiomers of a series of twelve O,O-diethyl, (p-methyl-benzenesulfonamido), aryl(alkyl)-methylphosphonates have been separated by high performance liquid chromatography on a Pirkle model chiral stationary phase. Chromatographic data and a chiral recognition model are presented for the separation of these organic phosphorus enantiomers on the chiral phase. The influence of column temperature and flow rate of mobile phase has been described.

INTRODUCTION

Optical resolution methods using high performance liquid chromatography (HPLC) have been extensively developed. There are three

methods of separation available in liquid chromatography. 1. Formation of diasteroisomeric derivatives; 2. addition of chiral discriminating agents to the mobile phase and, 3. use of chiral stationary phases (CSP). In particular, CSP methods have become of interest.

For the direct HPLC resolution of enantiomers, various kinds of enantioselective chiral stationary phases (CSP's) and packings have been developed. They are described comprehensively in several recent reviews and books.[1-5]

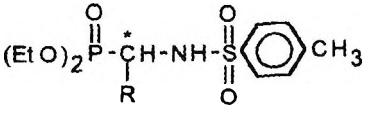
Separation based on complementary donor-acceptor rearrangements such as π-donor-acceptor, dipole-dipole (dipole-stacking), hydrogen bonding and steric interaction have been investigated. π-Donor-acceptor type phases, which also contain amide functionalities, are especially successful, and highly efficient HPLC columns can be obtained. These have been rationally designed by Pirkle and co-workers utilizing chiral recognition models.[4]

In this paper, we descibe the synthesis of a chiral, L-leucine tert-butylamide bonded phase. This synthesized phase has been used in the resolution of enantiomers of a series of twelve O,O-diethyl, (p-methyl-benzenesulfonamido)-, aryl (alkyl)-methylphosphonates. We discuss how column temperature, volume fraction of mobile phase and flow rate variation affect retention and selectivety for chiral resolution.

EXPERIMENTAL

Materials

The study was performed with a series of twelve O,O-diethyl, (p-methyl-benzenesulfonamido)-, aryl (alkyl)-methylphosphonates, prepared by us; the spectra and elemental analyses were recorded. The general formula of the compounds is



The structures of the substituents " R " are (1) C_6H_5, (2) p-$CH_3OC_6H_4$, (3) p-ClC_6H_4, (4) p-$NO_2C_6H_4$, (5) p-$CH_3C_6H_4$, (6) m-$NO_2C_6H_4$, (7) m-ClC_6H_4, (8) o-$NO_2C_6H_4$, (9) o-$CH_3C_6H_4$, (10) 2,4-$Cl_2C_6H_3$, (11) ET and (12) i-Pr. These compounds were dissolved in acetone, then diluted with eluent solvent. Solutions with approximate concentration of 0.1 mg/mL in eluent solvent were used for injection. All solvents were redistilled and filtered through a 0.45 μ filter and degassed in vacuo before use.

Apparatus

The HPLC system was composed of a Model 2010 liquid chromatograph (Varian, Northeast Florham Park, NJ, USA) with a Model 2050 UV detector and HP-3392 integrator (Hewlett packard, Palo Alto, CA USA).

Chromatography

During the chromatographic runs, all experimental variables, except those being investigated, were carefully controlled at fixed values. The variables held constant included the sample size (2 μL) and UV detector wavelength (230 nm). The range of the mobile phase compositions were 15, 20, 25 and 30 % of isopropyl alcohol-water, the column temperatures were 16, 25 and 35 °C and the flow rates were 1.0, 1.5 and 2.0 mL/min.

Pirkle-Type Column Preparation

For 3,5-Dinitrobenzoylleucine preparation refer to Reference 6. A solution of 3.5 g of L-3,5-dinitrobenzoylleucine in 200 ml of dry THF was poured over 5 g of dry aminopropyl silica gel, YWG-NH (Second Reagent Factory, Tianjin, P. R. China) and 3 g of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was added with swirling. After 8 h at room temperature, the silica was isolated by filtration and washed repeatedly with methanol, acetone and ether. These last washings employed centrifugation-decantation and some fines were thus removed. After drying, ca. 5.6 g of silica bonded phase was obtained. Anal. Found: C, 10.10; H, 1.80; N, 2.5 %.

RESULTS AND DISCUSSION

In order to optimize separation, the experiment was under various mobile phase compositions. The separation data, capacity factor k', and α values, are indicated in Table 1. These results evidently prove that 15% isopropyl alcohol mobile phase gave the best α values for O,O-diethyl, (p-methyl-benzenesulfonamido)-, aryl-methylphosphonate enantiomers on the CSP, which all gave baseline separation of compounds 11 and 12 (R all is aliphatic group). Isopropyl alcohol, in the concentration range 15 - 30 %, produced α values for the enantiomers which decrease with increasing concentration of isopropyl alcohol. But the effect was not large. The maximum α value was obtained with 15% isopropyl alcohol and minimum analytical time with 30 % in all the enantiomers. The result is listed in Table 1. Chromatograms of compounds Nos. 1 and 2 are shown in Figure 1.

Table 1

k'_2 , k'_1 and α Values of Twelve O,O-Diethyl, (p-Methyl-Benzenesulfonamido)-, Aryl (alkyl)-Methylphosphonate at Different Mobile Phase Compositions (Column Temperature: 16°C, Flow Rate: 1.0 ml/min)

No*	15 % i-PrOH			20 % i-PrOH			25 % i-PrOH			30 % i-PrOH		
	k'_2	k'_1	α	k'_2	k'_1	α	k'_2	k'_1	α	k'_2	k'_1	α
1	4.307	2.702	1.591	3.042	1.924	1.572	2.329	1.517	1.535	1.863	1.23	1.508
2	7.610	3.750	2.209	5.075	2.592	1.959	3.969	2.070	1.917	3.184	1.656	1.889
3	4.281	2.302	1.860	2.983	1.657	1.799	2.462	1.381	1.782	2.011	1.146	1.754
4	6.712	3.949	1.700	4.779	2.879	1.660	3.860	2.357	1.638	3.102	1.926	1.611
5	4.376	2.483	1.763	3.569	2.026	1.762	2.769	1.609	1.720	2.415	1.417	1.705
6	8.094	5.493	1.621	6.498	4.069	1.597	4.822	3.052	1.580	3.484	2.477	1.552
7	3.682	2.623	1.404	2.751	1.974	1.393	2.129	1.549	1.375	1.746	1.279	1.365
8	9.541	5.445	1.752	6.493	3.782	1.717	4.902	2.944	1.665	3.809	2.329	1.636
9	5.839	4.007	1.457	4.126	2.857	1.445	2.997	2.115	1.417	2.300	1.654	1.391
10	3.348	1.827	1.832	2.724	1.519	1.794	2.196	1.257	1.747	1.975	1.133	1.744
11	1.720	1.569	1.096	1.404	1.293	1.086	1.105	1.026	1.077	0.965	0.908	1.063
12	1.457	1.261	1.156	1.201	1.048	1.145	0.932	0.819	1.139	0.859	0.760	1.130

* Substituents R number (see text).

The efficiency of column temperatures was investigated in all the compounds for the range of 16 - 35 °C. The capacity factor , k', decreased with

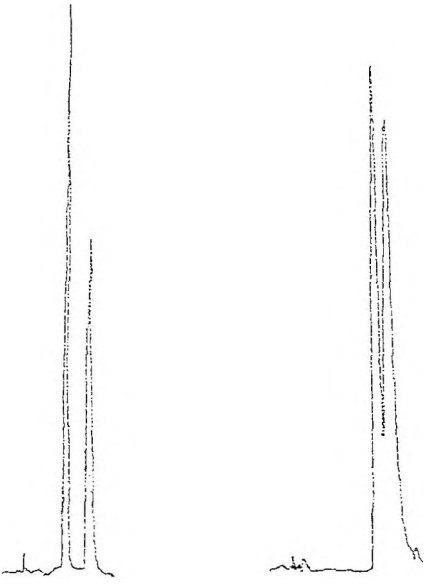


Figure 1. Chromatogram for compounds 1 (left) and 12 (right) enantiomers (mobile phase: 20 % isopropyl alcohol-water, column temperature: 16°C, flow rate: 1mL/min).

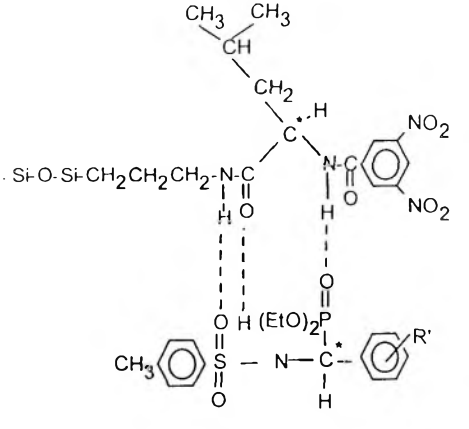


Figure 2. Proposed mechanism for chiral recognition.

Table 2

k'_2 k'_1 and α Values of Twelve O,O-Diethyl, (p-Methyl-Benzenesulfonamido)-Aryl (Alkyl)-Methylphosphonate at Different Column Temperatures (Mobile Phase: 20 % Isopropyl Alcohol, Flow Rate : 1.0 ml/min)

No*	16°C			25°C			35°C		
	k'_2	k'_1	α	k'_2	k'_1	α	k'_2	k'_1	α
1	3.024	1.924	1.572	2.893	1.886	1.534	2.266	1.536	1.475
2	5.075	2.592	1.959	5.000	2.624	1.905	3.730	2.080	1.794
3	2.983	1.657	1.799	2.910	1.652	1.762	2.218	1.336	1.660
4	4.779	2.879	1.660	4.586	2.841	1.614	3.474	2.263	1.535
5	3.569	2.026	1.762	3.248	1.893	1.716	2.588	1.564	1.655
6	6.498	4.069	1.597	5.948	3.790	1.570	4.533	2.972	1.525
7	2.751	1.974	1.393	2.586	1.886	1.371	2.028	1.526	1.329
8	6.493	3.782	1.717	6.270	3.759	1.652	4.696	2.990	1.571
9	4.126	2.857	1.445	3.941	2.690	1.413	3.059	2.246	1.362
10	2.724	1.519	1.794	2.597	1.466	1.771	2.048	1.218	1.682
11	1.404	1.293	1.086	1.338	1.238	1.081	1.118	1.038	1.077
12	1.201	1.048	1.145	1.152	1.003	1.148	0.962	0.848	1.135

* same as Table 1.

a rise in temperature, but the separation factor, α, was small decreased, the result was shown in Table 2. The effect of mobile phase flow rate was investigaed in the range of 1 - 2 ml / min in step of 0.5 mL / min. for all compounds. The result of separation was hardly affected which was listed in Table 3.

The results suggest the mechanism for chiral recognition depicted in Figure 2. This model involves the formation of a charge-transfer complex between the π-electron acceptor 3,5-dinitrobenzoyl group of the CSP and the π-electron donor aryl substitument R of the organic phosphorus compounds and three hydrogen bonding of the 3,5-dinitrobenzamide hydrogen of the CSP and the phosphorous compounds. Obviously the π-bonding is more important, so the enantiomers of a series ten compounds (No. 1-10) have good separation. When R is an aliphatic group (No. 11-12), the π-bond disappears and the results of separation are not as good as with an aromatic group.

Table 3

k'_2 k'_1 and α Values of Twelve O,O-Diethyl, (p-Methyl-Benzenesulfonamido)-Aryl (Alkyl)-Methylphosphonate at Different Mobile Phase Flow Rate (Mobile Phase: 20 % Isopropyl Alcohol, Column Temperture:16^{0}C)

No*	1.0 mL/min			1.5 mL/min			2.0 mL/min		
	k'_2	k'_1	α	k'_2	k'_1	α	k'_2	k'_1	α
1	3.024	1.924	1.572	3.383	2.150	1.573	3.188	2.049	1.556
2	5.075	2.592	1.959	6.047	3.047	1,985	5.583	2.861	1.951
3	2.983	1.657	1.799	3.466	1.902	1.823	3.215	1.799	1.788
4	4.779	2.879	1.660	5.528	3.321	1.665	5.049	3.083	1.637
5	3.569	2.026	1.762	3.736	2.130	1.754	3.576	2.056	1.740
6	6.498	4.069	1.597	6.943	4.347	1.597	6.528	4.118	1.585
7	2.751	1.974	1.393	3.026	2.171	1.394	2.840	2.046	1.382
8	6.493	3.782	1.717	7.591	4.389	1.730	6.993	4.118	1.698
9	4.126	2.857	1.445	4.554	3.161	1.441	4.306	3.014	1.429
10	2.724	1.519	1.794	2.886	1.606	1.797	2.813	1.576	1.784
11	1.404	1.293	1.086	1.497	1.378	1.806	1.451	1.340	1.083
12	1.201	1.048	1.145	1.285	1.114	1.153	1.250	1.087	1.153

*Same as Table 1

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QUANTITATIVE ANALYSIS OF THE LOOP DIURETIC TORASEMIDE IN TABLETS AND HUMAN URINE BY HPLC-EC

M. Begoña Barroso, Rosa M. Alonso, Rosa M. Jiménez

Departamento de Química Analítica
Facultad de Ciencias
Universidad del País Vasco/EHU
Apdo. 644, 48080 Bilbao, Spain

ABSTRACT

A High Performance Liquid Chromatographic method with amperometric detection for the determination of the loop diuretic 1-Isopropyl-3-[4 - (3-methylphenylamino)-3-pyridinesulphonyl] urea, torasemide, is reported using a μ-Bondapak C_{18} column and a mobile phase water:acetonitrile (65:35), 5 mM in potassium dihydrogenphosphate/dipotassium hydrogenphosphate at pH 5.3. Eluant is monitored at +1300 mV with an amperometric detector equipped with a glassy carbon working electrode. The method showed a determination limit of 8 ppb and a reproducibility in terms of relative standard deviation lower than 2% in intra-day assays and 5 % in inter-day assays. The HPLC-EC method was applied to urine samples obtained from a healthy volunteer. Concentration levels of torasemide at different time intervals were monitorized and results were in agreement with the pharmacokinetic parameters of this diuretic. The determination of torasemide in urine required a liquid-liquid extraction prior to chromatographic analysis due to the interferences found in urine matrix. With this simple clean-up

procedure, recoveries around 70% were achieved. Torasemide was directly determined in tablets after dissolution of the powder in a methanolic solution.

INTRODUCTION

1-Isopropyl-3-[4-(3-methyl phenyl amino)-3-pyridinesulphonyl]urea, torasemide is a high-ceiling loop diuretic of the pyridil sulfonylurea class, structurally related to furosemide, azosemide and bumetanide[1]. This long-acting loop diuretic has a bioavailability of about 90 %[2,3] and undergoes oxidative biotransformation, while furosemide is excreted unchanged. In healthy volunteers given torasemide 20 mg, about 25 % was recovered unchanged in urine within 24 hours[3-6]. Due to its diuretic properties, torasemide is a compound forbidden in sports, and it is included by the International Olympic Committee in the list of banned substances in sports.[7]

Since torasemide is a relatively new diuretic (J. Delarge and C. L. Lapière 1978)[8], in comparison with other loop such as furosemide, only a few papers can be found in literature dealing with its analysis. Methods reported for the determination of torasemide include preferently chromatographic procedures with photometric detection[9-12] and voltammetric analysis on different electrodes[13,14].

The aim of this work is the application of an HPLC system with amperometric detection for the determination of the diuretic torasemide in tablets and real urine samples.

MATERIALS AND METHODS

Reagents, Chemicals and Standard Solutions

Pure torasemide and tablets containing 10 mg of torasemide, were very kindly supplied by Boehringer Mannheim (Barcelona, Spain). HPLC grade solvents were purchased from Lab-Scan (Dublin, Ireland), and water obtained from the Milli-RO and Milli-Q Waters systems. All the reagents used were Merck Suprapur (Bilbao, Spain).

A stock solution of torasemide 1000 μg/mL was prepared in pure acetonitrile and stored in the dark under refrigeration.

Procedure for Tablets and Urine Samples

The tablets were pulverized and dissolved in methanol. The resulting solution was filtered through the 0.45 μm membrane and an aliquot diluted with the mobile phase to provide the concentration required for the injection.

The clean-up procedure for urine samples was a liquid-liquid alkaline extraction with NaCl and ethyl acetate based on the method proposed by Ventura et al[15].

Chromatographic Conditions

The HPLC system consisted of a Model 2150-LKB (Pharmacia, Barcelona, Spain) HPLC pump, and a Rheodyne (Pharmacia) Model 7125 injector with a loop of 20μL. The electrochemical detector (PAR Model 400) equipped with a glassy carbon cell (EG&G Princeton Applied Research, Madrid, Spain). It was operated at +1300 mV vs a Ag/AgCl electrode, in the DC mode with a 5-s low-pass filter time constant, and a current range between 0.2 and 100 nA. Chromatograms were recorded using an LKB Model 2221 integrator. A 125Å μBondapak C_{18}, 30 cm x 3.9 mm I.D., 10-μm, (Waters Assoc.) column with a μBondapak C_{18} precolumn module (Waters Assoc.) were used. To study the influence of the temperature, a Waters TMC temperature control system was used.

The mobile phase was a mixture acetonitrile-water (35:65) containing 5 mM potassium dihydrogenphosphate/dipotassium hydrogenphosphate. pH was adjusted to 5.3 and the buffer served as the supporting electrolyte. The μBondapak C_{18} column head-pressure was 69 bar at a flow rate of 1.0 mL/min. The injection volume was 20 μL. The work was made at room temperature.

RESULTS AND DISCUSSION

In order to choose the optimum potential value for the amperometric detection of this diuretic, hydrodynamic voltammetry of the compound was carried out. An oxidative potential of 1300 mV was chosen as the working potential, since it was the potential which provided the maximum sensitivity for torasemide.

The study of the influence of pH gave an optimum value of 5.3 which

allowed the separation of torasemide from the electrooxidable interferences found in urine, keeping a low retention time.

The buffer potassium hydrogen phosphate/dipotassium hydrogen phosphate was used as supporting electrolite providing the best signal to noise ratio at a concentration of 5mM.

A variation in temperature from 26 to 55 °C produced small variations on the peak area of chromatograms. A linear relationship between k'-log 1/T was obtained. Since the influence of the temperature was not very relevant, the work was carried out at room temperature and with a flow rate of 1 mL/min.

When optimum chromatographic conditions had been established, a quantitative method for the determination of torasemide was developed at two concentration levels: ppm and ppb. The calibration curves for both concentration levels showed that the detector response was lineal up to 12 mg/l. The reproducibility studies (intra-day and inter-day assays) made on n=10 solutions for both concentration levels gave rise to the following results in terms of relative standard deviations: 1.1% (at day) and 5.6% (inter-day) at ppm level and 0.7% (at day) and 2.9% (inter-day) at ppb level. The experimental quantitation limit, defined as the minimum concentration of torasemide which gives rise to a signal able to be quantified for the integrator, was 8.5 ng/mL.

Analytical Applications

The method developed was applied to the determination of torasemide in tablets. Since torasemide was not commercially available in Spain, the pharmaceutical Company (Boehringer Mannheim) supplied the tablets containing 10 mg of torasemide (torasemide 10 mg tablets GA-1/3. Sample for research). Values obtained were in accordance with those certificated, as can be seen in Table 1, with errors lower than 1%, which demonstrate the accuracy of the method.

In order to calculate the percentages of recovery, the method was applied to urine samples spiked with this diuretic. Quantitative recoveries calculated from urine samples spiked with 0.5μg/mL and 1μg/mL were (71.02±2.9)% and (70.1±3.0)% respectively.

The chromatographic method has been applied to the analysis of torasemide in real urine samples obtained from a healthy volunteer after a

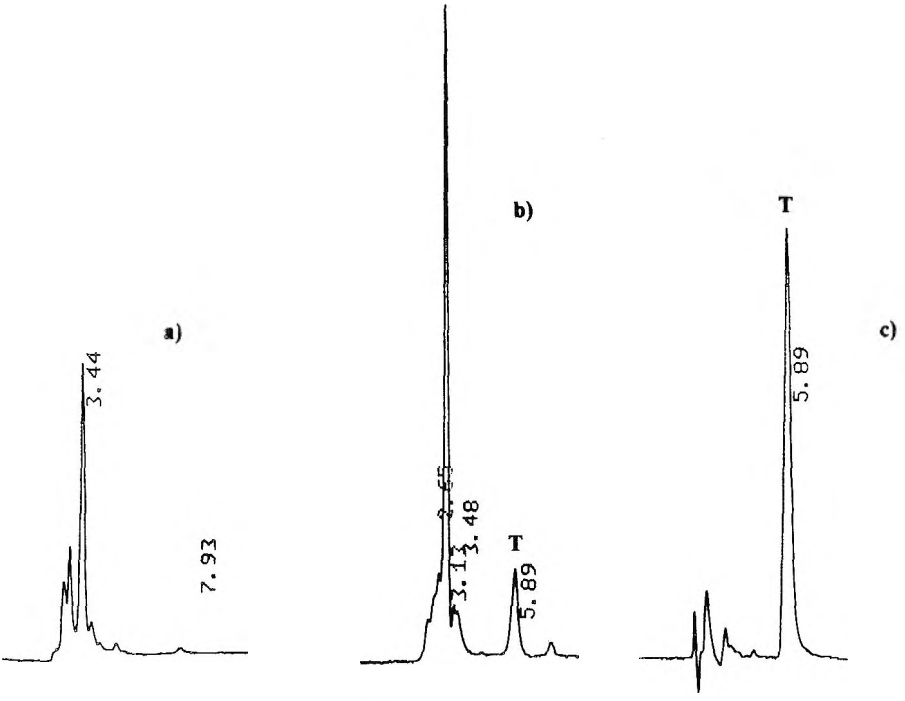

Figure 1. Chromatograms obtained from an extract of: a) blank urine sample, b)urine sample 2-8 hours after oral administration of 1 tablet of torasemide 10 mg to a healthy male volunteer and c) a diluted solution of a tablet containing 10 mg of torasemide. Potential +1300 mV, full current scale: 50nA. Mobile phase: acetonitrile-water (35:65), 5mM KH_2PO_4/K_2HPO_4, pH 5.3.

Table 1

Determination of Torasemide in Pharmaceutical Formulations and Urine

Tablet Formulation	Component	Found (mg)[a]	Nominal (mg)
Torasemida GA-1/3 Sample for Research	torasemide	9.98 ± 0.02	10.00
Urine Samples			
Time interval	0-2 hours	2-8 hours	8-24 hours
Torasemide conc. (μg/mL):	0.84	0.89	0.12

[a] amount ± ts/√n. n=4 different samples and 3 replicates of each sample.

single dose of a tablet containing torasemide 10 mg. Urine was collected at different time intervals for the quantitative determination of the diuretic: 0-2 hours, 2-8 hours and 8-24 hours. The compound was easily detected at the different times and the concentrations determined, collected in Table 1, were in agreement with the pharmacokinetic data[3]. Urine samples were treated following the clean-up procedure described in the experimental section and Fig. 1 shows the chromatograms corresponding to a blank urine, a real urine sample obtained 2-8 hours after administration of a single dose of torasemide 10 mg and a diluted solution of a tablet (Torasemida GA-1/3 Sample for research).

CONCLUSIONS

In a previous work we described a static voltammetric method for the determination of torasemide based on its oxidation peak[14]. Since the diuretic was not commercialized in Spain, the method was applied to standard solutions and spiked urine samples. The static method allowed the determination of torasemide without problems using the standard aditions method.

But when this method was applied to real urine samples obtained after the oral ingestion of a tablet of torasemide, some matrix interferences found made impossible the determination of the diuretic and led us to develop a chromatographic method.

The clean-up procedure used in static conditions was an acid extraction using ethyl acetate. Although this method allowed a good recovery, the chromatographic peak of torasemide was very close to the peaks from endogenous compounds, and the quantification of the diuretic was very difficult. So other procedures had to be tried. Changing the organic solvent for the extraction lower recoveries were obtained and the interferences could not be eliminated.

A method based on the one proposed by Ventura et al.[15] allowed the elimination of most of the interferences . Although the percentage of recovery was lower than with the acid extraction, it was enough for the quantitation of torasemide at the usual levels found in urine samples.

The chromatographic method with amperometric detection developed present some advantages over other reported methods: the low retention time for the diuretic, as well as the low detection limit achieved. The compound is eluted in less than 6 min, while other authors[9-12] have reported methods ranging from 10 to 25 min. The determination limit achieved for torasemide in

urine with the HPLC-EC method developed (8 ng/mL with an injection volume of 20 μL) is lower than the one achieved with the HPLC-UV system (10 ng/mL with an injection volume of 50 μL), reported by March et al.[12], and makes possible the application of the method developed to the monitoring of low levels of torasemide for pharmacokinetic and pharmacodynamic purpouses, as well as for doping control.

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INFLUENCE OF SEVERAL PARAMETERS ON EFFICIENCY AND PEAK SHAPE IN THE MICELLAR LIQUID CHROMATOGRAPHY OF POLYNUCLEAR AROMATIC HYDROCARBONS

M. A. Rodríguez Delgado, M. J. Sánchez, V. González,
F. García Montelongo

Department of Analytical Chemistry,
Food Science and Toxicology
University of La Laguna
38071-La Laguna, Spain

ABSTRACT

The influence of different factors on efficiency and peak asymmetry for a wide group of PAHs in micellar liquid chromatography (MLC) is studied. One can see that efficiency increases as the concentration of surfactant in the mobile phase increases. The addition of 2-propanol increases efficiency up to a 15%. The simultaneous effect of temperature and flow rate show the influence of hydrophobicity of solutes on efficiency. H-μ plots show a different behavior of PAHs in MLC as compared to RP-HPLC.

INTRODUCTION

The development of Micellar Liquid Chromatography (MLC) has experienced a solid growth in recent years. Since the first report by Armstrong and Henry[1], most of the work has been focused on exploring the advantages of

this technique.[2-12] It offers the advantages of the feasibility of optimizing parameters due to the linear retention behavior, the capability of simultaneous separation of ionic and nonionic compounds, performing gradient elution without a need for column reequilibration, gradient compatibility with electrochemical detection, direct injection of biological fluid (serum and/or plasma), usefulness in quantitative structure-biological activity relationship (QSAR studies), etc. These capabilities combined with the low toxicity and low cost of these mobile phases provide compelling reasons to consider MLC as a strong alternative to reverse phase liquid chromatography (RP-HPLC) with hydro-organic mobile phases.

The most noticeable drawback of MLC are, on the one hand, the slow resistance to mass transfer from the surfactant-modified stationary phase which leads to a poor efficiency and, on the other hand, the weak solvent strength of purely micellar mobile phases. Dorsey et al.[13] have reported that the addition of a small amount of propanol and raising the temperature to 40°C would improve the column efficiency. Yarmchuk et al.[14] have reported that poor efficiency is due to slow mass transfer of solute from micelles as well as from stationary phase to the bulk solvent. Armstrong et al.[15] noted that poor efficiency is due to a poor transfer from the surfactant modified stationary phase. All these authors suggested that chromatographic efficiency is improved with the addition of organic modifier until a determined percentage, which does not bring the system closer to a hydro-organic system, and increasing the operating temperature.

When using micellar phases in RP-HPLC the solute may interact with both the stationary and the mobile phases and thus partition equilibria are established between water and stationary phase (P_{sw}), between water and micelles (P_{mw}) and between micelles and stationary phases (P_{sm}). It is also commonly understood that only two of these three partition coefficients are necessary for a complete description of the solutes in the micelles. However, the retention mechanisms in MLC are not yet well known. Surfactant molecules adsorb on bonded stationary phases, altering the properties of the stationary phases. The addition of an alcohol modifies the surfactant adsorption, producing an alteration in the selectivity of the stationary phase as a function of the percentage and type of alcoholic modifier.[11]

Although the effect of organic modifiers on chromatographic efficiency[13] and the effect of temperature on selectivity separation[16] in MLC has been studied, the simultaneous effect of the presence of organic modifier and of temperature on efficiency and selectivity of separation has not been reported yet.

In this paper we report the results of the effect of adding alcohols to micellar eluents on the chromatographic efficiency of a series of PAHs of environmental concern. In this way the effect of the nature and percentage of alcohol as well as the influence of the temperature on the chromatographic efficiency in MLC are studied with the aim of developing separations with optimum chromatographic efficiencies in the presence of micelles of sodium dodecyl sulfate.

EXPERIMENTAL

Apparatus

All high-performance liquid chromatographic measurements were made with a Waters 600 Multisolvent Delivery System, equipped with a U6K sample injector, and a Waters Lambda-Max 481 LC variable wavelength spectrophotometric detector operating at 254 nm. Data collection and processing were provided by a Baseline 810 Waters Chromatography Workstation. The analytical column was a Waters Nova-Pak C_{18}, 150 x 3.9 mm i.d., 4 μm particle diameter. A silica precolumn was used to saturate the mobile phase with silicate to protect the analytical column and to avoid hydrolysis of the bonded stationary phase. The analytical column and the mobile phase reservoir were water-jacketed and temperature controlled with a circulating bath.

Reagents

The surfactant sodium dodecylsulfate (SDS) was of electrophoresis grade obtained from Aldrich and used as received. Methanol (MeOH), ethanol, 2-propanol (PrOH) and n-butanol (BuOH) were Merck, pro analysi products. Naphthalene (1), acenaphthylene (2), fluorene (3), anthracene (4), phenanthrene (5), 9-methylanthracene (6), fluoranthene (7), pyrene (8), chrysene (9), benzo[*b*]fluoranthene (10), benzo[*a*]pyrene (11) and dibenz[*ac*]anthracene (12) were Aldrich products. Numbers identify the compound in tables and figures.

Procedure

The appropriate weight of SDS was dissolved in Milli-Q (Millipore) water or in water with the desired alcohol content, and the solution filtered through

a 0.45μm nylon membrane filter (Whatman) to remove particulate matter. The mobile phase was degassed in an ultrasonic bath prior to use. Stock solutions of PAHs were prepared in ethanol and diluted with the mobile phase to obtain the desired concentration. These solutions were stored in the dark at 0°C to avoid possible degradation of PAHs.

The column temperature was controlled by immersion in a circulating water bath. Temperature was varied in the range 30°C to 60°C and adjusted to ±0.1°C. All temperature studies were performed in sequence from high to low temperature, according to other workers[17, 18] as this procedure eliminates solvent entrapment in the stationary phase. For chromatographic measurements the column was equilibrated to the desired temperature with at least 100 column volumes of micellar mobile phase until the detector signal was constant. Additionally, the mobile phase reservoir was thermostated during the experiments in order to obtain proper and quick temperature equilibration, and the injection volume was of 20μL.

Chromatograms shown were obtained with different solutions obtained by dilution of a stock solution of the following composition ($\mu g.mL^{-1}$): naphthalene (0.256), acenaphthylene (0.308), fluorene (0.332), anthracene (0.356), phenanthrene (0.356), 9-methylanthracene (0.384), fluoranthene (0.404), pyrene (0.405), chrysene (0.454), benzo[*b*]fluoranthene (0.126), benzo[*a*]pyrene (0.505), dibenz[*ac*]anthracene (0.139).

RESULTS AND DISCUSSION

The most serious problem described in the published reports on micellar liquid chromatography is the loss of efficiency when compared to traditional hydroorganic mobile phases. The plots of the height equivalent to theoretical plate (H) versus reduced velocity are very useful to compare efficiencies of chromatographic systems. From the chromatogram obtained the theoretical plate number (N) can be calculated for each peak according to the following equation 1

$$N = 5.54 \left(\frac{t_r}{w_{1/2}} \right)^2 \qquad (1)$$

where t_r is retention time and $w_{1/2}$ is the peak width at half peak height. However, this equation is highly inaccurate for skewed peaks.[19]

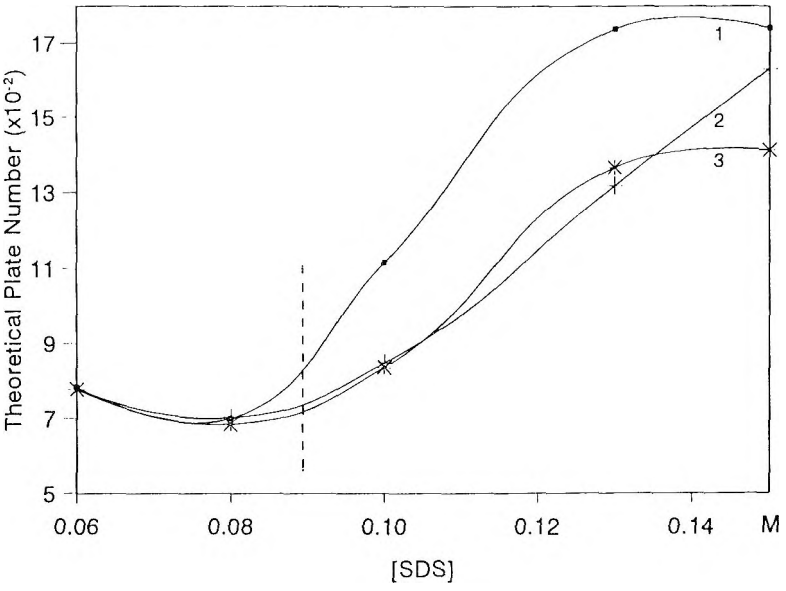

Figure 1. Variation of efficiency as a function of the concentration of SDS in the mobile phase for: 1) fluorene, 2) chrysene and 3) dibenz[*ac*]anthracene.

To determine column efficiency using information regarding peak asymmetry, the equation described by Foley and Dorsey[20] gives a more accurate measurement of column efficiency as the accuracy and precision values determined were a factor of two better than those found by Barber and Carr[21] equation. Thus Equation 2 [20]

$$N = \frac{41.7(t_r / w_{1/2})^2}{A/B + 1.25} \qquad (2)$$

was used in this work, where A/B is the asymmetry ratio measured at 10% peak height. The height equivalent to theoretical plate (H) can be calculated from the ratio H=L/N.

Effect of Concentration of Surfactant

Figure 1 shows the plot of the theoretical plate number (N) vs SDS concentration for three selected PAHs of different hydrophobicity. One can

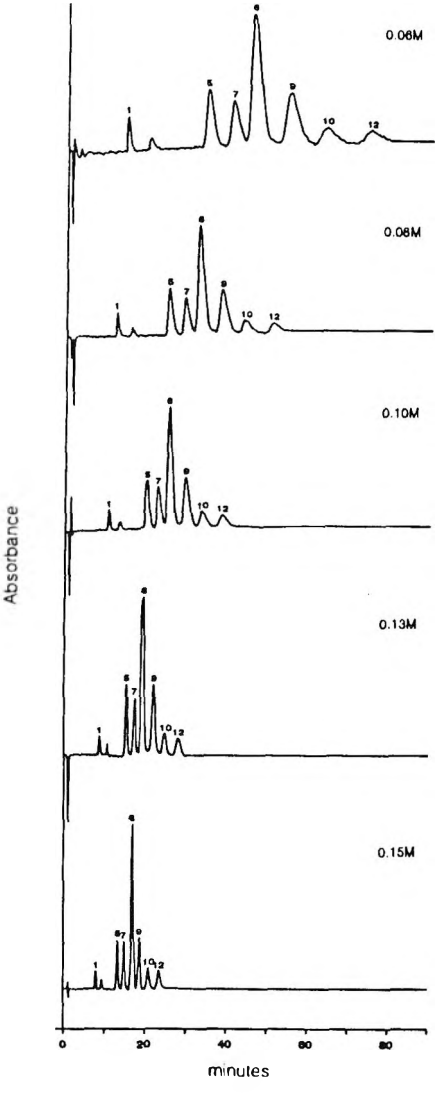

Figure 2. Chromatograms of a mixture of PAHs with different micellar mobile phases containing XM SDS + 15% 2-propanol. Flow rate 1.0 mL min^{-1} and column temperature 60°C.

observe that N increases drastically when increasing the concentration of the surfactant in the micellar mobile phase. This increment of efficiency is more pronounced for the less hydrophobic solute fluorene than for the more hydrophobic chrysene and dibenz[*ac*]anthracene. The degree of retention was large enough to keep the influence of extracolumn band broadening at an insignificant level, since k' values were greater than 8 for any solute at all concentrations. Figure 2 presents typical chromatograms for a mixture of seven PAHs to show the influence of the concentration of SDS on the efficiency of separation. One can observe that better separations can be obtained with high concentrations of SDS, showing a considerable change in the efficiency up to 0.13M SDS. From 0.13M to 0.15M SDS the variation in the effciency is minimum and only a slight improvement in the analysis time exists.

Effect of Type of Alcohol

The addition of small amounts of alcohols to MLC systems has been shown to improve column efficiencies significantly, especially when measured with hydrophobic analytes.[11,12] The most common reasoning is that the addition of alcohols reduces the loading of surfactant in the stationary phase which leads to improvements in the mass transfer and wetting of the stationary phase.[13] The concentration of alcohol, however, must not be very high because it might reduce the role of micelles and bring the system closer to a hydroorganic system. Notwithstanding, it has been described that percentages of 2-propanol up to ≈20% mantain the integrity of micelles, although in this case mixed micelles are formed.[22,23]

Table 1 shows plate counts and asymmetries for fluorene, chrysene and dibenz[*ac*]anthracene in a 0.15M SDS mobile phase, with 3% alcohol modifier. An improvement in both peak symmetry and efficiency can be observed from methanol to 2-propanol for the less hydrophobic fluorene and chrysene, while for the most hydrophobic dibenz[*ac*]anthracene the best symmetry is obtained using butanol as the modifier, even though the variations in efficiency are smaller. Thus, the value of efficiency is directly related to hydrophobicity of solutes. From Table I, one can deduce that higher efficiency will be obtained with an alcohol of intermediate chain such as 2-propanol.

Figure 3 shows an increase in efficiency when increasing the percentage of 2-propanol in the mobile phase up to 15%, from which an almost constant plate count is seen, and only a reduction in the analysis time exists.

To illustrate the efficiencies attainable with micellar mobile phases a

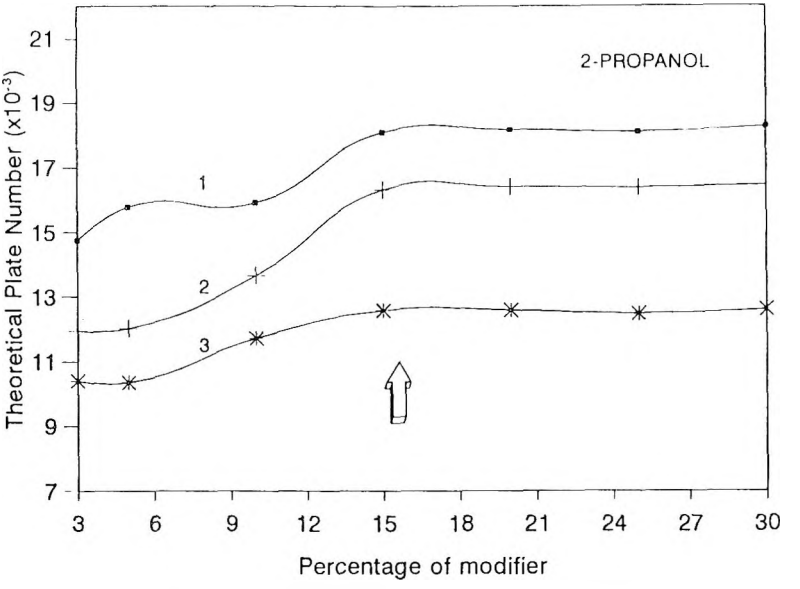

Figure 3. Variation of efficiency as a function of the percentage of 2-propanol in the mobile phases for: 1) fluorene, 2) chrysene and 3) dibenz[*ac*]anthracene.

Table 1

Variation of Efficiency and Asymmetry with Organic Modifiera

Solute	Organic Modifier					
	Methanol		2-Propanol		Butanol	
	N	A/B	N	A/B	N	A/B
Fluorene	890	1.36	1112	1.09	552	1.12
Chrysene	940	1.12	1195	1.03	644	1.32
Dibenz[ac]-anthracene	1133	1.66	1209	1.60	1092	1.17

Mobile phase 0.15M SDS with 3% of organic modifier, 30^{0}C

series of chromatograms were run. Figure 4 is the separation of a mixture of seven PAHs using a 0.15M SDS mobile phase with variable percentage of 2-propanol. The separation efficiency improves substantially as the percentage

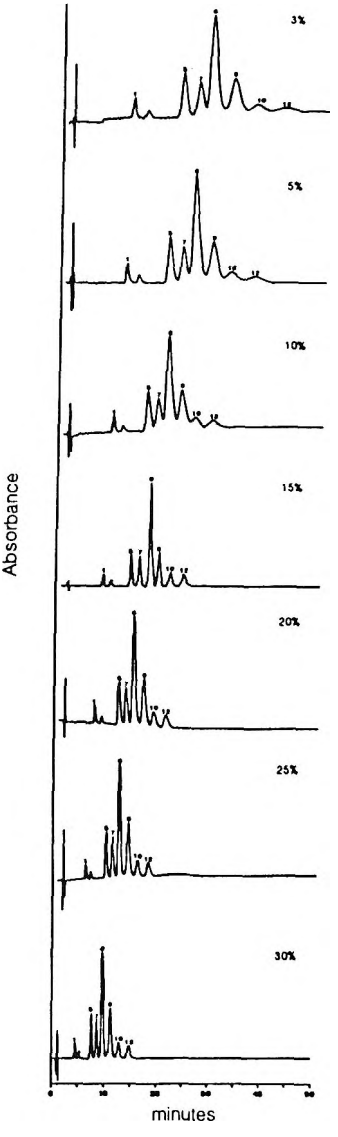

Figure 4. Chromatograms of a mixture of PAHs with a mobile phase of 0.15M SDS and variable percentages of 2-propanol. Flow rate 1.0 mL min^{-1} and column temperature 60°C.

of 2-propanol in the mobile phase increases, with a maximum at 15% 2-propanol. From this point up only slight improvements are obtained, while analysis time is reduced.

Effect of Temperature

An important parameter which can be adjusted to improve mass transfer characteristics is the temperature, because increasing temperature diminishes the viscosity of micellar mobile phases and improves mass transfer. We chose to investigate the influence of temperature on column efficiency across a wide range of eluent flow rates. The experimental data are used to construct H-μ plots, where H is the height equivalent to a theoretical plate and μ is the eluent linear velocity determined as the column length divided by the void time. Since the proper method for determination of the void time is not correct enough, particularly when a range of temperature is to be used, the linear velocity has been substituted for the flow rate ($mL.min^{-1}$).

Figure 5 shows plots of plate height versus flow rate for PAHs of different molecular weight at different temperatures. Contrary to what occurs in RP-HPLC, where an increase in column temperature leads to higher H values regardless of eluent flow rate, MLC is characterized by a general diminution of H as temperature increases. However, a different behavior exists as a function of the flow rate depending on the type of solute. For the lower molecular weight acenaphthylene and fluorene, Figures 2a and 2b show that H increases as flow rate increases up to 0.5 $mL.min^{-1}$, with a reversal order in the magnitude of H as temperature goes from 50 to 60°C. Greater increases in H from 1 $mL.min^{-1}$ on are observed at lower temperatures for these compounds. For higher molecular weight compounds such as anthracene and chrysene, Figs. 5c and 5d, this increase in plate height at lower temperatures is even sharper, and no reversal in H values exists at higher temperatures.

Table 2 shows the variation in asymmetry and plate counts for several PAHs using a 0.15M SDS+15% 2-propanol as mobile phase, as temperature is changed from 30 to 60°C. While the efficiency increases slightly, an improvement of peak shape is seen at higher temperatures.

Figure 6 illustrates the separation of nine PAHs using a mobile phase of 0.15M SDS + 15% 2-propanol at a constant temperature of 60°C. It is possible to see that efficiency increase from a flow rate of 0.5 $mL.min^{-1}$ to 1.0$mL.min^{-1}$, and that the resolution of separation as well as the analysis time is satisfactory. At flow rates higher than 1.5$mL\ min^{-1}$ the resolution is lost for the less hydrophobic solutes, being even much worse at the highest flow rates.

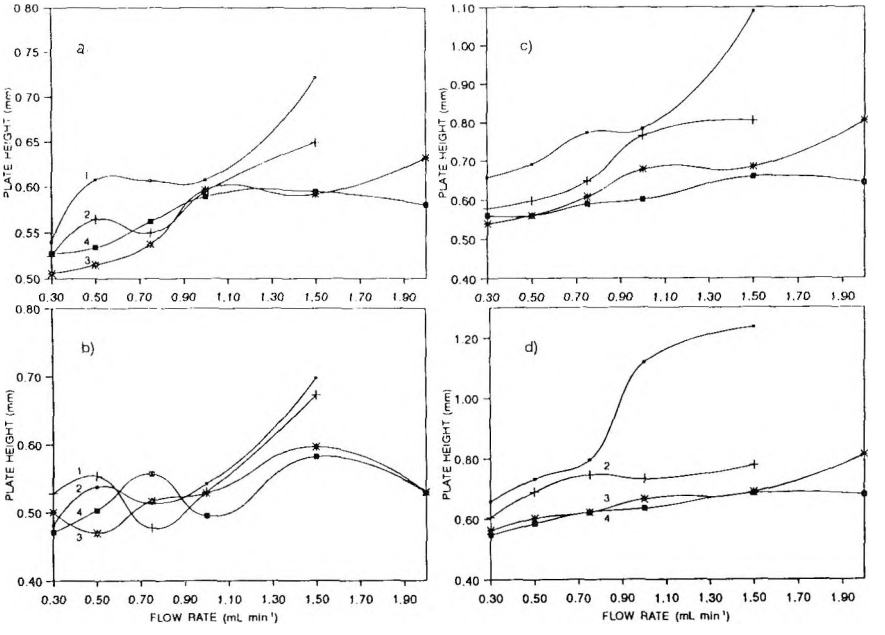

Figure 5. Experimental H,μ curves for: a) acenaphthylene, b) fluorene, c) anthracene, d) chrysene at different temperatures: 1) 30°C, 2) 40°C, 3) 50°C and 4) 60°C.

Table 2

Variation of Efficiency andAsymmetry with Temperature*

	Temperature (^{0}C)							
Solute	**30**		**40**		**50**		**60**	
	N	**A/B**	**N**	**A/B**	**N**	**A/B**	**N**	**A/B**
Fluorene	1846	1.03	1889	1.02	1893	1.04	2021	1.03
Chrysene	1408	1.24	1367	1.12	1503	1.19	1577	1.17
Dibenz[ac]-anthracene	1290	1.20	1355	1.18	1281	1.19	1411	1.02

*Mobile phase 0.15M SDS with 15% of 2-propanol

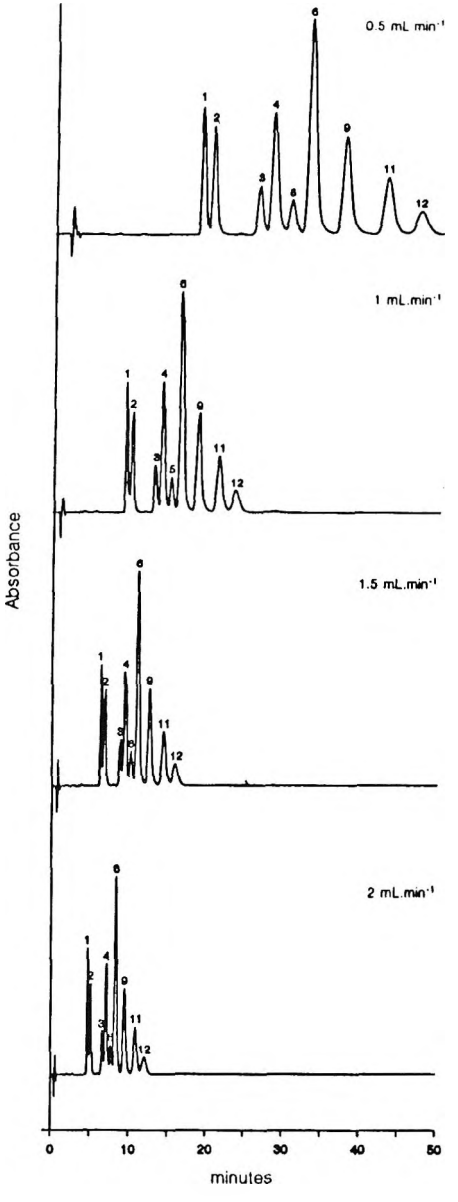

Figure 6. Chromatograms of a mixture of PAHs at different flow rates. Column temperature was 60°C and all mobile phases were modified with 15% 2-propanol.

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AN EASY WAY TO ENHANCE ABSORBANCE DETECTION SENSITIVITY OF WATERS QUANTA-4000 CAPILLARY ELECTROPHORESIS SYSTEM

Nebojsa M. Djordjevic,* Kieran Ryan

Sandoz Pharma
Preclinical Research
Bldg. 882, Muttenz 1,
CH 4132, Switzerland

ABSTRACT

Capillary electrophoresis (CE) has established itself as a powerful and widely utilized technique for routine analytical separations, characterized by short run times and high efficiencies. A major drawback to CE is its low concentration sensitivity. Because total system volume is generally only a few μl, the detector flow cell should have a small volume, which imposes a limit on detection sensitivities. In this study, we report a simple method for creating a region of extended path length for absorbance detection in capillary electrophoresis. Up to a six-fold gain in sensitivity, compared to on-column detection, was accomplished when the sleeve-cell was utilized at the detection point.

INTRODUCTION

Detection of the solute zone in CE can be achieved either while it is migrating through the capillary (on-column detection), or as it elutes from the capillary (off-column detection). In off-column detection, the detector region usually

contributes to band broadening, while in on-column detection the detection cell is part of the separation capillary. The design of the detector cell is of great importance in ensuring high sensitivity and low background noise.

Several attempts have been made to extend the path length for UV detection in capillary separation techniques by changing the design of the detection cell or by utilizing optical fiber bundles.[1] Chervet et al.[2] and Liu et al.[3] have demonstrated the usefulness of a Z-shaped longitudinal capillary flow cell for micro separation techniques. Another possibility to increase the effective path length across the capillary is the use of an on-column multireflection absorbance cell.[4] Tsuda et al.[5] have evaluated the use of a rectangular glass capillary. Liu and Dasgupta[6] have reported a three-fold gain in sensitivity, without significant loss in efficiency (in CE), by connecting a large diameter capillary to a separation column at the measurement point. Recently introduced capillaries[7,8] with a bubble blown at the detection point (bubble cell) offer a unique approach to extending the optical path. In-depth reviews about detection techniques in CE are given elsewhere.[9-11]

In this study, we report a six-fold increase in detection sensitivity, compared to on-column detection, which was realized when a sleeve capillary (i.d. 220 μm) was utilized at the detection point. The loss of separation efficiency, about 50%, could be minimized by optimizing separation parameters and by improving the design of the sleeve cell.

EXPERIMENTAL

The capillary electrophoresis system used was the model Quanta 4000 from Waters Chromatography Division, Millipore Corporation, Milford, MA, USA.

The capillary columns utilized in this work were an AccuSep™ (75 μm i.d., 350 μm o.d., total length 60 cm) from Waters Chromatography Division, Millipore Corporation, Milford, MA, USA, and an analytical column 75 μm i.d., and 200 μm o.d. from Scientific Glass Engineering (SGE), Weiterstadt, Germany. The capillary used as a sleeve had a 220 μm i.d. and 350 μm o.d. The polyimide coating was removed (ca. 3 mm) from the sleeve capillary to create a detection window. The sleeve cell arrangement is depicted in Figure 1. The SGE analytical column was divided into two parts. The longer section, labeled as A in Figure 1 (approximately 45 cm) was used as the separation column. One end was immersed into a buffer solution and the other end was inserted into the sleeve cell (B) up to the detector window. The other piece (C) from the 75 μm i.d. column (7.5 cm long) was inserted into the other side of the sleeve with the free end immersed in buffer. The

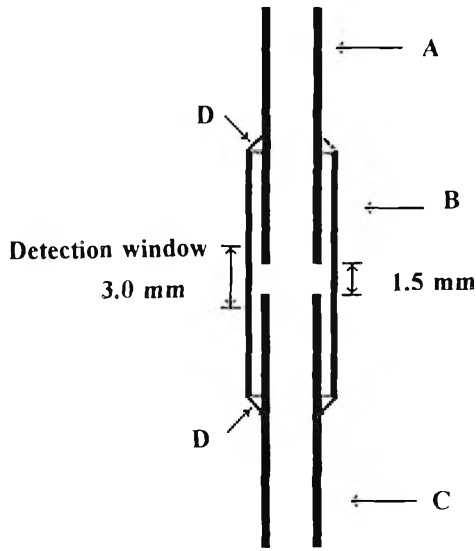

Figure 1. Experimental arrangement of the sleeve-cell (as described in the experimental section).

distance between the two pieces (A and C) in the sleeve capillary (was approximately 1.5 mm). A detector window already built in the capillary holder for Quanta 4000 is approximately 3 mm. The capillaries A and C were sealed (point D in Figure 1) to the sleeve cell with a polyimide sealing resin, Alltech Associates, Inc., Deerfield, IL, USA. Throughout this work, hydrodynamic sample injection (by siphoning action) was utilized. The running buffer was 20 mM phosphate buffer at pH 7. The vitamin samples (thiamine hydrochloride, nicotinamide and nicotinic acid) were from Hewlett-Packard GmBH, Waldbrunn, Germany.

RESULTS AND DISCUSSION

In Figure 2 are depicted electropherograms obtained, on the one hand utilizing on-column detection with AccuSep^{TM} column, and on the other the SGE column combined into the sleeve-cell (as described in the experimental section). A six-fold increase in normalized peak area (peak area/migration time) was observed when the sleeve-cell was utilized. A 50 % plate loss in going from a 75 μm on-column detection to a 220 μm i.d. sleeve-cell was noted. By mass balance theory, there should be peak compression when the solute z one moves from 75 μm i.d into a 220

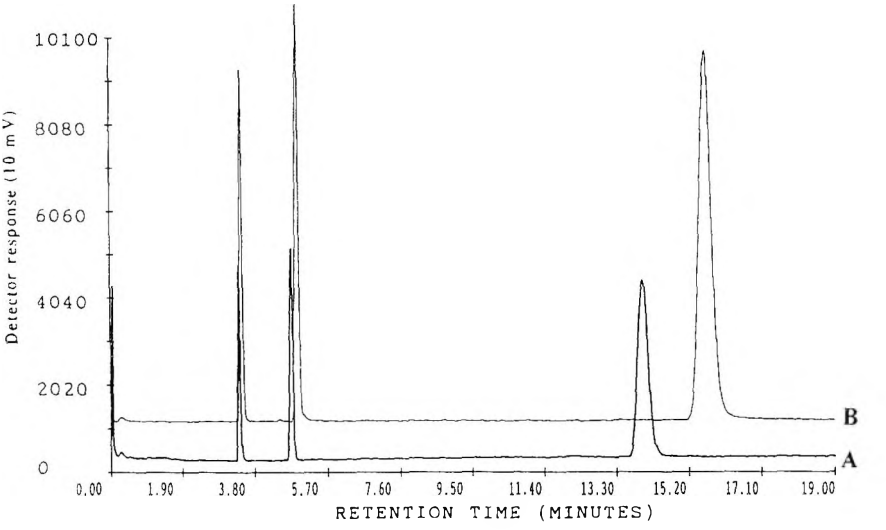

Figure 2. Electropherograms of vitamin sample (thiamine hydrochloride, nicotinamide and nicotinic acid) obtained with AccuSep™ column (A), and SGE column combined into the sleeve-cell (B). Hydrodynamic sample injection for 7 sec, Voltage 15 kV, Detection wavelength 214 nm.

μm i.d. column, where the sample zone expands rapidly to fill the increased volume.[8] This is true if diffusion can be ignored. In the sleeve-cell, the sample was present in the 1.5 mm gap long enough to allow diffusion to take place. We were anticipating this peak broadening, and because of that, we did not extend the gap between capillaries to the full size of the detector slitwidth (3 mm). Already 1.5 mm gap was too wide and longitudinal solute diffusion in a 220 μm i.d. capillary degraded peak shape and efficiency. This effect is more pronounced for a component with a long migration time (peak 3 in Figure 2). Kuhr[12] investigated sample transfer, at different ionic strengths, across a 50-200 μm gap (without boundary) between two capillaries. There was no significant distortion in the sample zone when the gap between the capillaries was 150 μm or less. As mentioned earlier, the sample zone slows down in the sleeve cell, which explains the difference in migration times obtained in the 75 μm i.d. AccuSep™ column compared with the 75 μm i.d. combined with the 220 i.d. μm sleeve cell.

The asymmetry factors (measure of a peak shape distortion) for thiamine, nicotinamide and nicotinic acid on AccuSep™ column were 3.78, 0.45 and 0.35, respectively. The same solutes had asymmetry factors of 1.26, 1.00 and 1.34 on the

SGE column integrated into the sleeve-cell. This proves that the sample zone does slow down and that peak compression occurrs when the solute moves from the 75 μm column into the 220 μm region. In the cell, the gap between the capillaries is long (1.5 mm), and the internal diameter of the cell is large; the diffusion leads to peak broadening with an improved peak symmetry but reduced efficiency.

This cell design has to be optimized. The use of sleeve capillaries more than three times in diameter than the principal analytical column does not appear to be very practical for CE. However, for capillary analysis systems where the above degree of plate loss is tolerable, this approach may still be attractive for improving detection sensitivity. The heat effect should not be neglected. In systems where the detector itself is thermostated, the results should be better.

CONCLUSION

When the sleeve-cell was utilized at the detection point, a six-fold gain in sensitivity, compared to on-column detection, was accomplished. By optimizing the gap between the capillaries and the detector slit-width, it should be possible to minimize the loss of efficiency. We envision that the sleeve-cell arrangement can substantially increase sensitivity, especially in capillary gel electrophoresis (with cross-linked polymers), where the polyacrylamide gel interferes with the UV absorbance detection of proteins at 214 nm. Furthermore, the cell arrangement described can be produced for any type of CE instruments with any desired combination of i.d. ratio (analytical column to sleeve cell). There is almost no additional cost in producing the cell.

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SIMULTANEOUS DETERMINATION OF METSULFURON-METHYL, CHLORSULFURON AND BENSULFURON-METHYL IN VARIOUS FORMULATIONS OF SULFONYLUREA HERBICIDES BY HPLC-UV DETECTION

Hong-Zhen Lian, Wen Bin Zhang, Qin Jiang, Jin Miao

Center of Materials Analysis
Nanjing University
22 Hankou Road
Nanjing 210093, P. R. China

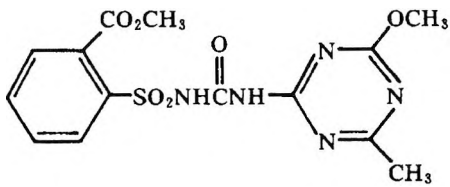
ABSTRACT

A reliable method is proposed for simultaneous determination of sulfonylureas in various formulation of commercial products by high performance liquid chromatography. HPLC was achieved on a reverse phase C_{18} column by isocratic elution using water (pH 4.3 with acetic acid) / methanol, and detection was by UV absorption at a wavelength of 254nm. This method has been applied for the quality control of sulfonylurea herbicides in the laboratories of agricultural chemical plants.

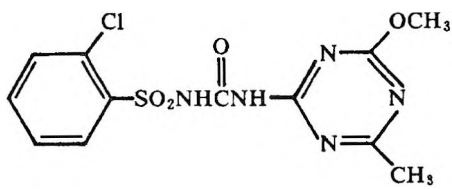
INTRODUCTION

Sulfonylurea herbicides were first introduced by DuPont in 1982, and since then many new sulfonylureas have been developed for controlling broad spectrum broadleaf weeds. Of these herbicides, the most common are

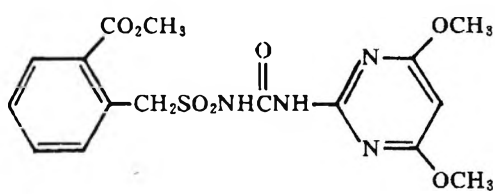
metsulfuron-methyl, chlorsulfuron and bensulfuron-methyl, which have the following structural formulae:



metsulfuron—methyl (M)



chlorsulfuron (C)



bensulfuron—methyl (B)

The first two were mainly applied to cereal grain fields, and the last one to rice grain fields. In recent years, these herbicides were widely used for their high herbicidal activity at extremely low application rate.[1-5] Methods developed for sulfonylureas analysis include bioassay,[6,7] enzyme immunoassay,[8,9] gas chromatography with derivatization,[10-15] high performance liquid chromatography-photoconductivity detection[16-20] and UV detection,[21-25]

packed capillary liquid chromatography coupled with fast atom bombardment mass spectrometry.[26] But most of these aimed at determination of single sulfonylurea in a herbicide, or residue level of these sulfonylureas and their metabolites in soil, water, plant materials such as grain and straw after application.

These herbicides may exist as sulfonylurea or its sodium salt. The final products from manufacturers can be made into emulsifiable concentrate, wettable powder, or many other formulations. Metsulfuron-methyl, chlorsulfuron and bensulfuron-methyl can not only be used independently but also be used after mixing with each other, or with other kind of herbicides such as chlortoluron, benthiocarb, etc. according to practical needs. In order to control the product quality, therefore, it is essential to develop an efficient and accurate method for separation and quantitative analysis of sulfonylureas in various formulation of products. The results from the determination by high performance liquid chromatography with UV detection are satisfactory.

EXPERIMENTAL

Apparatus

The liquid chromatograph was a Varian LC 5060 with a UV-100 spectrophotometric detector, a VISTA-401 data system (Varian Instrument division, Walnut Creek, CA, U. S. A.), and a Yokogawa Hokuskin Electric Type 3066 pen recorder (Sino-Japanese No. 4 Meter Factory of Sichuan, Chongging, P. R. C.). Chromatograms were recorded by monitoring absorption at 254nm. The analytical column was a stainless steel tube (15cmX4mm I. D.) packed with E. Merck LiChrosorb RP-18, 5μm (Shanghai Institute of Materia Medica, The Chinese Academy of Sciences, Shanghai, P. R. C.). The column temperature was held at 30^0 C to maintain the solubility of sulfonylurea in the mobile phase.

Reagents and Materials

Methanol was HPLC grade (Institute for Fine Chemical Engineering of Huaiyin Plastic Product Factory, Huaiyin, P. R. C., WHO Collaborating Center for Research in Human Reproduction, Tianjing, P. R. C.). Glacical acetic acid was Analytical grade (Nanjing Chemical Reagent Factory, Nanjing, P. R. C.). Water was distilled twice. Methanol and water were purified with the Millipore Milli-Q system (Bedford, MA, U. S. A.). The pH value of aqueous

phase was adjusted to 4.3 by using glacical acetic acid. The mobile phase was water (pH 4.3 with acetic acid) -methanol (45/55 V/V) at a flow rate of 1.0mL/min and the injection loop 10μl.

All samples and reference substances of sulfonylurea herbicide were obtained from various manufacturers. A mixture of metsulfuron-methyl, chlorsulfuron and bensulfuron-methyl reference substances was used as a standard solution to optimize the chromatographic separation and to allow the qualitative analysis of the chromatograms of the real samples. The stock solution of individual sulfonylurea was prepared by taking 40mg of each reference substance and dissolving it in 100mL of methanol in a volumetric flask and filtering it on cellulose 0.5μm filter (Millipore) by means of syringe before analysis. Standards at concentrations of 0.02~0.40mg/mL of sulfonylureas were prepared by serial dilutions of these stock solutions with methanol and used for calibration purpose.

Procedure

Technical powder - A 40mg of sample was weighed accurately into a 100mL volumetric flask. After adding methanol to mark and shaking vigorously, the sample solution was filtered through a membrane with 0.5μm micropore. 10μl of the filtered solution was injected to the reverse phase C_{18} column and analyzed under the conditions represented above.

Emulsifiable concentration - An accurately measured volume of liquid sample, equivalent to about 40mg of sulfonylurea, was transferred to a 100mL volumetric flask, diluted to volume with methanol, and mixed. This solution was filtered, injected to the column as described above.

Wettable powder - A 200mg of powder was weighed, transferred to a 100mL volumetric flask, mixed with about 80mL of methanol, and sonicated for about 15min . After bringing to volume with methanol and mixing, the resulting slurry was centrifuged for 10 min and the supernatant liquid was also analyzed as described above.

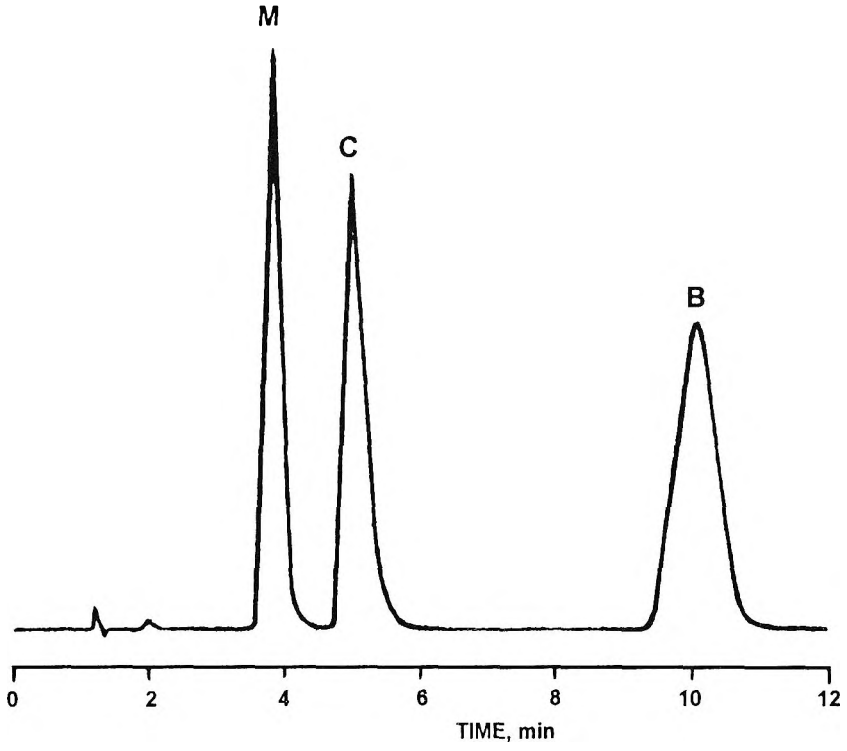


Figure 1. Chromatogram of standard solution containing metsulfuron-methyl (M), chlorsulfuron (C) and bensulfuron-methyl (B).

RESULTS AND DISCUSSION

Chromatogram and Calibration Curve

An HPLC chromatogram demonstrating the separation of three sulfonylureas is shown in Figure 1. As can be seen the order of the retention is metsulfuron-methyl < chlorsulfuron < bensulfuron-methyl, and the resolution is satisfactory for every mixture component. This figure will be the reference chromatogram for the subsequent HPLC analysis of sulfonylurea herbicides produced by various manufacturers.

The quantitation was based on a calibration by series of dilution from primary standard. Linear regression analyses of the relationship between peak area versus amount of standard were carried out within the range 0.20~4.00 μg

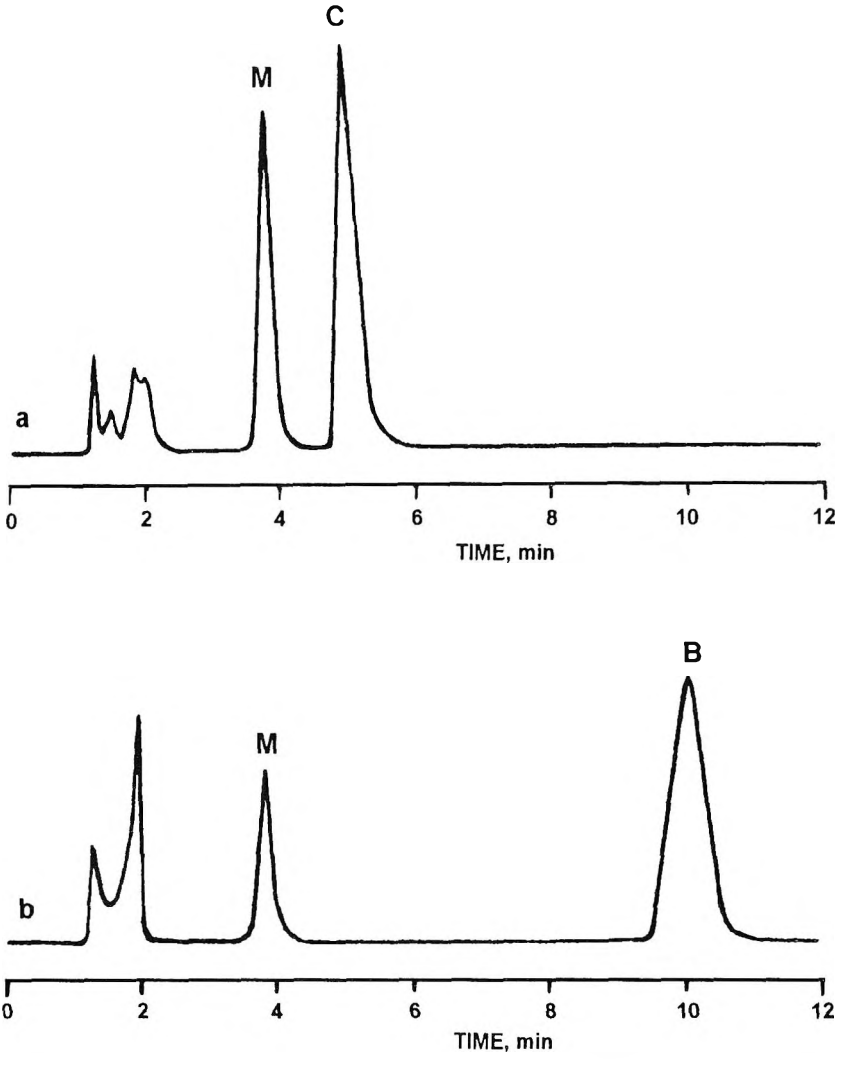


Figure 2. Chromatograms of commercial sulfonylurea herbicide products. a) Maicaoke wettable powder; b) Xindeli wettable powder (for abbreviations see legend to Figure 1).

in 10μl injection volume. The regression equations were y=1760328.30x-593.38 for metsulfuron-methyl, y=1885869.50x+1408.63 for chlorsulfuron, and y=1550125.00x+300.36 for bensulfuron-methyl, with correlation coefficients of 0.9999, 0.9999 and 0.9996 respectively, where y equaled peak area and x

equaled final standard concentration (mg/mL). The limits of detection (S/N=2) is 1.2ng, 1.5ng and 2.0ng for metsulfuron-methyl, chlorsulfuron, bensulfuron-methyl respectively.

Analysis of Commercial Samples

Typical chromatograms for metsulfuron-methyl, chlorsulfuron and bensulfuron-methyl in various sulfonylurea herbicides are given in Figure 2. In the present study, 10 random samples of commercial products were analyzed for three sulfonylureas content. The results are summarized in Table 1.

Table 1

Analysis of Sulfonylureas in Products from Several Manufacturers

			---------- $\bar{X} \pm SD$ ----------		
Herbicide products	**Form**	**Unit**	**Metsulfuron-methyl**	**Chlorsulfuron**	**Bensulfuron-methyl**
Jiahuanglong	TP	g/100g	95.53±0.47		
Luhuanglong				97.19±0.44	
Jiahuanglong	EC	g/100mL	2.50±0.07		
Luhuanglong				3.53±0.10	
Jiahuanglong	WP	g/100g	10.24±0.21		
Luhuanglong				19.98±0.39	
Luhuanglong (Sodium salt)				70.52±0.58 (Sodium salt)	
Maicaoke			6.32±0.17	8.68±0.17	
Maicaoling			4.47±0.14	15.27±0.32	
Xindeli			1.81±0.12		8.08±0.18

TP - Technical powder
EC - Emulsifiable concentrate
WP - Wettable powder
$\bar{X}$ - Mean value of 6 determinations
SD - Standard deviation

Table 2

Recovery of Sulfonylureas from Emulsifiable Concentrate and Wettable Powder

Form	Unit	Initial account $\bar{X} \pm$ SD	Amount added	Amount determined $\bar{X} \pm$ SD	Recovery %
			(Metsulfuron-methyl)		
EC	g/100mL	2.50	0.50	3.02±0.09	100.7
			3.00	5.56±0.12	101.1
WP	g/100g	10.24	2.00	12.08±0.17	98.7
			10.00	20.15±0.46	99.6
			(Chlorsulfuron)		
EC	g/100mL	3.53	0.50	4.05±0.11	100.5
			3.00	6.50±0.11	99.5
WP	g/100g	19.98	2.00	21.74±0.48	98.9
			10.00	29.55±0.52	98.6
			(Bensulfuron-methyl)		
WP		8.08	2.00	10.01±0.15	99.3
			10.00	17.85±0.28	98.7

For abbreviations see legend to Table 1

In order to estimate the efficiency of the recovery the samples were spiked with sulfonylureas. The initial amounts of metsulfuron-methyl, chlorsulfuron and bensulfuron-methyl were previously determined using standard curves. Table 2 shows the amounts added of sulfonylureas to these samples and the percentage recoveries.

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SEPARATION OF ETHOXYLATED TRIBUTYLPHENOL OLIGOMERS ON POROUS GRAPHITIC CARBON COLUMN

Veronika Németh-Kiss

Central Research Institute for Chemistry
Hungarian Academy of Sciences
P.O. Box 17
H-1525 Budapest, Hungary

ABSTRACT

Commercial ethoxylated tributylphenol oligomers were separated according to the number of ethylene oxide (EO) units by reverse phase high performance liquid chromatography on porous graphitic carbon column at different eluent concentration (methanol-water). The retention time increased with decreasing methanol concentration of the eluent and increased with the ethylene oxide unit number. Linear correlation was found between $\lg k_0'$ and fraction of EO number. Stepwise regression analysis indicated the appearance of the helical form of the oligomers.

INTRODUCTION

Nonionic surfactants generally consist of a polar chain containing some ethylene oxide (EO) units, and an apolar hydrocarbon moiety.[1,2]

Ethoxylated alkylphenols belonging to nonionic surfactants have been used in a wide variety of commercial products such as detergents,[3,4,5,6] cosmetic preparations,[4] industrial formulations,[4,6] emulsifying agents.[5,6,7]

Commercial polyethoxylated alkylphenols are manufactured by reacting

alkylphenols with an excess of ethylene oxide. Like many condensation and polymerization reactions, the final product is a complex mixture of oligomers in which the number of EO units varies over a considerable range. The distribution of ethylene oxide oligomers in a commercial product often follows the Poisson law. The length of ethylene oxide chain exerts a considerable influence on the physical and chemical properties, and even on the biodegradability in the environment.[5,8,9,10]

Normally, surfactants are discharged with waste water after application. Depending on their structure, they have a more or less toxic effect on aquatic life forms such as fish, daphnia and algae.[11]

Various chromatographic procedures have been developed for the separation of the commercial ethoxylated alkylphenols into their different oligomers.

Thin layer chromatography is of limited applicability in this area because of its poor reproducibility and lack of quantitation.[12,13] Using gas chromatography, only a limited number of oligomers are eluted from the column, even if the volatility of the sample is increased by derivatization.[14,15] High performance liquid chromatography has been shown to be a suitable technique for separating the different oligomers.[16] Most of the investigations that have been done so far, using normal phase HPLC with ultraviolet detection, the applied column types were : Spherosil silica and Kieselguhr diatomaceous earth product. With isocratic elution, the eluent was carbon-tetrachloride and a mixture of carbon-tetrachloride-i-octane in 1:1 ratio,[4] LiChrosorb-NH_2, with the gradient elution, the eluent concentration was changed from 98 % n-hexane, 2 % (1:1 n-hexane-2-propanol) to 30 % n-hexane, 70 % (1:1 n-hexane-2-propanol),[5] LiChrosorb-NH_2, with gradient elution, the eluent was a mixture of isooctane-methylene chloride-methanol and the ratio of the components was changed from 95:5:3 to 60:40:7.5[7]. Alumina, with isocratic elution, the eluent was a mixture of ethyl acetate-n-hexane, where the ethyl acetate concentration was varied from 100 % to 50 %,[18] Zorbax-CN, with gradient elution the eluent concentration was changed from 2 % to 50 % (75:25 2-methoxy-ethanol-isopropanol) in n-hexane,[19] and fluorescence detection on Zorbax-NH_2, Partisil-5PAC, with gradient elution the eluent concentration was changed from 0.1 % acetic-acid in methyl tert.-butyl ether to 0.1 % acetic-acid in acetonitrile-methanol (95:5).[3] LiChrosorb-Si60, gradient elution was used, the eluent was changed from n-hexane to ethanol-tetrahydrofuran-water (60:40:1),[20] only a few researchers used reverse phase HPLC for samples up to 9 or 10 EO units using C_{18} coated silica column; with isocratic elution the eluent was a mixture of methanol-water (60:40) and 0.1 M ammonium acetate in water in the ratio of 8:7,[11] μBondapak-C_{18} and μBondapak-NH_2, gradient elution was used, the concentration of the eluents were changed from 50 % to 85 % p-dioxane in water, from 10 % to 55 % of (55 % tetrahydrofuran in water) in (10 % tetrahydrofuran in water), from 15 % to 100 %

hexafluoroisopropanol in water[17]; LiChrospher RP-18, with gradient elution the eluents were acetonitrile-water and methanol-water containing alternatively $NaClO_4$, TFA, $TBAH_2PO_4$ as phase modifiers;[21] Porasil A(60), gradient elution was used, the eluent concentration was changed from ethyl acetate-acetic acid-water (100:32:30) to ethyl acetate.[22]

Isocratic mode with mixed solvent on silica column allows separation of oligomers up to 10 EO units; gradient programming moves the limit up to 15 EO units. For higher EO values (up to 25) a Zorbax-NH_2 column can be used either with isocratic or gradient mode.[10]

Porous graphitic carbon (PGC) is a non-polar adsorbent, which can be used in both the normal and reverse phase modes and is stable across the whole pH range from 0-14. The uniqueness of PGC compared to conventional reverse phase HPLC supports is due to its delocalized band of electrons, available for electronic interactions, especially donor-acceptor (charge transfer) interactions and direct π-electron overlap. It possesses a rigid, planar surface, which is capable of dispersion and charge-transfer interactions.[23,24] The planar surfaces of the PGC Hypercarb-S allows special stereoselectivity.[25] The retention on a carbon phase seems to be determined by how much contact is possible between a solute and carbon surface.[26,27]

The aim of this study was to determine the relationship between the molecular structure of the surfactant and its retention behaviour on porous graphitic carbon column; to separate the different oligomers of polyethoxylated alkylphenol according to the EO number. As the PGC column is available for electronic interactions, and the separation is influenced by the steric effects on its surface, these properties made us suppose that PGC column would be suitable for separating the oligomers of different ethylene oxide units.

EXPERIMENTAL

Measurements were carried out on a porous graphitic carbon (PGC) column – Shandon Hypercarb 100 x 4.6 mm I.D., partical diameter 7 μm (Shandon Scientific Ltd., Cheshire, England). The applied equipment consisted of a Gilson pump 307 (Gilson, Villiers-le-Bel, France), a Valco injector with a 20 μl sample loop (Valco Instruments Co. Inc., Houston, USA), a Biotronik UV detector BT 3030 (Wissenschaaftliche Geräte GmbH, Frankfurt, Germany) and a Hewlett Packard integrator HP 3396A (Hewlett-Packard company, Avondale, USA). The detection wavelength was 220 nm and the flow rate was 1 mL/min.

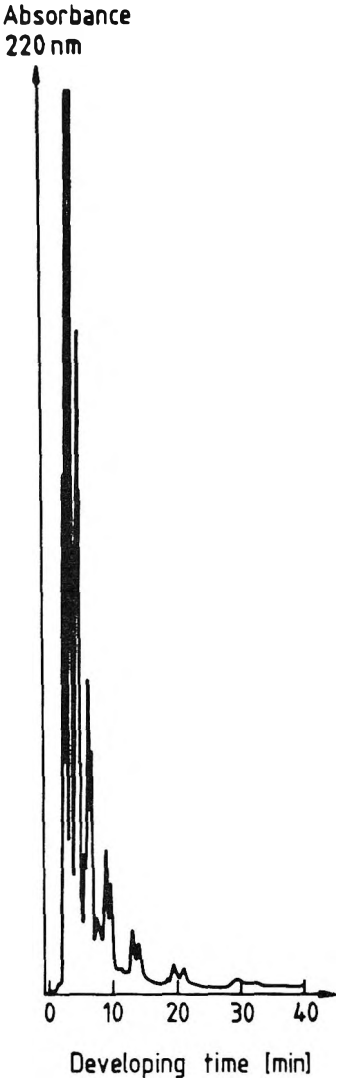

Figure 1. Chromatogram of ethoxylated tributylphenol oligomers with average EO unit number of 4 on a PGC column. Eluent 97.5 % methanol+2.5 % distillated water. Detection wavelength 220 nm. Flow rate 1 mL/min.

Eluent was water-methanol mixture, the methanol concentration varying between 80 %-97.5 % v/v in steps of 2.5 %, v/v.

Table 1.

The lgk' Values Depending on the Methanol Concentration (v/v %) and the Number of Ethylene Oxide (EO) Units.

Fraction (EO) Number	lgk[1] Methanol Concentration [Volume %] 97.5	95.0	92.5	90.0	87.5	85.0	82.5	80.0
1	-0.24	-0.16	0.01	0.03	0.12	0.21	0.31	0.43
s.d.*	0.003	0.009	0.001	0.033	0.005	0.003	0.011	0.001
2	-0.03	0.06	0.20	0.22	0.42	0.43	0.54	0.68
s.d	0.007	0.007	0.001	0.032	0.006	0.007	0.000	0.003
3	0.09	0.18	0.26	0.34	0.48	0.58	0.69	0.80
s.d.	0.011	0.006	0.000	0.024	0.008	0.009	0.001	0.004
4	0.18	0.29	0.37	0.46	0.60	0.70	0.81	0.93
s.d.	0.014	0.003	0.001	0.015	0.006	0.009	0.007	0.005
5	0.33	0.43	0.52	0.61	0.74	0.84	0.95	1.07
s.d.	0.019	0.003	0.018	0.006	0.005	0.009	0.011	0.004
6	0.50	0.60	0.67	0.78	0.90	1.01	1.12	1.24
s.d.	0.024	0.002	0.001	0.002	0.004	0.010	0.013	0.003
7	0.67	0.76	0.84	0.95	1.07	1.17	1.28	1.41
s.d.	0.028	0.002	0.001	0.007	0.003	0.011	0.014	0.003
8	0.84	0.94	1.01	1.12	1.24	1.34	1.45	1.58
s.d.	0.033	0.003	0.001	0.011	0.003	0.012	0.012	0.003
9	1.02	1.11	1.18	1.30	1.41	1.51	1.63	1.75
s.d.	0.037	0.002	0.002	0.014	0.003	0.012	0.009	0.006
10	1.20	1.29	1.36	1.47	1.58	1.68	1.81	1.94
s.d.	0.042	0.002	0.002	0.014	0.004	0.017	0.011	0.010
11	1.38	1.47	1.53	1.64	1.76	1.87		
s.d.	0.046	0.008	0.000	0.009	0.010	0.010		

*s.d. Standard Deviation

The sample was a commercial ethoxylated tributylphenol oligomer (Hoechst, Frankfurt, Germany). That surfactant was a mixture of ethylene oxide (EO) oligomers, its average number of EO units was 4 per molecule. The hydrophilic moiety (tributylphenol) had isomers depending on the position of butyl groups. The sample was a methanolic solution with 0.1 mg/1 mL methanol concentration. The experiments were carried out at room temperature (22-24 °C), and 3-4 paralell

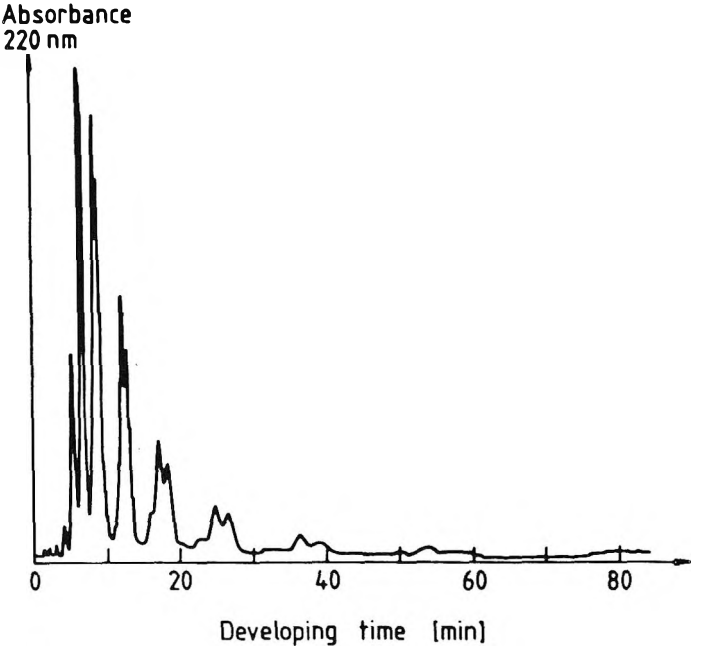

Figure 2. Chromatogram of ethoxylated tributylphenol oligomers with average EO unit number of 4 on a PGC column. Eluent 85 % methanol+15 % distillated water. Detection wavelength 220 nm. Flow rate 1 mL/min.

measurements were made. Retention time of 1 % $NaNO_3$ solution was consider as dead time.

Linear correlations were calculated between the logarithm of the capacity factors and the concentration of the methanol in the eluent :

$$\lg k' = \lg k'_0 + b \cdot C \qquad (1)$$

where k' is the actual capacity factor of an ethoxylated tributylphenol oligomer at a given methanol concentration in the eluent, k_o' is the theoretical capacity factor of an ethoxylated tributylphenol oligomer at 0 % (v/v) methanol (100 % (v/v) distillated water) concentration, b is the change in the logarithm of capacity factor caused by a 1 % (v/v) change in methanol concentration in the eluent (related to the surface area of the solute in contact with the stationary phase), C is the concentration (v/v %) of methanol in the eluent.

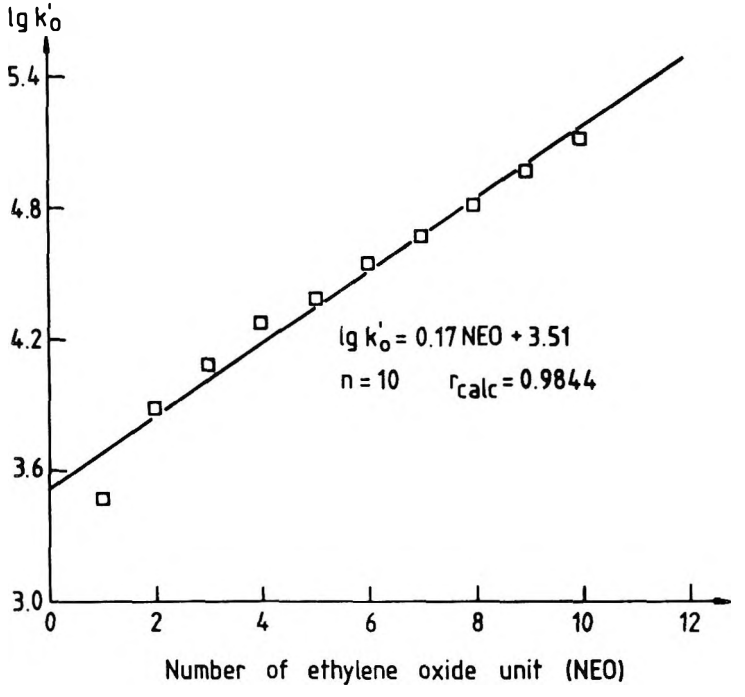

Figure 3. Relationship between the variable lgk_o' and the number of ethylene oxide units (NEO).

To determine the dependence of the retention on the number of ethylene oxide units per molecule and on the eluent concentration, five stepwise regression analysis were calculated. Linear correlation was calculated between lgk_0' and the number of ethylene oxide units (NEO) :

$$lgk_0' = BNEO + C \quad (2)$$

and between lgk_0' and lgNEO :

$$lgk_0' = BlgNEO + C \quad (3)$$

Logarithmic and quadratic correlations were also applied between the contact surface area (b) and lgk_0' :

$$b = A(lgk_0')^2 + Blgk_0' + C \quad (4)$$

between the contact surface area (b) and the number of ethylene oxide units (NEO) :

$$b=ANEO^2+BNEO+C \quad (5)$$

and between b and lgNEO :

$$b=AlgNEO^2+BlgNEO+C \quad (6)$$

Table 2

Relationship Between the Number of Ethylene Oxide (EO) Units, lgk_0' and b Values

Fraction (EO) Number	lgk_0'	$-b\cdot10^2$	$s_b\cdot10^3$	r_{calc}
1	3.46	3.86	1.47	0.9942
2	3.89	4.06	1.52	0.9944
3	4.07	4.13	1.04	0.9975
4	4.29	4.25	0.94	0.9980
5	4.38	4.19	1.08	0.9974
6	4.54	4.18	1.19	0.9968
7	4.67	4.14	1.31	0.9960
8	4.82	4.11	1.45	0.9951
9	4.96	4.07	1.58	0.9940
10	5.12	4.05	1.72	0.9929
11	4.99	3.72	2.77	0.9838

k_0' = theoretical capacity factor of an ethoxylated tributylphenol oligomer at 0% (v/v) methanol (100 % (v/v) distilled water); b = change in the logarithm of capacity factor caused by a 1% (v/v) change in methanol concentration in the eluent; s_b = standard deviation of b value; r_{calc} -= correlation coefficient.

because we supposed, that the oligomers would have special retention behaviour, and on the other hand coiling helically of oligomers was likely to appear causing extreme retention behaviour, and the quadratic correlation was suitable to describe that shape.

Even a structure analysis was also calculated by computer (Alchemy software) on the ethoxylated tributylphenol oligomer at all EO unit numbers from 1 to 11. It seemed to be suitable to determine the probability of coiling helically of the molecule. The software calculated the structure of the lowest energy in all case of the different EO units.

RESULTS AND DISCUSSION

Eleven fractions of ethoxylated tributylphenol were detected on the PGC column. Each of the fractions was supposed to belong to one EO number. The peaks were symmetric. The fractions were separated well. (see Figures 1., 2.)

As it was expected the number of ethylene oxide units effected on the retention behaviour. Higher retention time belonged to longer polyethylene oxide chain.

Each of the mentioned fractions is supposed to belong to one EO number. The equation (1) was suitable to describe the results. The Table 1. contains the lgk' values calculated by the equation (1) depending on the methanol concentration (v/v %) and the EO number. The correlation between the logarithm of actual capacity factor (lgk') and the concentration of methanol (C) was significant. The lgk' values are increasing by decreasing the methanol concentration and increasing the EO number as it was expected.

The relationship between the fraction of EO number and lgk_0' values was significant. The results of the linear correlation based on the equation (1) are collected in Table 2.. It can be seen in Table 2., that extreme retention behaviour started at the EO unit number of 4. After that fraction the properties of the oligomers became opposite ones. Before that point the b value increased with lgk_o' and NEO, but after it b decreased with them. Here presumably the helical form of the EO chain appeared and that event had a prominent effect on the electronic interactions between the stationary phase and the solute. The stepwise regression analysis between lgk_o'-NEO (2) — the lgk_o'-NEO correlation (2) as a linear one wasn't so significant, but the lgk_o'-lgNEO (3) gave a better correlation coefficient for the linear correlation — proved, that one fraction belongs to one EO number. (see Figure 3.) Quadratic correlation was found between b and lgk_o', and between b and NEO, which proved, that a helical form of oligomers appeared and the change

in the retention behaviour was at the EO number of 4. (see Figure 4., 5.) In addition to the b-lgNEO parabolic correlation was more significant, than the b-NEO. Results of the stepwise analysis are summarized in the Table 3. (the general forms of the equations (2,3,4,5,6) see above in the section "**EXPERIMENTAL**"). The significant level of variables was 99.9 %, except in the case of b-NEO, where it was 99 % (based on F value). The significant level of correlation was 99.9 % in every case (based on t – Student – value).

Table 3

Results of Stepwise Analysis on b, lgk_0' and the Number of Ethylene Oxide Units (NEO) (General Form : $Y=AX^2+BX+C$).

Parameter	Equation Number				
	2	**3**	**4**	**5**	**6**
A	–	–	-0.36	-0.01	-0.92
s_A	–	–	0.125	0.004	0.205
B	0.17	1.58	3.16	0.14	1.13
s_B	0.004	0.022	0.978	0.044	0.207
C	3.51	3.38	-2.81	3.79	3.84
s_C	0.049	0.059	1.968	0.184	0.135
r	0.9844	0.9869	0.9614	0.8927	0.9648
n	10	10	10	10	10
$F_{99.9\%}$	21.69	21.69	23.70	23.70	23.70
F_{calc}	250.86	300.25	42.73	13.74*	47.17

* = that correlation was significant at the level of 99 %, as $F_{99\%} = 9.78$.
A, B, C = coefficients of the equations; s_A, s_B, s_C = standard deviations of coefficients; r = correlation coefficient; n = number of samples; $F_{99.9\%}$ = F value belonging to the 99.9% significance level; F_{calc} = calculated F value.

Structure analysis was calculated by computer (Alchemy software). As the software couldn't consider the effects of the environment, the result wasn't suitable for representing the events of the experiments, but it was determined, that without any environmental effects the molecule doesn't tend to coil helically, so the reason for coiling must be the environment — the methanol and the water in the eluent, the possibility of coiling changes with the methanol concentration.

It can be concluded from our data, that the retention time of ethoxylated

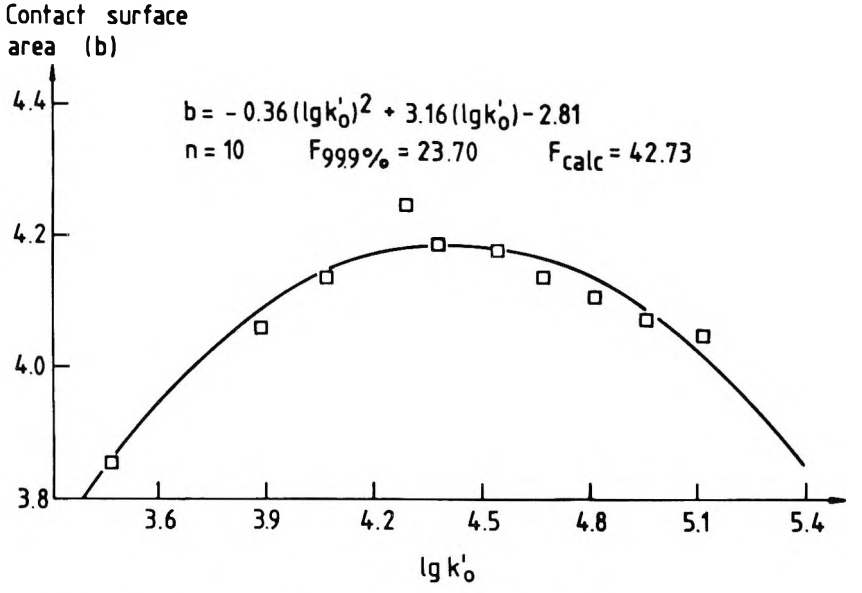

Figure 4. Relationship between the contact surface area (b) and lgk_o'

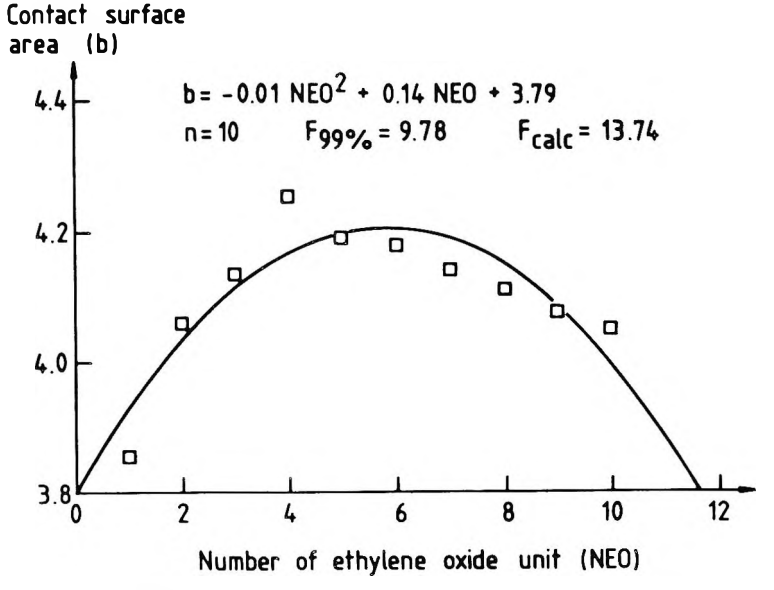

Figure 5. Relationship between the contact surface area (b) and the number of oxylated units (NEO).

tributylphenol oligomer on PGC column increased with decreasing methanol concentration of the eluent and increased with the ethylene oxide unit number. That means it managed to separate well the ethoxylated tributylphenol oligomers. The regression analysis indicated the appearance of the helical form of the oligomers. The same conclusion can be established by observing of lgk_0' and b values in Table 2. It was also proved, that coiling helically started at the EO unit of 4.

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SIMULTANEOUS DETERMINATION OF THE DIURETICS TRIAMTERENE AND FUROSEMIDE IN PHARMACEUTICAL FORMULATIONS AND URINE BY HPLC-EC

M. Begoña Barroso, Rosa M. Alonso, Rosa M. Jiménez

Departamento de Química Analítica
Facultad de Ciencias
Universidad del País Vasco/EHU
Apdo. 644, 48080 Bilbao, Spain.

ABSTRACT

A high performance liquid chromatographic method with amperometric detection has been developed for the simultaneous determination of two diuretics: triamterene and furosemide, using a μ-Bondapak C_{18} column. The mobile phase consisted of a mixture water:acetonitrile, 30:70, 5mM in KH_2PO_4/K_2HPO_4, pH 5.5 pumped at a flow rate of 1 mL/min. The amperometric detector, equipped with a glassy carbon electrode, was operated at +1300 mV.

The method was applied to the determination of both diuretics in the pharmaceutical formulation Salidur (triamterene 25 mg and furosemide-xanthinol 77.6 mg) and real urine samples obtained from a healthy volunteer after the ingestion of a single dose of Salidur. Using a simple liquid-liquid extraction procedure, good recovery and separation from interferences found in urine matrix is achieved and the simultaneous determination of both compounds is possible. Reproducibility is good with relative standard deviations lower than 1.5% intra-day

and 5% inter-day, and the method is accurate since errors obtained for tablets are lower than 1 %. The determination limits are 15 ng/mL for furosemide and 0.1 ng/mL for triamterene. Results obtained from urine samples are in accordance with those expected from pharmacokinetic studies.

INTRODUCTION

The combination of drugs with different mechanisms of action is a common therapeutic procedure for the treatment of hypertension, since sometimes the use of a single compound can lead to secondary effects in susceptible patients.

4-Chloro-2-furfurylamino-5-sulphamoylbenzoic acid (furosemide), an anthranilic acid derivative, is one of the most potent diuretics available. It inhibits the active reabsorption of chloride in the diluting segment of the loop of Henle, thus preventing the reabsorption of sodium which passively follows chloride.[1] This loop diuretic is commonly used for the treatment of renal disease, congestive heart failure and hypertension.[2] The continous administration of diuretics, especially furosemide, thiazides and related compounds, leads to a significant loss of potassium which can lead to hypokalemia and hypochloremic alkalosis.[3] Due to this fact, they must be given with a potassium replenisher or with potassium-sparing diuretics.[4] That is why pharmaceutical formulations containing more than one type of diuretic are used in the treatment of certain diseases.

6-Phenylpteridine-2,4,7-triamine (triamterene), is the most widely prescribed member of the group of potassium-sparing diuretics. It permits the reabsorption of potassium, but not sodium ions, in the tubules and it does not cause serious uric acid retention, which is a problem with some of the other diuretics.[5] Because of these properties, it is used mainly as an adjusment to thiazide and loop diuretics such as hydrochlorothiazide and furosemide in the treatment of edema and hypertension, to increase natriuresis and reduce kaliuresis.[6]

The pharmacokinetic and pharmacodynamics of the two diuretics are different. While furosemide is found unchanged in urine in a 60 %,[7] less than 10 % of the dose of triamterene is excreted unchanged.[8]

Some methods have been reported for the determination of triamterene in plasma and urine. Most of them use high performance liquid chromatography with photometric[9,10] and fluorimetric[11-13] detection. Gonzalez et al.[14] describe a method for the separation of three drugs, including triamterene, by capillary zone electrophoresis with pulsed-laser fluorescence detection, but only as an example to show the application of this type of detection and not focused on the determination of the compounds.

For furosemide determination, various methods are available, including mostly high performance liquid chromatographic separation followed by photometric[15-24] or fluorimetric[25-34] detection of the eluted fractions. Some simultaneous determination of furosemide with other compounds can be found in the literature. The determination of furosemide and metolazone in plasma and urine has been reported by Farthing et al.[35] and furosemide, phenylbutazone and oxyphenbutazone in plasma by Pinkerton et al.[19]

The aim of this work is the development of a simple and reliable HPLC-EC method for the simultaneous determination of triamterene and furosemide in pharmaceutical formulations and real urine samples obtained from a healthy volunteer after administration of tablets which contain both diuretics.

MATERIALS AND METHODS

Apparatus and Column

The HPLC system consisted of a Model 2150-LKB (Pharmacia, Barcelona, Spain) HPLC pump, and a Rheodyne (Pharmacia) Model 7125 injector with a loop of 20μl.

Electrochemical detection was carried out using an amperometric detector, PAR Model 400, equipped with a glassy carbon working electrode (EG&G Princeton Applied Research, Madrid, Spain). It was operated at +1300 mV vs a Ag/AgCl electrode, in the DC mode, with a 5-s low-pass filter time constant, and a current range between 0.2 and 100 nA. Chromatograms were recorded using an LKB Model 2221 integrator. The chart speed was 0.5 cm/min and the attenuation was 8 mV, full scale.

The column was a μBondapak C_{18}, 30cm x 3.9mm I.D., 10-μm, 125 Å, (Waters Assoc.).

A precolumn module packed with μBondapak C_{18} (Waters Assoc.) was used to protect the column from degradation. All the measurements were made at room temperature.

The extracted urine samples were evaporated to dryness using a Zymark TurboVap LV evaporator (Barcelona, Spain).

Reagents and Chemicals

Triamterene was obtained from Aldrich (Milwaukee WI 53233, USA) and furosemide from Hoechst Ibérica (Barcelona, Spain). Solvents were Lab-Scan HPLC grade, and the water which was used was obtained from Milli-RO and Milli-Q Waters systems. All the reagents used, as well as the salts for the supporting electrolyte, were Merck Suprapur (Bilbao, Spain).

Standard Solutions

Stock solutions of furosemide and triamterene were prepared separately. 50 mg of furosemide were weighted into a volumetric flask, and made up to 100 mL with acetonitrile. The standard solution of triamterene was prepared by weighing 10 mg of the compound, making up to 100 mL with methanol and ultrasonicating for 5 min.

Chromatographic Conditions

The mobile phase was a mixture acetonitrile-water (30:70) containing 5 mM potassium dihydrogenphosphate/dipotassium hydrogenphosphate. The pH was adjusted to 5.5 and the buffer also served as the supporting electrolyte. This phase was filtered through Millipore membrane filters of 0.45 μm porosity, and the filtrate was degassed by bubbing helium through it.

The μBondapak C_{18} column head-pressure was 69 bar at a flow rate of 1.0 mL/min. The system was operated at room temperature and the injection volume was 20 μL.

Care of the Working Electrode

At the end of each working day, the electrode was cleaned electrochemically by keeping it at -800 mV for 2 min and, after that, at +1600 mV for 5 min. This operation was carried out using a mobile phase of pure methanol at a flow rate of 1.5 mL/min.

When the baseline was unstable or a drift could be observed, the glassy carbon electrode was removed from the cell compartment and rinsed with distilled water to remove any encrusted buffer salts from its surface. Once this was done, the electrode was cleaned with a tissue wet with methanol to remove possibly adsorbed compounds.

Procedure for Tablets

The pharmaceutical formulation analyzed in this work was presented as tablets. In order to perform the determinations, the tablets were pulverized and an adequate amount weighted out. Methanol was added and the sample shaken for 30 min. The mixture was then immersed for 5 min in an ultrasonic bath to facilitate dissolution, since triamterene is quite insoluble. Filtration of the dissolved sample through a filter paper, Albet 242, was necessary in order to avoid plugging the column. The residual solid was washed with more methanol to prevent the loss of analytes. The filtered solution was made up to 50 mL with methanol, and an aliquot of this one was diluted with mobile phase to provide the concentration required for the injection.

Different amounts of powder from each of the tablets were weighed and analyzed, and this operation was repeated for different tablets to calculate a mean value.

Procedure for Urine Samples

The clean-up procedure for urine samples was based on the method proposed for Ventura et al.[36]: 2 mL of urine were alkalinized with KOH 2M and adjusted to a pH value of 10.0. Then, 1.5 mg NaCl (s) were added. To this solution, 4 mL of ethyl acetate were added and it was shaken for 10 min. After that, the mixture was centrifugated at 2500 rpm for 5 min and the organic layer

was separated and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was dissolved in 2 mL of the mobile phase with ultrasonification, to facilitate dissolution, just before the analysis on the chromatograph, with a volume injected of 20 μL.

RESULTS AND DISCUSSION

Triamterene and furosemide are oxidized at a glassy carbon electrode.[37] Although triamterene gives rise to a unique voltammetric peak, furosemide produces two waves with a second peak potential which overlaps with the one of triamterene. This fact makes impossible the determination of triamterene in presence of furosemide, but this diuretic could easily be detected in presence of triamterene, using the first oxidation wave. The combined administration of these two diuretics in tablets, together with the impossibility of their simultaneous determination by voltammetric methods in static conditions, led us to the development of a chromatographic method to achieve their simultaneous determination.

Optimization of the Chromatographic System

In order to choose the optimum potential value to apply for the determination of these diuretics, a hydrodynamic voltammogram of each compound was obtained (Figure 1). An oxidative potential of 1300 mV was chosen as the working potential, since it was the lowest potential necessary to produce the oxidation of both diuretics, giving rise to reproducible peak areas. A higher potential would produce an increase of the background current together with a loss of reproducibility.

Because of the different acid-base characteristics and pK_a values of these drugs (triamterene 6.2[38] and furosemide 1.64, 3.97, 9.40[37]), their chromatographic behaviour was affected by the pH of the mobile phase. With an increase in the pH value of the mobile phase, an increase in the retention time of triamterene was observed, but it produced a large decrease in the retention time of furosemide. The study of the influence of pH gave an optimum value of 5.5, since the chromatograms of the two compounds were becoming too close when the pH was increased above this value while, with a lower pH, the retention time of furosemide was too large. This pH was also

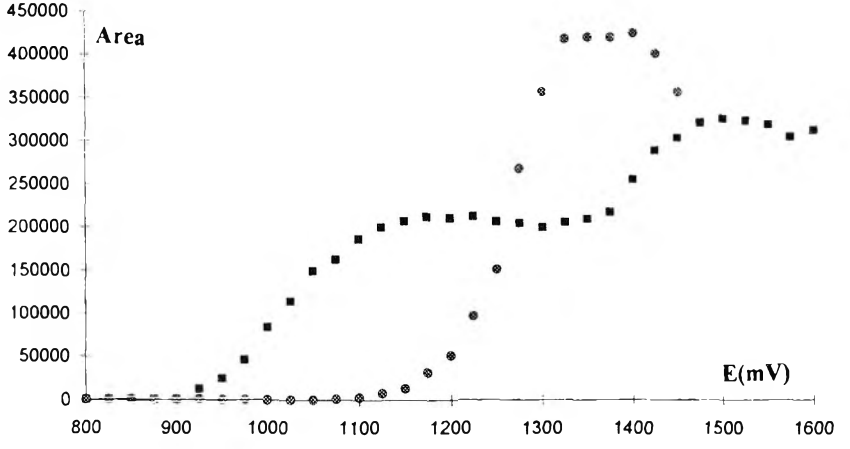

Figure 1. Hydrodynamic voltammograms of (●) triamterene and (■) furosemide. Amount of drug injected: triamterene 10 ng and furosemide 100ng in acetonitrile-water (30:70) containing 5mM KH_2PO_4/K_2HPO_4, pH 5.5 and flow rate 1 mL/min.

adequate for the separation of the electrooxidable interferences found in the urine matrix.

The supporting electrolyte used, which is necessary for the amperometric detection, was the buffer potassium hydrogen phosphate/dipotassium hydrogen phosphate. Other buffers, such as acetate were tried, but the area under the chromatographic peaks was higher with phosphate buffer. The best signal-to-noise ratio was achieved with an electrolyte concentration of 5 mM.

Different ratios of methanol-water and acetonitrile-water, containing 5 mM potassium dihydrogen phosphate/dipotassium hydrogen phosphate were tested as the mobile phase. Acetonitrile produced better defined chromatographic peaks than methanol, with less peak broadening. The ratio 60:40 (water-acetonitrile), used for the determination of furosemide in other work,[39] achieved a good resolution of both compounds (k'=1.56 for triamterene and k'=2.30 for furosemide), but it did not allow the separation from the urine interferences, so a ratio 70:30 water-acetonitrile had to be chosen as the best one, although the elution times were a little bit higher (k'= 1.81 for triamterene and 2.41 for furosemide); see Figure 2.

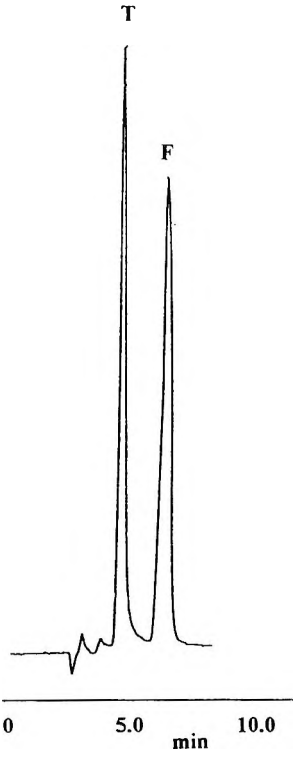

Figure 2. Separation of triamterene (T) and furosemide (F), using amperometric detection and a μBondapak C_{18} column; mobile phase acetonitrile-water (30:70) containing 5 mM KH_2PO_4/K_2HPO_4, pH 5.5 and flow rate 1mL/min; oxidation potential: 1.3 V vs Ag/AgCl/KCl. Amount of drug injected: triamterene 10 ng and furosemide 100 ng. Full scale current: 100 nA.

A study of the influence of the flow rate on the chromatographic separation was carried out. As was expected, the peak area decreased with an increase in flow rate, while the effect on k′ was practically negiglible. A value of 1mL/min was chosen as optimum. All the measurements were carried out at room temperature.

After establishing the optimum chromatographic conditions, a quantitative method for the simultaneous determination of both diuretics was developed. In Table 1 are collected the linear regresion for the calibration graphs, the intra-day and inter-day reproducibility studies made on n=10

Table 1

Determination of Triamterene and Furosemide.

Diuretic	**Triamterene**	**Furosemide**
Retention time (min)	5.01	6.70
Experimental determination limit (ng/mL)	0.1	15
Linear concentration range	Up to 5 mg/l	Up to 10 mg/l
Slopes of calibration graph	251454.9[a] (r^2=0.999)	26764.1[a] (r^2=0.999)
Reproducibility (%RSD)	1.4 (intra-day) 4.9 (inter-day)	0.85 (intra-day) 3.4 (inter-day)

[a] area/concentration (ppm)

solutions, as well as the experimental quantitation limit, defined as the minimum concentration which gives rise to a signal able to be quantified for the integrator.

Analytical Applications

In a first step, the method developed was applied to the determination of triamterene and furosemide in pharmaceutical formulations (Salidur: triamterene 25 mg and furosemide-xanthinol 77.6 mg) obtaining values in accordance with those certified, with relative errors lower than 1%. The results, collected in Table 2, show that accuracy is good for both diuretics. No interferences were noticed from the adjuncts used in the formulation with the present method, as can be observed in Figure 3.

Secondly, the method developed was applied to spiked urine samples of these diuretics in order to calculate the percentages of recovery, using the clean-up procedure described above. Quantitative recoveries, calculated from

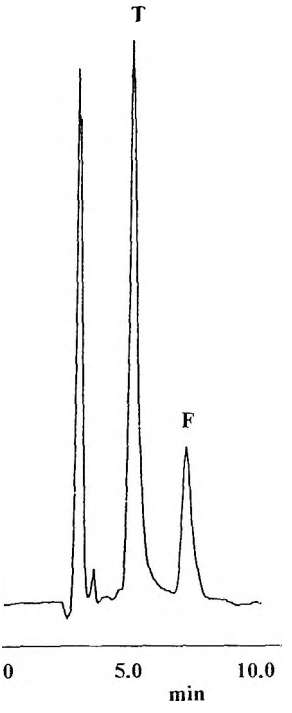

Figure 3. Chromatogram corresponding to a diluted solution of a tablet of Salidur (Furosemide-Xanthinol 77.6 mg and triamterene 25 mg). Full scale current: 100 nA. The same chromatographic conditions as in Figure 2.

Table 2

Determination of Triamterene and Furosemide in Pharmaceutical Formulations.

Formulation	Component	Found (μg)[a]	Nominal (μg)
Salidur	Triamterene	24.82 ± 0.91	25.00
Salidur	Furosemide-Xanthinol	77.02 ± 0.86	77.60

[a] amount ± ts/√n. n=3 different tablets and 3 replicates of each tablet.

urine samples spiked with 1μg/mL (triamterene) and 3 μg/mL (furosemide) were (98.7 ± 3.0)% for triamterene and (44.4 ± 3.5)% for furosemide. The high recovery for triamterene is in accordance with its basic character, which allows a good extraction from urine, while the acid characteristics of furosemide make it difficult to extract. This liquid-liquid extraction method, with these recoveries, allows the simultaneous determination of both compounds, given the different concentrations in which triamterene and furosemide are found.

The chromatographic method has been applied to the analysis of triamterene and furosemide in real urine samples obtained from a healthy female volunteer after a single dose of Salidur. Urine was collected at different time intervals for the quantitative determination of triamterene and furosemide: 0-2 hours, 2-8 hours and 8-24 hours. Following the clean-up procedure described in the experimental section, both compounds were easily detected at the different times. In Table 3 are collected the concentrations of unchanged drug found in urine (9.2 % for triamterene and 9.16 % for furosemide). Taking into account some reported works, triamterene concentrations are in accordance with the pharmacokinetic data,[7] while furosemide shows a lower percentage excreted,[8] perhaps due to the combination of both diuretics. The peak concentrations times for both drugs are in agreement with the literature data (2-8 hours for furosemide and 8-24 hours for triamterene).[7,8] Figure 4 shows the chromatograms corresponding to a blank urine and a real urine sample obtained from 2-8 hours after the administration of one tablet.

Table 3

Determination of Triamterene and Furosemide in Real Urine Collected at Different Time Intervals after the Ingestion of a Single Dose of Salidur (Triamterene, 25 mg and Furosemide-Xanthinol, 77.6 mg)

Time Intervals	Volume of Urine (mL)	Triamterene (mg/mL)	Furosemide (mg/mL)
0-2 hours	475	0.82	1.42
2-8 hours	560	1.45	3.58
8-24 hours	550	2.00	1.25

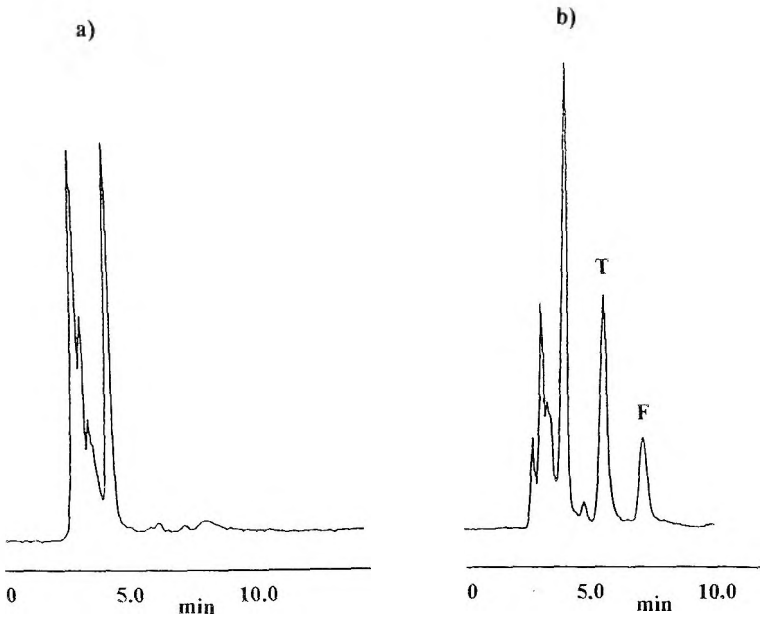

Figure 4. Chromatograms obtained from an extract of a) blank urine sample, and b) urine sample, 8 h after oral administration of 1 tablet of Salidur (Furosemide-Xanthinol 77.6 mg and triamterene 25 mg) to a healthy female volunteer. Full scale current: 100 nA. The same chromatographic conditions as in Figure 2.

High-performance liquid chromatography with amperometric detection has been shown to be a powerful method for the separation, identification and determination of the two diuretics triamterene and furosemide.

The main advantage of the method developed is the determination of triamterene in presence of furosemide, since it was impossible with static electrochemical methods. Moreover, as can be seen in the hydrodynamic voltammograms, it would be possible to choose a potential for the determination of furosemide without interferences from triamterene, for instance, 1100 mV.

The method shows a great sensitivity for the analysis of triamterene with a determination limit of 0.1 ppb, which is lower than the one reported by Sved et

al. using fluorimetric detection.[11]

The different chemical characteristics of these compounds makes it difficult to find an extraction procedure with good recoveries for both of them. On one hand, if an alkaline pH is used for the extraction, the recovery for triamterene is very high, but low for furosemide. On the other hand, if an acid pH is used, the recovery is high for furosemide but triamterene is almost not extracted. Taking into account the usual therapeutic doses and the percentage of unchanged drug excreted, a basic medium was chosen.

In our laboratory, new clean-up procedures based on the application of solid-liquid extraction are being evaluated in order to obtain good recoveries for both compounds without increasing the amount of interferences found in the chromatograms from the urine matrix.

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PHENOLS HPLC ANALYSIS BY DIRECT INJECTION OF SHERRY WINE VINEGAR

C. Garcia-Parrilla, F.J. Heredia, A. M. Troncoso

Area de Nutricion y Bromatologia
Facultad de Farmacia
c/P, Garcia Gonzalez s/n
Sevilla 41012, Spain

ABSTRACT

Analysis of sherry wine vinegars using reverse phase HPLC and diode array detection, via direct injection is performed. The technique proved to be useful to study phenolic composition, the identification of major peaks being achieved. The following compounds were detected in all the samples analyzed: gallic acid, caftaric acid, tyrosol, hydroxymethylfurfural and, in almost every sample analyzed, p-coumaroyltartaric glucosidic ester, caffeic acid, p-coumaric acid and caffeic ethyl ester. The results obtained may be helpful to establish a phenolic profile for sherry vinegars which may be used as an origin recognition pattern. These vinegars have been recently awarded their certification brand recognition.

INTRODUCTION

Due to the diversity of wine vinegars in the market and the increase in demand, it has been considered necessary to investigate reliable analytical methods to establish quality and origin criteria as the authentification remains unsolved. Among wine vinegars, sherry vinegars are specially recognized. Their elaboration from sherry wine and subsequent aging in oak barrels

following the 'solera' system gives singular organoleptic properties to the final product.

Polyphenolic compounds have been exhaustively reviewed in relation to origin, elaboration, aging and browning of wines.[1,2,3]

High Performance Liquid Chromatography has been employed for the study of phenols in vinegars.[4,5] Usually, the sample is subjected to a prior extraction before injection. However, substantial changes in sample composition may occur due to cis-trans isomerizations and hydrolysis phenomena occurring as a result of the extraction procedure.

To solve this problem in the analysis of phenolic compounds in wines, the direct injection of the wine onto the column with no other prior treatment but filtration was proposed.[6,7] These analytical methods allow a real and accurate knowledge of the sample, as no alteration in phenol composition occurs. Besides, the quantification is exact, as it avoids the losses associated with an extractive approach. However, this technique has not previously been applied to vinegars.

The characterization of phenolic compounds is enhanced by means of photodiode array detection, as it provides useful information related to the molecular structures.[8]

The aim of this work is to identify phenolic compounds of traditional sherry vinegars by means of HPLC with no handling of the sample, as well as the identification of other major peaks which remained unknown until now, in order to establish the whole phenolic profile of these vinegars. This point takes special interest since sherry vinegars have been recently awarded their certification brand recognition (appellation origin).

MATERIALS AND METHODS

Samples

The analyzed samples (n=22) are derived from different producers of Jerez wine vinegars and were purchased in local markets. The declared periods of aging were variable.

A wine vinegar sample obtained in an experimental bioreactor by quick

acetification process from sherry wine, with no subsequent aging, was also studied. It was included in this study for comparative purposes.

HPLC

The chromatograph employed was a Waters 600E system controller (Milford, Massachusetts, USA) connected to a Waters 996 photodiode array detector. Data treatment was performed with a Millenium 2.0 data station. The injection system is a syringe loading sample manual injector Model 7125 from Rheodyne (Cotati, California, USA). The temperature was controlled by a Waters Steel Column Heater Module.

The column was a Merck Superspher 100 RP-18 (250-4mm), particle size 4 μ, protected by a guard cartridge Nova-Pak C_{18} module from Waters. The volume injected was of 50 μL.

Chromatographic Conditions

The chromatographic conditions were originally described for the analysis of simple phenols and flavonols in wines.[6] Recently, the method was enhanced by changing the acetic content of the solvents used in the gradients.[7]

The vinegar was injected onto the column without any handling except for filtration with Millex-GV_{13} 0.22 μm filters.

The solvents:

A: acetic acid - water(1/99)
B: acetic acid - water (6/94)
C: acetic acid - water - acetonitrile (5/65/30)

The gradient profile was as follows:

Time	%A	%B	%C
0	100	0	0
15	0	100	0
30	0	100	0
50	0	90	10
60	0	80	20
80	0	70	30
120	0	0	100

The flow was 0.5 mL/min and the temperature was set at 22.5°C. Solvent is heated as it travels through the heater before entering the column. An internal cover maintains thermal stability during operation. The temperature stability within the column compartment is ±0.1 degree Celsius over the entire operating range.

Reagents

Simple phenols (gallic, caffeic, p-coumaric, ferulic...), catechin, vanillin, tyrosol and hydroxymetylfurfural (HMF) were purchased from Sigma (Milwaukee, USA), Carlo Erba (Milano, Italy), Fluka (Buchs, Switzerland) and Merck (Darmstadt, Germany). Standards were prepared in a concentration of 10 mg/L in water. The water employed was filtered through a Milli-Q system and the others solvents were furnished by Carlo Erba.

Hydroxycinnamic Ester Synthesis

Ethyl esters were synthetisized by the trifluorurum method. Two mL of trifluoruethane were added to a solution of 100 mg of acid in 50mL of absolute ethanol. After 48 hours, the amount of ester formed was enough for the identification.

Identification

Simple phenols were identified by matching their spectra with those of standards and by the comparison of their retention times with standards. In doubtful cases, the samples were spiked with standard and the purity of the peak obtained checked by comparison of spectra within the peak from peak liftoff to peak touchdown. Different shapes in overlayed spectra revealed any coelution. The Photodiode Array Detector together with the software uses a complex mathematical technique to automatically determine spectral matches of standard to sample spectra and compound purity testing. The overall shapes of spectra are converted into vectors in multidimensional space and then those vectors are compared. When a peak purity test is performed, the spectrum at the peak apex is the reference spectrum. All other spectral data contained within that peak are compared with the spectrum of the apex. Match results are reported in terms of angle between vectors' degrees. If two vectors (representing spectra) completely overlap, there is difference of zero degrees between them; therefore they represent the same compound.

The identification of mixed acids such as cafeoyltartaric acid, coumaroyltartaric, and the ester of glucose with this last one, was carried out using the spectra provided under the same conditions of the method described by Professor Dr. Roggero[7] (Laboratoire de Chimie Organique et Analytique, Faculte de Sciences de Avignon, France) to whom we are grateful.

Table 1

T_R, λ_{max}, of the Identified Peaks

Peak No.	T_R	λ_{max}	Identification
1	9.6	260	?
2	14.3	264	?
3	16.0	269	?
4	18.4	272	Gallic Acid
5	25.3	285	5-(Hydroxymethyl)-2-furaldehyde
6	26.8	262.3	?
7	27.8	312	Caffeoyl-tart acid
8	34.8	312	p-Coumaroyl-tart.ac caffeoyl glucosidic ester
9	37.6	314	p-Coutaric
10	38.0	278	2-Furaldehyde
11	43.6	278	Tyrosol
12	57.5	267	?
13	59.7	324	Caffeic Acid
14	69.4	276	?
15	70.4	271.7	?
16	86.2	309	?
17	89.5	310	p-Coumaric Acid
18	100.1	324	Ferulic Acid
19	108.1	255-355	Isoquercitrin
20	123.9	328	Caffeic Ethyl Ester
21	141.3	310	p-Coumaric Ethyl Ester

RESULTS AND DISCUSSION

More than 20 compounds have been identified in sherry vinegars. Their spectra have been recorded with the photodiode detector. Table 1 shows the retention time, the λmax value found and the identification of some peaks. Some of them have remained unidentified in sherry vinegars till now (HMF, furfural, tyrosol, cafeoyltartaric acid, p-coumaroyltartaric acid, isequercetrin).

Despite the complex chromatographic profile, as there are other compounds which absorb in the ultraviolet region besides the phenolic ones, it has been proved that the separation between peaks is adequate. Most of the peaks were shown to be pure by using the detector's peak purity test so that this technique is highly recommended for the study of phenolic compounds in sherry vinegars. The peak purity test is performed by taking spectra from peak apex through to the tail. The absorbance contribution of a second contribution compound, if any, reshapes the spectrum. It may be assumed that a peak which is spectrally homogeneous throughout the whole peak is a single and well separated compound.

Table 2 illustrates the compounds identified in the samples we analyzed. As can be observed, not all the compounds are present in every sample. However, there are some points in common that should help us to establish a phenolic pattern in sherry vinegars.

Among simple phenolic acids it can be noted that gallic acid is present in all the samples analyzed. p-Hydroxybenzoic and vanillic acids were not detected, although they have been reported by other authors in sherry wine.[9] Special attention was paid to protocathecuic acid which had been reported to be present in sherry vinegar (Galvez et al., 1993). A compound with very similar spectral characteristics (λ = 262, 293) was detected at a very close time to protocatechuic acid standard. When the samples were spiked with the standard, a unique peak was obtained, but the verification of purity (peak purity test) pointed out spectral inhomogeneity (Fig. 1).

Caffeic and p-coumaric acid were generally found in most sherry wine vinegars. On the other hand, ferulic was seldom identified. Cis-isomers of tartaric-hydroxycinnamoyl acids, whose retention times in reversed phase chromatography are slightly smaller than those of the *trans* compounds have been investigated without success. However, p-coumaroyl ethyl ester and caffeic ethyl ester have been properly identified.

Table 2

Compounds Identified in Sherry Wine Vinegars

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	+	+	+					+	+				
2	+	+	+	+		+	+	+					
3	+	+	+	+			+	+	+		+		
4	+	+	+	+	+	+	+			+	+	+	
5	+	+	+	+	+		+	+			+	+	+
6	+	+	+	+			+	+	+			+	+
7	+	+	+	+			+	+	+		+	+	
8	+	+	+				+	+	+				
9	+	+	+	+			+		+	+	+	+	+
10	+	+	+	+		+	+	+	+	+		+	+
11	+	+	+	+			+	+	+		+	+	+
12	+	+	+	+	+	+	+	+	+		+	+	+
13	+	+	+	+			+	+	+			+	+
14	+	+	+	+	+	+	+	+	+			+	+
15*	+	+	+	+			+	+	+			+	+
16	+	+	+	+			+	+					
17	+	+	+	+		+	+	+	+				
18	+	+	+	+	+	+	+	+	+			+	+
19	+	+	+	+	+	+	+	+	+			+	
20	+	+	+	+	+	+	+	+	+			+	+
21	+	+	+	+		+	+	+	+		+	+	
22	+	+	+	+		+	+	+	+			+	+

* Wine vinegar obtained in an experimental bioreactor

A: Gallic Acid; B: HMF; C: Cafaric Acid; D: p-Coumaric Glucoside Ester; E: p-Coumaric Acid; F: 2-Furaldehyde; G: Tyrosol; H: Caffeic Acid; I: p-Coumaric Acid; J: Ferulic Acid; K: Isoquercetrin; L: Caffeic Ethyl Ester; M: p-Coumaric Ethyl Ester.

With very few exceptions, the information provided according to the elaboration process is scarce. Besides, it was very difficult to obtain a large number of samples from different producers. Due to the above mentioned constraints, it was difficult to attribute the differences found either to the original subtract nor to the elaboration procedure.

As the caftaric acid is a substrate for the polyphenoloxidase and its

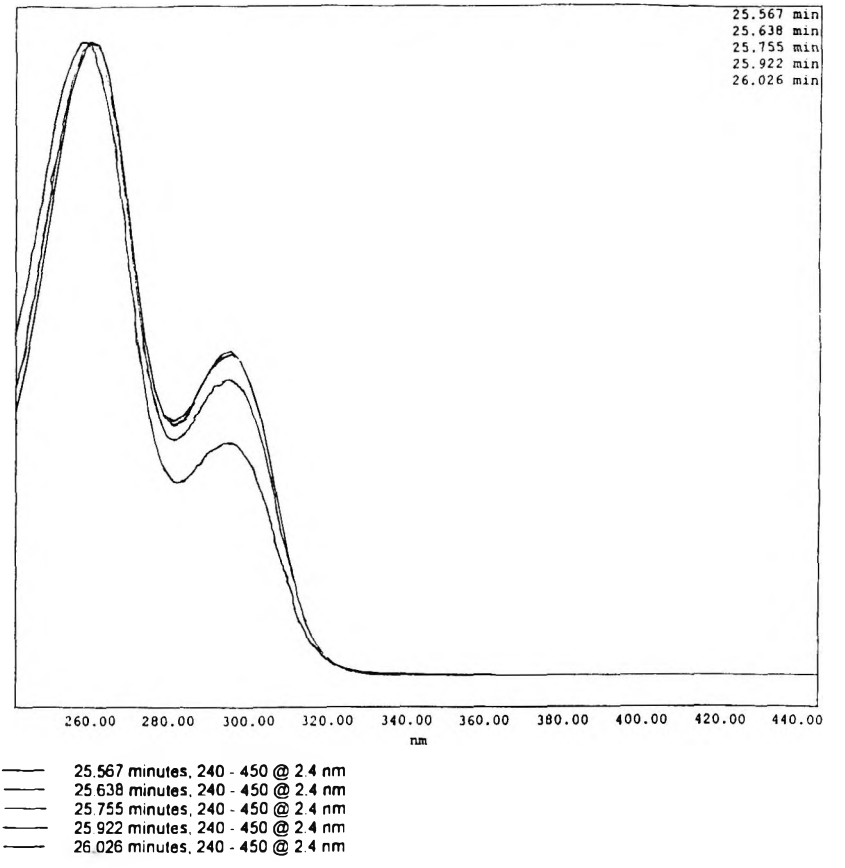

Figure 1. Spectra obtained by spiking the sample with protocatequic acid.

content in musts decreases during aging, and under oxidative conditions,[10] it is surprising to find this compound in almost every sherry vinegar analyzed, taking into account that vinegars are submitted to an oxidative medium and those of Jerez have suffered a browning process. This phenomenon should be explained by considering that a 'solera' is a dynamic system in which fresh wine is periodically added while a part of the most aged product is withdrawn and bottled. The presence of caftaric acid should be a useful indicator to differentiate sherry vinegars elaborated by a dynamic 'solera' system from those coming from a static one. Further research is required to confirm this point.

The largest peak in the 280 nm chromatogram is 5-hydroxymethylfurfural (HMF). Even before being identified, its value to differentiate sherry wine

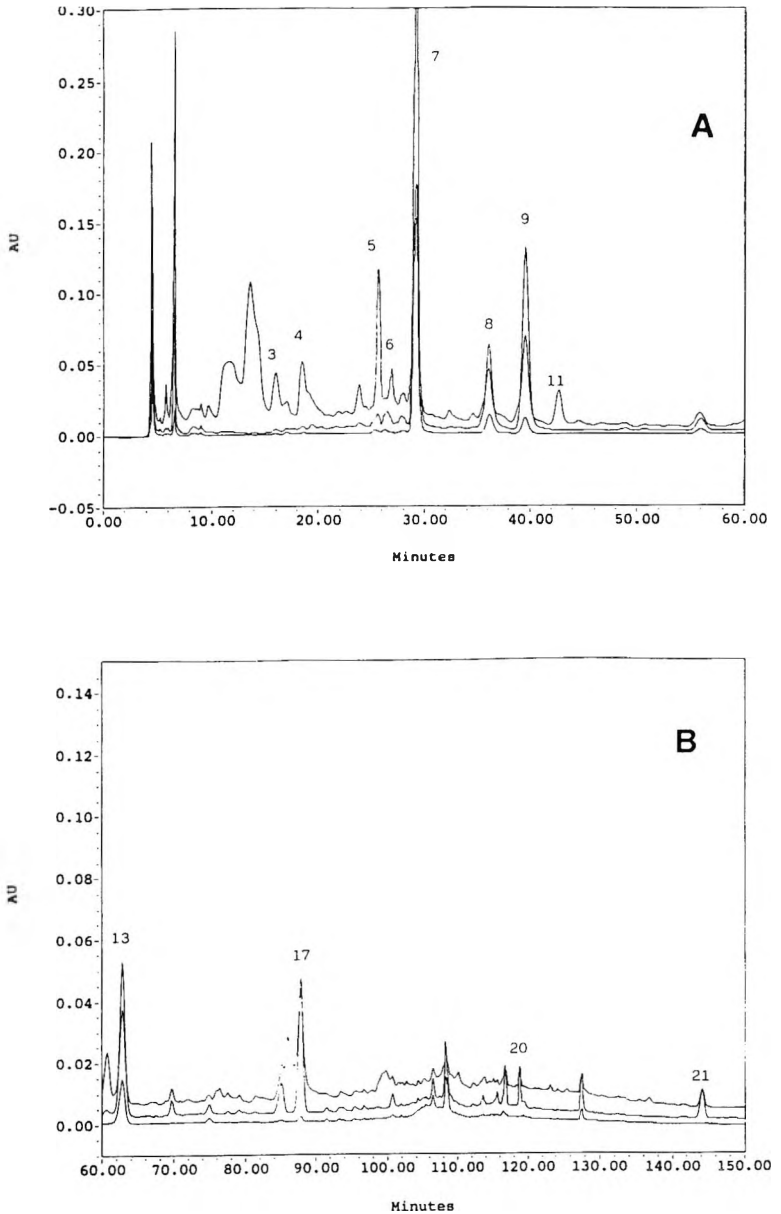

Figure 2 (a&b). Chromatogram corresponding to a sherry vinegar sample obtained by a quick acetification process.

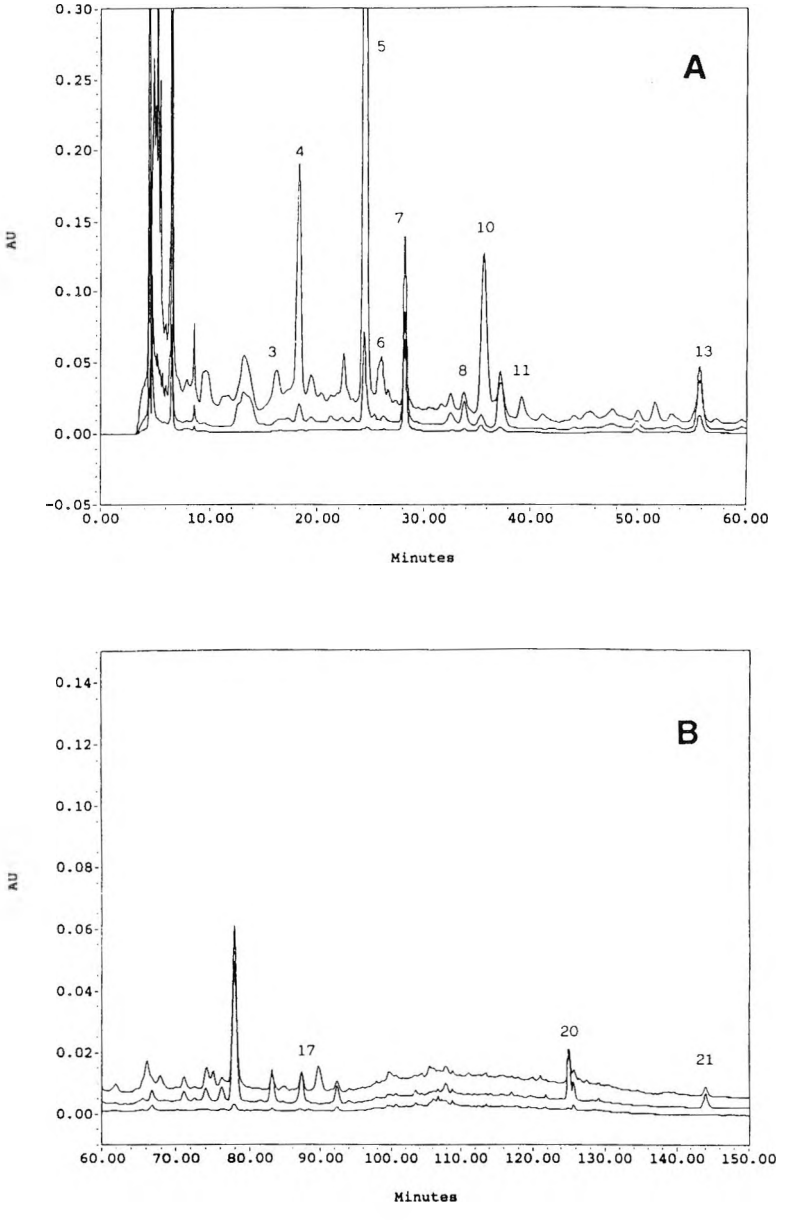

Figure 3 (a&b). Chromatogram corresponding to a sherry vinegar sample obtained by a"solera" system.

vinegars from those of other origin had been pointed out.[11]

Furfural and HMF have been included in the table despite their non-phenolic nature, due to the similiarity of their spectra with those of simple phenols.

HMF is barely detected in the sherry vinegar produced by a quick acetification process which has not been submitted to aging (Fig. 2), but its presence is very relevant in aged samples (Fig. 3).

Although these results are promising, the addition of must caramel requires one to be careful in drawing conclusions. This practice is well extended in vinegar wineries. Further research is necessary to prove the relationship of HMF to sherry vinegar's aging. But, it is clear that the absorbance at 280 nm proposed as measure of phenolic compounds in white wines[12] cannot be used as measure of phenolic compounds in sherry vinegars due to the enormous contribution of this compound to the global absorbance at 280 nm.

The utility of the technique to separate and characterize, not only the phenolic compounds, but also products of the Maillard reaction in sherry vinegars in a single run, has been proved. On the other hand, some compounds have been identified for the first time in vinegar and are essential to establish a fingerprint of sherry wine vinegar. Besides, there are others such as cafeoyltartaric acid and HMF which reveal differences in the elaboration process (aging and caramel addition). So that, the technique is of great interest to determine the origin, elaboration and aging of vinegars.

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OPTIMIZATION OF SAMPLE PREPARATION TECHNIQUES FOR THE DETERMINATION OF 4-NONYLPHENOL IN WATER AND SEDIMENT

K.K. Chee, M.K. Wong, H.K. Lee*

Department of Chemistry
National University of Singapore
Kent Ridge
Republic of Singapore 119260

ABSTRACT

Sample preparation techniques for the determination of 4-nonylphenol in water and sediments were optimized using two-level orthogonal array design (OAD). Comparison between the use of cartridge-based solid-phase extraction and membranous disk-based extraction was made for water samples. For sediment samples, microwave-assisted solvent extraction (MASE) was employed. A comparison between MASE and classical soxhlet extraction of spiked samples was also carried out. 4-Nonylphenol was determined using high performance liquid chromatography (HPLC) coupled to both ultraviolet and fluorescence detection

INTRODUCTION

The focus in trace organic pollutant analysis has shifted from analytical instrumentation to sample preparation.[1,2] Sample preparation is a critical stage

in the entire experimental procedure. It is both time-consuming and tedious as two-thirds of the analysis time is typically spent on it. The method detection limits for the determination of many organic pollutants are greatly dependent on the sample preparation conditions used. Loss of samples at this stage of the experiment is usually inevitable, e.g. adsorption of analytes onto the glassware, inadequate transfer of samples, inefficient extraction of compounds from the samples and matrix effects. However, sample losses can be largely avoided or at least minimized by careful selection of optimum sample preparation conditions derived from appropriate optimization procedures.

Recently, orthogonal array design (OAD) has been used as a chemometric method for the optimization of analytical procedures.[3-9] OAD is a sophisticated and cost-effective optimization strategy that is used to assign factors to a series of experimental combinations whose results can then be analyzed by using a common mathematical process. e.g. analysis of variance (ANOVA). The main effects of the factors and two-variable interactions can be considered separately as different factors and estimated by OAD along with the corresponding linear graphs or triangular tables.[10-11]

During the last 40 years, 4-nonylphenol (4-NP) has been widely used for the production of non-ionic surfactants, e.g. nonylphenol ethoxylates (NPE) in industrial detergents formulations. The biodegradable non-ionic surfactants had replaced the non-biodegradable synthetic types which were responsible for severe environmental problems such as foaming. However, the former surfactants themselves are of concern owing to their release into the environment through industrial runoff. The biodegradability of the NPE under laboratory and natural conditions has been well studied.[12-14] During anaerobic sewage treatment, NPE is degraded to its refractory metabolite, 4-NP, and concern has arisen owing to the recognition of the increased aquatic toxicity of this species over that of the parent surfactants. The toxicity of this hydrophobic metabolite towards marine organisms is well-documented.[15-18] Due to their hydrophobicity, 4-NP can associate with suspended matter and eventually with sediments. Recently, Soto and co-workers found that nonylphenol is strongly oestrogenic in cultured human breast cells and in rodents.[19] The occurrence and potential hazardous effects of 4-NP or other alkyphenols have thus stimulated research on their presence in environmental samples.[20-23]

For the extraction of 4-NP from water and sewage effluent, liquid-liquid partition using methylene chloride is usually used.[15] Recently, solid-phase extraction (SPE) using C_{18}-packed cartridges has been used for the trace enrichment of nonylphenols in water.[19]

For extracting 4-NP from sediment and sewage sludge, soxhlet extraction is the most common technique. Different extracting solvents including methanol,[15] dichloromethane[16] and hexane[18] have been used. Recently, microwave-assisted solvent extraction (MASE) has been applied for the extraction of several classes of organic compounds (pesticides, polyaromatic hydrocarbons, acidic/neutral/basic organics, biological compounds and stabilizers) from solid matrices such as soil, sediment, plant and animal tissues, and polymers.[24-30] Through microwave irradiation, the temperatures of the extracting solvents can be increased by more than 100^0 C above their normal boiling points, thus allowing much shorter extraction times (minutes, compared to >16 hours for soxhlet extraction). The desorption process during the irradiation does not cause breakdown of organic compounds of interest, thus further enhancing the usefulness of this method of extraction. The reduction in extraction time and solvent consumption in MASE are obvious advantages over soxhlet extraction as the latter technique requires 16-24 hours and 200-300 mL of organic solvent in order to extract one sample. The parameters which can be optimized for the MASE of 4-NP in sediment samples include types of extracting solvents, extraction temperature and duration of extraction.

In the present study, 2 two-level orthogonal array designs using an OA_8 (2^7) matrix is used to optimize conditions for three sample preparation procedures, namely C_{18}-packed cartridge SPE, C_{18}-impregnated disk SPE and MASE, in the determination of 4-NP in water and sediment.

MATERIALS AND METHODS

Instrumentation and Chemicals

The microwave-assisted solvent extraction was carried out using a microwave extraction system (Model MES-1000, CEM, Matthews, NC, USA), equipped with a solvent detector, a safety feature. The MES-1000 is able to extract 12 solid samples at a time.

HPLC analysis was performed on a Waters (Milford, MA, USA) Powerline system comprising the Waters 600E system controller, Waters 486 tunable absorbance detector, Waters 470 scanning fluorescence detector and Waters 700 Satellite WISP autosampler. The Maxima 825/Baseline 815 Powerline HPLC software was used to control the system. The HPLC conditions used were: initial eluent of 80%:20% acetonitrile:water; programmed linearly to 100% acetonitrile over 20 min at a flow rate of 1.2

mL/min. Chromatographic separation was achieved on a 25 cm x 4.6 cm I.D. (5-μm particle size) Spherex C_{18} column (Phenomenex, Torrance, CA, USA). For UV detection, the wavelength was set at 225 nm. For fluorescence detection, the excitation (E_x) wavelength was 224 nm and emission (E_m) wavelength 308 nm.

All pesticide-grade and HPLC-grade organic solvents were purchased from Fischer Scienific (Pittsburgh, PA, USA). The water used was taken from a Milli-Q purification system (Millipore, Bedford, MA, USA). Technical grade 4-NP was purchased from Tokyo Kasei Kyogo (Tokyo, Japan). A standard solution of 4-NP containing 1000 ppm was prepared in methanol. Appropriate dilutions were made from this stock solution for calibration and spiking purposes. Analytical-grade sodium chloride and sodium dodecyl sulphate were purchased from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland), respectively.

Bakerbond Spe-500 cartridges (C_{18}-bonded silica gel containing 16% carbon) used in the present optimization procedure were purchased from J.T. Baker (Philipsburg, NJ, USA). 47-mm Empore (3M, St. Paul's, MN, USA) membranes with Bakerbond (J.T. Baker) C_{18}-bonded silica were used for disk extraction.

Optimization Strategy

On the basis of previously published information relating to the sample preparation techniques for 4-NP in water and sediment,[23] two different experiments were designed for optimization. All the parameters and their possible interactions in these two experiments were examined by two-level orthogonal array design with an $OA_8(2^7)$ matrix. Details on the assignment of factors and their interactions in the two-level OAD have been described elsewhere.[3-6] In the application of OAD, prior knowledge of the parameters most likely to be influential in the analysis is very helpful as certain two-variable interactions can be neglected.

The first experiment was designed to optimize the extraction of 4-NP using C_{18} cartridges. Three parameters likely to affect the extraction efficiency include: eluting solvent (Factor A), concentration of sodium chloride (Factor B) assignment of the factors and their levels is shown in Table 1. For a more direct and concentration of sodium dodecyl sulphate (SDS) (Factor C). The comparison between the membranous C_{18} disk and SPE cartride in terms of extraction efficiency, the optimum SPE conditions for cartridge extraction

Table 1

Assignment of Factors and their Levels Using an OA_8 (2^7) Matrix for the Extraction of Water

	Column*						Response
Trial No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**PR**
1	Methanol	0		0				70.5
2	Methanol	0		$1x10^{-4}$				88.7
3	Methanol	10		0				90.5
4	Methanol	10		$1x10^{-4}$				96.5
5	Acetonitrile	0		0				69.0
6	Acetronitrile	0		$1x10^{-4}$				83.6
7	Acetronitrile	10		0				84.0
8	Acetronitrile	10		$1x10^{-4}$				88.0

*Column 1 is assigned to eluting solvent (Factor A); column 2 is assigned to the concentration of sodium chloride (Factor B) [0-10% (v/v)]; column 3 is the interaction between Factor A and Factor B (AxB); column 4 is assigned to concentration of sodium dodecyl sulphate (Factor C) [0-$1x10^{-4}$M]; column 5 is the interaction between the Factor A and Factor C (AxC); column 6 is the interaction between Factor B and Factor C (BxC) and column 7 is unassigned.

were also used for disk-based extractions.

In the second experiment, the following parameters affecting the extraction efficiency of 4-NP present in sediment by MASE were selected: extracting solvent (Factor D), extraction temperature (Factor E) and duration of extraction (Factor F). The duration of extraction was defined as that from whence the pre-set temperature was reached. The microwave power was not considered for optimization because it was dependent upon the number of samples in one run. In this second experiment, the power was maintained at 50% and the temperature of the extracting solvent reached the required level within 5 minutes. The assignment of factors and their levels are provided in Table 2.

The percentage recovery (PR) of 4-NP was used as the response function in both experiments because it is convenient and time-saving especially when considering the effect of changes the parameters have on the extraction

Table 2

Assignment of Factors and their Levels Using an OA_8 (2^7) Matrix for the Extraction of Water

	Column*						Response
Trial No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**PR**
1	Methylene chloride	100°C		5				95.4
2	Methylene chloride	100°C		15				86.1
3	Methylene chloride	120°C		5				80.2
4	Methylene chloride	120°C		15				80.0
5	Acetone/ Petroleum ether	100°C		5				79.2
6	Acetone/ Petroleum ether	100°C		15				75.0
7	Acetone/ Petroleum ether	120°C		5				69.0
8	Acetone/ Petroleum ether	120°C		15				72.0

*Column 1 is assigned to the extracting solvents (methylene chloride and acetone/petroleum (1:1, v/v) (Factor D); column 2 is assigned to extraction temperature (100°C to 120°C) (Factor E); column 3 is the interaction between factor D and factor E; column 4 is assigned to the duration of extraction (Factor F); column 5 is the interaction between the factor D and factor F; column 6 is the interaction between the factor E and factor F; column 7 is unassigned.

efficiency. The PR results relating to extraction of 4-NP in water and in sediment are shown in Tables 1 and 2, respectively.

Sample Preparation

Solid phase extraction of water

SPE of 4-NP was carried out in a Supelco (Bellefonte, PA, USA) vacuum

manifold. 0.5 μg of 4-NP was spiked into 0.5 L of Milli-Q water. Prior to use, SPE cartridges were conditioned with 5 mL of the eluting solvents, followed by 5-mL aliquots of their respective aqueous media as shown in the experimental design (Table 1) for washing. The vacuum was applied at 68kPa, gauge reading. After spiked samples had been processed, the sorbent was air dried for 1 min. The adsorbed analyte was then eluted with one aliquot of 4 mL of the appropriate solvents. 20 μL of the extract was injected for HPLC analysis. The procedure was identical for disk extraction except that a solvent filtration apparatus was used.

Soxhlet extraction

5 g of sediment samples were carefully weighed and quantitatively transferred into an extraction thimble pre-rinsed with methylene chloride. The sample was extracted in a soxhlet apparatus with 300 mL of methylene chloride for 16 hours.

Microwave-assisted solvent extraction of sediment

5 g samples were accurately weighed out and quantitatively transferred into Teflon-lined extraction vessels of the MES-1000. The volume of extracting solvent was 30 mL. The operating conditions are given in Table 2.

Real sample analysis

Different types of water samples (tap water, sea water and wastewater) were extracted using the optimum SPE conditions established from the OAD exercise. Sediment was sampled from locations *ca.* 1 km from the shore of the primary industrial areas (Jurong and Tuas regions) of Singapore. The samples were extracted using the optimum MASE conditions and the extracts then analyzed with HPLC/fluorescence.

RESULTS AND DISCUSSION

Linear Range, Detection Limits and Precision

The linearities of the calibration graphs for 4-NP were in the range 50-5000 ng for UV detection and 0.5-100 ng for fluorescence detection. Fluorescence

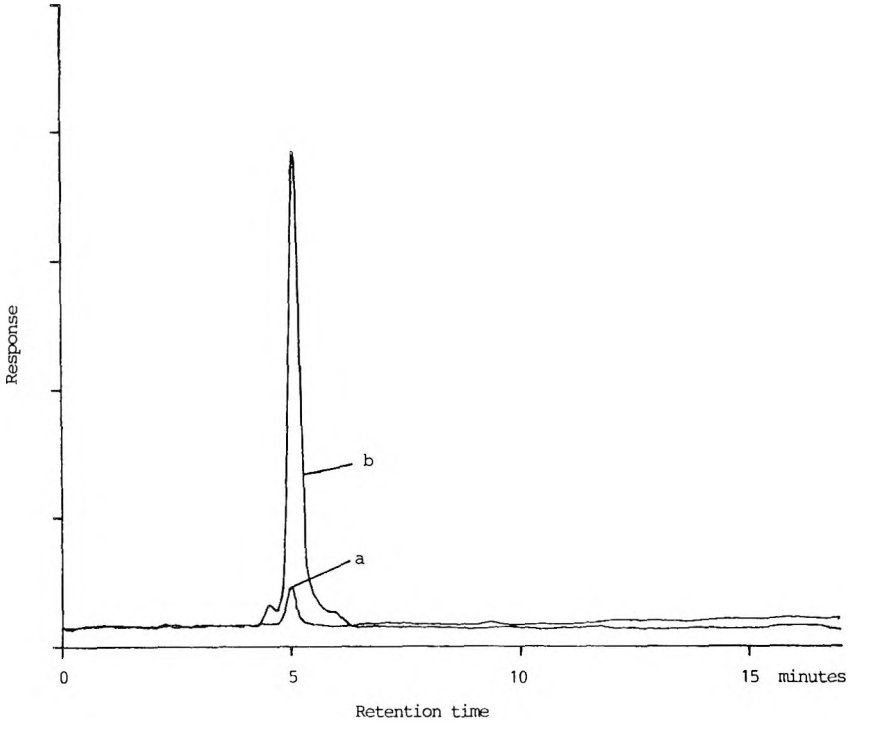

Figure 1. Superimposed high performance liquid chromatograms of a standard solution of 4-nonylphenol using (a) UV detection and (b) fluorescence detection. (See text for conditions).

detection exhibited at least 100-fold greater sensitivity than UV detection. Thus, quantitation for 4-NP was carried out with this mode of detection. The detection limits were determined based on a signal-to-noise ratio of 3. The detection limits for the 4-NP with fluorescence and UV detection were 0.02 ng and 25 ng, respectively. The retention time reproducibility was 0.05%. Figure 1(a) shows a liquid chromatogram of a standard solution of 4-NP in methanol using UV detection while Figure 1(b) gives that with fluorescence detection.

Optimization of Analytical Conditions for 4-NP

A total of 16 experimental trials pre-designed according to the 2-level OA_8 (2^7) matrix for the two optimization experiments were carried out. Corresponding chromatograms were subsequently obtained to derive percentage recoveries (PRs) based on peak areas. The PRs obtained for water and

sediment are tabulated in Tables 1 and 2 respectively. Based on the method presented previously[3], the results of the sum of squares (SS) for PRs for different variables and so-called two-variable interactions in both experiments were first calculated (see Tables 3 and 4, respectively). Three possible two-variable interactions are assigned to columns 3, 5 and 6 by using associated triangular tables[3]. Column 7 is unassigned to any variable or two-variable interaction in this optimization exercise. Thus, it is not necessary to carry out repetition of any experimental trials. However, data from previous publications and also the computed results of SS given in the ANOVA table (Table 3) indicate that 2 two-variable interactions assigned to columns 3 and 5 are negligible except for interaction BxC (column 6). In the case of the second experiment, interactions assigned to columns 5 and 6 are ignored. Finally, columns 3, 5 and 7 for the first experiment, and columns 5, 6 and 7 for the second experiment have been treated as dummies amd the error variance must be pooled from the SS for the total of the respective columns in each experiment.

From the ANOVA Table 3, it is seen that variable B (concentration of NaCl), variable C (concentration of SDS), variable A (eluting solvents) and interaction BxC are statistically significant at $P<0.05$ in the first experiment. (Variable B was the most significant factor ($P<0.005$) in the first experiment). Both variables B and C have been found previously to have significant influence on the recoveries of 4-NP during SPE[23]. Marcomini and his co-workers have mentioned that at least 3% NaCl (w/w) or $3x10^{-3}$ M SDS was necessary to prevent losses occurring in the filtration step during sample preparation.[23] In their work, recoveries of above 82% were obtained during SPE of water and industrial effluent spiked with 4-NP in the presence of 8% NaCl (w/w) or $7x10^{-3}$ M SDS. A relatively higher recovery was observed when NaCl instead of SDS was added. The same observation has also been made by Marcomini and his coworkers.[23] In our present work, the use of 10% (w/w) of NaCl and $1x10^{-4}$ M SDS during extraction of water gave good recoveries of 4-NP in the range of 83.6-96.5%. Thus, the optimum conditions for SPE of 4-NP are A_1, B_2 and C_2, namely, methanol as the eluting solvent, 10% (w/w) of sodium chloride and $1x10^{-4}$ M of SDS. It is affirmed that the above conclusion from ANOVA is the same as that from the direct observation method, which indicates that optimum conditions were obtained from experimental trial no. 4 in the first experiment.

In the case of the second experiment (MASE), only variable D (extracting solvent) and interaction DxE were statistically significant above $P<0.05$ (ANOVA Table 4). For the MASE of 4 - NP, methylene chloride is a better extracting solvent compared to 1:1(v/v) acetone/petroleum ether. The

Table 3

An ANOVA Table for Results Obtained in the Extraction of Water

Source	Sum of Squares	Degrees of Freedom	Mean Square	F ratio[a]	Significance
A (Solvent)	58.3	1	58.3	13.6	*P<0.05
B (NaCl)	278.5	1	278.5	64.8	***P<0.005
C (SDS)	229.0	1	229.0	53.1	**P<0.01
BxC	65.0	1	65.0	15.1	*P<0.05
Pooled Error[b]	12.9	3	4.3		
Total	643.7	7			

[a]The critical F ratio is 55.55 (***P<0.005), 34.12 (**P<0.01) and 10.13 (*P<0.05).

[b]Pooled error result from the pooling negligible effects from columns 3 and 5 (AxB and AxC) and unassigned column effect (column 7).

influence of the solvent on the extraction of organics has also been mentioned previously.[26,30] With the help of microwave irradiation, temperatures of the extracting solvents can be raised 100^0 C above their respective boiling points. The importance of interaction between the extracting solvent and the extraction temperature on the recoveries of 4-NP can only be an observation here. The extraction temperature from 100^0C to 120^0C and duration of extraction from 5 to 15 mins were not statistically significant to affect the recoveries of 4-NP from sediment samples. Similarly, the optimum conditions for SPE of 4-NP are D_1, E_1 and F_1, namely, methanol as extracting solvent, extraction temperature of 100 C and duration of extraction of 5 mins (experimental trial no. 1 in the second experiment).

Analytical Results of Real Samples

Using the optimum conditions obtained in the optimization procedures, analysis of genuine samples was carried out and the results tabulated in Tables 5 and 6.

Table 4

An ANOVA Table for Results Obtained in the Extraction of Sediment

Source	Sum of Squares	Degrees of Freedom	Mean Square	F ratio[a]	Significance
D (Solvent)	270.3	1	270.3	18.8	**P<0.025
E (Temp)	14.6	1	14.6	1.0	
F (Duration)	8.0	1	0.6		
DxE	147.9	1	10.3		*P<0.05
Pooled Error[b]	43.2	3	14.4		
Total	484.0	7			

[a]The critical F ratio is 17.44 (**P<0.025) and 10.13 (*P<0.05). [b]Pooled error results from the pooling negligible effects from column 5 & 6 (DxF and ExF) and unassigned column effect from column 7.

For the analysis of different types of water, a comparison between cartridge extraction and disk extraction of 4-NP using identical optimum conditions was carried out. Table 5 shows that both methods gave comparable recoveries for different types of spiked water samples. However, lower recoveries were obtained for unfiltered sea water and untreated industrial wastewater samples. Losses of 4-NP during extraction may be due to adsorption to the particulate matter present in these water samples. The high sedimentation observed in the untreated industrial wastewater may be the cause of the low recovery of 4-NP from this matrix. Basically, the problem of clogging of the SPE sorbents by particulate matter is also another possible reason for the lower recoveries in these water samples. However, this problem was less significant when disks, which are less prone to clogging, were used with the result that relatively higher recoveries were obtained from sea water and wastewater samples. Thus, in summary, membranous disk is more suitable for the extraction of high volumes of 'dirty' water samples.

Chromatograms of extracts of an untreated industrial wastewater sample and its spiked counterpart are shown in Figures 2(a) & (b).

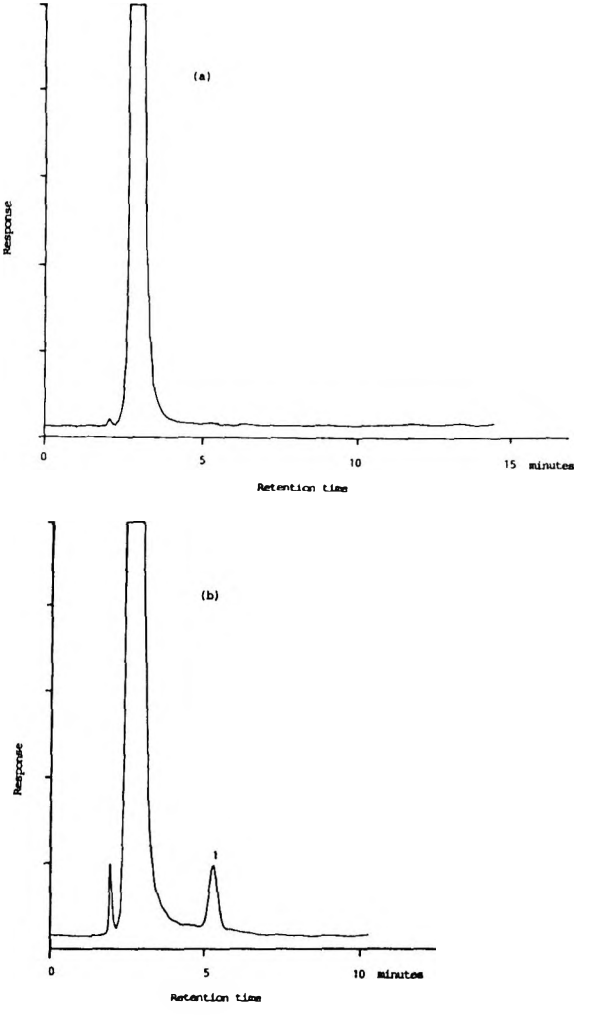

Figure 2. High performance liquid chromatogram with fluorescence detection of extract of (a) untreated wastewater extracted with C_{18} SPE cartridges and (b) spiked untreated wastewater with 50 ppb of 4-nonylphenol and extracted with C_{18} SPE cartridges. Peak 1 = 4-nonylphenol (retention time: 5.1 minutes). (See text for conditions.)

Table 5

Percent Recoveries of 4-nonylphenol Spiked into Different Water Samples using Conditions Optimized by OAD

	Recovery (%)[a]	
Sample	**A***	**B**
Distilled water	96.5±2.5	95.±3.0
Tap water	96.0±2.5	96.3±4.1
Seawater (unfiltered)	85.5±7.1	89.3±6.9
Seawater (filtered)	89.0±4.7	91.0±3.7
Wastewater (pH2)	82.9±8.4	84.0±7.1

[a]Mean %RSD (n=4)

*Method A: optimum SPE conditions using SPE C_{18} cartridges. Method B: same optimum SPE conditions using membranous C_{18} disks

Table 6

Comparison of Percent Recoveries Between Optimum MASE Conditions and Soxhlet Extraction for 4-Nonylphenol Spiked into Marine Sediments at 0.5 µg Levels

	Recovery (%)[a]	
Sample[b]	**MASE**	**Soxhlet Extraction**
Sediment (A)	95.4±3.0	92.3±6.1
Sediment (B)	89.6±4.1	87.9±3.0
Sediment (C)	85.1±8.3	80.8±11.6

[a]Mean %RSD (n=4)

[b]Genuine sediment samples collected from different coastal region around Singapore, A=east coast (minimal industrial activities), B=Straits of Johore (some industrial activities) and C=Jurong (heavy industrial activities)

Table 6 shows the recoveries of 4-NP spiked into different marine sediment samples, using MASE and soxhlet extraction. Both techniques gave satisfactory and comparable results for the recovery of 4-NP from marine sediment samples collected from several coastal locations. Recoveries were in the range 85.1-95.4% (%RSD 3.0-8.3) for MASE, and 80.8-92.3% (%RSD 3.0-11.6) for soxhlet extraction. Lower recoveries were observed for 4-NP spiked in marine sediments sampled from a heavy industrial area in Jurong to the west of Singapore island. On the other hand, higher recoveries were obtained from marine sediments sampled near the east coast of the island, a recreational area with minimal industrial activity. It was observed that the levels of recoveries might be affected by the quality of marine sediment in terms of the level of organics present in the sediment samples, e.g. organic carbon and humic acid. One possible reason may be the adsorption of 4-NP to the organic carbon present within the sediment particulates. Using the optimum conditions for MASE, no 4-NP was detected in the marine sediments sampled in this study. In summary, based on recovery studies of spiked samples, MASE is a better extraction technique, in terms of time and costs, when compared to soxhlet extraction as the latter requires the consumption of large volumes of hazardous organic solvents and prolonged hours of extraction time for one sample. With MASE, one may carry out extraction for 12 samples in one run and with minimal losses of the compound of interest.

CONCLUSION

This paper is concerned with the optimization of the sample preparation procedures using OAD for the determination of 4-NP in water and sediment. All three factors under consideration for solid phase extraction of 4-NP from water samples, namely concentration of sodium chloride, concentration of SDS and eluting solvents were found to be statistically significant. In the case of MASE of 4-NP in sediment, only the type of extracting solvent and its interaction with extraction temperature were found to be statistically significant. In this study, two-level OAD was used as a testing strategy for screening all factors and their interactions using fewer experimental trials so as to achieve the optimum analytical conditions without affecting the quality of the results. However, it should be emphasized that adequate judgement and pre-experience is necessary in identifying the factors and their levels, and output reponse for a given sample preparation procedure, and this procedure is normally subject to a case-by-case consideration. Under the optimum conditions for both sample preparation procedures, good recoveries of above 80% can be obtained for 4-NP in water and sediment. A preliminary survey of some areas of the Singapore marine environment indicated that there was no contamination of water and sediment samples by 4-NP.

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DETERMINATION OF SOLVENT BASICITY SCALE, β, OF MIXED SOLVENTS FOR THREE CHROMATOGRAPHIC SOLVENT SYSTEMS: 2-PROPANOL/HEXANE, ETHYL ACETATE/HEXANE, AND METHANOL/WATER

Won Jo Cheong*, Sung Hyun Chun, Gong Yeal Lee

Department of Chemistry,
Inha University,
Incheon 402-751, South Korea

ABSTRACT

We have determined β values of mixed solvents for the 2-propanol/hexane, ethyl acetate/hexane, and methanol/water systems using totally 5 indicator pairs (10 dyes) three of which were synthesized in this study. The variations of β vs. solvent composition for the RPLC solvent system are in a striking constrast with those for the NPLC systems. While a definite maximum was recognized in the plots of β vs. φ for the NPLC systems, a linear correlation of β with φ was observed for the RPLC solvent system. The appearance of maximum is probably due to formation of aggregates of the more polar solvent when its composition gets higher.

INTRODUCTION

Linear solvation energy comparison methods based on Kamlet/Taft polarity scales[1-5] have been known to be very useful in exploring linear solvation energy relationships (LSER) in reverse phase liquid chromatography (RPLC)[6-12]. Application of LSER to normal phase liquid chromatography (NPLC)[13] has been recently reported, too. The basic idea of such approaches is that a distribution of a solute between two immisicible phases is governed by the cavity formation energy of the solute and by the solute-solvent interaction energies in each phase and that the solute-solvent interaction energies are the linear sum of several independent terms each of which corresponds to a characteristic solute-solvent interaction. Each interaction energy is proportional to the cross product of the semiempirical polarities of the solute and the solvent.

In LSER studies of chromatography, one needs polarity data of mixed solvents. Some research groups have reported polarity scales of mixed solvents for RPLC[14-16] and NPLC[17-18].

In this study, hydrogen bond accepting basicity (β) values of binary solvents have been determined for a typical RPLC solvent system (methanol/water) and two NPLC solvent systems (2-propanol/ hexane and ethyl acetate/hexane) using five pairs of indicator dyes three of which were directly synthesized in this study. Determination of β is based on the assumption that β is linearly correlated with the difference between absorption frequencies of a pair of indicators comprising of a dye with a hydrogen bond donating group (-OH or $-NH_2$) and another one with its alkylated group (-OR or $-NR_2$).

The basicity of cyclohexane is defined 0, and the basicity of hexamethylphosphoramide, 1. Normalization schemes for individual indicator pairs were well documented in the literature[2]. Only one research group[16] has reported β values of binary solvents for RPLC including methanol/water mixtures. Their data, however, were based on one pair of indicators, and subject to some uncertainties, thus we redetermined them in this study. β values of mixed solvents for normal phase liquid chromatography were previously measured in our laboratory[18]. In that study, we used only two indicator pairs and suggested that β values of the mixed solvents be reestimated with more indicators. We have redetermined β values of such solvents in this study using totally five indicator pairs.

EXPERIMENTAL

All solvents were HPLC grade from Fisher Scientific Inc. (Pittsburg, PA, U.S.A.) and were used without further purification. The binary solvent mixtures were prepared by mixing a known volume of each liquid, and the composition (ϕ, volume fraction) is defined based on the volume before mixing. The indicator pairs are as follows: the pair of 4-nitrophenol [p-nitrophenol, PNP] and 4-nitroanisole [1], the pair of 4-nitroaniline [14] and N,N-diethyl-4-nitroaniline [6], the pair of 2-nitro-p-toluidine [23] and N,N-dimethyl-o-nitro-p-toluidine [22], the pair of 2-nitroaniline [33] and o-nitrodimethyl aniline [31], and the pair of 2-nitro-p-anisidine [35] and N,N-dimethyl-o-nitro-p-anisidine [34]. The numbers in brackets refer to the Kamlet/Taft indicator designations. Each β determined for an indicator pair is named after the Kamlet/Taft designations. Thus, the five β's based on individual indicator pairs are defined as β_{PNP-1}, β_{14-6}, β_{23-22}, β_{33-31}, β_{35-34}, respectively. The solutes, 22, 31, and 34, were synthesized.

Other chemicals were either kindly donated from professor Peter W. Carr (Department of Chemistry, U of Minnesota, Minneapolis, U.S.A.) or purchased from Aldrich Chemical Co. (Milwakee, WI, U.S.A.) Each solute was tested by HPLC for impurities before spectroscopic measurements, and recrystallized if UV/VIS absorbing impurities existed.

All spectroscopic measurements were carried out by the previously reported procedures[1-5]. A Perkin Elmer UV/VIS double beam spectrophotometer (Model 552S, Perkin Elmer, England) was used to make the measurements to $\pm$ 0.2nm with a bandwidth of 1nm. The solute concentration in each mixed solvent is carefully controlled to give absorbance within the range from 0.5 to 1.5. The method of Campbell[19] for synthesis of o-nitro dimethylaniline was extensively modified and applied to syntheses of three indicators 22,31, and 34.

Synthesis of o-Nitrodimethylaniline[31]

A mixture of 10g o-nitrochlorobenzene, 15g sodium bicarbonate, and 80ml pyridine was poured into a 250ml three-neck round bottom flask, then 8g dimethyl hydrochloride dissolved in 3ml warm water was added through a dropping funnel in vigorous agitation. The bottle was heated to the reflux temperature, and the reflux, maintained for 10 hrs. The content was filtered to remove inorganic salts while it was hot, and the filtrate was allowed to

evaporate to lose two thirds of its original volume. The crude oily o-nitrodimethyl aniline precipitated upon addition of excess water (ca. 500mL). To the solidified bottom layer (0-5°C) separated from the mother liquid by decantation, 30mL water was added, the temperature, raised to 40°C, and dilute hydrochloric acid, added slowly to give a clear solution, then the pH of the solution was adjusted to pH3. The solution was mixed with 50mL methanol and allowed to stand overnight at 5°C to give needle-like crystal precipitations. The crystals were filtered, dissolved in dilute hydrochloric acid, and recrystallized. The crystals were filtered, dissolved in distilled water, and neutralized to pH7. Pure liquid o-nitrodimethylaniline formed in the bottom was extracted with chloroform. The chloroform solution was washed with distilled water several times, dehydrated in a dessicator, and evaporated to give ca. 4g orange color product. ^{1}H-NMR ($CDCl_3$):δ 2.8 (s, 6H), 6.8-7.7 (m, 4H); MS (EI 30eV): m/z 166 (M , 100), 149 (96.0), 134 (37.2), 121 (68.0), 120 (37.3), 119 (78.0), 118 (80.2), 107 (42.5), 106 (36.4), 105 (73.6), 104 (83.7), 94 (64.3), 92 (69.2), 91 (85.1), 78 (68.1), 77 (77.8); IR (neat): 3090 (w), 2965 (w), 1605, 1564 (s), 1520 (vs), 1477 (s), 1458 (s), 1362 (s), 1348 (s), 1303 (s), 1272 (s), 1120, 1014 (s), 956, 918, 902, 846 (w) cm^{-1}.

Synthesis of N,N-Dimethyl-o-Nitro-p-Toluidine[22]

The procedure was generally similar to that of synthesis of o-nitrodimethylaniline except for use of cyclohexanol instead of pyridine as the reflux solvent since a much higher temperature was required. Dimethylamine hydrochloride was continuously added in a powder form to the dropping funnel and allowed to be washed down by the reflux condensate. An aliquot of cyclohexanol solution was taken for HPLC analysis periodically. The reaction was terminated when the yield reached 90%. We obtained ca. 3g pure cardinal color oily product from 10g N,N-dimethyl-o-nitro-ptoludine. ^{1}H-NMR ($CDCl_3$): δ 2.3 (s, 3H), 2.8 (s, 6H), 6.9-7.5 (m, 3H); MS (EI 30eV): m/z 180 (M^+, 35.1), 163 (35.5), 135 (19.2), 133 (38.3), 132 (27.2), 131 (28.3), 119 (42.4), 118 (58.2), 105 (100), 91 (59.0), 77 (18.1), 69 (49.6), 65 (20.0); IR (neat): 2930, 2905, 2875, 2700 (w), 1619, 1522 (vs), 1448, 1438, 1340 (s), 1278 (s), 1200, 1160, 1149, 1060 (w), 953 (w), 907 (w), 798, 757 (w) cm^{-1}.

Synthesis of N,N-Dimethyl-o-Nitro-p-Anisidine[34]

The procedure was very similar to that of synthesis of N,N-dimethyl-o-nitro-p-toluidine except that the reaction was terminated when the yield reached 75%. We obtained ca. 2g vermilion color oily product from 10g 3-

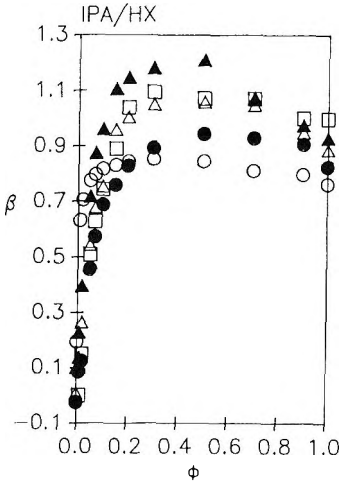

Figure 1. Variation trends of individual β's of 2-propanol/hexane mixtures obtained with 5 indicator pairs with respect to volume fraction of 2-propanol. Symbols; o: β_{PNP-1}, • : β_{14-6}, Δ:β_{23-22}, ▲ :β_{33-31}, □ : β_{35-34}.

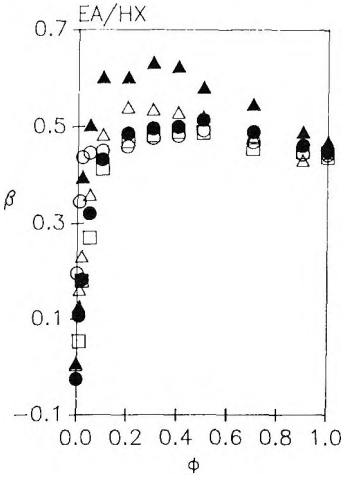

Figure 2. Variation trends of individual β's of ethyl acetate/ hexane mixtures obtained with 5 indicator pairs with respect to volume fraction of ethyl acetate. Symbols; o:β_{PNP-1}, • : β_{14-6}, Δ : β_{23-22}, ▲ : β_{33-31}, □ : β_{35-34}.

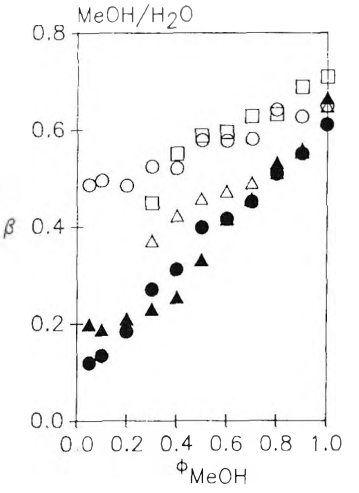

Figure 3. Variation trends of individual β's of methanol/water mixtures obtained with 5 indicator pairs with respect to volume fractin of methanol. Symbols; o: β_{PNP-1}, • : β_{14-6}, Δ : β_{23-22}, ▲ : β_{33-31}, □ : β_{35-34}.

nitro-4-chloroanisole by following the adequate procedures. ^{1}H-NMR ($CDCl_3$): δ 2.7 (s, 6H), 3.8 (s, 3H), 6.9-7.1 (m, 3H); MS (EI 30eV): m/z 196 (M^+, 53.5), 179 (32.9), 164 (8.0), 151 (34.1), 150 (15.3), 149 (32.2), 135 (77.1), 134 (76.8), 121 (83.7), 120 (100), 106 (27.0), 92 (23.4), 79 (10.0), 77 (18.5); IR (neat): 2920, 2885, 2830, 2795, 1558 (s), 1520 (vs, broad), 1452 (s), 1435 (s), 1323 (s), 1290 (vs,broad), 1237 (s), 1194, 1158, 1142, 1058, 1034 (s), 950 (w), 918 (w), 903 (w), 847 (w), 798 (s), 758 (w), 723 (w), 680 (w) cm^{-1}.

RESULTS AND DISCUSSION

The individual β values obtained with 5 indicator pairs are comparatively plotted with respect to solvent composition in Figure 1 for the 2-propanol/hexane system, in Figure 2 for the ethyl acetate/hexane system, and in Figure 3 for the methanol/water system, respectively. The variation trends of β for the NPLC solvent systems (2-propanol/hexane and ethyl acetate/hexane mixtures) are in a striking contrast to those for the RPLC solvent system (methanol/water mixtures).

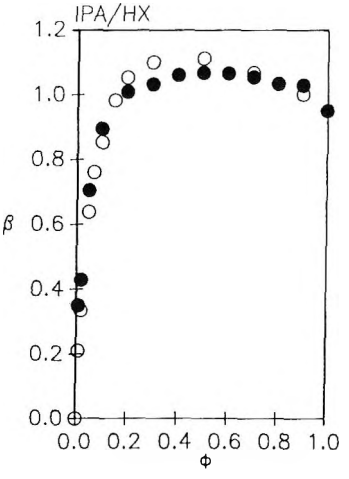

Figure 4. Comparison of the new β values with the previous ones for the 2-propanol/hexane system. Open circle: new values, Closed circle: old values.

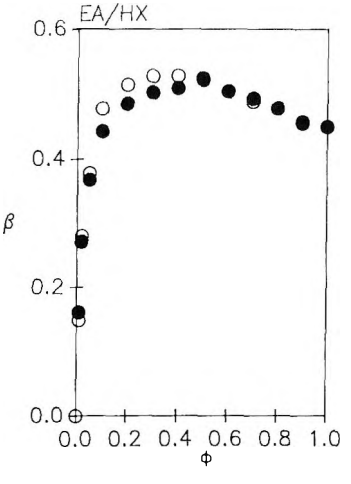

Figure 5. Comparison of the new β values with the previous ones for the ethyl acetate/hexane system. Open circle: new values. Closed circle: old values.

In the 2-propanol/hexane and ethyl acetate/hexane systems, every β follows the same trend; it sharply increases at the beginning, levels off later, reaches a maximum, and decreases as the composition of the more polar solvent varies from 0 to 100%. On the other hand, β's of methanol/water mixtures monotomcally increase with methanol content (Figure 3). We observed some scatter for 5 individual β values. The extent of scatter among the 5 data sets remains virtually invariant with respect to solvent composition for the NPLC solvent systems, while the extent of scatter at compositions of lower methanol content (higher water content) tends to be much larger than that at compositions of higher methanol content for the methanol/water system.

There were difficulties in preparing solutions of indicator 22 and 34 in mixed solvents of high water content because of their limited solubilities, and therefore $\beta_{23\text{-}22}$ and $\beta_{35\text{-}34}$ could not be measured for solvents of 0-20% methanol. Nevertheless, $\beta_{23\text{-}22}$ and $\beta_{35\text{-}34}$, tend to fall in the middle of the scatter span of 5 data sets, and the average β values of the 3 data sets for the solvents of 0-20% methanol are regarded to be consistent with the average β values of the 5 data sets for solvents of other compositions.

The averaged (5 data sets) and corrected β values for the 2-propanol/hexane and ethyl acetate/hexane systems are assembled in Table 1. The correction of averaged β values was executed to make the measured β values of mixtures be consistent with the literature βvalues of pure solvents. We obtained β value of 0.027 for pure hexane, 0.880 for pure 2-propanol, and 0.449 for pure ethyl acetate while the literature β values for hexane, 2-propanol, and ethyl acetate are 0, 0.950, and 0.45, respectively. The literature β values are based on much more extended data sets, and are likely to be different from our measured values based on 5 data sets. We believed the literature values are more reliable and made our data be corrected assuming a good linear correlation between the literature and measured β values. Let us define a_1, a_2, as the measured and literature values of pure hexane, b_1, b_2, as the measured and literature values of the pure polar solvent, and x_1, as the measured β value of a mixture, then the corrected β value (x_2) consistent with the literature data is obtained as follows:

$$x_2 = a_2 + \frac{b_2 - a_2}{b_1 - a_1}(x_1 - a_1)$$

Such correction was not applied to the methanol/water system since we were not able to measure reliable β value of pure water. The measured values

Table 1

The Averaged and Corrected β Values of 2-Propanol/Hexane and Ethyl Acetate/Hexane Mixtures

2-Propanol/Hexane		Ethyl Acetate/Hexane	
ϕ^a	β	ϕ^b	β
0.0	0.0	0.0	0.0
0.01	0.211	0.01	0.149
0.02	0.335	0.02	0.280
0.05	0.638	0.05	0.378
0.07	0.761		
0.1	0.853	0.1	0.478
0.15	0.982	0.2	0.515
0.2	1.052	0.3	0.528
0.3	1.099	0.4	0.529
0.5	1.111	0.5	0.522
0.7	1.067	0.7	0.489
0.9	1.001	0.9	0.455
1.0	0.950	1.0	0.450

a. Volume fraction of 2-propanol
b. Volume fraction of ethyl acetate

of methanol/water mixtures do not seem to be much deviated from the literature values, anyway. The averaged β values for the methanol/water systems are summarized in Table 2.

We mentioned that we had determined β values of 2-propanol/hexane and ethyl acetate/hexane mixtures using only 2 indicator pairs in the previous study[18]. We compared the new β values with the previous ones in Figure 4 (2-propanol/hexane mixtures) and in Figure 5 (ethyl acetate/hexane mixtures). Assuming that the new data based on 5 indicator pairs are more reliable than the old data based on 2 indicator pairs, we noted that the old β values are a little overestimated at lower content of the more polar solvent and that the values around the maximum are more or less underestimated (See Figures 4 and 5).

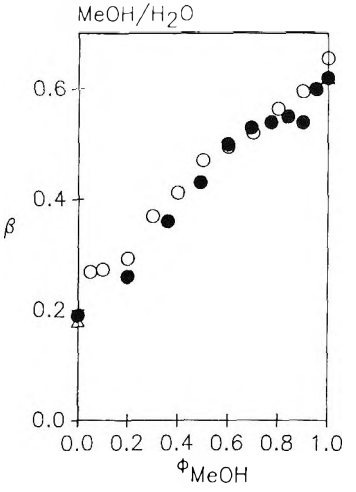

Figure 6. Comparison of the new βvalues with the previously reported ones. Open circle: new values, Closed circle: old values, Open triangle: the original literature value for pure solvents.

Table 2

The Averaged β Values of Methanol/Water Mixtures

ϕ^a	β^b
0.05	0.269 (0.193)
0.1	0.273 (0.195)
0.2	0.293 (0.166)
0.3	0.369 (0.122)
0.4	0.412 (0.128)
0.5	0.470 (0.112)
0.6	0.495 (0.077)
0.7	0.521 (0.071)
0.8	0.564 (0.059)
0.9	0.596 (0.054)
1.0	0.656 (0.032)

a. Volume fraction of methanol
b. Standard deviations are given in parentheses

We also mentioned that a research group had reported β values of methanol/water mixtures using only one indicator pair[16]. The newly determined and previously reported values are compared in Figure 6. They are roughly identical but the irregular fluctuation present in the plot of old data disappears in the plot of new data. We can note that the β of methanol/water mixtures are almost linearly correlated with volume fraction of methanol.

We believe that the appearance of a maximum in the plot of β against solvent composition for the 2-propanol/hexane and ethyl acetate/hexane systems is real considering that all of the five data sets show exactly the same trend. We now turn to rationalizing how a mixture of two solvents can have higher basicity than either of the pure solvents does. Appearance of extrema in plots of polarity scales of mixed solvents vs. solvent composition is occasionally observed when the system is composed of a polar and a nonpolar solvents[20-22]. We first emphasize that all indicator dyes used in solvatochromic measurements are moderately polar in general[1-5].

We should also note that a polarity scale of a solvent , whether pure or mixed, is monitored by an indicator, and that the polarity determined by the indicator actually refers to the local environments around the indicator. It is very likely that the environments of an indicator get more polar as the polar solvent is added to the pure nonpolar solvent, and it actually happens at the initial stage of addition. The more polar solvent molecules seem to behave in two different fashions when the more polar solvent is introduced into the nonpolar solvent. First, they coordinate the indicator molecules to give strong solute-solvent interactions. Second, they coordinate themselves one another to yield strong solvent-solvent interactions forming dimeric or polymeric aggregates. In the latter case, strong functional groups interact one another inside the aggregates, then the aggregates will not be capable of strong solute-solvent interactions, which leads to reduction of solvent polarity. The former process prevails when the composition of more polar solvent is low since formation of aggregates of polar solvent molecules is thermodynamically forbidden (very high negative entropy change).

The free monomeric polar solvent molecules selectively coordinate solute molecules, and addition of a small amount of the polar solvent to the nonpolar solvent causes a sharp increase of polarity as is shown in Figures 4 and 5. As the more polar solvent is further added, the second process gets more favorable, thus the basicity of the mixed solvent reaches a maximum and reduces after the composition pass the limit where the number density of monomeric polar solvent molecules hits its maximum. Hurtubise et al.[17] proposed similar arguments in their sovatochromic study for 2-propanol/heptane and ethyl

acetate/ heptane mixtures. They noted that there were two major hydrogen bondings- a region where monomeric hydrogen-bonding solvent molecules were interacting with the solute molecules, and a region where dimeric or polymeric hydrogen-bonding solvent molecules were interacting with the solute molecules.

On the other hand, β monotonically and linearly varies with respect to methanol composition for the RPLC solvent system (methanol/water). We can expect a similar variation trend to those of the NPLC solvent systems if we merely consider that there is large difference in β between water (0.18) and methanol (0.62). We suggest a very crude and qualitative explanation to account for the linear variation of β with ϕ for methanol/water mixtures. Not difference in β but difference in the overall molecular polarity contributes to formation of dimeric or polymeric aggregates of the more polar solvent. Considering that π^* (dipolarity/ polarizability, 1.09) of water is greater than π^* (0.60) of methanol, α (hydrogen bond donating acidity, 1.17) of water, a little greater than α (0.93) of methanol, and β (0.18) of water, smaller than β (0.62) of methanol, we may conclude that the overall polarity of water is roughly comparable to that of methanol.

This argument is against the general conception that water is much more polar than methanol as we note that addition of water to pure methanol yields a striking increase in solute retention in RPLC and that nonpolar solutes do not dissolve in water but moderately dissolve in methanol. But the peculiar polarity of water in phase-transfer-related processes is largerly due to the very high cohesive energy density of water which causes a very high positive cavity formation energy when a solute is introduced in water. When only molecular interactions are considered, as in solvatochromic measurements, the overall polarity of water could be regarded to be roughly similar to that of methanol. In such a situation, both methanol and water will not form aggregates of one kind but yield random and uniform mixing. Therefore β of the mixture linearly correlates with volume fraction of methanol.

It is interesting to note that Katz et al.[23] proposed that a mixtures of water and methanol is composed of three species, that is, water, methanol, and 1:1 complex of water and methanol. Their view also seems to support random and uniform mixing. Cheong et al.[24] reported a possibility of existence of a minimum in the plot of α (hydrogen bond donating acidity) of aqueous methanol mixtures vs. methanol volume fraction based on solvatochromatic data obtained with a zwitterion type betaine dye, which might support nonrandom mixing between water and methanol, but later Park et al.[25] showed

that use of a non-zwitterion type indicator free of specific interactions yielded a monotonous variation of α with respect to methanol volume fraction. There have been some reports that allude similarity in polarity (excluding cohesive energy density effect) between water and methanol. Some workers[26-27] observed a linear trend in plots of ln k' vs. methanol volume fraction. A linear relationship of the logarithmic solute activity coefficient in aqueous methanol mixtures with methanol volume fraction was also observed[28]. Street et al.[29] obtained a linear correlation between Py (solvatochromic polarity scale based on light absorption of pyrene) and methanol volume fraction. Krygowski et al[16]. and Johnson et al.[30] showed an almost linear correlation between E_T (solvatochromic polarity scale based on a betaine dye) and methanol volume fraction. On the other hand, in other RPLC solvent systems such as acetonitrile/water, 2-propanol/water, or tetrahydrofuran/water, nonlinear relationships were observed between a solvatochromatic or chromatographic/ thermodynamic property and ϕ.[16,26-30]

CONCLUSIONS

The appearance of a maximum in the plots of β vs. solvent composition for NPLC solvent systems seems to be related to the twofold behaviors of the more polar solvent molecules: formation of solute-solvent interactions (monomeric solvent) and aggregation of solvent molecules (polymeric solvent). The averaged and corrected β data reported in this study could be useful for LSER applications in liquid chromatography. The linear correlation of β with volume fraction of methanol for the methanol/water system leads to the conclusion that either water or methanol molecules do not form polymeric aggregates of one kind but randomly mix with the other solvent molecules.

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SIMULTANEOUS DETERMINATION OF QUININE AND A MAJOR METABOLITE 3-HYDROXYQUININE IN BIOLOGICAL FLUIDS BY HPLC WITHOUT EXTRACTION

S. Wanwimolruk,* S. M. Wong,
H. Zhang, P. F. Coville

School of Pharmacy
University of Otago
P O Box 913,
Dunedin, New Zealand

ABSTRACT

A reverse phase, isocratic HPLC method has been developed for the quantitation of quinine and its major metabolite, 3-hydroxyquinine in human plasma, urine and hepatic microsomal samples. The method involves simple protein precipitation for sample treatment and ion-paired chromatography. The chromatographic separation is accomplished with a mobile phase comprising acetonitrile-aqueous phosphate buffer (40:60, v/v) containing 10 mM sodium dodecyl sulphate and 0.1 mM tetrabutylammonium bromide and adjusted to pH 2.1. The mobile phase is pumped at a flow rate of 0.5 mL/min. A microbore column is used (2 mm I.D. x 100 mm) packed with a C_{18} reverse phase material (5 μm ODS Hypersil). Biological samples (100-500 μL) were precipitated with two volumes of cold methanol, vortexed and then centrifuged at 1500 g for 10 min. The supernatant (30 μL) was injected into the HPLC column. The chromatograms were monitored using a fluorescence detector setting with excitation and emission wavelenths of 350 and 450 nm, respectively. Under

these conditions, the lower limit of detection was 0.1 μM (0.034 μg/mL) for the major metabolite 3-hydroxyquinine, and 0.5 μM (0.16 μg/mL) for quinine. The inter- and intra-assay coefficients of variation were found to be less than 7%. The assay procedure is applicable for studying the pharmacokinetics and metabolism of quinine.

INTRODUCTION

Quinine is one of the oldest drugs in the pharmacopoeia. While synthetic antimalarial drugs have largely replaced quinine, the emergence of strains of *Plasmodium falciparum* resistant to chloroquine in Southern Asia, South America and East Africa, has necessitated its continued therapeutic use.[1] Some 42% (2,117 million) of the world population live in endemic malaria area.[2] Quinine is also widely used for the prevention of night cramps in the elderly. Despite its long history in the treatment of malaria, the metabolism of quinine in man has not been fully elucidated. In man, quinine undergoes extensive oxidative metabolism with approximately 10-20% of an oral dose being excreted in the urine unchanged.[1,3,4]

In contrast, the metabolism of its diastereoisomer, quinidine, has been well investigated. Major metabolites of quinidine appear to be 3-hydroxyquinidine, quinidine-*N*-oxide and 2'-quinidinone.[5,6] The *in vitro* hepatic microsomal metabolism of quinidine to form 3-hydroxyquinidine and quinidine-*N*-oxide metabolites was shown to be catalysed by P450IIIA4.[5] A polar product of quinine was also detected after incubation with human liver microsomes. This had a similar retention time to 3-hydroxyquinidine, but this product was not identified.[5] Liddle et al.[7] identified 3-hydroxyquinine in human urine after administration of quinine. They also identified five other quinine metabolites which were 6'-hydroxycinchonidine (*O*-desmethylquinine), 6'-hydroxydihydrocinchonidine, 3-hydroxydihydroquinine, quinine-10,11-epoxide and quinine-10,11-dihydrodiol.

The lack of information on the pharmacokinetics and metabolism of quinine is largely due to non-availability of synthetic metabolites. In addition, there is no specific analytical method available for the determination of quinine metabolites in biological samples. A number of high performance liquid chromatography (HPLC) methods for determination of quinine have been published.[8-15] Most of these employ solvent extraction as a sample preparation, thus being time consuming, and none of these measured quinine metabolites. Therefore, this study was conducted to develop a simple and specific HPLC

assay for the simultaneous determination of quinine and its metabolites in biological fluids without solvent extraction.

MATERIALS AND METHODS

Reagents and Chemicals

All chemicals were of analytical grade. Quinine bisulphate (M.W. of quinine free base = 324.4), was kindly supplied by Kimia Pharma, Indonesia. 3-Hydroxyquinine, M.W. of 340.4 (free base), was a gift from Dr. P Winstanley, Department of Pharmacology and Therapeutics, University of Liverpool, UK. Tetrabutylammonium bromide (TBA) was purchased from Sigma Chemical Co (St Louis, MO, USA). Sodium dodecyl sulphate (SDS), HPLC-grade methanol and acetonitrile were purchased from BDH Chemicals Ltd (Poole, UK). All water was double glass distilled and MilliQ® filtered.

Stock solutions of 1 mg/mL quinine free base and 0.5 mg/mL of 3-hydroxyquinine were prepared in 50% (v/v) methanol-water. The solutions were protected from light and stored at -20 °C until required. These solutions were found to be stable for at least 1 month. Standard solutions (in plasma, urine, or hepatic microsomal samples) with known concentrations of quinine and its metabolite were prepared by mixing stock solutions of quinine, 3-hydroxyquinine and drug-free samples.

Identification of Urinary Metabolites of Quinine

When this study was commenced, synthetic metabolites of quinine were not available. *The metabolites of its diastereoisomer quinidine were therefore used as reference compounds* to identify possible metabolites of quinine in the human urine, plasma samples and the samples from human liver microsomal studies. Quinidine metabolites, 2'-oxoquinidine (2'-oxoquinidinone), quinidine-*N*-oxide, 3-hydroxyquinidine and *O*-desmethylquinidine (6'-hydroxycinchonidine) were kindly provided by Professor I A Blair, Vanderbilt University School of Medicine, Tennessee, USA. Later, when synthetic 3-hydroxyquinine (kindly donated by Dr P Winstanley, University of Liverpool, UK) became available, it was used as a reference standard instead of using 3-hydroxyquinidine.

Stock solutions of 10 μg/mL of each metabolite of quinidine (2'-oxo-quinidinone, quinidine-*N*-oxide, 3-hydroxyquinidine and *O*-

desmethylquinidine) were prepared in 96:4 (v/v) methanol-water. The solutions were further diluted with distilled water and mixed to give a final concentration of 0.1 μg/mL of all the metabolites except for *O*-desmethylquinidine (7 μg/mL). The mixture of metabolites was injected into the HPLC column and used as a reference to identify the possible metabolites of quinine in the biological samples.

The concentration of quinine and its metabolite 3-hydroxyquinine were determined from calibration plots of the chromatographic peak heights versus drug or metabolite concentration.

Biological Samples

Samples for development and evaluation of the method were obtained both by spiking drug-free plasma and urine with known amounts of quinine and its metabolite 3-hydroxyquinine, and by collecting blood and urine samples from healthy volunteers participating in a pharmacokinetic study.[4] These volunteers took a single oral dose of 600 mg quinine sulphate. Venous blood was obtained in a 5 mL heparinised tubes and plasma was prepared by centrifuged blood at 1500g for 10 min. All samples collected were kept at -20°C until analysis. Microsomal samples were obtained after incubation of quinine (as a substrate) with human liver microsomes. Procedures for the microsomal incubation have been described previously.[16]

Sample Preparation

To 100 μL of plasma was added 200 μL of cold methanol. The samples were vortexed for 10 seconds, then centrifuged at 1500g for 10 minutes. After centrifugation, an aliquot (30 μL) of the supernatant was injected into the HPLC column.

It was found that the concentrations of quinine and its metabolites in urine samples (0-48 h) collected from healthy volunteers who took a single oral dose of 600 mg quinine sulphate, were too high. So it was necessary to dilute the urine samples 1 in 10 with HPLC water before the sample preparation. In brief, 100 μL of urine samples were diluted by adding 900 μL of HPLC water. Then, to 100 μL of the diluted urine samples was added 200 μL of cold

methanol. The samples were vortexed, then centrifuged and processed as above.

For hepatic microsomal samples, the samples were obtained after incubation of quinine (500 μM) with 1 mg/mL human liver microsomes and 1 mM NADPH, in a final 500 μL volume, for 30 minutes as described previously.[16] In order to terminate the metabolic reactions, two volumes of cold methanol (i.e. 1 mL) was added to the microsomal samples. The samples were then vortexed for 10 seconds, and centrifuged at 1500 g for 10 minutes. The resultant supernatant was diluted 1 in 4 with HPLC water and the aliquot (30 μL) of this diluted supernatant was injected into the HPLC column. Dilution was made to allow the detection of both quinine and its major metabolite 3-hydroxyquinine within the appropriate attenuation of the HPLC integrator.

Chromatographic Conditions

The HPLC system consisted of a Model 250 Perkin-Elmer LC pump (Perkin-Elmer Corporation, Norwalk, CT, USA) equipped with a WISP 712 autoinjector (Waters Assoc., Milford, MA, USA). A Hitachi fluorescence detector (Hitachi, Tokyo, Japan) was used and operated with exitation and emission wavelengths of 350 and 450 nm, respectively. The chromatographic response was recorded by a Hitachi D2500 integrator (Hitachi, Tokyo, Japan). A mocrobore HPLC column (2 mm I.D. x 100 mm) packed with a reverse phase C_{18} material, 5 μm ODS Hypersil (Shandon, London, UK) was used.

Analysis of the samples of quinine and its major metabolite 3-hydroxyquinine was performed using a mobile phase consisting of an acetonitrile-aqueous (40:60, v/v) containing 10 mM Na_2HPO_4, 10 mM sodium dodecyl sulphate (SDS) and 0.1 mM tetrabutylammonium bromide (TBA). The pH of mobile phase was finally adjusted to 2.1 with orthophosphoric acid. The flow rate was 0.5 mL/min. Chromatographic separations were performed at room temperature.

RESULTS AND DISCUSSION

An initial attempt had been made to identify possible metabolites of quinine in human urine by using the original HPLC mobile phase as previously described.[10] Initially, since the metabolites of quinine were not available,

metabolites of quinidine were used as reference compounds based on the assumption that there would be a similarity in the retention times of corresponding metabolites of quinine and quinidine. Admittedly, there was no evidence showing that the metabolites would necessarily have similar retention times. However, this assumption was supported by the evidence that quinine and quinidine had very similar retention times of 8.2 and 8.3 min, respectively, when the original mobile phase was used. Under the chromatographic conditions used by Zoest et al.,[10] i.e., using the original mobile phase consisting of an acetonitrile-aqueous phosphate buffer (10 mM) mixture (50:50, v/v) containing 25 mM SDS and 3 mM TBA, there was no resolution between the peaks of 3-hydroxyquinidine and quinidine-*N*-oxide. They had retention times of 3.9 and 4.4 min, respectively.

Therefore, the HPLC assay was further developed by modification of the original mobile phase. The mobile phase was selected on the basis of the retention behaviour of quinine and metabolites of quinidine (used as reference compounds) as a function of ion-pairing agent (SDS). Chromatographic separation of quinine and metabolites of quinidine was accomplished with a mobile phase of acetonitrile-aqueous phosphate buffer mixture (40:60, v/v) containing 10 mM disodium hydrogen phosphate, 10 mM SDS and 0.1 mM TBA, adjusted to pH 2.1. With these chromatographic conditions, a good resolution of 3-hydroxyquinidine and quinidine-*N*-oxide was achieved. Optimum separation of other quinidine metabolites (*O*-desmethylquinidine and 2'-oxoquinidine) was also obtained using this new modified mobile phase (Figure 1). Under these chromatographic conditions, 2'-oxoquinidine was eluted first, followed by 3-hydroxyquinidine, quinidine-*N*-oxide, *O*-desmethylquinidine and quinine. Their retention times were 4.0, 17.7, 21.0, 29.1 and 47.7 min, respectively. Under this modified mobile phase, 3-hydroxyquinine had a retention time of 17.8 min, and co-eluted with the 3-hydroxyquinidine peak. Quinine and quinidine also had very similar retention times of 47.7 and 47.9 min, respectively, when this modified mobile phase was used. These support the assumption that quinidine metabolites could be used as reference compounds.

Possible interference 3-hydroxyquinine and quinine by other drugs was tested. None of the following drugs tested (at a concentration of 10 μg/mL) had a fluorescence response under the chromatographic conditions used, thus they do not interfere with the assay: chloroquinine, mefloquinine, primaquine, artemether, arteether, proguanil and its active metabolite cycloguanil, isoniazid, rifampicin, streptomycin, ethambutol, pyrazinamide, propranolol, debrisoquinine and its 4-hydroxy debrisoquinine. Piroxicam had a retention time of 2.3 minutes and did not interfere with the analytes of

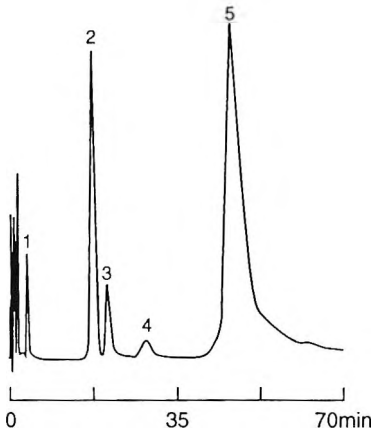

Figure 1. HPLC separation of quinine and metabolites of quinidine using a modified mobile phase consisting of an acetonitrile aqueous phosphate buffer (10 mM, pH 2.1) mixture (40:60 v/v) containing 10 mM SDS and 0.1 mM TBA. Other conditions are as described in the Methods section. Concentrations of 2'-oxoquinidine, quinidine-*N*-oxide, 3-hydroxyquinidine and quinine were 0.1 μg/mL and concentration of *O*-desmethylquinidine was 7 μg/mL. Peak identification: 1 = 2'-oxoquinidine; 2 = 3-hydroxyquinidine; 3 = quinidine-*N*-oxide; 4 = *O*-desmethylquinidine; and 5 = quinine.

interest. Two alkaloids, cinchonine and cinchocaine, had retention times of 45.1 and 52.3 minutes, respectively, which overlapped that of quinine. Consequently, the search for a suitable internal standard for the assay was not successful. Therefore, an internal standard was not used in the present method. Despite this, the reproducibility of the assay was good for both 3-hydroxyquinine and quinine (see Table 1 and further discussion), because the sample preparation only involved direct protein precipitation.

Plasma and urine, collected before administration of quinine tablets (i.e. blank samples), showed no endogenous sources of interference with the analytical assay (Figures 2A and 3A). More than 20 human blank plasma and urine samples were analysed in the study and no endogenous peaks with retention time similar to 3-hydroxyquinine and quinine were detected. Representative chromatograms of blank plasma, and a plasma sample obtained (2.5 h after dose) from a volunteer who took a single oral dose of quinine sulphate (600 mg) are shown in Figure 2. As can be seen, good

Table 1

Within-Day Reproducibility and Accuracy of Analysis for 3-Hydroxyquinine and Quinine in Human Urine Using the Method Described

Nominal Concentration (μM)	Observed Concentration (μM)[1]	C.V.[2] (%)	Accuracy[3] (%)
3-Hydroxyquinine			
0.1 μM (0.034 μg/mL)	0.091 ± 0.006	6.6	91
30 μM (10.2 μg/mL)	28.4 ± 1.3	4.6	94.7
Quinine			
0.5 μM (0.16 μg/mL)	0.48 ± 0.03	6.3	96
30 μM (9.6 μg/mL)	32.0 ± 1.5	4.7	106.7

[1] Results are given as mean ± S.D. (n = 5).
[2] C.V. is a coefficient of variation.
[3] Accuracy (%) = $\frac{\text{observed concentration}}{\text{nominal concentration}} \times 100$

separation of 3-hydroxyquinine, quinine and other possible unidentified metabolites with no interference from plasma was observed in the blank sample. There are at least 7 possible metabolites of quinine detected in urine samples from volunteers who were given a single oral dose of 600 mg quinine sulphate (Figure 3). A major peak identified as 3-hydroxyquinine was detected in all plasma and urine samples from the volunteers who took a single oral dose of quinine sulphate. This suggests that 3-hydroxyquinine is a major metabolite of quinine in human.

The assay procedure was also used to quantitate formation of 3-hydroxyquinine in the *in vitro* metabolism of quinine by human liver microsomes. There was no endogenous interference from the blank liver microsomal samples (Figure 4A). After incubation of quinine with human liver microsomes in the presence of NADPH for 30 minutes, a major peak was formed, having a retention time of 18.0 minutes (Figure 4B) which was similar to that of the reference compound. This peak co-eluted with 3-hydroxyquinine when the samples were spiked with standard 3-hydroxyquinine solution,

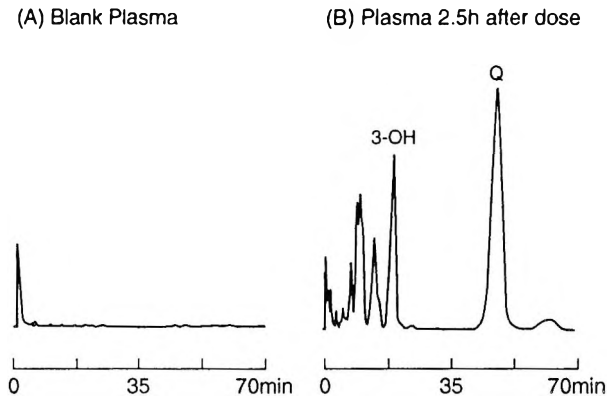

Figure 2. Typical chromatograms of blank plasma (A); and a plasma sample obtained from a volunteer, 2.5 hours after a single oral dose of quinine sulphate (600 mg). Chromatographic conditions used as described in Figure 1. Peaks : 3-OH = 3-hydroxyquinine (7.6 μM); Q = quinine (8.9 μM).

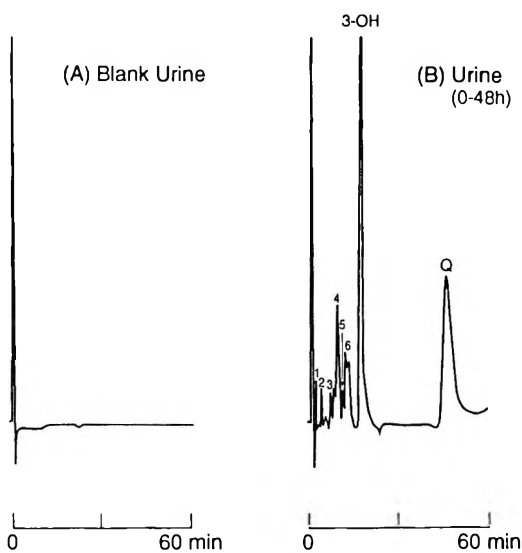

Figure 3. HPLC analysis of quinine and its metabolites in subject H's urine samples : (A) blank urine; and (B) urine sample collected from 0-48 hours after a single dose of 600 mg quinine sulphate, using the modified mobile phase. Chromatographic conditions used as described in Figure 1. Peaks : 1 = 2'-oxoquininone; 3 = quinine glucuronide; 3-OH = 3-hydroxyquinine (15.9 μM); 2, 4, 5 and 6 = unidentified metabolites of quinine; and Q = quinine (4.1 μM).

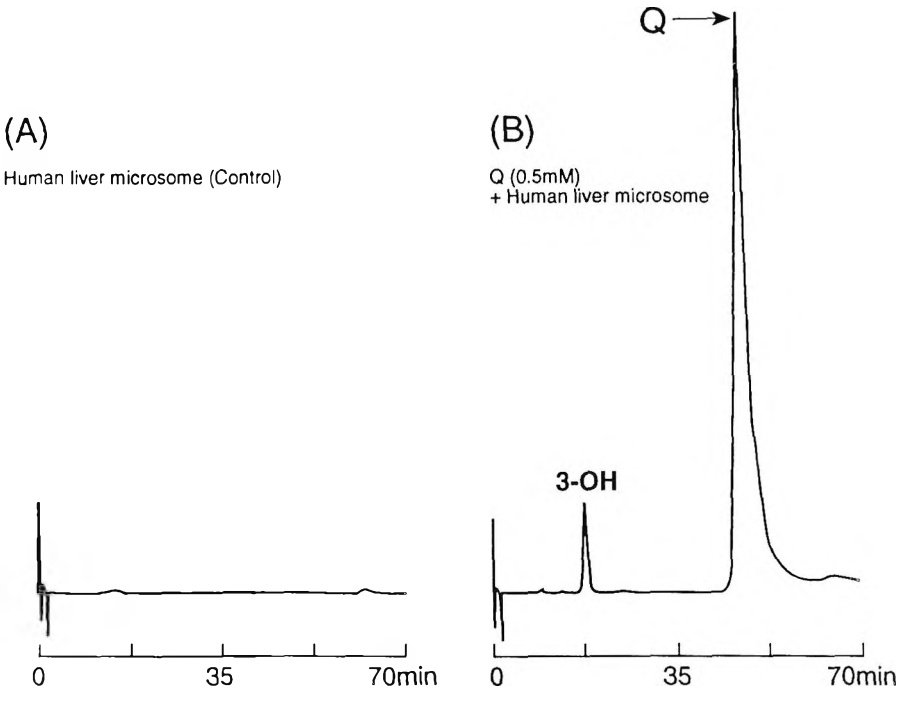

Figure 4. HPLC chromatograms showing the formation of the major metabolite (3-hydroxyquinine) by human liver microsomes : (A) blank liver microsomal sample; and (B) quinine (0.5 mM) was incubated with human liver microsomes (1 mg/mL) in the presence of 1 mM NADPH, at 37°C for 30 min. Chromatographic separation was performed by using the modified mobile phase as described in Figure 1. Peaks : 3-OH = 3-hydroxyquinine (19.5 μM); and Q = quinine (not determined

suggesting this metabolite is 3-hydroxyquinine. Evidence obtained from the inhibition study and enzyme activity correlation study also suggests that the formation of 3-hydroxyquinine is catalysed by P450IIIA.[16,17]

Detector response of 3-hydroxyquinine and quinine was found to be linear covering a concentration range of 0.1 to 30 μM (0.034 to 10.2 μg/mL) for 3-hydroxyquinine and 0.5 to 30 μM (0.16 to 9.6 μg/mL) for quinine. These were observed in plasma, urine and liver microsomal samples. The linear calibration curves for both 3-hydroxyquinine and quinine were obtained with square of the

correlation coefficient (r^2) greater than 0.99. The day-to-day coefficients of variation (C.V.) of the slope of the calibration curves for both compounds were less than 5% (n = 5).

The within-day reproducibility of the method was determined by replicate analyses (n = 5-6) of drug-free urine spiked with known concentrations of 3-hydroxyquinine and quinine. The results, expressed as mean values of the concentrations found, are given in Table 1. The within-day coefficients of variation (C.V.) for both 3-hydroxyquinine and quinine were less than 7%, illustrating the precision of a method suitable for metabolism and pharmacokinetic studies. The C.V. values of the assay at a concentration of 0.1 μM 3-hydroxyquinine was 6.6%, and for quinine at a concentration of 0.5 μM was 6.3%, which are lower than the generally accepted C.V. of 20% for minimum quantifiable concentration (MQC). Thus, the detection limit of sensitivity for this assay for 3-hydroxyquinine and quinine was assigned at 0.1 μM (0.034 μg/mL) and 0.5 μM (0.16 μg/mL), respectively. The results with respect to reproducibility and precision of the assay in plasma and liver microsomal samples were also similar to those of urine samples. Although the detection limit for quinine is much higher than that reported in previous methods[8-11, 13-15] this limit was found to be sufficient for determination of quinine concentrations in urine and hepatic micrsomal samples. If very low detection limits are desirable, e.g. for plasma quinine concentrations, the simple HPLC method by Zoest et al.[10] can be used.

The absolute recovery from biological samples of 3-hydroxyquinine and quinine was assessed by comparing the peaks of 3-hydroxyquinine and quinine with those obtained from direct injection of the pure standards of the drugs of equivalent quantities. The mean recovery for 3-hydroxyquinine (n = 4) from plasma samples was 90 ± 8% at 0.1 μM and 94 ± 5% at 10 μM. The mean recovery for 3-hydroxy-quinine (n = 4) from urine samples was 94 ± 4% at 0.1 μM and 97 ± 3% at 10 μM. The mean recovery for 3-hydroxyquinine (n = 4) from human liver microsomal samples was also similar, i.e. 89 ± 5% at 0.1 μM and 97 ± 4% at 10 μM.

Plasma and urine samples, and also the liver microsomal samples (after treatment with methanol) stored at -20°C for up to two months, showed no signs of decomposition and practically the same concentration values were obtained (n = 5). This suggests that 3-hydroxyquinine is stable under these storage conditions for at least two months.

This HPLC method is now intensively used in our laboratories to analyse the plasma and urine samples from healthy volunteers receiving oral doses of quinine in pharmacokinetic studies and to analyse the hepatic microsomal samples from metabolism studies. The method described here has proved to be simple as it does not involve solvent extraction. This specific HPLC assay is suitable for simultaneous quantitation of 3-hydroxyquinine and quinine concentrations in biological fluids. The limit of detection may be further improved by doubling the injection volume from 30 μL to 60 μL without overloading the column.

In summary, the method presented in this communication is simple. It is sufficiently reproducible and sensitive to be used both in metabolic and pharmacokinetic studies. With ease of analytical assay for 3-hydroxyquinine, it is suggested that quinine could be used as an alternative model drug for P450IIIA.

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PERFLUOROOCTYL AND PERFLUOROBUTYL BONDED ALUMINA STATIONARY PHASES FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Jerome E. Haky,[1] Tina M. Blauvelt,[1] Larry F. Wieserman[2]

[1]Department of Chemistry
Florida Atlantic University
Boca Raton, FL 33431

[2]Surface Technology Division
Aluminum Company of America
Alcoa Center, PA 15069

ABSTRACT

The preparation and properties of perfluorooctylalumina (PFOA) and perfluorobutylalumina (PFBA) high performance liquid chromatographic stationary phases have been investigated. The PFOA phase was produced by chemisorption of perfluorooctanoic acid onto the surface of alumina. The PFBA phase was produced by a similar adsorption of perfluorobutylphosphonic acid onto alumina. Both phases exhibit reverse phase liquid chromatographic properties. Elemental analyses of these materials indicated that alkyl group surface coverage of the PFBA phase is higher than that of the PFOA phase. In contrast, retention of solutes on the PFBA phase is lower than that of PFOA. Isocratic capacity factors of over 20 compounds on the PFOA and PFBA phases were determined and compared with those obtained on octadecylalumina (ODA) and octadecylsilica (ODS) phases. In contrast to the greater

retention of phenols than other compounds that was evident on the unfluorinated ODA phase, the retention of phenols on the PFOA and PFBA phases was not found to be significantly different from that of other compounds. These results are attributed to a reduced degree of hydrogen bonding interactions between phenolic solutes and the PFOA and PFBA phases compared to those which occur between phenols and the ODA phase. Preliminary investigations of the utilization of the PFOA phase for the separation of peptides and the employment of the PFBA phase for the rapid separation of phenols are also described.

INTRODUCTION

Aluminum oxide has long been used as a stationary phase in liquid chromatography. Its unique molecular structure, amphoteric nature and high mechanical stability imparts to it a unique selectivity, which has enabled alumina to be employed for separations of a variety of organic compounds under normal phase and ion exchange conditions.[1] Only recently, however, chemical processes have been developed which allow the modification of the surface of alumina with nonpolar alkyl functionalities, including octyl and octadecyl moieties.[2-7] Such surface modified aluminas have been employed as stationary phases in high performance liquid chromatography (HPLC) under reverse phase conditions. Owing to their stability in mobile phases over a wide range of mobile phase pH's, the unique shape of their particles and the absence of interfering acidic sites on their surfaces, these new reverse phase aluminas have been demonstrated to be equal or superior to commonly-used silica based phases for a variety of applications, including the separations of alkaloids,[4,5] peptides and proteins,[6,7] and the determination of octanol-water partition coefficients of organic compounds.[5]

Surface modified alumina HPLC stationary phases may be prepared by adsorbing organic phosphonic or carboxylic acids onto chromatographic-grade alumina.[3,8] The adsorbed acids create a hydrophobic surface on the alumina, enabling it to be employed in reverse phase HPLC. This method of preparation allows for the modification of the surface of alumina by any organic moiety which can be attached to a carboxylic acid (- COOH) or phosphonic acid ($-PO_3H_2$) functional group.

In the past, we have reported on the chromatographic properties of octyl-

bonded alumina (OCA) and octadecyl-bonded alumina (ODA) stationary phases prepared by the process described above.[4-7] We have recently utilized similar processes to prepare perfluorooctyl bonded alumina (PFOA) and perfluorobutyl bonded alumina (PFBA) stationary phases, which have selectivity and stability which are somewhat different from those of the previously prepared unfluorinated OCA and ODA phases. In this report, we describe the preparation of the PFOA and PFBA phases, and discuss some of their chromatographic properties and applications.

EXPERIMENTAL

Materials

The octapeptide samples listed in Table 1 were obtained from the Protein Chemistry Core Facility of the University of Florida (Gainesville, FL, USA). Glass distilled acetonitrile and trifluoroacetic acid (TFA) was obtained from Fisher Scientific (Fair Lawn, NJ, USA). The reverse phase test mixture D consisted of approximately 0.1 mg/mL each of uracil, phenol, benzaldehyde, N-N,diethyltoluamide, toluene, and ethyl benzene in acetonitrile. It was obtained from Alltech Associates (Deerfield, IL, USA). Perfluorooctanoic acid was obtained from Aldrich Chemical Company (Milwaukee, WI, USA) and Fluowet PL 80™ (perfluorobutylphosphonic acid, 80% aqueous solution) was obtained as a gift from Hoechst Celanese Corp. (Charlotte, NC, USA). Other organic compounds used in this study were obtained from various sources, mostly from Aldrich Chemical Company (Milwaukee, WI, USA). Spherisorb™ spherical alumina, (A5Y: 5 micron particle diameter; A10Y: 10 micron particle diameter) was purchased from Phase Separations Inc.

Table 1

Octapeptide Standards

Peptide	Amino Acid Sequence	Molecular Weight
Lys-Ser-R	Lys-Ser-Ala-Lys-Phe-Nph-Arg-Leu	1166
Lys-Ala-R	Lys-Ala--Ala-Lys-Phe-Nph-Arg-Leu	1150
Leu-Pro-R	Leu-Pro--Ala-Lys-Phe-Nph-Arg-Leu	1151

Nph = nitrophenylalanine

(Norwalk, CT, USA) and the Unisphere™ fused microplatelet alumina (particle diameter: 5.6 microns) was purchased from Biotage (Charlottesville, VA, USA). Potassium bromide was purchased from Aldrich Chemical Company (Milwaukee, WI, USA).

Instrumentation

Infrared spectra of stationary phases and other materials were obtained as KBr pellets on a Mattson Instruments Model 4020 Fourier Transform Infrared Spectrometer. All columns were packed using an Alltech Slurry Column Packer.

The HPLC system consisted of a Perkin-Elmer Series 410 quaternary solvent delivery system, a Rheodyne Model 7125 injector (10 microliter loop) and a Perkin-Elmer Model LC-135 diode array UV-VIS detector. All chromatographic data were recorded and processed on a Perkin-Elmer Omega Data System.

Elemental analyses of PFOA and PFBA phases were performed by Atlantic Microlabs, Inc. (Norcross, GA, USA).

Sample Preparation and Analysis

The octapeptide samples (Table 1) were prepared as 1 mg/mL solutions in 0.1% aqueous TFA. All other compounds and mixtures to be analyzed were prepared as 1 mg/mL solutions in acetonitrile. All samples were analyzed by injecting 10 uL of the solutions into the HPLC system. The wavelength monitored for the octapeptide analyses was 280 nm. A wavelength of 255 nm was employed during the analyses of all other compounds. Except where noted, the mobile phase flow rate was set at 1 mL/min.

Columns

Four different columns were used in these evaluations. Each column consisted of stainless steel and had an internal diameter of 4.6 mm and a length of 150 mm. The slurry method, described by Snyder and Kirkland,[1] was used to pack all of the columns.

The Adsorbosphere™ octadecylsilica (ODS) column was obtained from Alltech Associates (Deerfield, IL, USA). The particle diameter of this material was 5 microns. An experimental octadecylalumina (ODA) column (not available for purchase), packed with surface modified Spherisorb A5Y spherical alumina, was obtained from the Aluminum Company of America (Alcoa Center, PA).

The method which was used to modify the alumina with monomeric octadecyl groups is described by Wieserman et al.[3] and Haky et al.[4] The PFOA and PFBA columns were packed with the stationary phases described below.

Synthesis of the PFOA and PFBA Phases

The perfluorooctylalumina (PFOA) phase consisted of Spherisorb A10Y spherical alumina which had been chemically modified with a perfluorooctanoic acid using the method described by Wieserman et al.[8] Approximately 10 g of the hydrated alumina was combined with 40 mL of a 0.06 M aqueous solution of perfluorooctanoic acid. The reaction mixture was heated for 2 hours at 50°C with constant stirring. The product was isolated by vacuum filtration and then washed with 100 mL of 0.1 M aqueous sodium bicarbonate in order to remove excess acid which was weakly adsorbed. The product was then washed with water and dried for 1 hour at 60°C.

The perfluorobutylalumina (PFBA) phase was prepared from Unisphere fused microplatelet alumina particles which were surface modified with perfluorobutylphosphonic acid by the process described by Wieserman et al.[3,8] Approximately 10 g of the hydrated alumina were mixed with 40 mL of a 0.06 M aqueous solution of perfluorobutylphosphonic acid and stirred for 2 hours at 25°C. The product was then washed and dried by the same method used for the PFOA phase, as described above.

Characteristic infrared spectral bands and assignments for the PFOA and PFBA phases are shown in Table 2.

Stability Study of PFOA

As part of the stability study of the PFOA phase, TFA was adsorbed to the surface of alumina by reacting it with solutions of TFA as described below. Approximately 100 mg of Spherisorb A10Y spherical alumina was mixed with

Table 2

Characteristic Infrared Spectral Band Assignments for PFOA, PFBA and TFA-Treated Alumina

PFOA	PFBA	TFA-Treated Alumina	Mode
3400	3400	3400	O-H stretch
1248, 1213, 1153	1244, 1215, 1115	1221, 1159	C-F stretch
825, 543	825, 543	825, 543	Al-O stretch

All spectral bands are expressed in cm^{-1}

20 mL of 0.1% TFA in ACN and stirred for one day . The solid material was isolated by filtration, washed with water and dried in an oven at 120°C for 2 hours. Characteristic infrared spectral bands and assignments for this material are shown in Table 2.

The PFOA phase itself was then also reacted with 0.1% TFA in ACN, using the same procedure described above. The infrared spectra of the resulting material was identical to that of the alumina which had been reacted with the TFA, as described in Table 2.

Calculations

Capacity factor (k') values were calculated by using the equation $k' = (t_r - t_o) / t_r$ where t_r is the retention time of the compound and t_o is the retention time of an unretained solute. The value of t_o was determined by injecting a 1 mg/mL aqueous solution of sodium nitrate which is not retained on any column under any of experimental conditions.

Reduced capacity factor (k'_r) values were calculated by the equation: $k'_r = k'/ A$, where A is the surface area (in m^2 / g) of the stationary phase support, as supplied by the manufacturers.

The surface coverage, SC, for each phase, expressed in terms of micromoles of monomeric sites per unit area, was calculated by equation $SC = (10^6) (\%C) / [(100)(12.01)(M)(A)]$, where %C is the percentage of

carbon in the stationary phase obtained from elemental analysis and M is the number of carbon atoms in the monomeric unit (i.e., M = 8 for PFOA; M= 4 for PFBA).

Peak asymmetry factors (AF's) were calculated from the equation AF = b/a, where b is the distance between the end of the peak and a vertical line which bisects the top of the peak, measured at 10% of the peak height and a is the distance between the beginning of the peak and the vertical line which bisects the top of the peak, measured at 10% of the peak height.

RESULTS AND DISCUSSION

We chose to investigate the chromatographic properties of fluorinated alkyl-bonded alumina stationary phases because of their potentially unique and interesting properties. On the basis of previous work with perfluoroalkyl-bonded silica phases,[9-18] the perfluoroalkyl alumina phases could be expected to have selectivities which are different from the unfluorinated alumina-based phases that had been previously investigated. Moreover, the high polarity of the C-F bond in such phases would be expected to minimize van der Waals interactions between such phases and solutes interacting with them, thus minimizing problematical irreversible adsorption caused by such interactions during HPLC separations of hydrophobic compounds on unfluorinated phases.[19-22]

The perfluoroalkyl alumina-based stationary phases examined in this study were perfluorooctylalumina (PFOA) and perfluorobutylalumina (PFBA). These phases can be easily prepared by using simple and efficient reactions which result in alumina containing strongly bonded fluoro-alkyl groups on the surface. The PFOA phase was prepared by reacting hydrated alumina with perfluorooctanoic acid. Synthesis of the PFBA phase was accomplished by reacting hydrated alumina with perfluorobutylphosphonic acid. These two particular acids were chosen for this study because they were commercially available and enable comparisons between fluorinated alkyl groups of different lengths bonded to the surface of the alumina.

Surface Alkyl Group Coverage

The conditions for the preparation of the PFOA and PFBA phases shown in the experimental section are optimized to produce the highest surface alkyl group coverage possible. Adsorption of carboxylic acids onto alumina is not as

strong or as fast as the corresponding adsorption of phosphonic acids.[23-25] For this reason, the preparation of the PFOA phase, produced by carboxylic acid adsorption onto alumina, required higher reaction temperatures than the preparation of the PFBA phase, which is produced by a phosphonic acid adsorption.[3,8] For the same reason, the PFOA phase was also found to be less stable than PFBA in mobile phases containing organic acids, as will be discussed later.

As shown in previous studies, alkyl group surface coverage can affect the reverse phase properties of an HPLC column.[1] Table 3 lists the alkyl group surface coverages and related data for the PFOA and PFBA phases , along with similar data for an unfluorinated octadecylalumina (ODA) phase and a typical octadecylsilica (ODS) phase. Expressed in terms of monomeric sites per unit surface area, the surface coverages of all 3 alumina-based phases are higher than that of the silica-based phase. As might be expected, surface coverage is highest among the alumina-based phases for PFBA, whose alkyl groups are the smallest and thus interfere the least with each other during the bonding process.

While the alkyl groups of the PFOA phase are also smaller than those of the ODA phase and thus might also be expected to have higher surface coverage than the octadecyl-bonded alumina phase, this was not observed. As shown in Table 3, surface coverage is nearly equivalent for the ODA and PFOA phases. Apparently the weaker bond which is formed between the carboxyl group of perfluorooctanoic acid and alumina in the PFOA phase reduces the effect of the diminished steric crowding on the alumina surface. This results in the PFOA phase having a similar surface alkyl coverage to the ODA phase, which has a bulkier alkyl group but is prepared by forming a stronger bond between alumina and the phosphoryl group of octadecylphosphonic acid.

Table 3

Surface Coverage Data

Phase	Surface Area (m^2/g)	Weight % Carbon	Surface Coverage ($mmoles/m^2$)
ODA	93	7.2	3.6
ODS	200	12	2.8
PFOA	90	3.0	3.5
PFBA	105	4.5	8.9

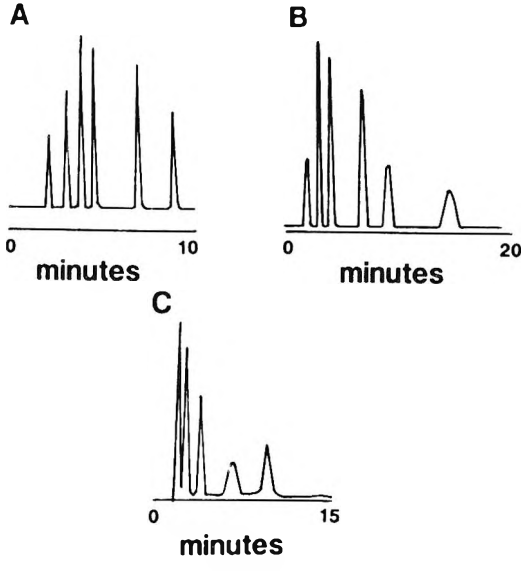

Figure 1. Chromatograms of reverse phase test mixture D on three columns. A: ODS column; B: PFOA column; C: PFBA column. Mobile phases: ODS: 65% acetonitrile, 35% water; PFOA: 30% acetonitrile, 70% water; PFBA: 10% acetonitrile, 90% water. Elution order for ODS and PFOA columns is uracil, phenol, benzaldehyde, N,N-diethyltoluamide, toluene and ethyl benzene. Elution order for PFBA column is the same as that described above, except that uracil and phenol coelute.

Comparison of Solute Retention

Since their surfaces are both less polar than that of their alumina backbones, the PFOA and PFBA phases can generally be used in the reverse phase mode, employing aqueous mobile phases similar to those employed with conventional ODS phases. Figure 1 shows chromatograms for a common reversed test mixture obtained on the PFOA and PFBA columns, along with a chromatogram obtained of the same test mixture on a commercial ODS column. Similar separations of the components of these mixtures were obtained on all three columns, indicating a general similarity of the retention mechanisms in all three phases. More significant, however, is the need to employ mobile phases containing lower percentages of organic modifier to achieve these separations on the PFOA and PFBA columns than on the conventional ODS column. Indeed, even with a mobile phase containing only

Table 4

Capacity Factor Data for Comparison of Relative Solute Retention

Column	ODS		ODA		PFOA		PFBA	
Parameter	k'	k'_r	k'	k'_r	k'	k'_r	k'	k'_r
Phenol	1.78	.0089	1.44	.0155	.351	.0039	.189	.0018
Benzaldehyde	3.76	.0188	7.28	.0783	.702	.0078	.386	.0035
Toluene	4.34	.0217	14.46	.1555	2.69	.0299	1.28	.0122
Mean	3.29	.0165	7.73	.0831	1.25	.0139	.612	.0058

Mobile phase: 30% acetonitrile, 70% water

10% acetonitrile, the two least hydrophobic compounds in the mixture, phenol and uracil, were not separated on the PFBA column at all. These results suggest that the perfluoroalkylalumina phases PFOA and PFBA are significantly less retentive than the conventional ODS phase.

A more realistic method of comparing the relative retention of stationary phases is to compare isocratic capacity factors (k's) of test solutes on each column employing aqueous mobile phases of identical compositions.[26] Table 4 shows such capacity factor data for three test compounds on the PFOA and PFBA phases, along with similar data obtained for the unfluorinated octadecylalumina (ODA) and ODS columns.

Average k' s of the three test solutes for all four columns are displayed in the bar graph in Figure 2A. On the basis of the relative magnitudes of these average k's, these data indicate that the ODA phase is the most retentive phase of those investigated, followed by ODS, PFOA and PFBA, respectively. However, retention of solutes can be influenced by a stationary phase's surface area, which differs among each of the phases investigated here. A more useful measure of relative retention of these phases, therefore, are the reduced capacity factors (k'_r), which can be obtained by dividing the capacity factors by the respective surface areas of stationary phase particles.[12] These k'_r data are displayed in Table 4, and the average k'_rs are displayed in bar graph form in Figure 2B. The capacity factors corrected in this manner still indicate ODA phase is the most retentive of the phases tested in this study. Average k'_rs for the alumina-based PFOA and silica-based ODS are very similar, indicating

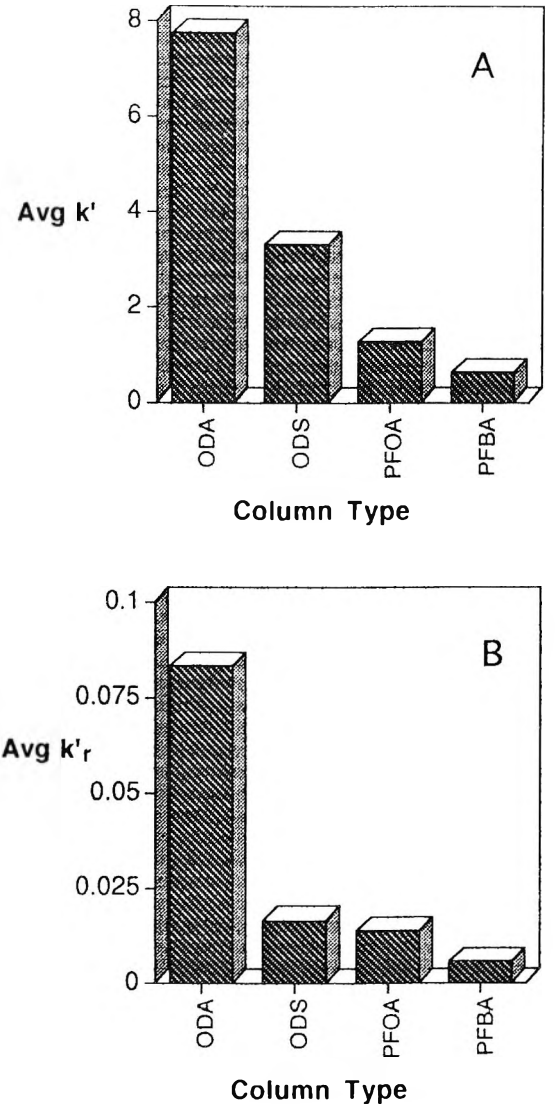

Figure 2. Bar graphs comparing the average capacity factors (A) and average reduced capacity factors (B) for three compounds on four columns using the mobile phase described in Table 4.

similar solute retention for these two phases in spite of PFOA's lower alkyl chain length. On the other hand, the PFBA column has the lowest verage k'_r,

Table 5

Asymmetry Factors for Test Compounds

Compound	AF (ODS)	AF (PFOA)	AF (PFBA)
Uracil	2.2	1.5	1.0
Phenol	1.4	1.0	1.0
Benzaldehyde	1.9	1.5	1.02
N,N-Diethyltoluamide	2.7	1.1	0.80
Toluene	1.2	1.3	0.60
Ethylbenzene	1.1	1.2	1.0
Mean value	1.7	1.3	0.90

Conditions: Same as in Figure 1.

consistent with low solute retention on other reverse phase columns with short alkyl chain lengths.[1]

Peak Symmetry and Interfering Mechanisms

One of the demonstrated advantages of alumina-based HPLC stationary phases is the absence of interferences of unbonded acidic sites on their surfaces. The presence of such sites in reverse phase silica-based columns often causes substantial peak tailing, especially during the analysis of basic solutes.[1,4,6] Similar to alkyl-bonded silica, reverse phase alumina surfaces might contain unbonded hydroxyl groups. However, if they are present, these groups are not nearly as acidic as those in silica. Earlier studies have demonstrated this by comparing symmetry factors for chromatographic peaks for a wide variety of compounds on ODA and ODS phases. Substantially higher degrees of peak symmetry were found on the alumina-based ODA column, especially for basic solutes.[4,6]

Table 5 lists asymmetry factors (AF's) for six compounds on PFOA and PFBA phases used in the present study, along with corresponding values determined for a conventional ODS phase. Consistent with earlier results, peak symmetries for all compounds are equal or superior for the alumina-based PFOA column than for the silica-based ODS column. Especially significant is the excellent symmetry of the peak corresponding basic solute N,N-

diethyltoluamide (AF = 1.1), in contrast to its poor symmetry on the ODS column (AF = 2.7). This again confirms the absence of acidic unbonded sites on alkyl-bonded alumina phases.

While average peak symmetry on the PFBA phase is also superior to that found on the ODS column (Table 5), asymmetry factors for some of the test compounds are less than unity, indicating peak fronting, an unusual occurrence in HPLC. This phenomenon results when solute molecules in the beginning of a band passing through an HPLC column interact with the stationary phase and enhance retention of solute molecules passing through later in the same band.[27] Such peak fronting has been observed during the normal phase liquid chromatographic analysis of some polar compounds on unbonded alumina.[28] Analogous to what was proposed for that phenomenon, a possible explanation for peak fronting occurring on the PFBA column is that retention is enhanced by solute molecules in the leading edge of their bands as they pass through the HPLC column, resulting in the increased retention of solute molecules in the middle and end of the solute bands. This explanation is especially appealing, considering the inherently low solute retention on the PFBA phase, as discussed earlier.

Selectivity Comparison

While comparison of reduced capacity factors of a few compounds provided a measure of the relative retention of solutes on the PFOA and PFBA phases, a detailed evaluation of selectivities of these phases requires additional studies involving a larger number of compounds. The method used to compare the selectivities of the phases investigated in the present work is based on one used in an earlier investigation of the chromatographic properties of the octadecyl-bonded alumina (ODA) phase.[4] In that study, isocratic capacity factors of over 20 compounds of different structure and functionality were determined on an ODA column and compared with those obtained on a standard ODS column. A logarithmic plot of the capacity factors of the compounds on the ODA column vs the capacity factors on the ODS column gave a straight line with excellent correlation for all compounds with the exception of the phenols. These compounds appeared to form a separate correlation line slightly above the line for the other compounds.

In the present study, a similar comparison of capacity factors of compounds on the PFOA and PFBA phases with those obtained on an ODS column was performed. Capacity factor data were also obtained on an ODA column to confirm the results found in the earlier study. The compounds used

Table 6

Capacity Factor Data for Correlation Study

Compound	Log k' (ODS)	Log k' (ODA)	Log k' (PFOA)	Log k' (PFBA)
m-Aminophenol	-0.334	-0.383	-0.795	--
Benzamide	-0.081	-0.019	-0.324	-0.719
Resorcinol	-0.082	0.021	-0.943	-1.128
Aniline	0.262	0.172	0.036	-0.738
Benzyl Alcohol	0.151	0.228	-0.228	-0.711
Phenol	0.254	0.366	-0.316	-0.699
Ethyl Propionate	0.485	0.254	0.165	-0.395
Benzonitrile	0.568	0.579	0.342	-0.210
Acetophenone	0.668	0.406	0.706	0.428
m-Cresol	0.427	0.713	0.033	-0.375
Benzene	0.797	0.786	0.265	-0.365
Quinoline	0.677	0.635	0.600	-0.910
Methyl Benzoate	0.751	0.921	0.571	0.101
Trichloroethylene	0.951	1.089	0.440	-0.113
Methyl Salicylate	0.862	1.294	0.038	0.313
Chlorobenzene	1.061	1.173	0.544	0.303
Ethyl Benzoate	0.963	1.113	0.631	0.412
1-Naphthol	0.710	1.447	0.425	0.032
Toluene	1.049	1.236	0.645	0.024
2-Naphthol	0.641	1.389	0.442	0.067
Bromobenzene	1.123	1.360	0.577	0.076
Thymol	1.029	1.146	0.979	0.468
Naphthalene	1.250	1.672	0.877	0.328
Anisole	0.773	0.870	0.344	-0.202

Mobile phases: ODS: 40% acetonitrile, 60% water; ODA: 20% acetonitrile, 80% water; PFOA: 10% acetonitrile, 90% water; PFBA: 10% acetonitrile, 90% water. Flow rate: 2 mL/min

in this study and k' data on all columns are shown in Table 6.

Logarithmic plots of k's of the test compounds of the ODA, PFOA and PFBA columns vs their k's on the ODS column are shown in Figures 3, 4 and 5

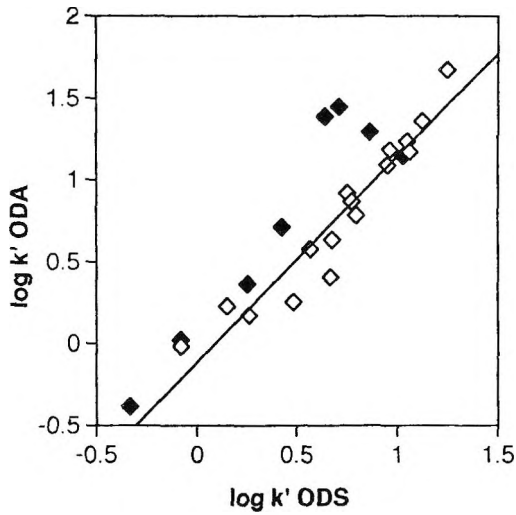

Figure 3. Logarithmic plot of the capacity factors of 24 compounds on the ODA column vs their capacity factors on the ODS column. Darkened squares correspond to phenols.

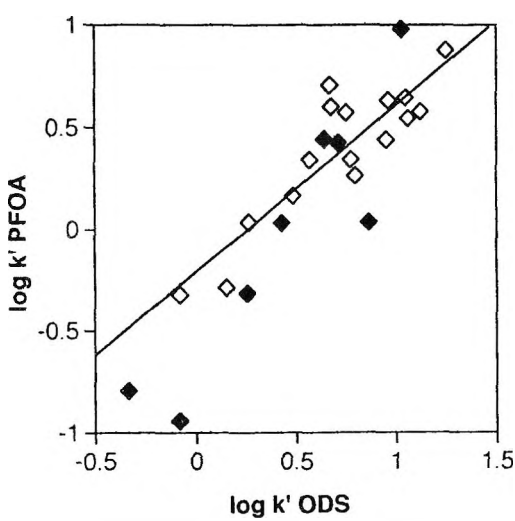

Figure 4. Logarithmic plot of the capacity factors of 24 compounds on the PFOA column vs their capacity factors on the ODS column. Darkened squares correspond to phenols.

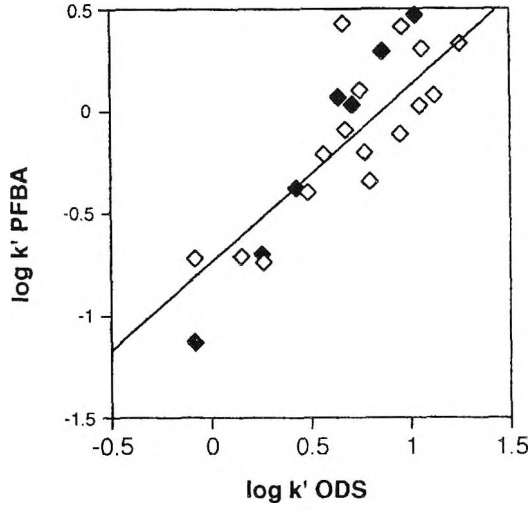

Figure 5. Logarithmic plot of the capacity factors of 23 compounds on the PFBA column vs their capacity factors on the ODS column. Darkened squares correspond to phenols.

respectively, and linear regression parameters for all graphs are shown in Table 7. In accord with the previous study, phenolic compounds are retained longer than other compounds on the ODA phase when compared with ODS, as indicated by their points generally being above the general correlation line for other compounds (see Figure 3) . Moreover, as shown in Table 7, removal of phenols from the ODA-ODS correlation substantially improves the degree of linear fit, as indicating by an increase in the correlation coefficient. Additionally, when the phenols are grouped together, they form an line with an excellent degree of correlation and a higher y-intercept than the line formed with the other compounds.

In contrast to the unique correlation of the phenolic compounds on the ODA-ODS curve, similar graphs produced for PFOA and PFBA columns exhibit no discernible unique retention behavior for phenols or any other class of compounds when compared to ODS. As shown in Figures 4 and 5, phenolic compounds do not appear to be consistently above or below the general correlation lines found for other compounds. Removal of the phenols does not substantially improve the correlation coefficients for these graphs (Table 7). These data indicate that neither phenols nor any class of compounds in consistently retained to a different degree or manner on the PFOA or PFBA phase than on a standard ODS phase.

Table 7

Linear Regression Parameters for Selectivity Comparison Study

Data set	Parameter	ODA/ODS	PFOA/ODS	PFBA/ODS	PFBA/PFOA
All Compounds	Slope	1.185	1.059	1.026	0.872
	Intercept	0.034	-0.411	-0.821	-0.396
	R^2	0.816	0.807	0.749	0.788
All except Phenols	Slope	1.261	0.847	0.866	1.049
	Intercept	-0.118	-0.285	-0.735	-0.518
	R^2	0.912	0.719	0.667	0.844
Phenols only	Slope	1.365	1.208	1.496	0.844
	Intercept	0.151	-0.567	-1.013	-0.271
	R^2	0.870	0.849	0.988	0.826

A comparison of selectivity of the PFOA with the PFBA phase is supplied by Figure 6, which is a plot of k's of the test compounds on the the PFOA column vs their k's on the PFBA column. This indicates a slightly enhanced retention of phenols on the PFOA phase than on the PFBA phase. Similar to the curve obtained using the ODA-ODS capacity factor data, the general correlation of the data improves when phenols are removed from the data set, and again the phenols appear to form their own unique correlation line (Table 7).

Hydrogen Bonding Retention Mechanism

The following conclusions can be reached from the analysis of the graphs and data discussed in the previous section:

1. Phenols are retained to a greater relative degree on the unfluorinated ODA stationary phase than on a standard ODS phase.

2. When compared to a standard ODS phase, the relative retention of phenols on the fluorinated PFOA and PFBA phases is not discernibly different from any other class of compounds.

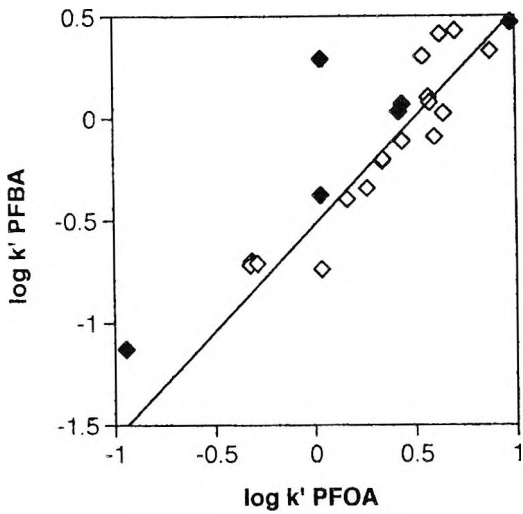

Figure 6. Logarithmic plot of the capacity factors of 23 compounds on the PFBA column vs their capacity factors on the PFOA column. Darkened squares correspond to phenols.

3. When compared to each other, the relative retention of phenols on the PFBA phase is slightly higher than on the PFOA phases.

In previous studies, differences in hydrogen bonding capabilities of the alumina backbone of the ODA phase and the silica backbone of the ODS phase were used to explain the different degrees of retention of phenols on the two phases.[4,29] The new data obtained in the present study indicate similar differences between the hydrogen-bonding capabilities of the ODA, PFOA and PFBA phases. Such differences can be justified through a consideration of the expected effects of the structural differences in the ODS, ODA , PFOA and PFBA phases on such hydrogen bonding interactions.

The most likely sites for hydrogen-bonding interactions between solutes and silica-based or alumina-based stationary phases are at the oxygen atoms directly bonded to the silicon or aluminum atoms of these materials. These oxygen atoms have unbonded electron pairs, which can serve as hydrogen-bond accepting sites for hydrogen-bond donating solutes, such as phenols. Such hydrogen-bonding would involve an interaction between a partial negative

charge on the oxygen atom bonded to alumina (or silica) and a partial positive charge on the hydrogen atom bonded to the oxygen atom of the phenol. These interactions would lead to increased retention of solutes such as phenols when compared to solutes which cannot be involved in hydrogen-bonding interactions.

Since phenols are retained to a greater relative degree on the ODA column than other compounds when compared to their retention on a silica-based ODS column, it appears that the potential for such hydrogen-bonding interactions is greater on the alumina-based ODA stationary phase than on ODS. This implies that a greater electron density (and a resulting more intense partial negative charge) exists on the oxygen atoms of the ODA phase than on the oxygen atoms of the ODS phase.

Since the structures and bonding chemistries of the ODA and ODS phases are so different, it is difficult to quantitatively assess the differences between electron densities and hydrogen-bonding capabilities of the oxygen atoms of the two phases on a theoretical basis. However, if such differences are primarily governed by the presence of either silicon or aluminum in the materials, a consideration of effects of these phases' silicon and aluminum atoms on the electron densities of adjacent oxygen atoms involved in hydrogen bonding may qualitatively indicate the relative hydrogen-bonding accepting abilities of the oxygen atoms of the ODA and ODS phases. Silicon is more electronegative than aluminum (Pauling electronegativities: 1.9 and 1.6 respectively[30]), and thus it should have a greater effect on reducing electron density around the oxygen atoms bonded to it. The intensity of any hydrogen bonding interactions to such oxygen atoms should be lower than interactions involving oxygen atoms bonded to aluminum. Thus, all else being equal, a silica-based phase such as ODS would be expected to have a lower degree cf hydrogen-bonding accepting interactions with solutes than an alumina-based phase such as ODA. On this basis, hydrogen-bond donors such as phenols should be expected to have greater relative retention than other compounds on the ODA column when they are compared to their retention on an ODS column. As shown in Figure 3 and in a previous study,[4] this is in accord with the experimental results.

In contrast to the results obtained the ODA phase, retention of phenols on the fluorinated PFOA and PFBA phases is not discernibly different from that of non-hydrogen bonding compounds, relative to their retention on the silica-based ODS column (Figures 4 and 5). On the basis of the discussion in the previous paragraph, this would indicate that the potential for hydrogen-bonding interactions between solutes and the oxygen atoms bonded to aluminum in

these fluorinated phases is lower than that in the unfluorinated ODA phase. It also implies that the electron density and resultant partial negative charges on these oxygen atoms of the PFOA and PFBA phases is lower than that on corresponding atoms of the ODA phase.

Since the ODA, PFOA and PFBA phases are all based upon an aluminum-containing backbone, the differences in the hydrogen bonding capabilities of these phases must be related to the differences in their alkyl-group containing substituents. In fact, on a theoretical basis, these substituents would be expected to have different effects on electron density around the oxygen atoms in these phases, and the resulting hydrogen bonding interactions of these phases with solutes. In contrast to ODA, the alkyl groups of the PFOA and PFBA phases contain highly electronegative fluorine atoms, which, by pulling electrons toward them, reduce electron density about the oxygen atoms in these phases. This in turn reduces the intensity of any hydrogen bonding interactions with solutes, compared to similar interactions which occur in the ODA phase. Thus retention of phenols on the PFOA and PFBA columns would not be expected to be increased by such hydrogen-bonding interactions as much as on the ODA column, which does not contain highly electronegative atoms in its alkyl groups. Relative retention of phenols on PFOA and PFBA should therefore be more similar to their retention on ODS, which has low hydrogen-bonding capabilities, than that on ODA, whose hydrogen bonding capabilities are inherently stronger. This prediction is, of course, in accord with the experimental results. Moreover, since the alkyl groups of the PFOA phase contain a larger number of electronegative fluorine atoms than the smaller alkyl groups of PFBA, reduction of electron density and hydrogen-bonding interactions around the oxygen atoms bonded to aluminum would be expected to be greater for the PFOA phase than for the PFBA phase. This should result in lower hydrogen-bonding interactions between phenols and the PFBA phase than between phenols and the PFOA phase. In accord with this prediction, slightly higher retention of phenols over other compounds on the PFOA column compared to their retention on the PFBA column is observed, as suggested by the graph in Figure 6.

Applications of the PFOA Phase

As discussed earlier, the high polarity of the C-F bond in the perfluoroalkylalumina phases reduces intermolecular interactions caused by induced dipoles, and thus should minimize such van der Waals interactions between these phases and solutes interacting with them. This in turn should reduce the degree of irreversible adsorption of solutes and increase rates of

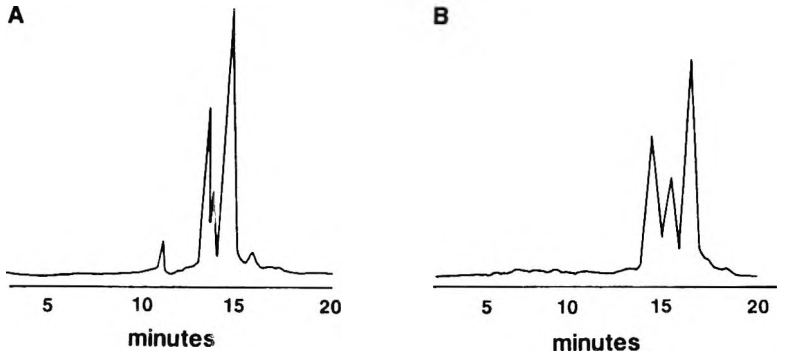

Figure 7. Chromatograms of mixture of octapeptide standards on PFOA column. Mobile phase gradients: A: 5 % - 95% aqueous acetonitrile containing 0.1% TFA; B: 5 % - 95% aqueous acetonitrile containing 0.1% perfluorooctanoic acid. Gradient time was 20 minutes for both chromatograms. Elution order corresponds to the order in which the octapeptides appear in Table 1. Minor peaks correspond to unknown impurities.

mass transfer between the stationary phase and the solutes. Irreversible adsorption and poor mass transfer is often a problem during the HPLC separation of high molecular weight proteins and peptides on conventional ODS and other nonpolar phases.[19-22] A potential application of the perfluoroalkylalumina phases, therefore, is the enhanced separation and preparative isolation of proteins and peptides by HPLC. For the same reasons, similar applications have been investigated for perfluoroalkyl-bonded silica-based phases.[12]

In a preliminary study, the HPLC separation of a mixture of three octapeptides shown in Table 1 was achieved using the PFOA column and a standard water-acetonitrile mobile phase gradient containing 0.1% trifluoroacetic acid (TFA). The TFA serves to protonate the peptides and act as an ion-pairing agent, which generally enhances the separations.[31,32] The chromatogram in Figure 7A shows that these conditions resulted in a reasonably good separation of the three octapeptides on the PFOA column. Unfortunately, the PFOA phase was not stable in this mobile phase. After about 5 hours of use with the TFA-containing mobile phase the PFOA column could no longer achieve separations of any kind.

As discussed in the experimental section , the infrared spectrum of the PFOA phase obtained after treatment with TFA-containing solvents was

virtually identical to that of unmodified alumina treated with the same solvents. This indicates that TFA had displaced the perfluorooctanoic acid from the surface of the PFOA phase, resulting in an unusable material in which trifluoroacetic acid is bonded to the alumina surface.

An easy solution to the problem described above was achieved by replacing the TFA in the mobile phase gradient with perfluorooctanoic acid. This acid has previously been demonstrated to serve the same role as TFA for protein and peptide separations by reverse phase HPLC.[32] Additionally, since perfluorooctanoic acid is one of the compounds from which the PFOA phase was produced, the phase could not degrade as a result of its presence in the mobile phase. Figure 7B shows a chromatogram of the octapeptide mixture on the PFOA column using an acetonitrile-water gradient containing 0.1% perfluorooctanoic acid. Good separation of the three peptides is evident. Additionally, the PFOA column remained stable for over 500 hours of use with the perfluorooctanoic acid-containing mobile phase.

Applications of the PFBA Phase

While the low solute retention of the PFBA phase rendered it unusable for separations of peptides and proteins under standard conditions, this same property enabled it to be employed for fast separations of smaller molecules using mobile phases little or no organic modifier. The advantages of reducing the percentage of organic modifier in the mobile phase are twofold: it reduces costs, and it minimizes waste disposal problems.

An example of such an application of the PFBA phase is shown in in Figure 8. It consists of a chromatogram of three phenols (m-cresol, 2-naphthol and thymol) obtained with the PFBA column using a mobile phase consisting of 99.8% water and 0.2% TFA. Good separation of these compounds was obtained in less than 6 minutes.

Unlike PFOA, which, as discussed earlier, would have been destroyed with a TFA-containing mobile phase such as that described above, the PFBA phase suffered no apparent short-term or long-term degradation when such mobile phases were employed. Apparently, the perfluorobutylphosphonic acid of PFBA is more strongly bound to the alumina surface than the octanoic acid of PFOA, and thus is more resistant to displacement by TFA. This is consistent with other studies which have demonstrated that phosphonic acids are more strongly adsorbed to alumina than carboxylic acids.[23-25]

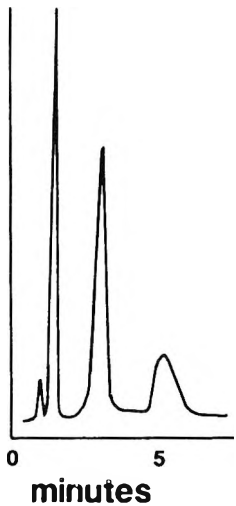

Figure 8. Chromatograms of phenol mixture on PFBA column. Mobile phase: 0.2% aqueous TFA. The flow rate was 2 mL/min for both chromatograms. Elution order: m-cresol, 2-naphthol and thymol.

CONCLUSIONS

In this study, perfluorooctylalumina and perfluorobutylalumina stationary phases were prepared and demonstrated to be useful for HPLC separations of compounds in the reverse phase mode. The mechanisms involving the retention of compounds on these and other alumina-based phases were demonstrated to be similar to those which are involved with separations on standard octadecylsilica phases. However, subtle differences can be detected for the retention of phenolic compounds on different alumina-based phases . These differences are apparently the result of hydrogen-bonding interactions of these phenolic solutes with oxygen atoms on the alumina surface. The extent of such interactions is at least in part controlled by the net electron-withdrawing power of the alkyl groups bonded to the alumina.

In the past, fluoroalkyl-bonded stationary phases have been used primarily for very specialized applications, such as the separation of fluorinated organic compounds. While they are only preliminary, the separation studies described in the present work have demonstrated potential applications of perfluoroalkylalumina stationary phases of much broader scope, such as the separation of peptides and the rapid separation of lower molecular weight compounds using low cost, nontoxic mobile phases. Further research is needed

to systematically evaluate the new perfluoroalkylalumina phases for these and other applications, and to compare their performance with more conventional stationary phases such as ODS. These studies are continuing in our laboratories.

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

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