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INVESTIGATION OF THE RETENTION BEHAVIOR AND STRUCTURAL CHANGE OF PROTEINS IN REVERSED PHASE AND HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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ABSTRACT

The retention behavior of proteins was investigated in reversed phase chromatography(RPC) and hydrophobic interaction chromatography(HIC). The observation was performed in three viewpoints : (1) What are the differences in the retention behavior between small molecules and proteins? (2) What are the differences between RPC and HIC of proteins and the reason of the differences? (3) How can be detected the structural changes of proteins eluted from the column?

The retention behavior of proteins was able to be understood by the parameters used in describing the retention behavior of small molecules. However, applicable range of the parameters for proteins was limited and, sometimes, peculier behavior appeard in RPC and HIC for proteins ; that is, the retention time of proteins showed extreme difference for the small change of organic fraction and showed the skewed U-shaped dependence on fraction of organic solvent. Even though the retention mechanism of proteins in RPC and HIC was based on the same fundamental principle relied on the hydrophobic properties of the proteins and stationary phase, the retention behavior of the proteins in RPC differed significantly from that in HIC. The difference between RPC and HIC resulted from adding organic solvent in mobile phase of RPC to elute the protein strongly bound to the ligand of the stationary phase. From the

view point of thermodynamics, the driving force of transferring proteins from the mobile phase to the stationary phase was ΔH° for RPC and ΔS° for HIC.

The investigation of structural change of the proteins eluted from the column was performed with UV and photodiode array detector as a function of the change of peak shape and the retention time. The number(Z) of solvent molecules required to displace the solute from the surface was useful for measuring the structural change of the proteins by combining the thermodynamic observation. For the identification of splitted peaks of hemeproteins eluted from RPC, apoproteins were prepared and compared with the retention time of heme proteins. K_D values were measured by using size-exclusion chromatography(SEC) with the same mobile phase used in RPC and HIC. From these values the phenomena such as aggregation or division into subunits of proteins were suggested.

INTRODUCTION

In the 1970s, HPLC has become a powerful technique widely used for the separation of large biological substance and for their purification. In particular, RPC has been the prime method of peptide analysis, while HIC has been an important separation mode for the purification and characterization of proteins [1]. The retention behavior of proteins has been investigated by the same method used for small molecules ; Snyder et al. applied the linear solvent strength (LSS) gradient elution theory developed for small molecules to characterizing the retention behavior of proteins [2], and Hearn et al. showed that van't Hoff plots confirmed significant changes in the free energy associated with retention of peptides and proteins [3]. However, there exist some differences between the elution behavior of proteins and low-molecular-weight molecules. Regnier et al. explained the fact as the structural changes of proteins during the chromatographic elution, particularly in RPC [4]. Guiochon et al. studied the changes in peak shape as a function of temperature and the type of the stationary phase [5]. Z value is in part related to the size of the protein molecules and the contact area between proteins and the support. Z value can be used as a measure of the structural change of proteins[6].

Even though the retention mechanism of RPC and HIC is based on the same fundamental principle relied on the hydrophobic interaction between the proteins and stationary phase, they show a quite different character. This has already been reported in our previous reports [7-8]. From those reports, the following facts have been found. The association of the proteins with the stationary phase in RPC was an enthalpy-driven process, and the retention of proteins was more favourable with decreasing temperature, while in HIC transfer of proteins from mobile phase to stationary phase was an entropy-driven process and the retention became longer with increasing temperature. Furthermore, the retention behaviors of hemoglobin and myoglobin were very similar in RPC, while the retention behaviors of two proteins were significantly different in HIC. This may be explained only by investigating the structures of those two proteins eluted through the column.

In the previous reports, structural change of proteins was observed along with the sudden change of retention time and the splitting and broadening of peaks by using UV and photodiode array detectors. The change of Z value with thermodynamic consideration was also used for the investigation of the structual change of proteins. However, precise structure of proteins eluted from the column and identification of splitted peaks were not clarified. For obtaining the information on the structural change of proteins, spectroscopic methods such as UV, fluorescence, and circular dichroism spectroscopy have been used[9-10]. When the structure of proteins changes, the UV and fluorescence spectra show changes in the aromatic acid on exposure to the solvent, while CD spectra can denote changes in secondary structures of the unfolded proteins. On-line conformational monitoring of proteins in HPLC is a proper method for the observation of the structural change of proteins during chromatographic elution[11]. SEC has been widely used for consideration of the change of molecular size and shape[12-14]. LC/mass spectroscopy[15] and LC/LALLS[16] may be applied to investigation of the change in the size of proteins by the occurrence of aggregation or division into subunits.

In this work, UV and photodiode array detectors were used to observe the structural change of proteins along with the changes in retention time and peak shape. For the identification of the splitted peaks, particularly in hemeproteins, apoproteins of hemoglobin and myoglobin were prepared and elution behavior of apoproteins was compared to that of native proteins. SEC was performed with the same solution conditions as employed in RPC and HIC to investigate the change in the size of proteins.

MATERIALS AND METHODS

Equipment

The chromatographic system employed in this work consists of a Waters M-6000A(Waters Associates Inc., Milford, Mass, U.S.A.) and a M-45 Solvent Delivery System with a Model 660 Solvent Programmer, a M-U6K Universial Injector, a M-740 Data Module, a series 440 Absorbance Detector (254, 280, and 405nm), and a Waters 991 Photodiode Array Detector. A SynChropak RP-P column, 25 X 0.46 cm I. D., from SynChrom (Lafayette, IN, U.S.A.) for RPC, a SynChropak propyl column, 25 X 0.46 cm I. D., from SynChrom for HIC, and two RPOTEIN-PAK 125 and a PROTEIN-PAK 300SW column, 30 X 0.78 cm I. D., from Waters for SEC, were employed.

Reagent

HPLC-grade acetonitrile and i-propanol from Merck (Hawthorne, NY, U.S.A.), HPLC-grade ethanol from Burdick & Jackson (Muskegon, MI, U.S.A.), trifluoroacetic acid (TFA), sodium phosphate monobasic, sodium phosphate dibasic, sodium sulfate, and proteins were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Proteins used in this work were lysozyme, hemoglobin, cytochrome

c, myoglobin, protease, transferrin, carbonic anhydrase, ovalbumin, α-lactoalbumin, and albumin. Water was purified using a Millipore Milli-Q water purification system (Bedford, MA).

Chromatographic Procedure

RPC was performed with 0.1% aq. TFA containing organic solvent such as i-propanol, acetonitrile, and ethanol whose compositions were varied from 30 to 40%. For HIC, concentrations of sodium sulfate were 0.50 to 0.85M in 0.05M phosphate buffer (pH 7.0). SEC was performed under the same solution conditions as used in RPC and HIC. The retention times of various proteins were measured over a range of temperature between 5 and 70°C. Proteins were prepared by dissolving in 0.05M phosphate buffer (pH 7.0) at a concentration of 3mg/ml. Injection volume was 5μl and the flow-rate was 1ml/min. Apoproteins of hemoglobin and myoglobin were prepared according to the reported method [17]. Distribution coefficient, K_D, was calculated using the following equation

$$K_D = (Ve-Vo)/(Vt-Vo) \qquad (1)$$

where Vo is the interstital volumn of the column, obtained from the retention volumn of blue dextrin (M.W.= 2,000,000), Ve is the retention volumn of protein, and Vt is the total liquid volumn of the column, obtained from the retention volumn of sodium azide (M.W.= 65).

RESULTS AND DISCUSSION

Application of UV Detector in Conformational Monitoring

UV spectrophotometry is one of the most important tools for the investigation of the changes in the molecular conformation and

refolding of proteins. In our previous report[7], it was not mentioned that the chromatograms for hemeproteins showed the peaks splitted into two at a certain condition of RPC. As shown in Figure 1 and Figure 2, retention time of one peak (smaller peak in Figure 1 and unfilled symbol in Figure 2) change more sensitively than that of the other peak with increasimg temperature (Figure 1) and organic modifier fraction (Figure 2). The more sensitive peak for the change of chromatographic condition showed greater absorbance at 280nm than at 254nm; it means that the peak is unfolded form. Futhermore the peak didn't appear at 405nm as in Figure 3. The maximum absoption wavelengh of heme group is 405nm and proteins do not show absorption at this wavelengh. Those results indicate that the more sensitive peak may be an apoprotein and the stucture seems very unstable. Table 1 shows a comparison of the values for change of enthalpy and entropy for myoglobin and apomyoglobin.

The fact that apomyoglobin has the greater negative value of ΔH° than myoglobin represents that apomyoglobin has the greater affinity for the stationary phase. The greater negative value of ΔS° for apomyoglobin suggests that apomyoglobin is more flexible in the mobile phase and more sensitive for the change of temperature ($\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$) than myoglobin. Two opposing processes may contribute to a change in entropy on unfolding process of protein [19]. First, the destruction of secondary structure requires increased solvation of the newly exposed hydrophobic amino acid residues which originally formed the internal core. This solvation would cause a corresponding decrease in entropy, owing to the ordering of water molecules around hydrophobic residues on the surface of the protein. However, this process will be compensated for by an increased disordering of the unbound structure due to the increase in the conformational freedom of the more flexible protein. The overall process would then be expected to yield negative ΔS° and this effect would be greater in apoprotein having the severe conformational disorder. Table 2 shows ΔG° values for hemoglobin and apohemoglobin at the various temperature.

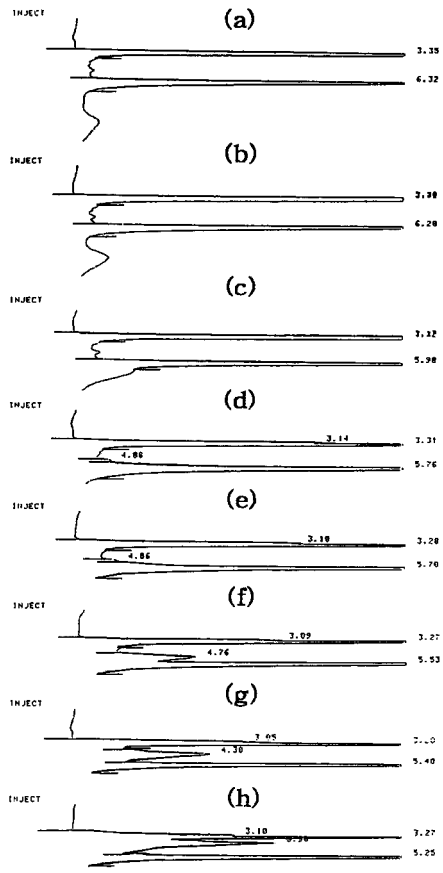

FIGURE 1. Chromatograms of myoglobin. Conditions: column, SynChropak RP-P; wavelength, 254nm; temperature, (a) 30, (b) 35, (c) 40, (d) 45, (e) 50, (f) 55, (g) 60, and (h) 65°C.

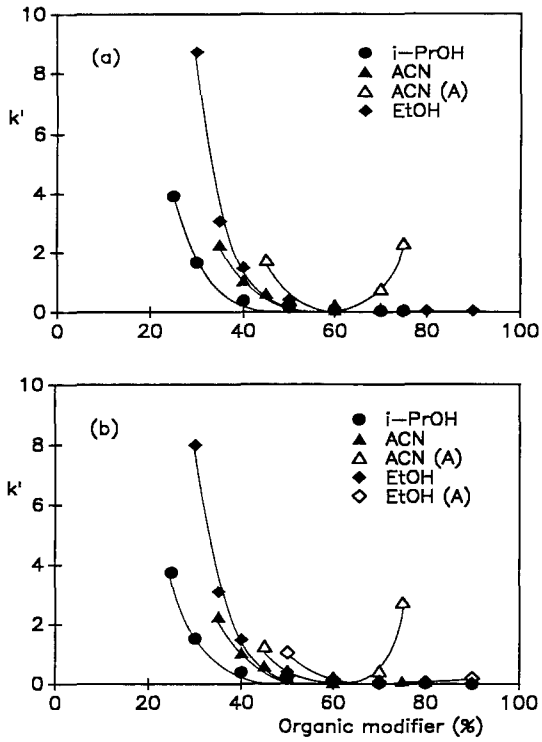

FIGURE 2. Plots of k' vs. volumn fraction of organic solvent in 0.1% aq. TFA. Condition: column, SynChropak RP-P; temperature, ambient; proteins, (a) hemoglobin and (b) myoglobin.

TABLE 1. The Values of Change of Enthalpy and Entropy[18] for Myoglobin and Apomyoglobin

Proteins	i-PrOH (%)	Temperature (°C)	ΔH° (kcal/mol)	ΔS° (cal/mol·K)
Myoglobin	30.0	30-70	-3.97	-9.12
	32.5	20-70	-2.96	-6.66
	35.0	25-70	-3.68	-9.58
	37.5	15-70	-3.68	-10.13
	40.0	20-70	-2.23	-5.94
Apo-myoglobin	30.0	55-70	-24.34	-68.99
	32.5	45-70	-23.82	-70.48
	35.0	30-50	-28.93	-93.81
	37.5	15-30	-19.27	-65.15

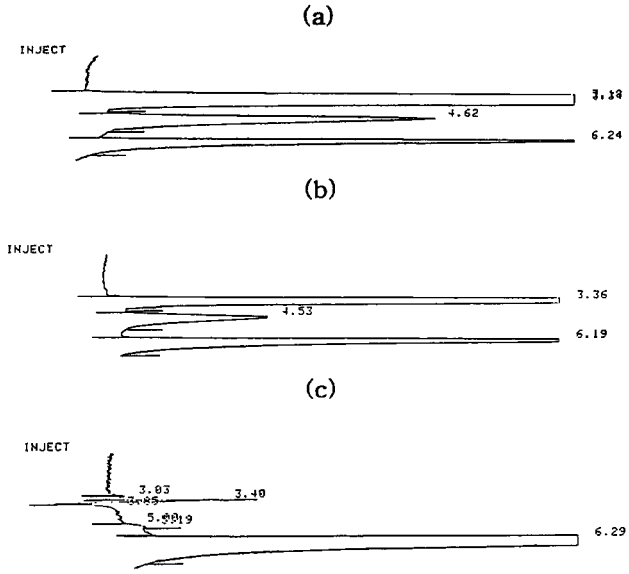

FIGURE 3. Chromatograms of myoglobin. Condition: column, SynChropak RP-P; temperature, 30°C; wavelength, (a) 280nm, (b) 254nm, and (c) 405nm.

TABLE 2. ΔG° Values for Hemoglobin and Apohemoglobin in 0.1% TFA containing 35% i-propanol

	ΔG° (kcal/mol)	
	Hemoglobin	Apohemoglobin
Temterature	ΔH°=-3.22, ΔS°=-8.15	ΔH°=-17.37, ΔS°=-53.41
(°C)	(kcal/mol) (cal/mol·K)	(kcal/mol) (cal/mol·K)
25	-0.79	-1.45
30	-0.75	-1.19
35	-0.71	-0.92
40	-0.67	-0.65
45	-0.63	-0.39
50	-0.59	-0.12
55	-0.55	0.15
60	-0.51	0.42
65	-0.47	

In Figure 4, ΔG° values calculated from Table 1 are plotted against temperature. The ΔG° values of apoproteins change more rapidly with the increase in temperature than those of proteins containing heme group. The range of chromatographic condition shown in the apoproteins was quite limited and Z values for apoproteins was not able to be calculated.

Application of Photodiode Array Detector for Conformational Monitoring

UV spectra with photodiode array detector would offer more information on conformational changes than conventional UV detector owing to the ability of monitoring the overall wavelengh showing the absorpion for proteins only by one injection. In Figure 5, chromatogram obtained by photodiode array detector shows a elution profile for cytochrome c eluted with 0.1% aq. TFA containing 30% acetonitrile at 50°C. A portion eluted at 4.5 minutes has two peaks and they appear around 200nm and 400nm, respectively. The apex showed around 400nm is a peculier peak for heme group. A portion eluted around 10.2 minutes has only one peak showed the maximum around 200nm, which is caused by absorpion of amino acid constituting cytochrome c. The peak may be an apoprotein losing the heme group during chromatographic elution. Figure 6 also shows UV spectra for hemoglobin eluted with 0.1% aq. TFA containing 35% i-propanol at 30°C and 50°C. In the chromatogram eluted at 30°C, a portion containing heme group was eluted earlier than apohemoglobin. On the other hand, apohemoglobin was eluted earlier around void volumn at 50°C. The retention time of apohemoglobin changes more sensitively with changing of temperature.

Identification of Splitted peaks

Apoproteins of hemoglobin and myoglobin were prepared by according to the reported method. Figure 7 shows the chromatograms

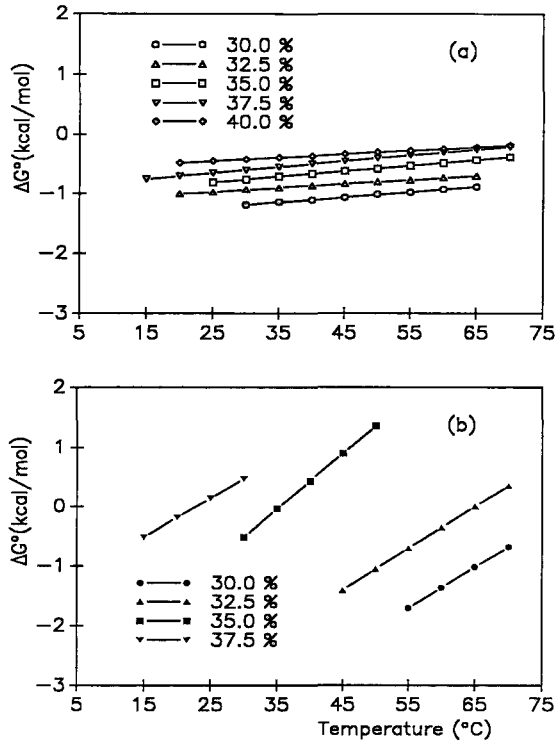

FIGURE 4. Plots of ΔG° vs. temperature for myoglobin (a) and apomyoglobin (b) under five different proportions of acetonitrile, 30.0, 32.5, 35.0, 37.5, and 40.0%.

of myoglobin (b) and apomyoglobin (c) eluted with 0.1% aq. TFA containing 35% i-propanol at 30°C. The chromatograms of left and right side were obtained at 280nm and 405nm, respectively. At 280nm myoglobin showed two peaks, at 3.64 and 4.89 minutes, respectively. However, at 405nm only a peak containing heme group showed at 4.88 minutes. In case of eluting the apomyoglobin, one peak appeared at the sameretention time as the first peak of myoglobin at 280nm. Because this peak didn't contain heme group,

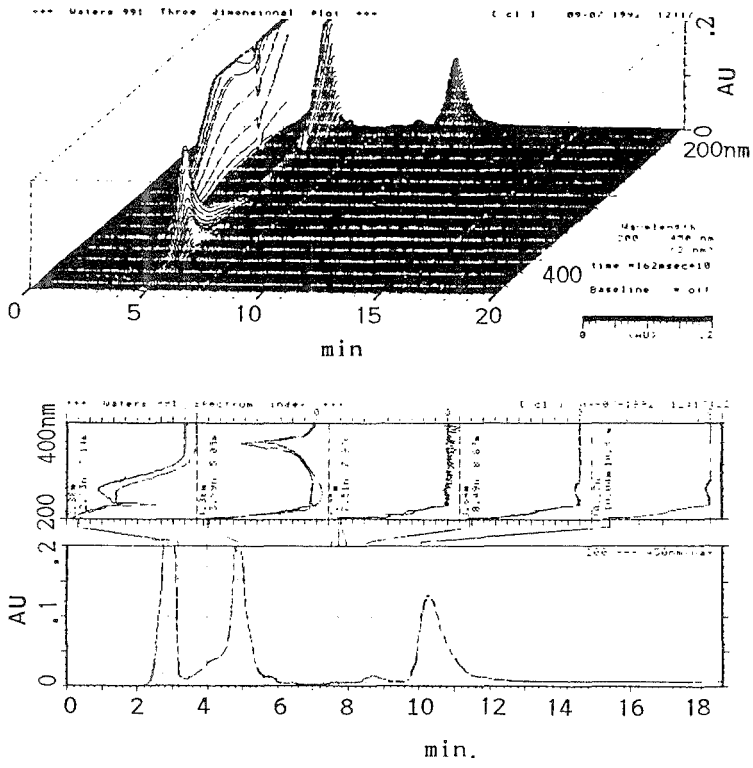

FIGURE 5. UV spectra with the photodiode array detector for cytochrome c eluted in 0.1% TFA containing 30% acetonitrile at 50° C.

only solvent peak appeared at 405nm. By the comparison of the elution behavior between native protein and apoprotein, splitted peaks of hemeproteins can be identified as one for hemeprotein and the other for apoprotein.

Application of Size-Exclusion Chromatograpy

SEC provides information on the structural change of proteins by the change of molecular size. SEC technique offers

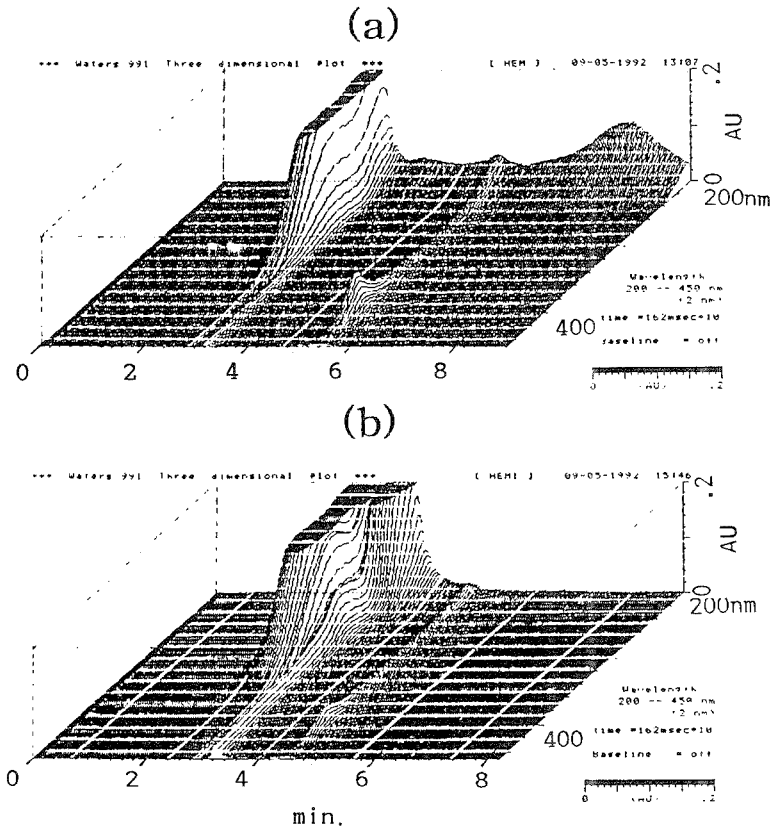

FIGURE 6. UV spectra with the photodiode array detector for hemoglobin eluted in 0.1% TFA containing 35% i-propanol at 30°C (a) and 50°C (b)

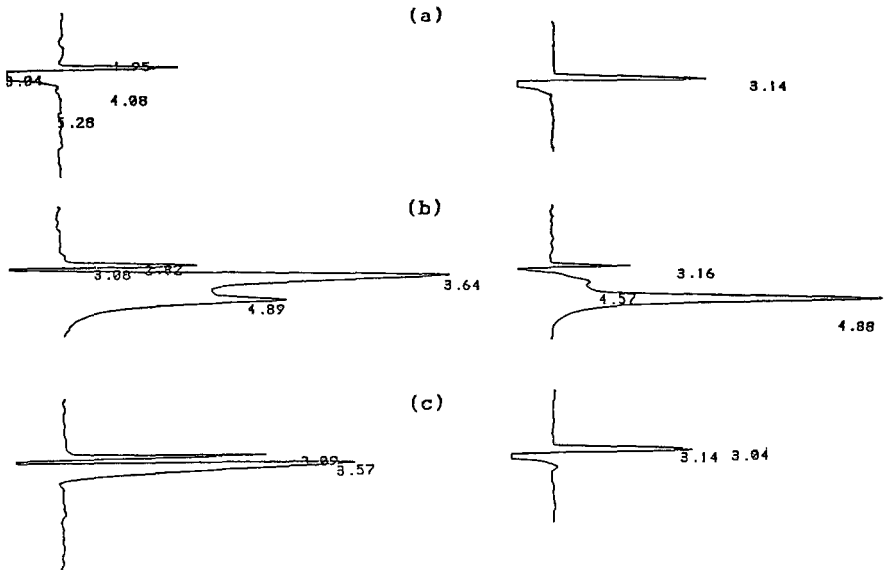

FIGURE 7. Chromatograms for water (a), myoglobin (b) and apomyoglobin (c) at 280nm (left), and 405nm (right).

several advantages [20] : (1) It is possible to monitor relatively rapid folding-unfolding transitions by using SEC. (2) In addition to monitoring denaturation by urea, SEC may be used to monitor thermal denaturation as well as denaturation by acids and ionic denaturants such as guanidium chloride. (3) A variety of optical detection methods could potentially be used to study the properties of the eluted species. (4) Protein samples can be recorvered at the end of the experiment.

Figure 8 shows the plots of K_D vs. log M.W. for proteins eluted in the same solution condition as employed in RPC, 0.1% aq. TFA (pH 2.0) and 0.1% aq. TFA containing 30% i-propanol. Under the condition of 0.1% aq. TFA, all proteins were eluted with the similar retention time. The K_D values were independent on the size

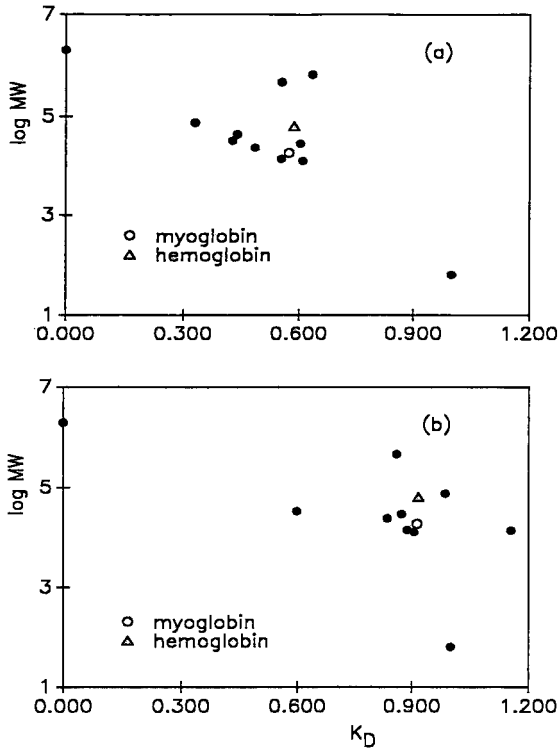

FIGURE 8. Plots of K_D vs. log M.W. for proteins eluted with 0.1% aq. TFA (a) and 0.1% aq. TFA containing 30% i-propanol (b).

for each protein and smaller than the literature values for proteins. It seems that proteins were aggregated under the acidic condition. In 0.1% aq. TFA containing 30% i-propanol, K_D values were greater than the values in 0.1% aq. TFA for all proteins. The result suggests that the addition of i-propanol in mobile phase promoted ionic interaction between the proteins and the column material. The K_D values for hemoglobin and myoglobin are little different in the both cases. The result may be due to one of the

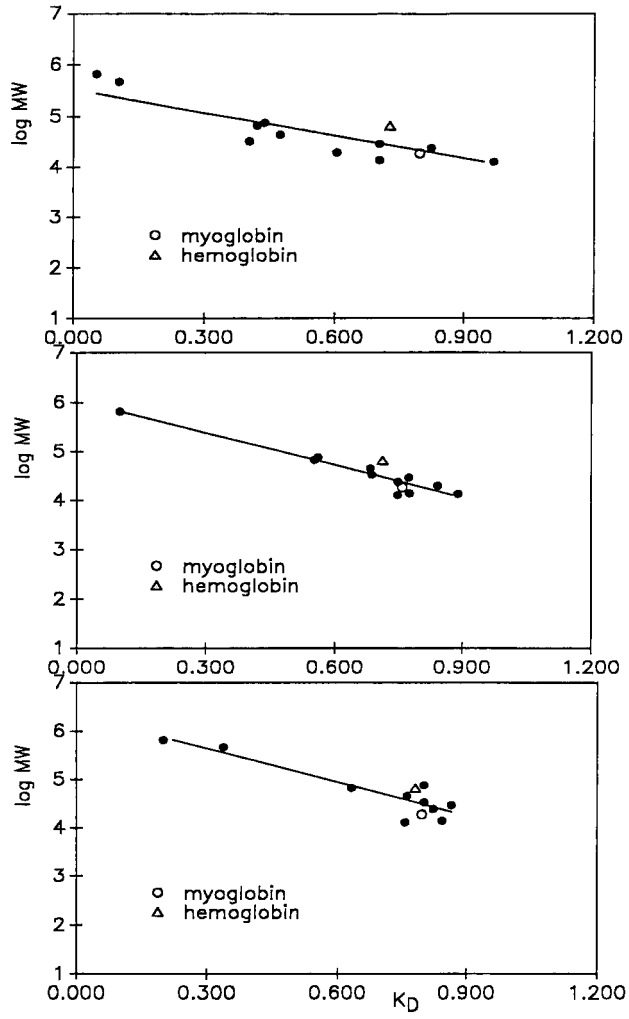

FIGURE 9. Plots of K_D vs. log M.W. for proteins eluted with 0.05M phosphate buffer (pH 7.0) containing 0M (a), 0.5M (b), and 0.85M (c) sodium sulfate.

reasons why two proteins had shown the same elution behavior in RPC[7]. Figure 9 shows the plots of K_D vs. log M.W. for proteins eluted with the same solution condition as employed in HIC [8], 0.05M phosphate buffer (pH 7.0) containing 0, 0.5, and 0.85M sodium sulfate. The correlation coefficients for each plot were 0.818 (n=10), 0.932 (n=10), and 0.605 (n=8), respectively. In the absence of a salt in buffer, electrostatic interaction between some proteins and column matrix may be occuring, which results in the poor relationship of the plot. In the buffer containing 0.85M sodium sulfate, K_D values increased for the most of proteins. The result demonstrates that increases in salt concentration facilitated hydrophobic interaction of proteins with the column material. In the buffer containing 0.5M sodium sulfate, the best relationship between log M. W. and K_D was obtained. It seems that this solution provides an approximative ideal SEC condition. In this condition, hemoglobin and myoglobin showed the difference in molecular size, in contrast to the result obtained in RPC. The difference in size may be due to one of the reason that two proteins showed the different elution behavior in HIC. However, K_D value for hemoglobin was larger than the value of the real size and hemoglobin deviated from the straight line. It may suggest that hemoglobin is divided into two subunits ($\alpha_1\beta_1$ and $\alpha_2\beta_2$). The precise information about molecular size may be obtained by using SEC/mass spctroscopy or SEC/LALLS.

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ENANTIOMERIC SEPARATION OF UNDERIVATIZED ALIPHATIC AND AROMATIC β-AMINOALCOHOLS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH A CHIRAL MOBILE PHASE

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ABSTRACT

Underivatized aliphatic and aromatic β-aminoalcohols with a primary or secondary alcohol moiety could be separated into enatiomers by revered-phase liquid chromatography with a chiral mobile phase containing copper(II), L-proline and barbital(or its analogues).

INTRODUCTION

Since the compounds containing chiral β-aminoalcohol moiety are important in pharmaceutical science[1-3] and organic chemistry[4,5], a number of enantiomeric separation

methods by high-performance liquid chromatography have been developed[6]. Indirect methods using chiral[7] or achiral[8] precolumn derivatizing reagents could be applicable in separating both aliphatic and aromatic β-aminoalcohols as into enantiomers. Direct methods using no precolumn derivatization have applicability that is limited to only aromatic β-aminoalcohols, such as ephedrine-like and propranolol-like compounds[9], and not to any aliphatic β-aminoalcohols.

Previous studies[10] in our laboratory showed that underivatized aromatic β-aminoalcohols could be separated into enantiomers by ligand exchange chromatography (LEC) using a copper(II) solution as a mobile phase and octadecylsilanized silica gel (ODS) coated with N-n-dodecyl-L-hydroxyproline(C12-Hyp) as a stationary phase.

On the other hand, for underivatized aliphatic β-aminoalcohols, our attempts using this methodology were unsuccessful, resulting in no retention or a low separation factor.

Gil-Av and coworkers reported enantiomeric separation of underivatized amino acids by LEC using an ODS column and a chiral mobile phase which was an aqueous solution of copper(II) and L-proline[11]. Their method was also

inapplicable to underivatized aliphatic and aromatic β-aminoalcohols. We discovered that addition of barbital to their mobile phase improved the separations of various β-aminoalcohols on ODS column.

EXPERIMENTAL

Samples

The β-aminoalcohols studied are listed in Table 1. Compound 1, 2, 3, (S)-1, (S)-2, (R)-4, and (S)-4 were purchased from commercial sources. Compound (R)-3 was prepared by amination of[12] of (R)-styrene oxide (commercially available).

Chromatography

Chromatography used in this work is described in the caption of Figure 1.

RESULTS AND DISCUSSION

Examples of the direct separation of aminopropanols using a mobile phase containing phenobarbital are shown in Figure 1. The β-aminoalcohols shown in Table 1 were all well separated. Compound 1 and 3 contain an amino group attached to a primary carbon atom and a secondary alcohol

TABLE 1 Enantiomeric Separation of β-Aminoalcohol[a]

$H_2NCHR_2CH(OH)R_1$

compound	R_1	R_2	k'_1[b]	EF[c]	α[d]	mobile phase
1	H	CH_3	2.77	R	1.34	a
2	CH_3	H	1.04	R	1.66	a
3	H	C_6H_5	3.96	R	1.26	b
4	C_6H_5	H	4.43	R	1.59	b

[a] Conditions are shown in Figure 2. [b] Taurine was used as a marker for t_0, $k'=(t_R-t_0)/t_0$, $\alpha=k'_1/k'_2$, where t_0 is the retention time of nonretarded solute, k'_1, the capacity factor of the first eluted enantiomer, [c] EF, the configuration of the first eluted enantiomer and [d] α, the separation factor. Mobile phase: a), 10 mM MOPS buffer solution (pH 7.5) containing 4 mM copper(II) acetate, 8 mM L-proline, 10 mM barbital and 20 mM triethylamine ; b), mixture of one volume of acetonitrile and 9 volumes of mobile phase a (pH 7.0). Other conditions are as in Figure 1.

group, conversely, compound 2 and 4 contain an amino group attached to a secondary carbon atom and a primary alcohol group. To shorten the retention time of the aromatic β-aminoalcohols, the mobile phase with minor modification was used. It should be notice that the elution order of all the enantiomers is R before S.

Figure 2 shows the effect of the barbital concentration in the mobile phase on the separation of aiphatic β-aminoalcohols. Though barbital is achiral, the barbital concentration in the mobile phase was critical for the

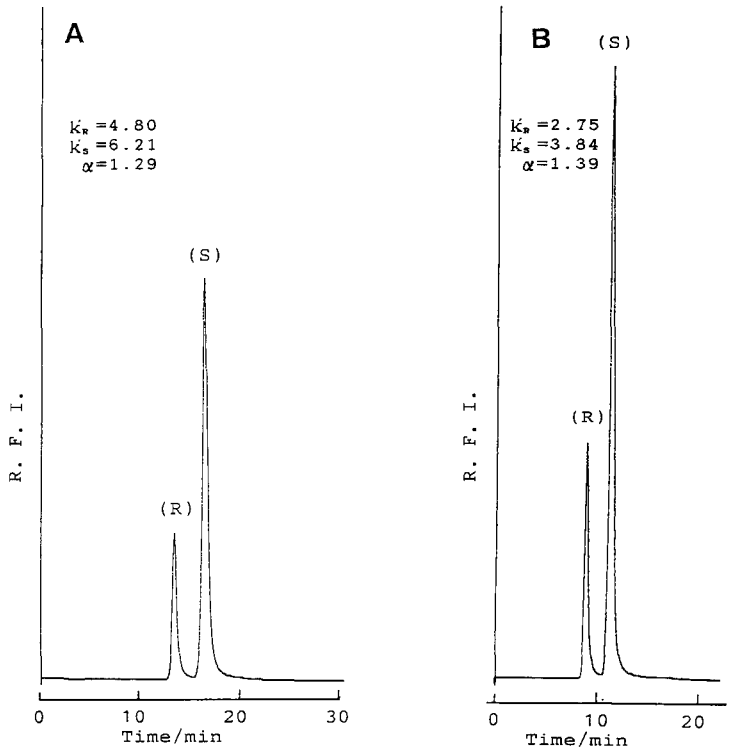

FIGURE 1. Chromatograms of (A) 1-amino-2-propanol and (B) 2-amino-1-propanol. Column: 150 × 4 (i.d.) mm self packed with Develosil ODS-5 (Nomura Chemicals, Gifu, Japan). Mobile phase: 4-morpholinepropanesulfonic acid(MOPS) buffer containing 4 mM copper(II) acetate, 8 mM L-proline, 2 mM phenobarbital and 20 mM triethylamine. The pH was adjusted with NaOH or acetic acid to 7.5. Flow rate, 0.5 ml/min; sample size, 20 μl containing 2μg of aminopropanol (R/S=1/3); detection, postcolumn reaction using o-phthaldehyde (See Reference 11). R. F. I. = relative fluorescence intensity.

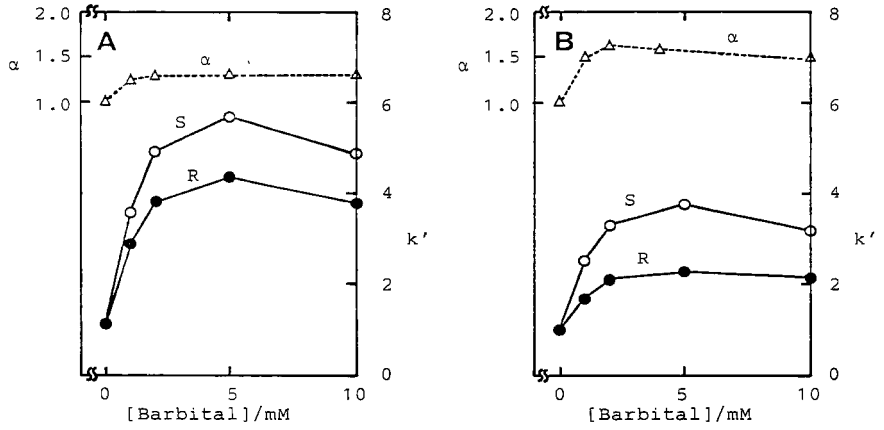

FIGURE 2. Effect of the barbital concentration in the mobile phase on the separation of (A) 1-amino-2-propanol and(B) 2-amino-1-propanol enantiomers. Mobile phase: MOPS buffer containing 4 mM copper(II) acetate, 8 mM L-proline, barbital of varying concentration(pH 7.5). Other conditions as in Figure 1.

separation. Separation did not occur in the absence of the barbital. Also, aromatic β-aminoalcohols could not be separated with a mobile phase containing no barbital. To secure the buffer action of the mobile phase containing no barbital, MOPS, which is one of Good's buffer reagent was used. Similar compound such as barbituric acid, ε-caprolactum, glycine anhydride and 3,3'-dimethylglutarimide were used as the barbital subsitutes. The capacity factors of aminopropanols under

the conditions described in Figure 1 using a mobile phase containing the subsitute(2 mM) were ≦0.6. Substantially, these compounds had no effect on the separations. Barbital can be replacedby other barbital analogues such as amobatbital and phenobarbital (Fig. 1). However, the mechanism of the barbital synergistic effect cannot be inferred from the present results.

The separation was also strongly dependent on the pH of the mobile phase. The influences on the separation of aminopropanol enantiomers were studied over the pH range of 5-8. Increasing the pH of the mobile phase results in greater retention and better separation of the enantiomers. To avoid the deterioration of the ODS, pH values above 8.0 were not studied. Because of smaller capacity factors and/or separation factors, the unsatisfactory separations were obtained at pH value below 6.0. The pH 7-7.5 was sufficient for the separation on the ODS column.

To decrease the tailing of the aminopropanol peak on a silica-base column trietylamine was added to the mobile phase. The triethylamine addition influenced the capacity factors for decreasing and did not the separation factors when triethylamine was used in the concentration

range 0-30 mM. Use of a 20 mM concentration of treiethylamine results in increased the separation.

This study has shown that the enantiomeric separation of underivatized aliphatic or aromatic β-aminoalcohols with a primary or secondary alcohol moiety on ODS column could be improved by barbital(or its analogues) addition to the mobile phase containing copper(II) and L-proline.

After this work, also we found that enanantiomeric separation of aiphatic aminoalcohols by LEC using C12-Hyp coated ODS as a stationary phase could be improved by barbital addtion to the mobile phase containing copper(II). The details will be published elsewhere.

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HPLC DETERMINATION OF TAXOL AND RELATED COMPOUNDS IN *TAXUS* PLANT EXTRACTS

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ABSTRACT

A sensitive and reproducible HPLC method, using a Curosil G column and gradient elution, was developed for the routine analysis of taxol (I) and six related taxanes, 10-desacetyl-7-epi-taxol (II), cephalomannine (III), 10-desacetyl taxol (IV), baccatin III (V), 10-desacetyl-7-epi-baccatin III (VI), and 10-desacetyl baccatin III (VII) in *Taxus* plant extracts. The method was linear for taxanes I-VII within the concentration range tested of 0.1μg - 2.4 μg injected. Purging and regeneration procedures were used which allowed more than 600 injections to be made onto the same column without the development of backpressure problems.

The day-to-day variation in the peak area of taxanes I-VII was minimum (C.V. = 9.8%, 9.1%, 5.8%, 7.7%, 7.4% and 7.8% respectively, n = 12). Variation within a day in the peak area of taxanes I-VII was even less (C.V. = 1.5%, 1.3%, 1.4%, 1.4%, 1.4%, 1.5% and 1.5%, respectively, n = 4).

The separation efficiency of the Curosil G column was compared to pentafluorophenyl (Taxil) and diphenyl (Supelcosil) bonded silica columns using 3 gradient elution systems.

INTRODUCTION

Taxol, a complex diterpene amide isolated from the bark of the Pacific yew tree *Taxus brevifolia* Nutt. (Taxaceae)(1), has been the subject of intensive research due

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to its unique cancer chemotherapeutic properties [2]. Since taxol occurs with a series of closely related taxanes in various species of *Taxus* [3-6], considerable work has been directed to devising effective methods of analysis and purification.

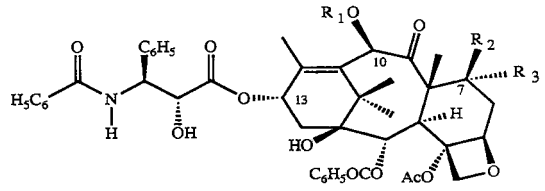
In early work high performance liquid chromatography (HPLC) with UV detection was used for the quantitation of taxol and the other taxanes in plant extracts [7-13], biological samples [14,15], and bulk taxol [16]. The HPLC columns used in these applications included bonded silica-C_{18} and silica-phenyl packing material operated in the reversed phase mode, and -CN columns operated both in the reversed and normal phase modes [7,17]. More recently, better resolution has been achieved with pentafluorophenyl (PFP) bonded silica as the column packing material [16]. There has, however, been a problem when a large number of plant extracts are analyzed with automated systems. After many injections the backpressure increases, resulting in a drastic decrease in column efficiency.

This paper describes a selective, reversed-phase HPLC method for *Taxus* plant extracts utilizing a Curosil G column, with a proprietary bonded phase packing material, for the routine determination of taxol (I) and six closely related taxanes: 10-deacetyl-7-*epi*-taxol (II), cephalomannine (III), 10-deacetyl taxol (IV), baccatin (V), 10-deacetyl-7-*epi*-baccatin III (VI) and 10-deacetyl baccatin III (VII). The backpressure problems were avoided by using a column purging procedure after each run. The separation efficiency of pentafluorophenyl (Taxil) and diphenyl (Supelcosil) columns is also presented and compared to that of the Curosil G column.

EXPERIMENTAL

Materials

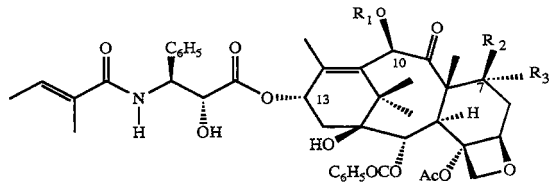
Curosil G HPLC column (4.6 x 250 mm, 6 µm) with a precolumn (4.6 x 30 mm) was purchased from Phenomenex (Torrance, CA). Taxil, a pentafluorophenyl



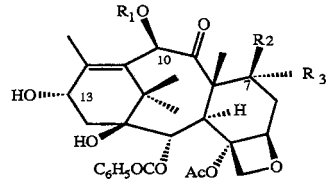
(I) ; R1=Ac, R2=OH, R3=H

(II) ; R1=Ac, R2=H, R3=OH

(IV) ; R1=H, R2=OH, R3=H



(III) ; R1=Ac, R2=OH, R3=H



(V) ; R1=Ac, R2=OH, R3=H

(VI) ; R1=H, R2=H, R3=OH

(VII) ; R1=H, R2=OH, R3=H

(PFP) HPLC column (4.6 x 250 mm, 5 μm), and a precolumn (4.6 x 13 mm) came from Metachem Technologies Inc. (Redondo Beach, CA). Supelcosil LC-DP diphenyl HPLC column (4.6 x 250 mm, 5 μm) was bought from Supelco Inc. (Bellefonte, PA) and used with a New Guard phenyl precolumn (3.2 x 15 mm, 7 μm) purchased from Applied Biosystems Inc. (San Jose, CA). A high pressure column pre-filter (0.5 μm) was purchased from Alltech Associates Inc. (Deerfield, IL) and used in all analyses.

HPLC grade acetonitrile, reagent alcohol, tetrahydrofuran, methyl-*t*-butyl ether and certified ethylene glycol monomethyl ether were purchased from Fisher Scientific Co. (Fair Lawn, NJ). The nanopure water (18 MΩ) was obtained in-house using a Barnstead nanopure purificator. All solvents and samples were filtered through a 0.45 μm nylon membrane (Alltech).

The seven taxane standards (I-VII) were provided by the National Cancer Institute through Dr. Kenneth Snader.

Apparatus

Instrumentation consisted of a Waters 600E Multisolvent Delivery System, Waters 712 WISP Auto sampler, Waters 991 Photodiode Array and NEC Power Mate SX Plus computer for controlling the analytical system and for data processing. The chromatograms and data reports were printed on a Waters 5200 Printer Plotter. Standards were weighed on a Mettler H51AR microbalance.

Methods

Sample preparation and injection

Powdered plant material (1g) was extracted twice with 10 ml of 95% ethanol by soaking with agitation for 16 hours. The combined extracts (25 ml) were evaporated to dryness, and the residue was partitioned 3 times between water (2 ml) and methylene chloride (4 ml). The organic fraction was evaporated and the

residue adsorbed on 1g of celite packed into a small column. The column was washed with 15 ml hexane until the eluate was colorless, followed by 15 ml methylene chloride. The methylene chloride wash was evaporated and dissolved in reagent alcohol to produce a solution containing ~ 20 mg/ml, from which 10 µl volume was injected.

The mixture of standards was prepared in reagent ethanol such that each standard was at a concentration of ~100 µg/ml and ten microliters were injected.

Chromatographic Conditions

Chromatography was performed using the following gradient programs (Table 1):

Gradient System 1. Linear gradient at a flow rate of 1.5 ml/min using a mobile phase starting with a 80:20 mixture of Solvent B (*reagent alcohol:water (6:94) and Solvent C (100% acetonitrile) going to 75:25, 60:40 and 55:45 (Solvent B:Solvent C) in 10, 30 and 40 minutes, respectively, and ending with a 50:50 mixture of (Solvent B:Solvent C) for 45 minutes. The system was purged for 3 minutes with a 50:50 mixture of Solvent A (reagent alcohol:tetrahydrofuran:methyl-*t*-butyl ether, 50:30:20) and Solvent C (100% acetonitrile), followed by 10 minutes equilibration with the initial solvent composition for a total of 1 hour/sample. (*N.B. reagent alcohol consists of a mixture of 90.6% ethanol, 4.5% methanol and 4.9% *iso*-propanol).

Gradient System 2. Linear gradient at a flow rate of 1.2 ml/min using a mobile phase starting with 75:25 mixture of Solvent B (reagent alcohol:water, 6:94) and Solvent C (100% acetonitrile) going to 70:30, 55:45 (Solvent B:Solvent C) in 12 and 35 minutes, respectively; and ending with a 50:50 mixture (Solvent B:Solvent C) for 45 minutes. The system was purged for 3 minutes using the same mobile phase described under Gradient System 1, followed by 10 minutes equilibration with the initial solvent composition for a total of 1 hour/sample.

TABLE 1

Gradient Tables for Gradient Systems 1-3

A. Gradient System 1 (Flow rate maintained at 1.5 ml/minute)

Gradient Time (min.)	Solvent A	Solvent B	Solvent C
0	0	80	20
10	0	75	25
30	0	60	40
40	0	55	45
45	0	50	50
46	50	0	50
49	50	0	50
50	0	80.0	20
60	0	80.0	20

B. Gradient System 2 (Flow rate maintained at 1.2 ml/minute)

0	0	75	25
12	0	70	30
35	0	55	45
45	0	50	50
46	50	0	50
49	50	0	50
50	0	75	25
60	0	75	25

C. Gradient System 3 (Flow rate maintained at 1.1 ml/minute)

0	0	76	24
12	0	70	30
36	0	55	45
45	0	50	50
46	50	0	50
49	50	0	50
50	0	76	24
60	0	76	24

Solvent A =*reagent alcohol:tetrahydrofuran:methyl-*t*-butyl ether (50:30:20)
Solvent B = reagent alcohol:water (6:94) for gradient system1 and 2; ethylene glycol monomethyl ether:water (8:92) for gradient system 3
Solvent C = acetonitrile (100%)

*Fisher Scientific Reagent Alcohol:90.6% ethanol, 4.5% methanol and 4.9% *iso*-propanol

Gradient System 3. Linear gradient at a flow rate of 1.1 ml/min using a mobile phase starting with 76:24 mixture of Solvent B (ethylene glycol monomethyl ether:water 8:92) and Solvent C (100% acetonitrile) going to 70:30, 55:45 (Solvent B:Solvent C) in 12 and 36 minutes, respectively, and ending with a 50:50 mixture (Solvent B:Solvent C) for 46 minutes. The system was purged for 3 minutes using the same mobile phase described under gradient system 1, followed by 10 minutes equilibration with the initial solvent composition for a total of 1 hour/sample.

After the analysis of 10-12 samples the column was flushed with the following regeneration system: starting at the flow rate and mobile phase mixture of Solvent B and Solvent C , and going to 50:50 (Solvent B:Solvent C) in 10 minutes, then a 50:50 mixture of Solvent A (reagent alcohol:tetrahydrofuran:methyl-*t*-butyl ether, 50:30:20) and Solvent C (100% acetonitrile) in 20 minutes,100% Solvent A from 25 to 35 minutes, a 50:50 mixture of Solvent A:Solvent C in 45 minutes, a 50:50 mixture of Solvent B:Solvent C in 50 minutes, and terminating with a starting mixture of Solvent B:Solvent C in 60 minutes.

The spectral data were collected over the 210-300 nm range of the absorption spectrum; chromatograms were plotted at the absorption maxima of taxol 227, and 273 nm.

The linearity of the detector response was determined for taxanes I-VII by injection of a series of standard solutions ranging in concentration from 10-240 μg/ml using the Curosil G column and gradient systems 1 and 3.

RESULTS AND DISCUSSION

A procedure was developed for the analysis of taxol (I) and 6 other related taxanes II-VII: 10-deacetyl-7-*epi*-taxol (II), cephalomannine (III), 10-deacetyl taxol (IV), baccatin III (V), 10-deacetyl-7-*epi*-baccatin III (VI) and 10-deacetyl

baccatin III (VII) in the needles of *Taxus* plants. Three gradient systems were tested, each using 3 different columns: Curosil G, Taxil (pentafluorophenyl, PFP) and Supelcosil (diphenyl, DP). The identity of the peaks was determined using retention times and confirmed by comparison of the UV spectra of peaks of interest with those of standard taxanes (Peak match see Table 3). In addition, the wavelength response ratio (spectral index) obtained for the different taxanes (A_{227}/A_{273}) provided an estimation of peak purity for the taxanes analyzed when compared with the values obtained for the standards I-VII (Table 2).

Since the taxanes studied represent compounds with a relatively wide range of polarity and molecular size, their separation in a single run was a challenging task. In all gradient systems 1-3 (Table 1) when Curosil G, Taxil and Supelcosil columns were used (Figures 1A-8A) a run time of 45 minutes resulted in a baseline separation of standard taxanes I-VII. However, when gradient system 3 and Supelcosil column were used, taxanes I and II appeared on a hump and this might be due to insufficient purging of the column (Figure 9A). The run time of 45 minutes was approximately equal to that obtained with gradient elution methods previously reported for the analysis of multitaxanes in extractives of the bark (7) and needles (8) using a phenyl column.

To avoid back pressure problems associated with multiple injections of plant extracts, the column was purged for 3 minutes after each run with a 50:50 mixture of Solvent A and Solvent C. Solvent A was a mixture of 50 parts reagent alcohol, 30 parts tetrahydrofuran, and 20 parts of methyl-t-butyl ether, while Solvent C was 100% acetonitrile. In addition, after the analysis of 10-12 samples the column was washed with the regeneration gradient system (see experimental). The purging and washing procedures described above allowed for the apparent removal of non-polar components in the plant extracts, the accumulation of which is responsible for the increase of back pressure. In this way, it was possible to perform more than 600 injections on the same column using the processed ethanol extracts.

TABLE 2

Wavelength Response Ratio and Response Factor for the Standards When 3 Different Columns Were Tested Each Using 3 Different Gradient Systems.

Wavelength Response Ratio[1] & Response Factor[2]

Column	Gradient System	DAB (VII)	DEAB (VI)	BIII (V)	DAT (IV)	CPN (III)	DAET (II)	TAX (I)
Curosil G	1	13.32(1.40)	14.23(1.65)	13.65(1.82)	15.49(2.10)	21.51(1.77)	15.91(1.85)	16.37(1.88)
	2	13.13(1.73)	14.18(2.04)	13.90(2.34)	15.60(2.57)	21.97(2.18)	15.51(2.27)	16.08(2.28)
	3	13.50(1.99)	14.57(2.04)	13.90(2.56)	15.38(2.90)	21.30(2.45)	15.84(2.54)	16.56(2.54)
Taxil	1	13.21(1.37)	14.38(1.54)	13.94(1.72)	15.55(1.98)	22.13(1.60)	15.80(1.73)	16.31(1.81)
	2	* *	13.98(1.95)	14.10(2.20)	14.48(2.49)	21.82(1.99)	15.78(2.14)	15.88(2.25)
	3	13.17(1.86)	14.25(2.22)	13.52(2.49)	15.36(2.95)	22.27(2.32)	15.60(2.49)	15.96(2.52)
Supelcosil	1	12.98(1.44)	14.73(1.64)	13.67(1.82)	15.78(2.05)	22.58(1.93)	15.88(1.79)	15.74(1.80)
	2	13.30(1.72)	14.36(2.04)	13.85(2.25)	15.62(2.54)	22.19(2.39)	15.78(2.21)	15.98(2.20)
	3	13.48(1.90)	14.55(2.21)	14.02(2.48)	15.67(2.86)	22.59(2.69)	16.99(2.17)	14.08(2.77)

[1] wavelength response ratio calculated as the ratio A_{227}/A_{273}

[2] values shown between brackets in (AU*min/μg)*E-2

* peak not detected at 273 nm

Taxanes Code: DAB=10-deacetyl baccatin III, DEAB=10-deacetyl-7-*epi*-baccatin III, BIII=baccatin III, DAT=10-deacetyl taxol, CPN=cephalomannine, DAET=10-deacetyl-7-*epi*-taxol and TAX=taxol.

TABLE 3

Wavelength Response Ratio and Peak Match for the Extracts When 3 Different Columns Were Tested Each Using 3 Different Gradient Systems.

		Wavelength Response Ratio[1] & Peak Match[2]						
Column	Gradient System	DAB (VII)	DEAB (VI)	BIII (V)	DAT (IV)	CPN (III)	DAET (II)	TAX (I)
Curosil G	1	8.32(870)	2.25(628)	* (769)	18.13(930)	26.00(937)	* (776)	15.40(970)
	2	10.71(857)	2.36(621)	* (798)	18.16(943)	21.42(944)	* (803)	13.73(975)
	3	11.32(880)	* (808)	* (866)	16.70(949)	32.06(934)	* (732)	14.25(969)
Taxil	1	19.74(635)	* (623)	* (764)	15.13(909)	25.38(932)	0.43(353)	1.75(857)
	2	13.00(832)	* (630)	* (811)	14.78(944)	27.67(962)	0.45(378)	1.84(709)
	3	9.00(828)	* (852)	* (907)	15.97(950)	21.63(940)	0.44(375)	1.88(671)
Supelcosil	1	6.28(829)	2.54(675)	5.19(762)	0.31(819)	32.05(901)	* (817)	14.57(968)
	2	* (853)	2.63(648)	4.65(739)	0.43(391)	12.05(932)	* (835)	13.98(968)
	3	12.82(869)	* (695)	* (828)	0.56(430)	28.25(921)	* (812)	17.62(950)

[1] wavelength response ratio (calculated as the ratio A_{227}/A_{273})

[2] values shown between brackets

* peak not detected at 273nm

Taxanes Code: DAB=10-deacetyl baccatin III, DEAB=10-deacetyl-7-*epi*-baccatin III, BIII=baccatin III, DAT=10-deacetyl taxol, CPN=cephalomannine, DAET=10-deacetyl-7-*epi*-taxol and TAX=taxol.

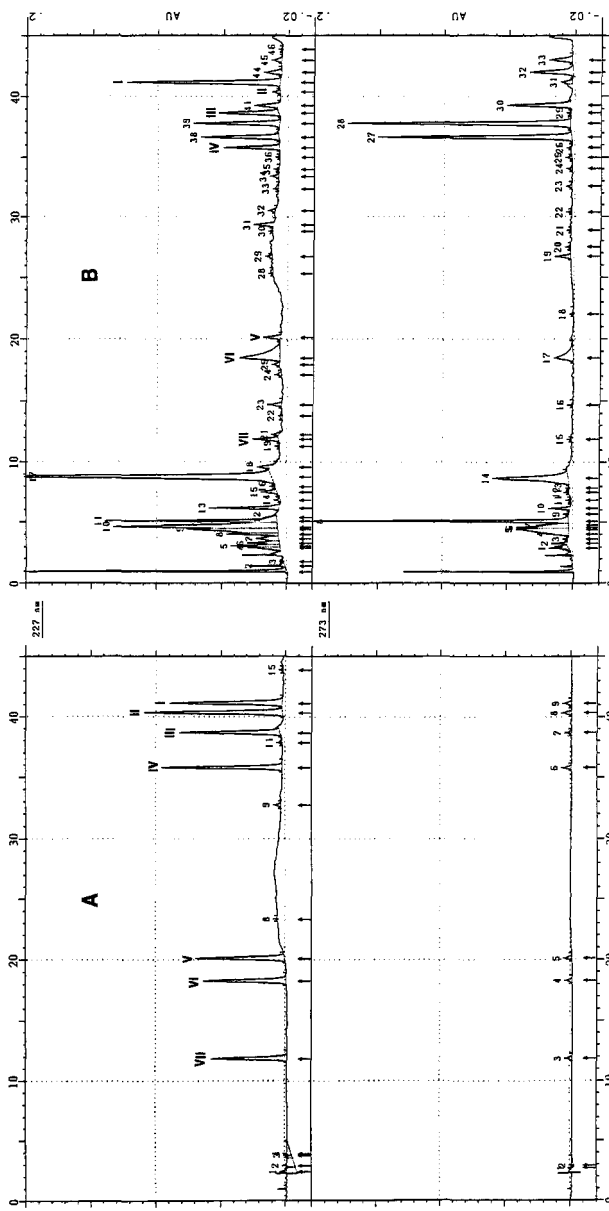


Figure 1. Chromatograms obtained on Curosil G column and gradient system 1.
A. Chromatogram of standards: I=taxol, II=10-deacetyl-7-*epi*-taxol, III=cephalomannine, IV=10-deacetyl taxol, V=baccatin III, VI=10-deacetyl-7-*epi*-baccatin III, VII=10-deacetyl baccatin III
B. Chromatogram of the purified ethanol extract of *T. x media* 'Nigra' needles.

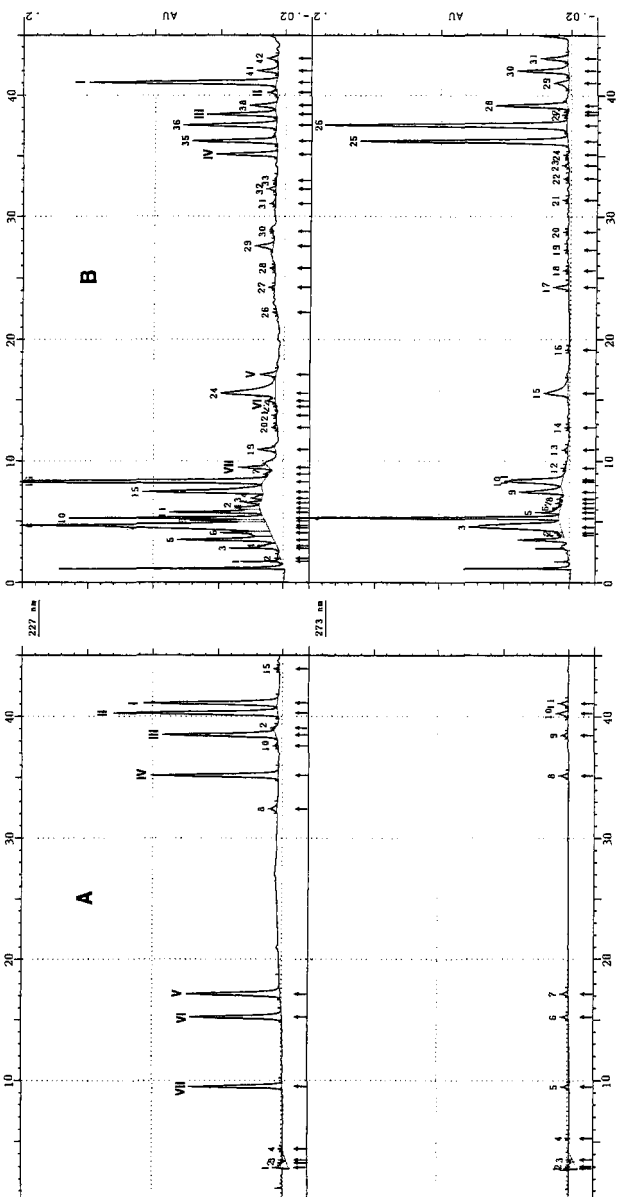


Figure 2. Chromatograms obtained on Curosil G column and gradient system 2.
A. Chromatogram of standards: I=taxol, II=10-deacetyl-7-*epi*-taxol, III=cephalomannine, IV=10-deacetyl taxol, V=baccatin III, VI=10-deacetyl-7-*epi*-baccatin III, VII=10-deacetyl baccatin III
B. Chromatogram of the purified ethanol extract of *T. x media* 'Nigra' needles.

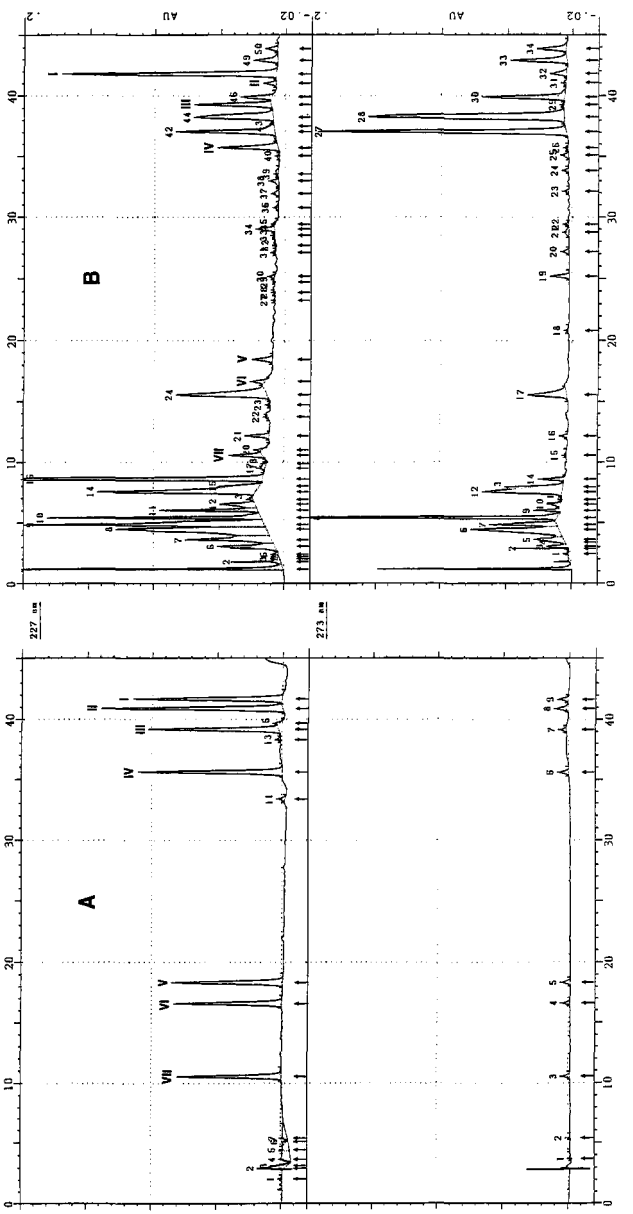


Figure 3. Chromatograms obtained on Curosil G column and gradient system 3.
A. Chromatogram of standards: I=taxol, II=10-deacetyl-7-*epi*-taxol, III=cephalomannine, IV=10-deacetyl taxol, V=baccatin III, VI=10-deacetyl-7-*epi*-baccatin III, VII=10-deacetyl baccatin III
B. Chromatogram of the purified ethanol extract of *T. x media* 'Nigra' needles.

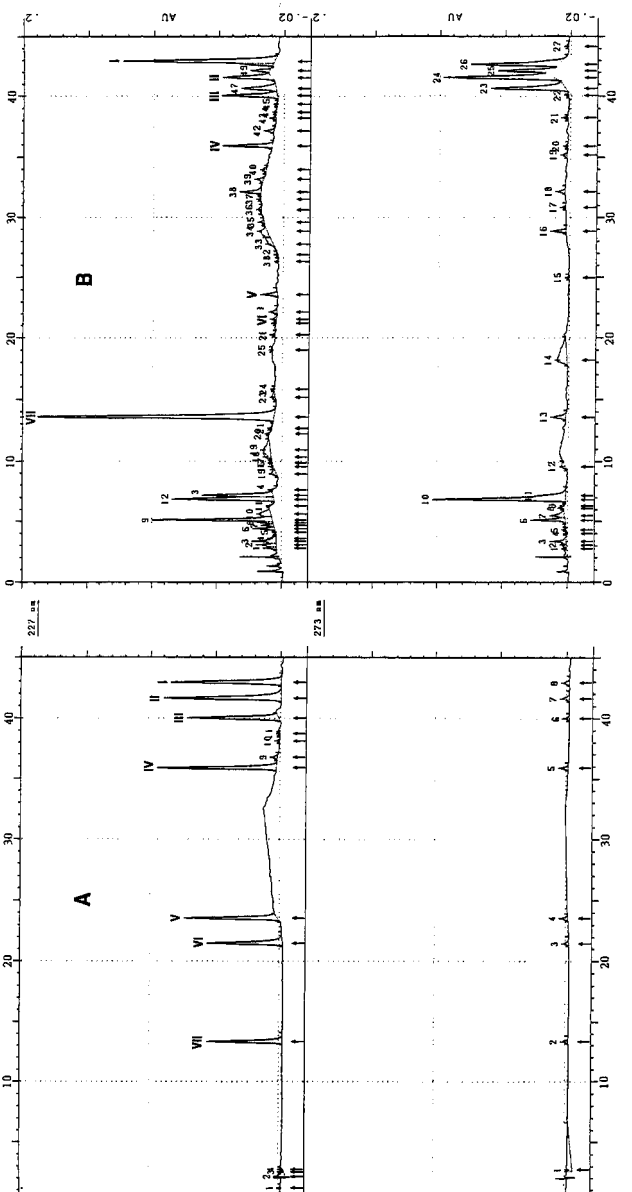


Figure 4. Chromatograms obtained on Taxil column and gradient system 1.
A. Chromatogram of standards: I=taxol, II=10-deacetyl-7-*epi*-taxol, III=cephalomannine, IV=10-deacetyl taxol, V=baccatin III, VI=10-deacetyl-7-*epi*-baccatin III, VII=10-deacetyl baccatin III
B. Chromatogram of the purified ethanol extract of *T. x media* 'Nigra' needles.

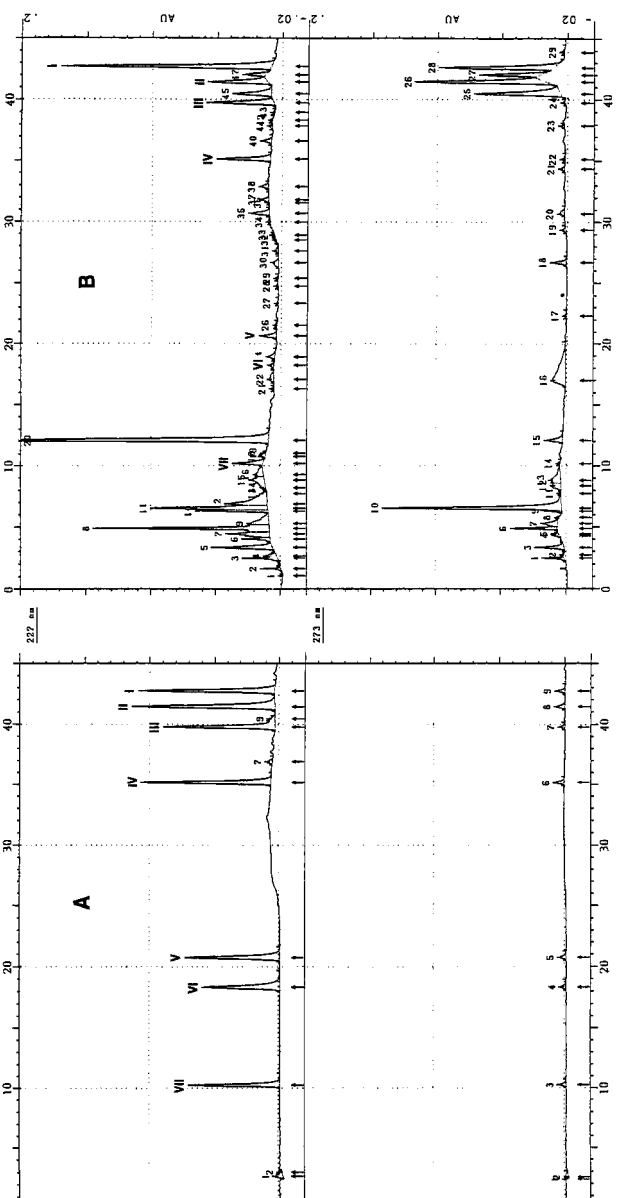


Figure 5. Chromatograms obtained on Taxil column and gradient system 2.
A. Chromatogram of standards: I=taxol, II=10-deacetyl-7-*epi*-taxol, III=cephalomannine, IV=10-deacetyl taxol, V=baccatin III, VI=10-deacetyl-7-*epi*-baccatin III, VII=10-deacetyl baccatin III
B. Chromatogram of the purified ethanol extract of *T. x media* 'Nigra' needles.

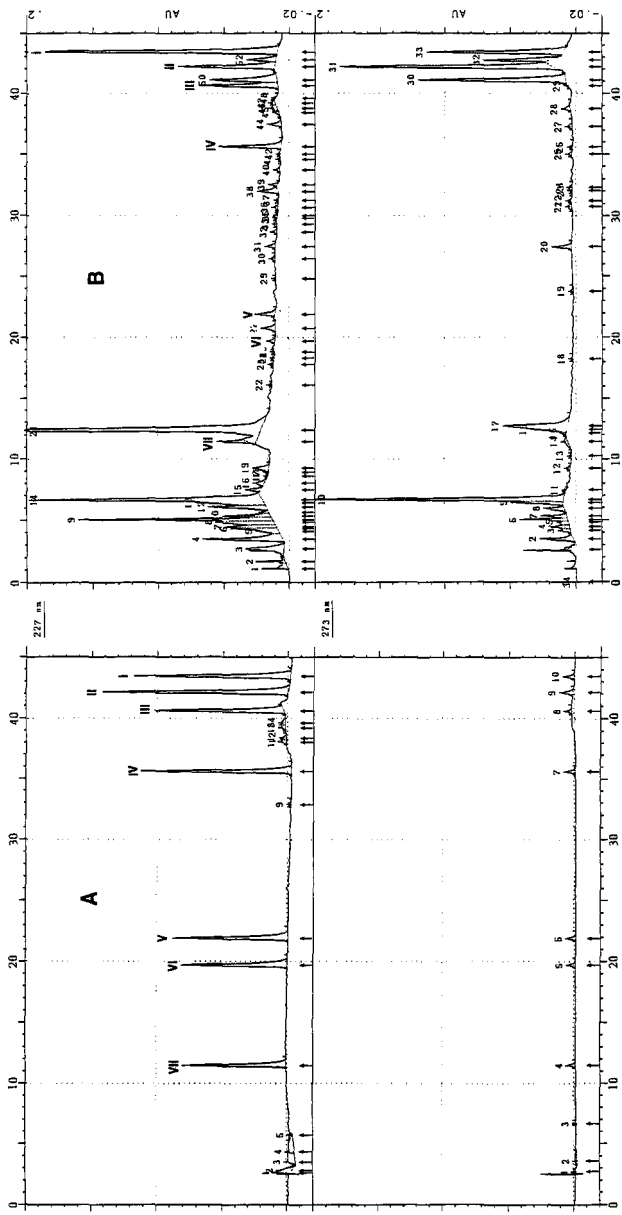


Figure 6. Chromatograms obtained on Taxil column and gradient system 3.
A. Chromatogram of standards: I=taxol, II=10-deacetyl-7-*epi*-taxol, III=cephalomannine, IV=10-deacetyl taxol, V=baccatin III, VI=10-deacetyl-7-*epi*-baccatin III, VII=10-deacetyl baccatin III
B. Chromatogram of the purified ethanol extract of *T. x media* 'Nigra' needles.

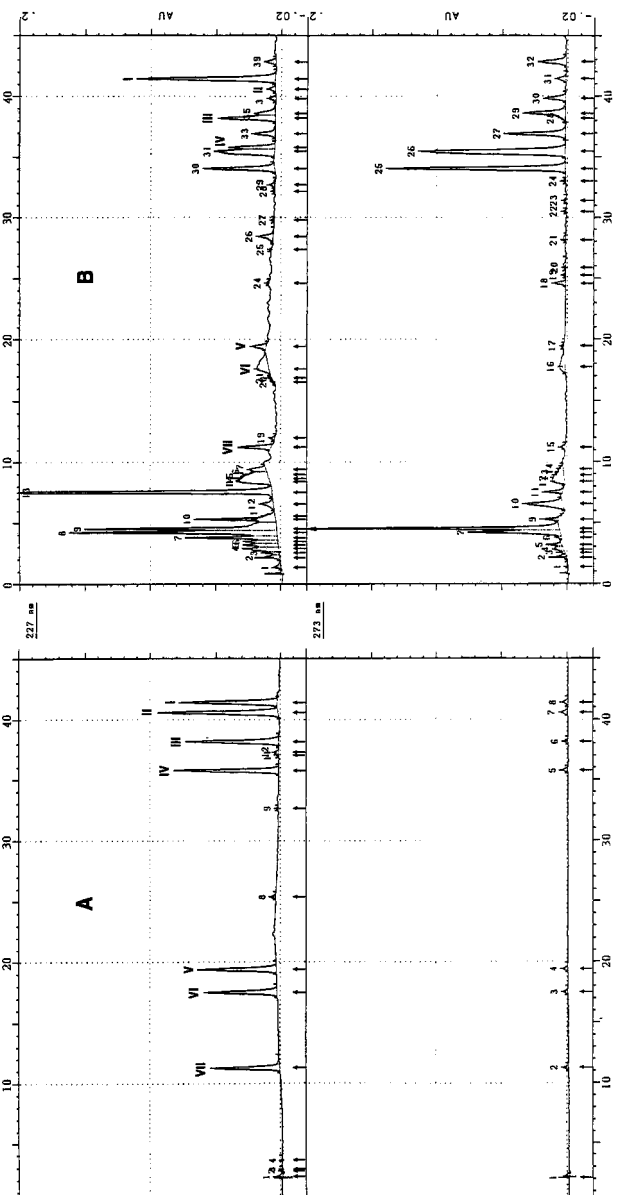


Figure 7. Chromatograms obtained on Supelcosil column and gradient system 1.
A. Chromatogram of standards: I=taxol, II=10-deacetyl-7-*epi*-taxol, III=cephalomannine, IV=10-deacetyl taxol, V=baccatin III, VI=10-deacetyl-7-*epi*-baccatin III, VII=10-deacetyl baccatin III
B. Chromatogram of the purified ethanol extract of *T. x media* 'Nigra' needles.

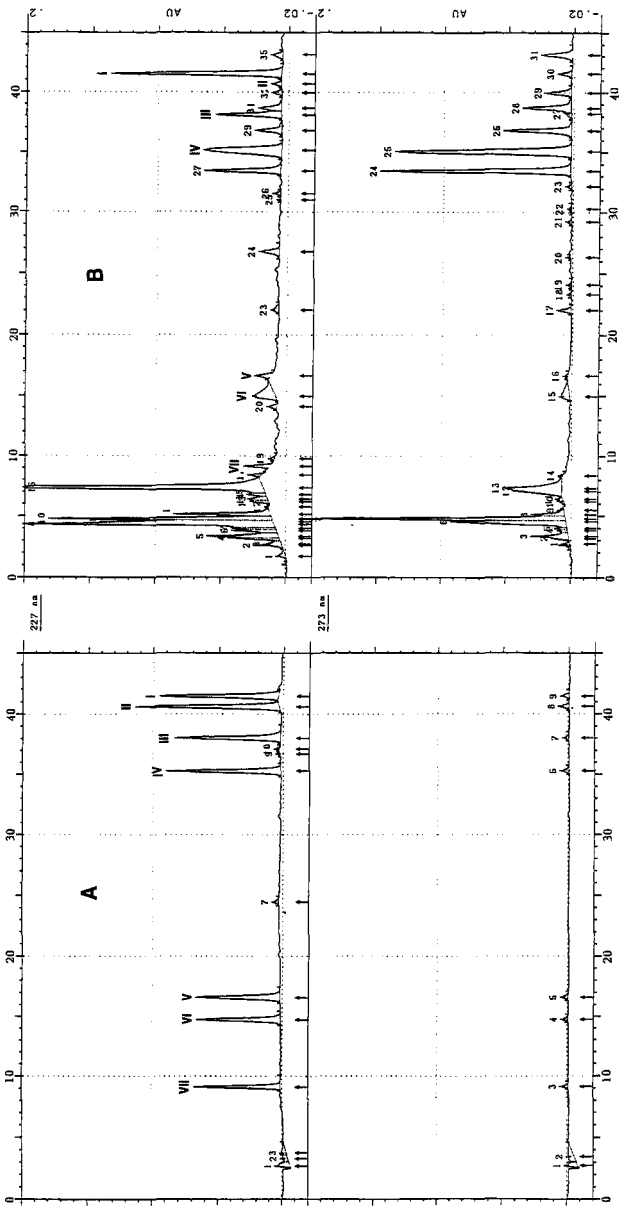


Figure 8. Chromatograms obtained on Supelcosil column and gradient system 2.
A. Chromatogram of standards: I=taxol, II=10-deacetyl-7-*epi*-taxol, III=cephalomannine, IV=10-deacetyl taxol, V=baccatin III, VI=10-deacetyl-7-*epi*-baccatin III, VII=10-deacetyl baccatin III
B. Chromatogram of the purified ethanol extract of *T. x media* 'Nigra' needles.

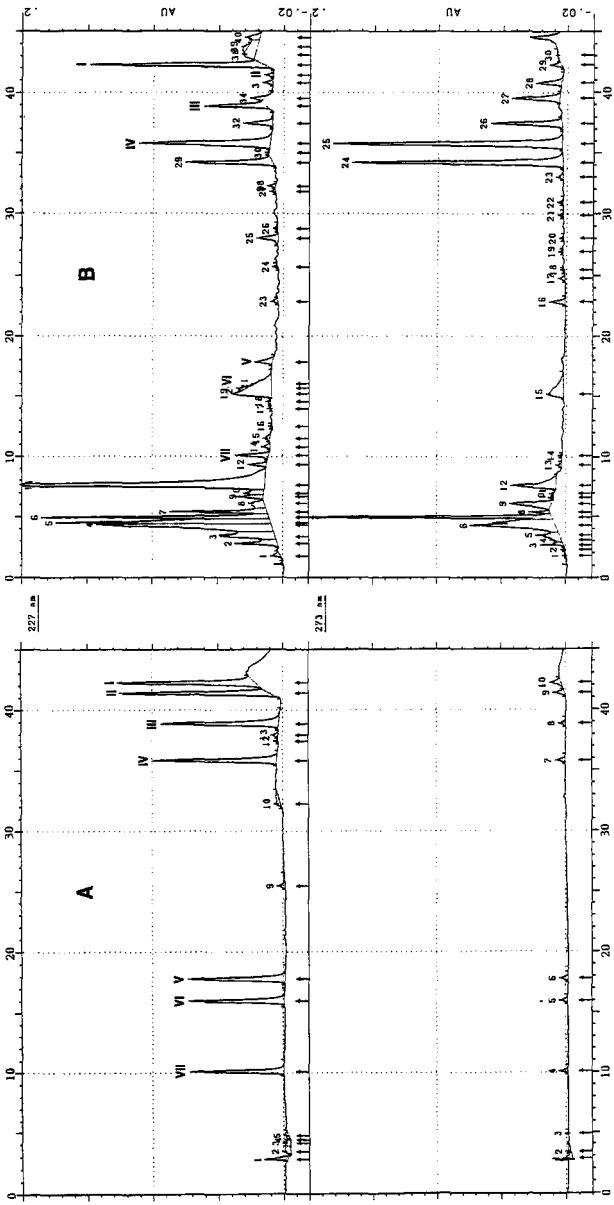


Figure 9. Chromatograms obtained on Supelcosil column and gradient system 3.
A. Chromatogram of standards: I=taxol, II=10-deacetyl-7-*epi*-taxol, III=cephalomannine, IV=10-deacetyl taxol, V=baccatin III, VI=10-deacetyl-7-*epi*-baccatin III, VII=10-deacetyl baccatin III
B. Chromatogram of the purified ethanol extract of *T. x media* 'Nigra' needles.

For HPLC analysis, the ethanolic extracts of *Taxus* needles were partitioned between water and methylene chloride. The methylene chloride phase was further purified by solid phase extraction with celite. Although the ethanol extract could be analyzed directly, it was found that the values of the taxanes studied in these extracts were about 20% higher than those obtained with the solid phase purification step. This apparent reduction in the taxanes content as a result of the celite purification step was found to be due to the removal of interfering substances and not due to incomplete recovery through spiking experiments. Most recently Castor and Tyler [18] reported that the analytical values for taxol in the needles of *T. x media* 'Hicksii' are inflated by 10% due to presence of a co-eluting substance.

The analysis of taxanes I-VII using the purified ethanolic extract of *T. x media* 'Nigra' needles was best achieved on the Curosil G column with all gradient elution systems (Table 3). Satisfactory peak match values (>900) were obtained for 10-deacetyl taxol, cephalomannine and taxol, while lower peak match values were seen for 10-deacetyl-baccatin III (857-880), 10-deacetyl-7-*epi*-baccatin III (621-808) and baccatin III (769-866). The wavelength response ratio values (A_{227}/A_{273}) calculated for standards I-VII and for same in the purified extract of *T. x media* 'Nigra' indicated the presence of small amounts of impurities co-eluting with each of these taxanes.

In general, the use of Taxil column (PFP packing material) resulted in a baseline separation of the polar taxanes, 10-deacetyl baccatin III, baccatin III, 10-deacetyl taxol and cephalomannine. The less polar taxanes, 10-deacetyl-7-*epi*-taxol and taxol, showed partial resolution and interfering peaks, indicated by the values for wavelength response ratio and peak match (Table 3).

Under the conditions of gradient systems 1-3, the Supelcosil column showed the least selectivity in the separation of taxanes III-VII (Table 3). A baseline separation was obtained for 10-deacetyl-7-*epi* taxol and taxol (Figures 7B-8B).

Although, the best separation selectivity for taxanes I-VII was obtained using Curosil G column and gradient system 1, the response factor for each of these taxanes was lower under the conditions of gradient system 1 when compared to that of gradient system 3 (Table 2). The reason for the better response with gradient system 3 may be attributed to the higher concentration of the organic solvent available at the start of the gradient elution which enhances the solubility of these taxanes. The samples analyzed contain a complex mixture of chemical compounds having a wide difference in chemical and physical properties, i.e., polarity, solubility and molecular weight. The use of methanol [7], which has a high solvent strength parameter ε^o = 0.95, to effect an increase in the solubility of those compounds resulted in a decrease in the separation selectivity for the structurally related taxanes. The solution of 8% ethylene glycol monomethyl ether (ε^o = 0.74) in water 76%, and 24% acetonitrile (ε^o= 0.65) at the start of the gradient elution in system 3 enhanced the solubility of sample material without affecting the separation selectivity of taxanes. Hence, a better response factor was attained for taxanes I-VII (Table 2).

The linearity of the detector response was also examined for the Curosil G column and gradient systems 1 and 3. A linear relationship (indicated by the correlation coefficient values shown in Table 4) was observed between the peak areas and the concentration of taxanes I-VII at the concentration range of 10-240 μg/ml. The day-to-day variation in the peak areas of standards I-VII was minimum (C.V.= 9.8%, 9.1%, 5.8%, 7.7%, 7.4%, and 7.8%, respectively, n=12), and the within-day variation was even less (C.V.=1.5%, 1.3%, 1.4%, 1.4%, 1.4%, 1.5%, and 1.5%, respectively, n=4).

In conclusion, a sensitive and reproducible HPLC method was developed for the quantitation of taxol and 6 related taxanes in the needles of *Taxus* plants using a Curosil G column and gradient system 3. The detection limit of the different taxanes

TABLE 4

Comparison of the Correlation Coefficient for the Different Taxanes using Curosil G Column and Two Gradient Systems.

	Correlation Coefficient						
Gradient system	DAB (VII)	DAEB (VI)	BIII (V)	DAT (IV)	CPN (III)	DEAT (II)	TAX (I)
1	0.999	0.998	0.998	0.999	0.999	0.994	0.998
3	0.995	0.999	0.999	0.999	0.999	0.999	0.999

Taxanes Code: DAB=10-deacetyl baccatin III, DAEB=10-deacetyl-7-*epi*-baccatin III, BIII=baccatin III, DAT=10-deacetyl taxol, CPN= cephalomannine, DAET=10-deacetyl-7-*epi*-taxol and TAX=taxol.

was determined to be below 100 ng for 10-deacetyl baccatin III, 10-deacetyl-7-*epi*-baccatin III and baccatin III and 50 ng for 10-deacetyl taxol, cephalomannine, 10-deacetyl-7-*epi*-taxol and taxol.

ACKNOWLEDGMENT

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COMPARISON OF ADSORPTION COEFFICIENT (K_{OC}) FOR SOILS AND HPLC RETENTION FACTORS OF AROMATIC HYDROCARBONS USING A CHEMICALLY IMMOBILIZED HUMIC ACID COLUMN IN RP-HPLC

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ABSTRACT

The determination of soil adsorption coefficients (K_{OC}) via HPLC capacity factors (k') has been studied, including the effect of column type and mobile phase composition on the correlation between log K_{OC} and log k'. K_{OC} values obtained by procedures other than HPLC correlate well with HPLC capacity factors determined on a chemically immobilized humic acid stationary phase, and it is suggested that this phase is a better model for the sorption onto soil or sediment than the octadecyl-, phenyl- and ethylsilica phases. By using log k'_w a theoretical capacity factor has been obtained by extrapolation of the retention data in a binary solvent system to pure aqueous eluent. There is a better correlation between log K_{OC} and log k'_w than the correlation between log K_{OC} and log k'.

INTRODUCTION

Many apolar organic contaminants commonly found in surface and subsurface waters are stable hydrophobic substances which readily partition into a variety of environmental compartments (sediments, biota, soils etc.)(1). Models for quantifying and predicting the partitioning processes of the compounds are frequently predicted by using aqueous solubilities (S) and n-octanol/water partition coefficients (K_{ow}). A very important parameter for the prediction of the movement of organic pollutants through the environment is K_{oc}, the ratio of adsorbed chemical per unit weight of organic carbon on the surface of soil or sediment to the concentration of the chemical in aqueous solution.

Although direct methods of measuring K_{oc} are to be preferred, its estimation from other physical properties can also be used (2-6). As values for K_{ow} and K_{oc} are difficult to determine, alternative methods for their determination have been considered. In view of this, it seemed worthwhile to investigate the relationship between soil or sediment partition coefficients and retention on stationary phases used in reverse-phase high-performance liquid chromatography (RP-HPLC) (7,8). From the theory of surface adsorption and the theory of partition it is evident that close parallels might be predicted for adsorption onto natural particulates and retention by lipophilic HPLC phases (9-11). Obviously, the closest prediction of the partitioning of chemical compounds by soil will be obtained from a stationary phase which bears a strong resemblance to soil.

This paper is an account of the evaluation of a new stationary phase, namely, chemically bound humic acid (**CBHA**). The potential of this new phase potential for the prediction of the soil adsorption coefficient K_{oc} has been evaluated by using a series of aromatic hydrocarbons. The K_{oc} values predicted on CBHA have been compared with the values determined on octadecyl- (ODS), phenyl- and ethylsilica stationary phases. A group of alkylbenzenes and polyaromatic hydrocarbons have been used as solutes in this study as they span a wide range of properties associated with the hydrocarbons that are toxic constituents of oil.

MATERIALS AND METHODS

Chromatographic retention data were measured with an LKB 2150 solvent delivery system and an LKB Wavescan Diode-array detector. Sample introduction was via a Rheodyne 7125 injection valve fitted with a 20 μl loop, and chromatograms were recorded on an Olivetti M24 personal computer. The phenyl- and ethyl-silica columns (250 x 4.6 mm) were obtained from Bio-Separation Technologies Co., Budapest, Hungary. The Partisil^R 10 ODS (250 x 4.6 mm) column was obtained from Hichrom Ltd. Reading, England. All other chemicals were obtained from various commercial sources and used as received.

Chromatography

Mobile phases (HPLC grade methanol and water from Hichrom Ltd.) were mixed, volume/volume, freed from dissolved air by helium entrainment and pumped at flow rates of 0.8 ml/min. The test solutes were dissolved in methanol at a concentration of 0.1 mg/ml. Typically 20 μl was injected. A laboratory temperature of 20-23°C was used for all HPLC measurements. The methanol content of the mobile phase was changed thus: (i) from 80% to 60% in 5% steps for the ODS and phenyl columns; (ii) from 60% to 40% in 5% steps for the immobilized humic acid phase; (iii) from 50% to 30% in 5% steps for the ethylsilica phase. Methanol was used for the measurement of the retention time (t_0) of an unretained compound. The relationship:

$$k' = (t_R - t_0)/t_0 \quad [\text{eqn. 1}]$$

was used to calculate the capacity factor k' from the retention time (t_R) of each compound. All capacity factors reported are the mean of at least three measurements. The correlation analysis for all compounds was made by linear regression analysis of log k' versus log K_{oc} and a least squares fit routine was used for curve fitting.

Selection of Log K_{oc} Values

The literature contains a limited number of compounds for which reliable log K_{oc} values have been reported. In order to have reliable K_{oc} data for the construction of the calibration graph, we have used selection criteria similar to those used by Brooke et al. (12) when they compiled K_{ow} data. These selection criteria are: (i) that the same log K_{oc} value (± 0.1 log K_{oc}) was reported in more than one independent study and (ii) that the procedure was well documented and precautions and checks were included in the study to ensure the validity of the result. In addition, it was required that the log K_{oc} value was obtained after plotting an adsorption isotherm with a soil or sediment in the temperature range of 18-25 °C. The chemicals having reliable log K_{oc} values are listed in Table 1.

Preparation of Chemically Bound Humic Acid Silica Gel

Dried silica gel (10g) was refluxed with 5% 3-aminopropyltriethoxysilane in anhydrous toluene (13) and the resulting aminopropyl silica gel was removed

TABLE 1. Reference Chemicals with Reliable log K_{oc} Values Obtained from the Literature

Chemicals	**Log K_{oc}**	**Literature**
Benzene	1.91	3
Toluene	2.18	12
Ethylbenzene	2.41*	7
Propylbenzene	2.86*	7
Butylbenzene	3.40*	7
o-Xylene	2.34*	7
Naphthalene	3.11	3
Phenanthrene	4.28*	7
Anthracene	4.41	3
Pyrene	4.83*	2

* NOTE: converted from literature log K_{oc} value using the relationship:

$K_{oc} = (K_p \times 100) / \%$ organic carbon

by filtration, washed with toluene, methanol and water, washed again with methanol and dried. The reaction product was activated with 10 volumes of 5% aqueous glutaraldehyde for 5 h to produce an activated gel which on isolation was washed with 15 volumes of distilled water. This purified gel was reacted with 100 mL of 1% aqueous solution of humic acid, pH 7.5, for 8 h at ambient temperature. After the chemically bound humic acid silica gel (CBHA) had been washed with 10 volumes of 0.5 M phosphate buffer and distilled water it was treated with 0.1 M buffered ethanolamine, pH 7.5, for 3 h. The reaction product was washed with a large excess of distilled water and dried to yield a dark-brown product. Elemental analysis of this new stationary phase was performed in the Micro Analytical Laboratory of the University of Manchester. This prepared silica gel was packed as a slurry under high pressure in a stainless steel column (250 x 4.6 mm) by Jones Chromatography, Hengoed, Wales.

RESULTS AND DISCUSSION

Elemental analysis of immobilized humic acid revealed a C, H and N composition of 4.7%, 0.7% and 0.5%, respectively

In order to eliminate selective solute-solvent interactions (14,15), we have used log k'_w, the capacity factor obtained by the extrapolation of retention data from binary eluents to 100% water instead of using log k', the capacity factor obtained from binary eluents. Snyder et al. (16) showed that the linear equation

$$\log k' = \log k'_w + S\Phi \quad \text{[eqn. 2]}$$

can be used to describe the relationship between log k' and log k'_w, where Φ is the volume fraction of the organic component in the water-organic compound mixture used as the mobile phase, k'_w represents the capacity factor of a solute with pure water as the mobile phase and S is a constant for a given solute-eluent combination. The log k'_w of 10 organic compounds calculated

TABLE 2. Log K_{oc} Values from the Literature and Log k'_w Values Obtained from Equation [2] on Different HPLC Stationary Phases

Solute	Log K_{oc}	ODS phase log k'_w	Phenyl phase log k'_w	Ethyl phase log k'_w	CBHA phase log k'_w
Benzene	1.91	1.352	1.756	0.060	0.096
Toluene	2.18	1.934	2.456	0.479	0.514
Ethylbenzene	2.41	2.142	2.892	0.739	0.782
Propylbenzene	2.86	2.786	3.447	1.156	1.107
Butylbenzene	3.40	3.163	3.978	1.577	1.407
o-Xylene	2.34	2.217	2.945	0.759	0.627
Naphthalene	3.11	2.272	2.962	0.934	1.452
Phenanthrene	4.28	3.007	3.723	1.859	2.569
Anthracene	4.41	3.137	3.918	1.961	2.907
Pyrene	4.83	3.531	4.395	2.374	3.212

from equation [2] on the ODS, phenyl, ethyl and CBHA phases are presented in Table 2, along with values of log K_{oc} for these chemicals.

The log K_{oc} versus log k'_w values on the ODS phase calibration curve for the chemicals listed in Table 2 is plotted in Fig. 1 and yields:

$$\log K_{oc} = 1.373 \log k'_w - 0.335 \quad [r^2 = 0.794 \quad n=10] \quad [\text{eqn } 3]$$

For the same 10 compounds the correlation between log K_{oc} and log k'_w on the phenyl phase is shown in Fig. 1 and gives:

$$\log K_{oc} = 1.149 \log k'_w - 0.560 \quad [r^2 = 0.816 \quad n=10] \quad [\text{eqn } 4]$$

For these same 10 chemicals listed in Table 2 the correlation between log K_{oc} and log k'_w on the ethyl phase is plotted in Fig. 2 and yields:

$$\log K_{oc} = 1.367 \log k'_w + 1.546 \quad [r^2 = 0.949 \quad n=10] \quad [\text{eqn } 5]$$

A similar treatment of the data for the humic acid phase (Fig. 2) yields:

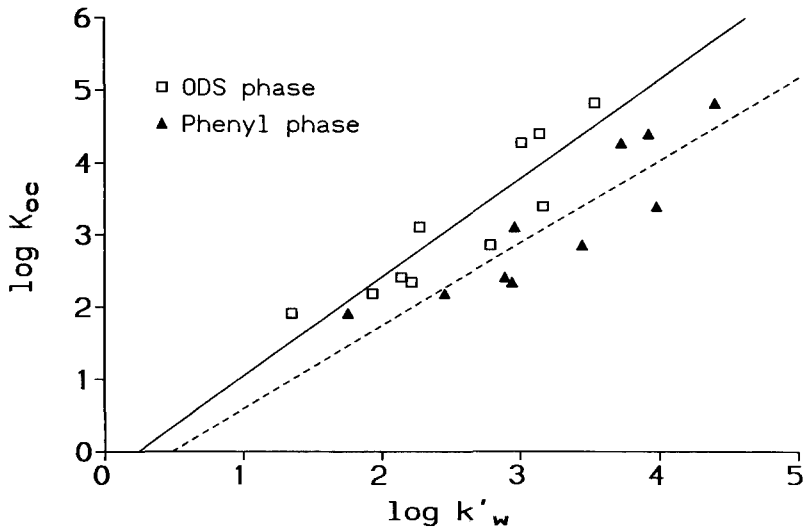

FIGURE 1 Relationship between the soil/water partition coefficient (K_{oc}) and the theoretical capacity factor (k'_w) for ODS and phenylsilica phases.

$$\log K_{oc} = 0.948 \log k'_w + 1.781 \; [r^2 = 0.986 \; n = 10] \; [\text{eqn } 6]$$

From a comparison of equations [3],[4],[5] and [6] it is evident that the best prediction of log K_{oc} from log k'_w arises from data obtained from the CBHA phase. On the basis of correlations of K_{oc} versus log k'_w, it is more accurate to estimate log K_{oc} from k'_w determined from the humic acid phase column than via a single relationship between log K_{oc} and log k'_w on the ODS, phenyl or ethyl phases. The differences for the 10 chemicals listed in Table 1 are small for the humic acid phase, suggesting that its adsorptive properties are similar to those of sediment and soil organic matter. Such similarity might be expected as humic acids represent a large proportion of the organic matter in soil. Thus the following order can be generated:

organic matter on the soil > CBHA > ethyl > phenyl > ODS

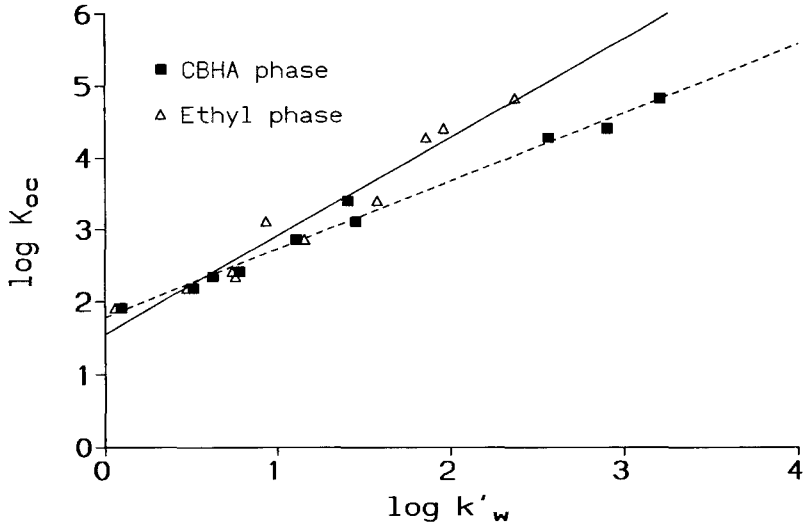

FIGURE 2 Relationship between the soil/water partition coefficient (K_{oc}) and the theoretical capacity factor (k'_w) for ethylsilica and CBHA phases.

This is the order that might be expected on the basis of the polarity of the media. ODS should be the least polar and organic matter of sediment or soil matter should be the most polar due to the presence of carboxyl, phenolic and amino moieties in humic substances.

It was noted that the correlation between log K_{ow} and log k' improves with an increase in the water content of the HPLC mobile phase (7). Harnisch et al. (17) specified a mobile phase water content of at least 30% v/v even for homologues and structurally related compounds. Several groups have demonstrated that an increase in the water content results in superior correlations for log K_{oc} and log k' for the octadecyl and cyanopropyl phases (7,8) and for the ethyl phase (18). In this study we have examined this correlation on the chemically bound humic acid column for mobile phases with varying water contents. The capacity factors determined for a range of water contents are given in Table 3 and the respective correlations are given below.

TABLE 3. Capacity Factors*(log k') Obtained on CBHA Using Different Mobile Phases.

Solute	40 % water	50 % water	60 % water	100 % water
Benzene	-0.744	-0.663	-0.537	0.096
Toluene	-0.705	-0.581	-0.291	0.514
Ethylbenzene	-0.687	-0.358	-0.152	0.782
Propylbenzene	-0.558	-0.287	0.008	1.107
Butylbenzene	-0.423	-0.125	0.204	1.407
o-Xylene	-0.559	-0.380	-0.155	0.627
Naphthalene	-0.222	0.006	0.322	1.452
Phenanthrene	0.221	0.826	1.123	2.569
Anthracene	0.274	0.871	1.293	2.907
Pyrene	0.672	0.999	1.392	3.212

*NOTE: calculated from equation [2]

Mobile phase: 40% water/60% methanol:

$$\log K_{oc} = 2.005 \log k' + 3.721 \quad [r^2 = 0.931]$$

Mobile phase: 50% water/50% methanol:

$$\log K_{oc} = 1.596 \log k' + 3.123 \quad [r^2 = 0.959]$$

Mobile phase: 60% water/40% methanol:

$$\log K_{oc} = 1.448 \log k' + 2.708 \quad [r^2 = 0.972]$$

Calculated results to 100% water:

$$\log K_{oc} = 0.948 \log k' + 1.781 \quad [r^2 = 0.986]$$

From an examination of the above series it is evident that there is an improvement in the correlation on increasing the water content of the mobile phase. Errors in calculating the capacity factor are higher for mobile phases of low water content than they are for high water contents. Reductions in these errors may be contributing to improvements in the correlation values. In addition, the high water content of the mobile phase is more like the conditions which will prevail in environmental processes. We suggest that our use of the calculated log k'_w for correlation is the best way to model the real conditions of soil/water sorption in the environment.

TABLE 4. Log K_{OC} Values Estimated from the CBHA Stationary Phase and Their Differences from Reported Values.

Solutes	Log K_{OC} from CBHA	Reported Log K_{OC}	Difference from reported value
Acenaphthene	3.79		
Acenaphthylene	3.83		
Antracene	4.53	4.41*	-0.12
Benzene	1.87	1.91*	0.04
Butylbenzene	3.15	3.40*	0.25
Chlorobenzene	2.28		
1,3-Dichlorobenzene	2.56		
1,4-Dichlorobenzene	2.56		
1,2-Dichlorobenzene	2.58		
Ethylbenzene	2.52	2.41*	-0.11
Fluoranthene	4.74	4.63$	-0.11
Fluorene	4.15		
9-Methylanthracene	4.78	4.81#	0.03
2-Methylnaphthalene	3.91	3.92#	0.01
Naphthalene	3.15	3.11*	-0.04
Phenanthrene	4.22	4.28*	0.06
Propylbenzene	2.83	2.86*	0.03
Pyrene	4.82	4.83*	0.01
Tetracene	5.77	5.81#	0.04
Toluene	2.26	2.18*	-0.08
1,2,5-Trichlorobenzene	3.45		
o-Xylene	2.37	2.34*	-0.03

* see Table 1 for origin; # reference 6; $ reference 19

By using equation [6] we have determined (Table 4) log K_{OC} values for the 10 chemicals listed in Table 1 by RP-HPLC on CBHA. In addition, we report log K_{OC} values for chlorobenzene, 1,2-, 1,3- and 1,4- dichlorobenzene, 1,2,5-trichlorobenzene, acenaphthene, acenaphthylene, 2-methylnaphthalene, fluorene, fluoranthene, 9-methylanthracene and tetracene. For 4 of this last group of chemicals we subsequently found in the literature log K_{OC} values and, as shown in Table 4, these values reported by others are exceedingly close to the values we have determined by RP-HPLC on CBHA.

CONCLUSION

This investigation has shown that it is possible to determine log K_{oc} values for aromatic hydrocarbons by measuring the HPLC capacity factors on CBHA. This evaluation of the four stationary phases has established that the best correlation between log K_{oc} and log k' was found for the chemically bound humic acid stationary phase. This study indicates that the best way to model the soil/water sorption of aromatics in the environment is to use log k'_w, the capacity factor obtained by extrapolation of retention data from binary eluents to 100% water. The best prediction of log K_{oc} is obtained by using log k'_w measured using binary eluents, instead of log k'.

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DETERMINATION OF OCTANOL-WATER PARTITION COEFFICIENTS FOR A SERIES OF IMIDAZOLIDINEDIONES BY A NOVEL COMBINATION OF MICRO SHAKE-FLASK AND HPLC TECHNIQUES

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ABSTRACT

Octanol-water partition coefficient (Log P) values for a series of 19 imidazolidinedione analogues have been determined by high performance liquid chromatography. To obtain a regression equation between log P and chromatographic log k' values, a modified micro shake-flask method has been developed. In the shaking step, a simple syringe and test tube system was devised and has proved sufficient to ensure the proper distribution of analytes between the two phases. In addition, HPLC was used not only as a detector for the shake-flask method, but also as a technique for determining the capacity factors of the compounds. Further technique modifications include alternatives to the use of octanol and dimethyl sulfoxide (DMSO) in HPLC thereby eliminating the detrimental effect of these solvents on the stationary phase. Our micro shake-flask method is a modified version of the technique developed by Ford et al. which used a Mixxor-separator device for distribution of solute and an HPLC apparatus for the determination of the ratio of solute concentrations in the two phases [H. Ford, Jr., C. L. Merski, and J. A. Kelley, J. Liq. Chromatogr., 14, 3365, 1991]. Finally,

calculated log P values for this imidazolidinedione series have also been determined and compared to experimental data. The relationship between lipophilicity and anticonvulsant activity for the molecules has thus been examined.

INTRODUCTION

The lipophilicity of a chemical compound is the single most important correlate in quantitative structure-activity relationship (QSAR) studies [1]. The most widely used measure of lipophilicity is the octanol-water partition coefficient, expressed as log P [2]. Experimental log P values can be determined either by the time-consuming shake-flask method or by a reversed-phase HPLC technique [3,4]. Although the HPLC determination is the more efficient of the two methods, it requires a reference system which is based on log P values determined by the classical shake-flask technique. Ford and coworkers introduced a rapid and simple microscale method, in which the conventional shake-flask was replaced by a Mixxor apparatus [5]. This technique requires only a 10 μg sample, making the distribution faster and more efficient. Since Ford et al used HPLC instead of other conventional methods to determine the contents of the two phases, the largest source of error in the experiment was eliminated. In our study, their technique was adopted for the shake-flask method. However, HPLC was used not only as a detector for the shake-flask method, but also as a technique for determining the capacity factors of the compounds. To our knowledge, this useful combination of the two techniques has not been previously reported. Other modifications to the micro scale method of Ford et al have also been included either to simplify equipment requirements or to alleviate other problems inherent in the use of some HPLC solvents.

Imidazolidinediones (hydantoins) constitute an important class of anticonvulsant compounds. It is well known that a correlation exists between the log P values of anticonvulsants and their activities [6]. However, no similar relationship has been demonstrated for hydantoins, despite numerous SAR studies for these compounds [7-9]. To examine whether the same relationship holds true for the hydantoin sub-class of anticonvulsants, we synthesized and determined the activity of 19 structurally related hydantoin derivatives. Their log P values were determined by both experimental and theoretical methods.

The purpose of this study was to develop a novel combination of micro shake-flask and HPLC techniques for the experimental determination of log P. Hydantoin anticonvulsant compounds were used to validate our novel method for log P determination. The relationship between hydantoin biological activities and log P values was also investigated.

MATERIALS

All hydantoin analogues were synthesized by standard methods and were characterized fully by ^{1}H, ^{13}C NMR, IR. The C5 monosubstituted hydantoins were prepared by the reaction of an appropriate α-amino acid with potassium cyanate [10]. Analogues having benzyl substitution at N3 were synthesized by benzylation using benzyl bromide [11]. The 3-ethyl and 3-phenyl analogues were obtained by the reaction of an appropriate alkyl or aryl isocyanate with an α-amino acid [12]. The C5 disubstituted compounds were produced by the Bucherer Bergs synthesis [13]. All chemicals and solvents were purchased from Aldrich or BDH as HPLC grade materials. Anticonvulant activities were measured using the picrotoxin method [14].

EXPERIMENTAL

A BECKMAN System Gold Module 126 liquid chromatograph equipped with a BECKMAN System Gold UV Module 166 detector operating at 215 nm was used for the analysis. The HPLC system was controlled by a NEC PC-8300 computer. The stationary phase was VYDAC ODS RP-C18 (4.6 mm x 250 mm, 5 μ, 300 Å) and the mobile phase was comprised of 30 (methanol):70 (aqueous at pH 2) v% mixture; the aqueous component is a 0.1 v% solution of trifluoroacetic acid (TFA). The flow rate was set to 1 mL/min. All experiments were performed at room temperature (23-25 C). Approximately 1 μg of each compound was dissolved in 0.5 mL pH 2 TFA solution. A 25 μL aliquot of this solution was injected onto the HPLC column by a 100 μL Hamilton 802 chromatographic syringe. The column dead time t_0 was measured as the first distortion of the baseline after injection of denaturated methanol (t_0= 2.01 min.).

TABLE 1. Experimental Parameters for Micro Shake-Flask Analysis.

Compounds	A (μL)	B (μL)
Hydantoin	300	300
5,5-diphenylhydantoin*	10	1000
3-methyl-5,5-diphenylhydantoin*	10	1000
5-methylhydantoin	300	300
5,5-dibenzylhydantoin*	10	1000
5-benzylhydantoin	300	300
3,5-dibenzylhydantoin*	10	1000

* These compounds were dissolved in MeOH instead of buffer (pH 7) at the beginning of the micro shake-flask method.

Experimental log P values were determined using a modified micro shake-flask method [5]. For each anticonvulsant, a 5 mg/mL solution was prepared in either a pH 7.0 potassium phosphate aqueous buffer or methanol (see Table 1). A 20 μL aliquot from this buffer was dissolved in 1.0 mL octanol-saturated pH 7.0 potassium phosphate buffer or methanol (Table 1). Then 1.0 mL buffer saturated n-octanol was added. To achieve solute distribution we departed from the previously described mixer apparatus of Ford at al. and employed a simple glass syringe and test tube system. This distribution was effected by applying 30 piston strokes of the glass syringe to the two phase mixture with repetitive ejection into and recovery from a glass test tube. After 15 mins, the two phases were separated, and each phase was transferred to a 1.5 mL Eppendorf microcentrifuge tube. They were centrifuged in a Hettich benchtop centrifuge at 2500xg for 5 mins. From the octanol phases, A mL (Table 1) was transferred into a centrifuge tube. This tube was placed in a LABLONCO Freeze Dry-50 lyophilizer connected to a SAVANT Speed Vac concentrator, and the octanol was removed. The dry compound was dissolved in B μL (Table 1) of a pH 7.0 buffer (henceforth this phase will be referred to as the "octanol" phase). 25 μL aliquots from both phases were injected into an HPLC chromatograph using a 25 μL loop injector. The relative concentration of the samples in each phase was then determined by an HPLC analysis. The chromatographic conditions were the same as described above. The P

value was obtained from the ratio of peak areas in the "octanol" and buffer phases, respectively. Three independent log P measurements were performed for each sample.

RESULTS AND DISCUSSION

Ford and coworkers used a mixer apparatus for partitioning the sample between octanol and water [5]. In our study, this apparatus was replaced by an ordinary 5 cm^3 glass syringe and a 10 cm^3 glass test tube. Log P results obtained using this simple equipment showed consistency and reproducibility as demonstrated by the standard deviations in log P's (see Table 1). This simple and inexpensive test tube and syringe system permits anyone with access to HPLC equipment to use this reliable micro shake-flask method for log P determination.

A major problem with the HPLC detection of analytes in the shake-flask technique arises if the UV absorption peak of the solute coincides with that of octanol. Unfortunately, this is the case for hydantoin compounds, whose λ_{max} values are close to 215 nm. This problem was solved by removing octanol from the 10 μL octanol phase aliquot containing the equilibrated solute by lyopilization. The resultant dry analyte was redissolved in a large amount (1000 μL) of pH 7 buffer to overcome precipitation of the analyte in the aqueous buffer. Henceforth this phase become the "octanol phase". The lyophilization of octanol also eliminated the need to wash the column regularly to remove detrimental octanol that would lead to peak distortions. If the UV absorption of the solute interferes with that of octanol, this procedure offers an excellent way of circumventing this problem.

We have also avoided using dimethyl sulfoxide (DMSO) as a preliminary solvent because its application would lead to more serious peak distortions in the chromatogram than those caused by octanol. Instead, we used a pH 7 buffer or methanol for less or more hydrophobic compounds, respectively.

In the HPLC technique, log k' is usually determined by either the isocratic or polycratic approach. In the isocratic approach, log k' is measured at a certain eluent composition, whereas in the polycratic approach log k'_ϕ values are determined at several ϕ, and the results are extrapolated to 100% water content. As opinions are divided as to the advantages and disadvantages of the two approaches, the isocratic method was used in this study. A 30% methanol solution

was employed as the mobile phase because this composition gave the most practical retention times. The advantages of using methanol as an organic modifier as opposed to other modifiers are well known [15]. The pH of the aqueous eluent was set to 2 to ensure that all compounds are fully protonated.

Table 2 shows the compounds with their measured and calculated log P values; bioactivities on a scale of 0 through 4 are also included. Five molecules which reflected the full spectrum of retention times were chosen as standards. For these five compounds, both the micro shake-flask log P and the HPLC log k' values were determined. For the remaining compounds, only their log k' values were determined; their log P's were obtained from the following regression equation:

$$\log P = a \log k' + b$$
$$(n=5,\ s=0.09,\ r^2=0.998)$$

As statistically shown, the correlation between log P and log k' is excellent for those five molecules. As a further check on the validity of our procedure, the obtained log P values for hydantoin and phenytoin (both were part of the standard set) were compared to earlier experimental data. In the literature, several log P's are available for phenytoin in the 2.23-2.47 range [16-18], and they compare well with our value of 2.27. Similarly, the hydantoin log P of -1.63 from this study is very close to the former value of -1.69 [18].

A regression analysis was also carried out between the measured and calculated log P values according to the following equation:

$$\log P = a\ \mathrm{ALOGP} + b$$
$$(n=19,\ s=0.06,\ r^2=0.95)$$

The two most conspicuous outliers are 3-phenylhydantoin and 1-methyl-3-hydantoin (Table 2). The structural similarity between these compounds suggests that some parameters associated with the N^3-C_{benzyl} link do not correctly take the electronic effects into consideration, thereby overestimating true log P values. (3-benzyl-5-phenylhydantoin possesses a somewhat high log P, although this is not out of line with the precision of the method [15]). Excluding those two outliners, one arrives at an improved r^2 value of 0.97.

TABLE 2.

Compounds	t_R (min)	log k'	log P	ALOGP	A‡
hy†	2.53	-0.69	-1.53, -1.63±0.01*	-1.18	0
5,5-diphenylhy	36.17	1.21	2.28, 2.29±0.01*	2.14	4
1-methyl-3-phenylhy	5.04	0.15	0.14	0.95	1
3,5-dibenzylhy	67.03	1.49	2.84	2.70	0
5-benzyl-3-phenylhy	35.86	1.21	2.27	2.71	0
3-ethyl-5-benzylhy	18.05	0.88	1.61	1.39	0
3-methyl-5,5-diphenylhy	70.64	1.51	2.88	2.38	3
5-isopropylhy	3.76	-0.10	-0.36	0.02	1
5-isobutylhy	5.84	0.25	0.35	0.44	1
5-methylhy	2.94	-0.40	-0.95, -0.86±0.02*	-0.86	0
3-benzylhy	8.53	0.49	0.82	0.79	2
5-phenyl-3-ethylhy	10.43	0.60	1.05	1.11	0
5,5-dimethylhy	3.19	-0.28	-0.72	-0.64	0
5-phenyl-3-methyl-5 ethylhy	19.45	0.92	1.69	1.73	2
3-phenylhy	4.21	0.00	-0.15	0.72	2
5-benzylhy	6.01	0.27	0.39, 0.44±0.02*	0.81	0
5-phenylhy	4.45	0.05	-0.05	0.53	3
5,5-diphenyl-2S-hy	47.81	1.34	2.53	2.69	3
5,5-dibenzylhy	44.22	1.30	2.46, 2.41±0.01*	2.78	0

* Log P's were measured by the micro shake-flask method.

† hy = hydantoin. ‡ Anticonvulant activities were measured using the picrotoxin method [14].

Regression analysis reveals no correlation between the anticonvulsant activities and the experimental log P values for the series (see eqn. (3)):

$$\log (\text{activity}) = a \log P + b$$

($n=19$, $r^2=0.13$)

This finding is in contrast to the strong dependence of general anticonvulsant activity on their log P values [6]. A possible explanation is that hydantoin binding to the receptor site is a specific geometric process. This is supported by the fact that whereas 5,5-diphenyl substitution seems necessary for high activity, the 5,5-dibenzyl compound is inactive.

CONCLUSION

A novel combination of HPLC measurement of log P values and the use of HPLC as a detection technique in a micro shake-flask method has been introduced and validated on a series of 19 hydantoin analogues. It is hoped that the technique described here will find widespread application due to its simplicity and reliability. No correlation between the activities and log P values for the series anticonvulsant compounds has been found.

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PREDICTION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC RETENTION DATA OF CARBOXAMIDES AND OXADIAZOLES

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ABSTRACT

Regression models that predict the high-performance liquid chromatographic retention behavior of carboxamides and oxadiazoles were proposed. A new intermolecular interaction parameter was developed that combines dispersion interaction and total water solvation shell surface energy ratio of structural(*o*, *m*, *p*) isomers to form nonpolar bonding constant descriptor. Also resonance effect constant and field effect constant were used as electronic descriptor. A three-variable model indicated high multiple correlation(R>0.996) between the observed and the calculated values.

INTRODUCTION

Quantitative structure-retention relationships(QSRRs) are the methodology of relating chemical structure with chromatographic retention parameter(1). QSRRs have two main goals, the prediction of chromatographic retention and the explanation of the chromatographic mechanism. Through the use of statistical

methods, retention data are characterized by various combinations of solute descriptors. Valuable QSRRs models obtained can be used to predict retention for new solutes, identify the most informative structural descriptors and suggest separation mechanism in a given chromatographic system.

In general, there are four available descriptors : topological, geometric, electronic, and physicochemical descriptors. Topological descriptors include molecular connectivity descriptors(2), κ indexes(3), path descriptors(4), and fragment descriptors. Fragment descriptors are evaluated from simple counts of atoms, bonds, rings, and substructures of the molecule. Geometric descriptors are evaluated from three-dimensional coordinates. They include principal moments of inertia, van der Waals molecular volumes(5), and length-to-breadth ratios(6). Physicochemical descriptors include molar refraction, polarizabilities(7), solubility parameters(8), Hammett constants, and boiling points. Electronic descriptors include σ electron density(9), dipole moments, orbital energies, and superdelocalizabilities. The QSRRs equations using these descriptors have been developed successfully for many compound classes and chromatographic systems(1).

Since the chromatographic retention is based on the intermolecular interactions such as solute-stationary phase, solute-mobile phase, and mobile phase-stationary phase interactions, the goal of this study is to find which of the available descriptors that describe the intermolecular interaction and discrimination of structural(*o,m,p*) isomers are related to the retention of the carboxamides and the oxadiazoles.

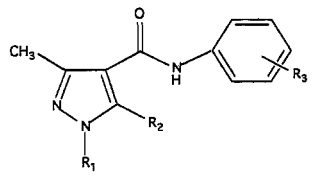
Multiple linear regression analysis was performed to find the regression model that correlates the retention data.

MATERIALS AND METHODS

Eight carboxamides and five oxadiazoles(10) were used for this study(Figure 1) and their retention data are listed in Table 1 and Table 2.

Methanol(HPLC grade) and acetonitrile(HPLC grade) were all from J.T. Baker (Phillipsburg, U.S.A.). Water was purified by using a Milli-Q water purification system(Millipore, Bedford, MA, U.S.A.).

A. Carboxamides



No.	R_1	R_2	R_3
1132	CH_3	Cl	*p*-Cl
1138	CH_3	Cl	*m*-OCH_3
1139	CH_3	Cl	*o*-OCH_3
1249	CH_3	CH_3	*m*-OCH_3
1250	CH_3	CH_3	*o*-OCH_3
1251	CH_3	CH_3	*o*-Cl
1253	CH_3	CH_3	*p*-Cl
1258	CH_3	CH_3	*p*-OCH_3

B. Oxadiazoles

No.	R_1	R_2	R_3	R_4
10392	*m*-Cl	*p*-CH_3	*i*-Pr	*m*-CH_3
10369	*m*-Cl	*p*-CH_3	*i*-Pr	*p*-CH_3
10397	*m*-Cl	*p*-CH_3	*i*-Pr	*p*-OCH_3
10380	*m*-Cl	*p*-CH_3	*i*-Pr	*o*-Cl
10378	*m*-Cl	*p*-CH_3	*i*-Pr	*m*-Cl

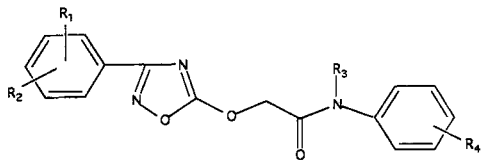


Figure 1. Compounds used in this study

TABLE 1
Observed and Calculated log k' of Carboxamides at 55% MeOH and 40% MeCN

	55% MeOH		40% MeCN	
No.	Obsd.	Calc.	Obsd.	Calc.
1258	0.129	0.128	0.045	0.061
1249	0.241	0.245	0.161	0.152
1138	0.454	0.463	0.431	0.431
1253	0.607	0.581	0.509	0.474
1250	0.677	0.668	0.487	0.483
1251	0.738	0.768	0.599	0.625
1132	0.800	0.795	0.751	0.754
1139	0.908	0.902	0.777	0.774

TABLE 2
Observed and Calculated log k' of Oxadiazoles at 75% MeOH and 65% MeCN

	75% MeOH		65% MeCN	
No.	Obsd.	Calc.	Obsd.	Calc.
10397	0.718	0.717	0.799	0.799
10380	0.790	0.788	0.934	0.932
10378	0.816	0.816	0.932	0.934
10392	0.835	0.837	0.952	0.957
10369	0.851	0.848	0.963	0.958

Apparatus

All chromatograms were obtained using a Shimadzu LC-10AD liquid chromatograph (Kyoto, Japan) equipped with a Shimadzu spectrophotometric detector SPD-10A at a wavelength 254nm. A Alltech NucleosilC18 column(250 × 4.6 mm i.d., Deerfield, Il, U.S.A.) was used. Isocratic elution was carried out with 55% and 75%(v/v) mixtures of methanol and water and 40% and 65%(v/v) mixtures of acetonitrile and water at 35°C. The flow rate was 1.0mL/min. The retention times for all solutes were measured three times and their average values were used for further analysis.

Descriptor Generation

Five descriptors were investigated : nonpolar bonding constant, polarizability, solubility parameter, resonance effect constant(11), and field effect constant(11). Nonpolar bonding constant descriptor is composed of two parts, the measure of the energy of dispersion forces acting between the solute and the stationary phase molecules(12) and the total water solvation shell surface energy ratio of structural(*o,m,p*) isomers. Calculation of the total water solvation shell surface energies were performed with a 33-MHz IBM compatible 486PC with the PCMODEL software. Polarizability descriptors were calculated using

Miller method(7) and solubility parameter descriptors were calculated using the group contribution additive method(8).

Regression Analysis

Multiple linear regression analysis(13) was used for descriptor selection and model construction. Preliminary information on the interrelationships among the descriptors was obtained from the correlation matrix. Also, multicollinearity was examined using tolerance. Multiple linear regression analysis was performed by a stepwise procedure, followed by model generation. The criteria for judging the best model were multiple correlation coefficient, standard error, and overall F-value for analysis of variance. Statistical calculations were performed with Macintosh Classic II using SYSTAT software.

RESULTS AND DISCUSSION

Since chromatogrpahic retention is basically dependent on the intermolecular interactions among solute, stationary phase, and mobile phase, it is necessary to develop the intermolecular interaction descriptor. The interaction acting between the solute and the stationary phase molecules has been calculated by dispersion interaction based on the group contributions(12). Structural(*o,m,p*) isomers had same values of dispersion interaction, because calculation method used is based on group contributions. The separation of the isomers could not be explained by dispersion interaction. The different retention behavior of the isomers in reverse-phase liquid chromatography is thought to occur through the interaction between the solute and the mobile phase rather than between the solute and the stationary phase.

The interaction between the solute and the mobile phase corresponds to the effect of solvation. However, since it was difficult to assess the extent of solvation of the solute in the mobile phase, the total water solvation shell surface energy of the isomers was calculated, followed by calculation of their relative ratio(Table 3), instead of determining the extent of the solvation of the solute in the mobile phase.

TABLE 3
Relative Isomer Ratios based on Total Water Solvation Shell Surface Energy

		Substituent		
Sample	Position	OCH_3	CH_3	Cl
Carboxamides	*o*-	1.565	1.037	1.187
	m-	1.123	1.038	1.051
	p-	1.000	1.000	1.000
Oxadiazoles	*o*-	1.213	1.236	1.013
	m-	0.979	1.014	0.980
	p-	1.000	1.000	1.000

TABLE 4
Resonance Effect Constants and Field Effect Constants for Substituents

Substituent	Resonance Effect Constant	Field Effect Constant
OCH3	-0.50	0.41
CH3	-0.14	-0.05
Cl	-0.16	0.69

Nonpolar bonding constant descriptor was determined by multiplying the value of dispersion interaction by the relative isomer ratio.

The solvation of solute may cause the change of the electronic environment of aromatic ring and the extent of the change is related to the electronic properties of substituents in the aromatic ring. The resonance effect constant and field effect constant describing the change separation were used as electronic descriptor(Table 4).

The best model selected for carboxamides and oxadiazoles is presented in Table 5 and Table 6, respectively. A plot of the observed vs. calculated retention data for carboxamides and oxadiazoles is presented in Figure 2 and Figure 3, respectively. They show the high degree of correlation.

TABLE 5
Regression Model Developed for the Carboxamides Separated at 55% MeOH

Coefficient	Std. Error	Tolerance	Descriptor
0.002	0.000	0.833	(Nonpolar bonding constant)/1000
-8.837	0.808	0.955	Resonance effect of pyrazole ring
1.184	0.050	0.869	Resonance effect of phenyl ring
-1.474	0.118		Constant
R=0.998	s=0.022	n=8	F=372 20

TABLE 6
Regression Model Developed for the Oxadiazoles Separated at 75% MeOH

Coefficient	Std. Error	Tolerance	Descriptor
1.862	0.322	0.574	(Nonpolar bonding constant)/1000
0.337	0.015	0.649	Resonance effect
-0.029	0.006	0.708	Field effect
1.691	0.140		Constant
R=0.999	s=0.004	n=5	F=268.11

In the correlation matrix of the descriptors used in the model, high correlation among nonpolar bonding constant, polarizability, and solubility parameter was observed. The evaluated model, keeping nonpolar bonding constant descriptor but eliminating polaizability and solubility parameters, gave a much better F-value.

In the model for carboxamides, resonance effect constant descriptors on phenyl ring and pyrazole ring are included, but field effect constant descriptors are excluded. It suggests that the polarization of charge density is influenced by both substituents of phenyl ring and pyrazole ring, because π electrons can transfer easily through amide bond.

In summary, each three-variable regression model for carboxamides and oxadiazoles showed a high degree of correlation between observed and

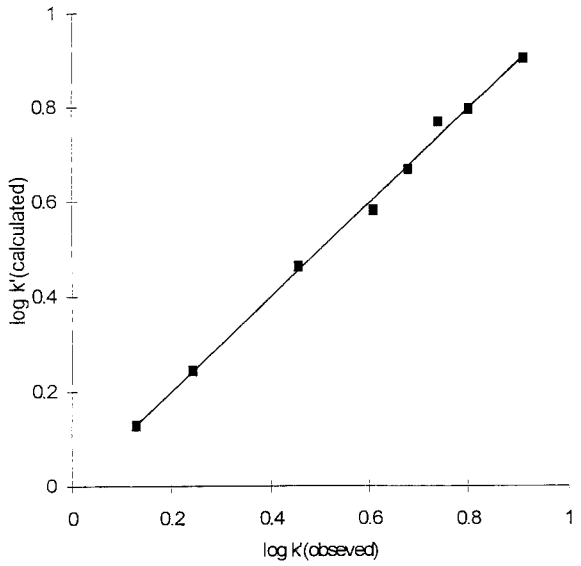


Figure 2. Plot of the Observed vs. Calculated Retention Data for the Carboxamides Separated at 55% MeOH

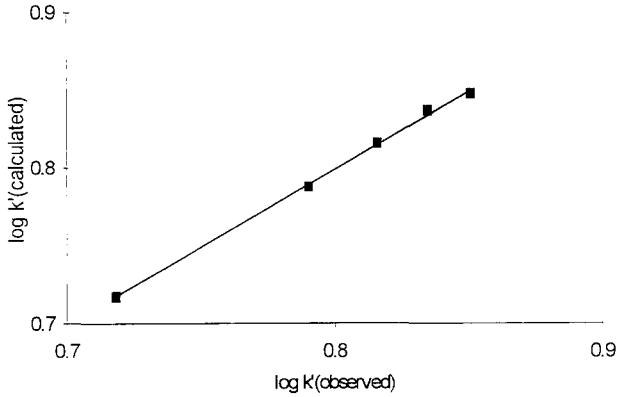


Figure 3. Plot of the Observed vs. Calculated Retention Data for the Oxadiazoles Separated at 75% MeOH

calculated values. This study showed that the models have a good predictive ability for application.

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PROCEDURE FOR LIQUID CHROMATOGRAPHIC DETERMINATION OF THIAMPHENICOL IN BOVINE SERUM AND MILK

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ABSTRACT

We describe a method for measuring thiamphenicol (TP) by high-performance liquid chromatography (HPLC). The assay involves a simple extraction procedure using Extrelut-3 with ethyl acetate, evaporation of the extract, and redissolution in 40% acetonitrile. Wakosil-II 5C18 HG is used as a stationary phase with an eluting solvent of acetonitrile/water (40/60 by vol). TP is eluted from the column in about 2.3 min and is well separated from the other components present in serum or milk. Absorption of the eluent at 224 nm is monitored and measured. After extraction from a 1-ml sample, the antibiotic can be analyzed within 30 min. The within-day recoveries for bovine serum and milk spiked with 1 ppm TP were 92.9% and 93.5%, respectively, with coefficients of variation of 3.18% and 3.21%, respectively. The between-day recoveries for the 1 ppm samples were 91.9% and 92.3%, respectively, with coefficients of variation of 4.50% and 4.40%, respectively. The method is suitable for rapid and specific analysis of the drug in serum and milk, and will be applicable to

other biological fluids, with a quantitative sensitivity of less than 0.1 ppm. We also analyzed TP by liquid chromatography-mass spectrometry with an atmospheric-pressure chemical-ionization interface system.

INTRODUCTION

Thiamphenicol (TP) has broad spectrum activity similar to that of chloramphenicol, without any serious side effect. Therefore, TP is an attractive drug for many veterinary applications.

Several assays have been reported for TP in biological fluids and tissue. These have been compiled, and include microbiological, colorimetric (8), and a gas-chromatographic (1,2,6,7) method both electron-capture and flame-ionization detection, and high-performance liquid chromatographic (HPLC) (3,4,5) methods. However, these methods have common disadvantages including a complicated extraction and clean-up method, and lengthy analysis time. In addition, there are no reports on the qualitative analysis of TP by LC-mass spectrometry.

We describe here a rapid, specific, and sensitive chromatographic procedure for analysis of TP. The method is suitable for routine analysis of TP in serum, milk and other biological fluids.

MATERIALS and METHODS

Reagents and apparatus

TP was obtained from Sigma Chemical Co., (St. Louis, MO 63178). Water used in the HPLC eluent was Milli-Q grade (Millipore). All other reagents used were of analytical grade.

Liquid chromatography was performed with a Model 501 pump equipped with a U6K universal liquid chromatography sample injector, and a multiple-wavelength detector, Model 481 (Waters Associates, Milford, MA 01756). A chromatopak C-R1B integrator (Shimadzu Seisaku Co., Ltd., Kyoto, Japan) was used as the recorder. The

chromatographic column used in the assay was a Wakosil-II 5C18 HG (15 cm x 4.6 mm I.D.; Wako Pure Chemical Co., Tokyo, Japan). The elution was carried out with acetonitrile-water (40:60, v/v) at a flow rate of 1.0 ml/min, and detection at 224 nm.

The LC-MS apparatus used was a Hitachi M-2008 mass spectrometer-computer system, equipped with a Hitachi M-8093 HPLC instrument through a Hitachi atmospheric-pressure chemical-ionization (APCI) interface system. The nebulizer and vaporizer temperatures were 260°C and 360°C, respectively.

Standard solutions and serum (milk) standards

A stock solution of TP was prepared in acetonitrile-water (40:60, v/v) at a concentration of 1 mg/ml. Serial dilution was performed to produce acetonitrile-water (40:60, v/v) spiking solutions ranging in concentration from 100 to 1 μg/ml. These solutions added to 1 ml of blank serum or milk produced final concentrations ranging from 0.1 to 10 μg/ml.

Extraction procedure

Biological samples (serum or milk, 1 ml) were diluted with 2 ml of distilled water and vortex-mixed. The total sample was applied to Extrelut-3 (Merck) and allowed completely enter the sorbent matrix. Next, a 25G x 1-needle (Terumo Co., Tokyo) was placed on the end of the Extrelut-3 and elution was performed with 10 ml of ethyl acetate. The organic phases were collected, evaporated under a stream of nitrogen at 40°C, and redissolved in 1 ml of mobile phase. A 20-μl volume was injected into the HPLC column. For quantitative analysis, the injection volume was increased to 50 μl. These all extraction procedures were completed within only 30 min.

RESULTS AND DISCUSSION

It was observed frequently that TP-containing serum or milk (pH 7.0) after deproteinization produced early interfering peaks on the chromatograms. To avoid this

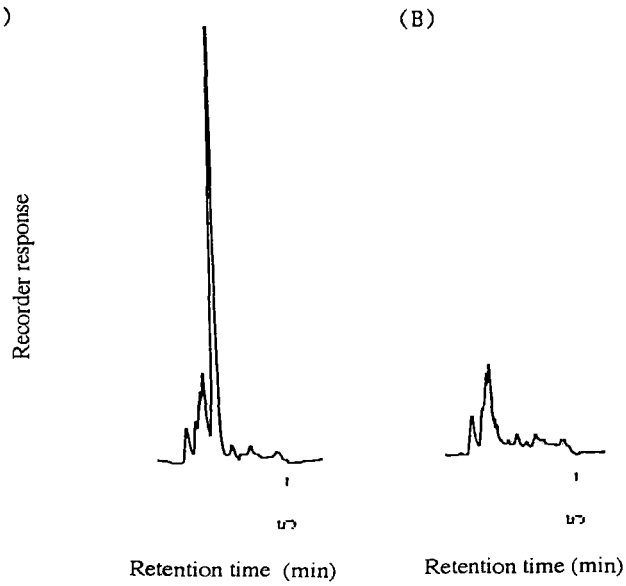

FIGURE 1. Typical chromatograms of (A) bovine serum spiked with 1.0 μg/ml thiamphenicol; and (B) bovine serum blank.

problem, we selected Extrelut-3 to extract TP from serum and milk. Extrelut-3 contains porous diatomaceous earth (Kieselguhr) and allows liquid-liquid extraction between the sample and organic solvent.

Typical chromatograms of serum blanks with and without TP supplementation are shown in Figure 1, and milk blanks with and without TP are shown in Figure 2. TP was identified by its retention time (2.3 min). Even though no internal standard was used in this method, there was a good relationship between peak heght and TP concentration. The limit of sensitivity of this method is about 0.1 μg/ml. Analytical recovery of TP added to serum and milk was determined by comparing the peak heights for serum and milk containing 1.0 μg of TP per ml with the results obtained from an

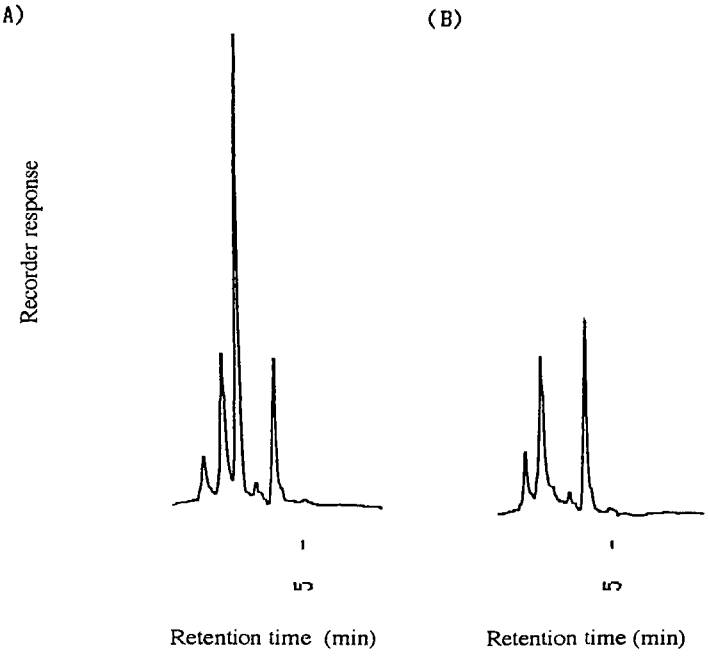

FIGURE 2. Typical chromatograms of (A) bovine milk spiked with 1.0 μg/ml thiamphenicol; and (B) bovine milk blank.

Table 1. Recovery rate of thiamphenicol in bovine serum and milk

Sample	Added (ppm)	Mean (%) (n=5)	
		Within-day	Between-day
Serum	10	93.9	-
	1.0	92.9	91.9
Milk	1.0	93.5	92.3

Table 2. Precision of assays for thiamphenicol in serum and milk

Sample	Concn (ppm)	Precision (n=5)			
		Within-day		Between-day	
		±SD (ppm)	CV (%)	±SD (ppm)	CV (%)
Serum	1.0	2.95	3.18	4.13	4.50
Milk	1.0	3.00	3.21	4.06	4.40

CV: Coefficient of variation.

aqueous standard of the same concentration. The within-day recoveries of serum and milk were 92.9% and 93.5%, respectively.

The precision of this method is summarized in Table 2. Precision for this assay was calculated for serum samples containing TP at concentrations of 1.0 and 10 μg/ml. Five samples at each concentration were extracted separately and chromatographed on the same day. The coefficient of variation (relative standard deviations) for TP at 1.0 μg/ml was 3.18%; at 10 μg/ml, the value was 2.77%. Between-day variations in recovery from serum samples containing 1.0 μg/ml was 4.5%. For five milk samples containing 1.0 μg/ml, the coefficients of within-day and between-day variation were 3.21% and 4.40%, respectively.

Our assay for TP is sufficiently sensitive for routine use. Its major advantages over other assays are its rapidity (assay can be performed in less than 30 min), accuracy, reproducibility and specificity. Preliminary studies suggest that it is highly specific for TP, and that tetracyclines such as oxytetracycline and chlortetracycline, sulfonamide, oxolinic acid and tylosin, do not interfere with the assay.

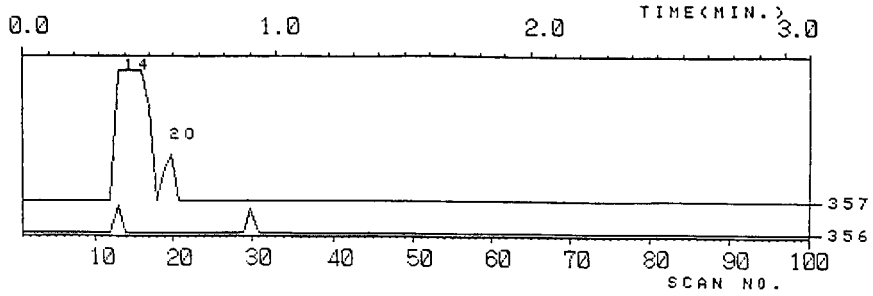

FIGURE 3. Mass chromatograms of thiamphenicol (m/z 356) and $[M+H]^+$ ions. The chromatographic conditions were: mobile phase, CH_3CN-H_2O (40:60, v/v); flow rate, 1.0 ml/min; injection volume 10 µl.

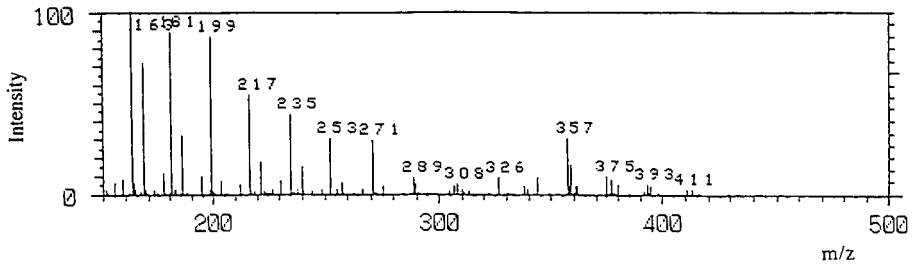

FIGURE 4. Mass spectra of thiamphenicol as scanned at the peak tops of the mass chromatograms in Figure 3. The mass spectrometer scanned from m/z 150 to 500.

Mass chromatograms and mass spectra of TP, obtained using the LC-APCI-MS system, are shown in Figure 3. In the LC-APCI-MS system, TP was determined by scanning the quasi-molecular ion $[M+H]^+$, and m/z 357 was specific for TP when selected ion monitoring was performed. The results suggest the possibility of qualitative analysis of other antibiotics and antibacterial agents. This is the first report of TP analysis using a LC-MS system.

In conclusion, this procedure appears to be potentially applicable for quantifying TP residue in stock farm products and the LC-APCI-MS method is applicable for qualitative analysis.

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REVERSED-PHASE LIQUID CHROMATOGRAPHIC ANALYSIS OF COENZYME Q10 AND STABILITY STUDY IN HUMAN PLASMA

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ABSTRACT

The objectives of this study were to design and validate a method for the assay of coenzyme Q10 (CoQ10) in human plasma and to evaluate the stability of CoQ10 in several conditions currently observed for routine determinations. CoQ10 was extracted from plasma with n-hexane after dissociation from lipoproteins with methanol. Ethoxy-CoQ10 was used as internal standard. CoQ10 was isolated and then quantitated by high performance liquid chromatography (HPLC) using a binary gradient. Linearity (r = 0.9999), recovery (97 %) and intra- and inter-run precision (1.7 and 2.0 % respectively) appeared to be satisfactory. The stability of CoQ10 in crude plasma was tested under various conditions (i.e. six hours at room temperature, freezing and thawing, storage for up to 24 weeks at - 20 °C), the stability of CoQ10 in n-hexane extracts was also tested (24 hours in autosampler rack at room temperature). CoQ10 content was found to be unaffected by any of the tested conditions.

INTRODUCTION

Coenzyme Q10 (CoQ10) is a lipid-soluble benzoquinone derivative having multiple functions within the cell (1), particularly as electron carrier within the respiratory chain of the mitochondria. This function has been proposed as the basis for energy transduction in heart cells (2). At physiological concentrations CoQ10 is also recognized as an effective lipid-soluble antioxidant (3). CoQ10 has been measured in plasma (6,7) and blood (8) by HPLC. In recent years increased

attention has been focused on the determination of plasma CoQ10 levels in humans. In particular, it has been demonstrated that low plasma CoQ10 levels were associated with an increased coronary risk factor in cardiac patients (4). Moreover a chronic treatment with statins, that are decreasing plasma cholesterol through inhibition of 3-hydroxy-3-methylglutaryl coenzyme A, has recently been shown as markedly decreasing plasma CoQ10 levels (5).

MATERIALS AND METHODS

Human plasma

Human plasma samples were obtained from two donors (Centre de tranfusion sanguine Dijon, France). One sample was used for method validation, the other sample being used for the CoQ10 stability study.

Chemicals and reagents

CoQ10 was obtained from Sigma. Ethoxy-CoQ10 was synthesized according to EDLUND (9) but purified using a flash chromatography technique instead of a preparative HPLC. Their chemical structures are shown in Figure 1. Bovine serum albumin was purchased from Sigma. All solvents were of HPLC grade: methanol, ethanol and isopropanol were supplied by Carlo Erba, n-hexane was obtained from SDS. Milli-Q™ grade water (Millipore) was used.

Chromatographic conditions

The chromatograph we used consisted of a 126 model binary HPLC pump (Beckman), a 460 autosampler (Kontron) and a 168 UV-VIS diode array detector (Beckman) operating at 275 nm. It was fitted with a Chromasil C_{18} column (150 x 4.6 mm, particle size 5 µm) maintained at 35 °C with a BAS LC 22A oven. Pump and detector control as well as UV signal acquisition and integration were

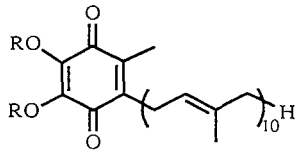

Figure 1. Structures of CoQ10 (R = CH_3) and Ethoxy-CoQ10 (R = C_2H_5).

performed by a Beckman Gold chromatography software running on a IBM PS/2 computer.

The solvents used for separation were A (methanol/isopropanol 95/5 v/v) and B (ethanol/isopropanol 95/5 v/v). The flow-rate was 1 ml/min with isocratic elution from 0 to 2 min (90% A and 10% B), linear gradient elution from 2 to 10 min (10 to 35 % B), isocratic elution from 10 to 20 min (35 % B) and return to initial conditions at 20 min for 5 min.

Plasma extraction procedure

Plasma sample (0.5 ml) was pipetted into a 10 ml amber glass centrifuge tube and then 2 µg of IS (100 µl of a 20 µg/ml solution in n-hexane), 2 ml of methanol and 5 ml of n-hexane were added. The tube was shaken horizontally at high speed for 10 min and centrifuged at 2500 x g for 5 min to separate the two phases. Most of the n-hexane layer was collected in a 10 ml conical glass tube. Five ml of n-hexane was then added to the first tube and the extraction was repeated. The n-hexane layers were combined and evaporated to dryness under nitrogen at 35 °C. The dry extract was re-dissolved in 50 µl of n-hexane and transferred into conical autosampler vials. 10 µl was injected into the column.

Calibration

Calibration standards were prepared by spiking 0.5 ml of a bovine serum albumin aqueous solution (40 g/l) with appropriate amounts of CoQ10 to obtain

0.25 - 0.50 - 1 and 2.5 μg/ml. The standards were processed as described above for plasma samples. Peak-height ratios following the IS method were used to quantify CoQ10 in the samples.

CoQ10 stability

CoQ10 stability in crude plasma

The plasma sample used for the stability study was analysed five times on day one and the mean value obtained was used as reference for all subsequent measurements in plasma, all carried out in quintuplicate. The same day (day one), CoQ10 stability was first assessed after a six hour storage period on the bench at room temperature. The influence of two consecutive freezing-thawing cycles on CoQ10 stability was also investigated on day one. Separate aliquots were frozen at - 20 °C and their CoQ10 content was measured at the end of the following storage periods at -20 °C: 4, 8, 12, 16, 20 and 24 weeks.

CoQ10 stability in n-hexane extracts

This was assessed on the plasma aliquots stored for 4 weeks at -20 °C. The n-hexane extracts were first injected immediately following extraction as described above. The same samples were then re-injected after a 24 hour storage period at room temperature in HPLC vials in the autosampler rack. In order to prevent the evaporation of n-hexane, the HPLC vials were recapped after the first injection. The reference sample for this experiment was that injected immediately after extraction.

For each condition tested (plasma and n-hexane extracts), the mean CoQ10 content was compared to the reference value and stability was considered acceptable if the relative variation did not exceed ± 15 %.

RESULTS AND DISCUSSION

Chromatograms

Figure 2 shows typical chromatograms of a 1 µg/ml standard extract (2A) and of plasma sample extract (2B). CoQ10 and IS eluted at 15 and 17 min respectively.

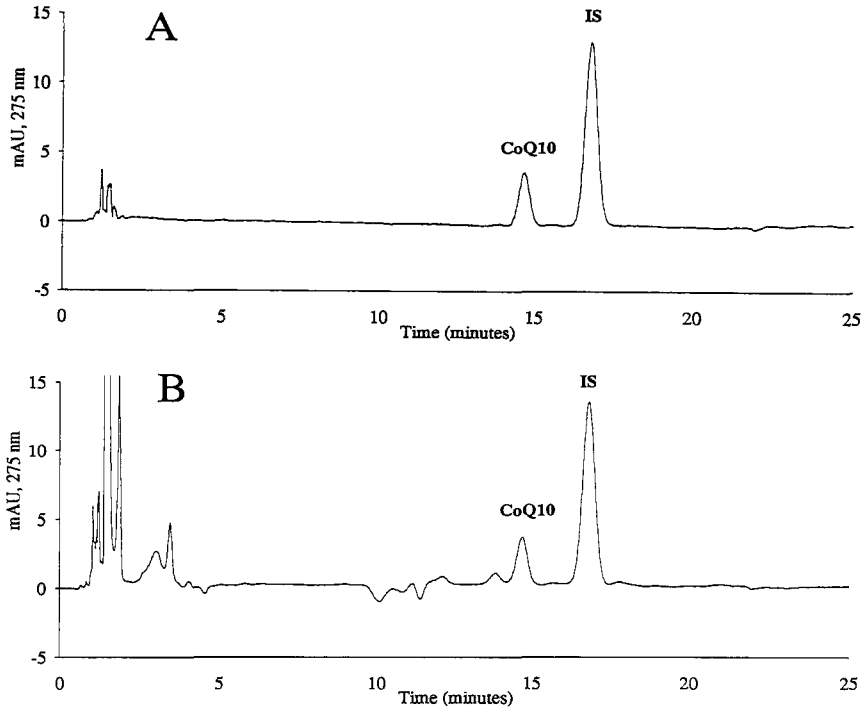

Figure 2. Chromatograms of 1 µg/ml standard extract (A) and plasma sample extract (B).
Conditions as described under Materials and Methods.

TABLE 1
Recovery of CoQ10 from Human Plasma

CoQ10 added µg/ml	Expected value µg/ml	Measured value µg/ml	Recovery %	n
0	-	0.99	-	6
0.25	1.24	1.21	97.2	2
0.50	1.49	1.45	97.0	2
1.00	1.99	1.95	97.7	2

TABLE 2
Intra and Inter-run Precision

Assay	Mean µg/ml	Standard deviation µg/ml	CV %	n
Intra-run	0.99	0.02	1.7	6
Inter-run	0.92	0.02	2.0	6

Method validation

Linearity

The method appears to be linear over the calibration concentration range (r = 0.9999).

Recovery

We evaluated the accuracy of the method by measuring the recovery of authentic CoQ10 added to human plasma samples (Table 1). Recoveries ranged from 97.0 % to 97.7 % (mean 97.3 %).

TABLE 3

CoQ10 Stability (all measurements n=5)

Testing conditions		Mean µg/ml	Standard deviation µg/ml	% relative variation
Plasma				
Reference		1.30	0.01	-
6 hours at room temperature		1.22	0.02	- 6.6
Freezing-thawing	one cycle	1.29	0.02	-1.0
	two cycles	1.40	0.06	+ 7.1
Storage -20 °C	4 weeks	1.37	0.09	+ 5.3
	8 weeks	1.32	0.04	+ 1.1
	12 weeks	1.33	0.04	+ 2.1
	6 weeks	1.34	0.04	+2.5
	20 weeks	1.28	0.04	- 1.9
	24 weeks	1.22	0.02	- 6.8
n-hexane extracts				
24 hours in autosampler vials *		1.42	0.08	+ 3.8

* reference value: plasma stored 4 weeks at -20 °C.

Precision

The coefficients of variation (CVs) observed for intra- and inter-run assays were ≤ 2.0 % (Table 2).

CoQ10 stability

The results for CoQ10 stability are shown in Table 3. Since relative variations ranged between - 6.8 and + 7.1 %, plasma CoQ10 content was considered unaffected under the studied conditions.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR BIOTINIDASE ACTIVITY IN THE HUMAN URINE

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ABSTRACT

To date, no detectable biotinidase activity has been reported in the human urine. In the present study, a simple and convenient HPLC-fluorimetric assay method was applied to biotinidase activity measurement of urine samples. Biotinyl-6-aminoquinoline was utilized as the enzyme substrate, and the liberated product (6-aminoquinoline) was monitored with a fluorimetric detector. Biotinidase activity was assessed in urine specimens from: twenty-five patients with various renal disorders associated with proteinuria, and forty age- and sex-matched healthy control subjects. The examined samples from the patients with

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renal diseases exhibited biotinidase activity with the exception of six of the patients (enzyme activity, median: 49.9 pmol · min^{-1} · ml^{-1} of urine; range: 0 – 2498 pmol · min^{-1} · ml^{-1}); specific activity, median: 19.4 pmol · min^{-1} · mg^{-1} of protein; range: 0 – 176 pmol · min^{-1} · mg^{-1}) while none of the urine specimens from the control subjects showed detectable enzyme activity. Thus, this method was shown to be a successfully applicable for the quantitative analysis of biotinidase activity in the human urine.

INTRODUCTION

Biotinidase (EC 3.5.1.12) is an amidase which mainly hydrolyzes biotinyl-amido compounds, such as biocytin [1], biotinyl-4-amino-benzoate [2], and biotinyl-6-aminoquinoline (BAQ)[3]. Although detectable biotinidase activity has been reported in most mammalian tissues and body fluids [4], the unequivocal demonstration of enzyme activity in human body fluids is hitherto limited to serum [5] and breast milk [6]. Although the molecular weight of the serum enzyme protein is known (M_r: 76 kDa) [1, 5], no information on its primary structure is to date available. Furthermore, to date there is no data present in the literature on the urinary excretion of this enzyme. Despite the detection of its high specific activity in the kidneys of several animal species, no biotinidase activity has previously been reported in the urine [4].

Recently, a HPLC-fluorimetric method for biotinidase assay, with BAQ as substrate, was developed by us [7] and applied to the enzyme determination in the cerebrospinal fluid [8]. Using a similar method the biotinidase activity in the urine of healthy subjects and patients with various renal diseases associated with proteinuria was investigated.

MATERIALS AND METHODS

Chemicals: BAQ was purchased from Sigma (St. Louis, MO, USA). 6-aminoquinoline (AQ) was obtained from Aldrich (Milwaukee, USA); 2-mercaptoethanol was from Wako (Osaka, Japan).

Urine specimens: A total of 65 subjects were involved in the study. Urine samples from 25 patients [10 male, 15 female, age (mean ± SD) 14.8 ± 4.8 years] with renal disorders (diagnoses are shown in Table I) and proteinuria in excess of 150 mg/24 h were collected. The patients were on a normal diet, presented no evidence for associated liver disease, and received no drugs known to interfere with biotin metabolism.

Urine samples from forty control subjects (17 male, 23 female, age: 15.1 ± 4.0 years) were also obtained. A 0.2 ml volume of fresh urine from each sample was stored at − 80 °C until the date of assay; before the assay, samples were thawed and filtered (Ekicrodisc 13, pore size: 0.2 μm, Gelman Sciences Japan, Ltd, Tokyo, Japan).

Biotinidase assay : Biotinidase activities were determined by a HPLC fluorimetric method [7] using BAQ as substrate. BAQ was dissolved at 44 μM (16.3 mg/l) in 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mM (452 mg/l) Na_4 EDTA and 10 mM (781 mg/l) 2-mercaptoethanol. Substrate containing 90 μl of the reaction buffer was mixed with 10 μl of enzyme solution. Thus, the reaction mixture (100 μl) contained 0.001468 mg (3.96 nmol) of BAQ, 0.04 mg of EDTA and 0.07 mg of 2-mercaptoethanol. The reaction proceeded for an appropriate time at 37 °C, and was stopped by adding 200 μl of methanol; the reaction mixture was diluted three-fold with methanol in order to precipitate the enzyme

TABLE I

Biotinidase activity in urine samples from patients with renal disease *

Sample no.	Diagnosis	Protein content	Activity	Specific activity
		$mg \cdot ml^{-1}$	$pmol \cdot min^{-1} \cdot ml^{-1}$	$pmol \cdot min^{-1} \cdot mg^{-1}$
1	HSPN	2.71	55.0	20.3
2	IgA-N	2.55	185	72.5
3	NS	3.33	269	80.8
4	SLE-MN	9.23	417	45.2
5	NS	20.1	463	23.0
6	IgA-N	4.21	333	79.1
7	MPGN	7.4	28.0	3.8
8	CRF	6.0	111	18.5
9	CNS	4.95	278	56.2
10	MPGN	12.6	17.0	1.4
11	HSPN	6.14	17.0	2.8
12	MN	10.8	259	23.9
13	FGS	10.8	57.0	5.3
14	FGS	31.4	2498	79.5
15	IgA-N	1.97	91.0	46.2
16	CRF	2.37	4.40	1.8
17	FGS	0.97	43.8	45.1
18	F-B2MG	1.56	44.8	28.7
19	CTA-I	1.48	261	176
20	CNS	13.6	n.d.	n.d.
21	ALP	2.53	n.d.	n.d.
22	CNS	1.45	n.d.	n.d.
23	IgA-N	1.45	n.d.	n.d.
24	CRF	0.72	n.d.	n.d.
25	IgA-N	2.89	n.d.	n.d.
Median		3.11	49.9	19.4
Range		0.97-31.4	0-2498	0-176

* Biotinidase activity was determined by measuring the hydrolysis rate of the enzyme substrate (BAQ).

n.d.: Not detectable

**

HSPN: Henoch-Schönlein purpura nephropathy; *IgA-N:* IgA nephropathy *NS:* nephrotic syndrome (idiopathic); *SLE-MN:* membranous lupus nephritis *MPGN:* membranoproliferative glomerulonephritis; *CRF:* chronic renal failure; *CNS:* congenital nephrotic syndrome; *MN:* membranous nephropathy; *FGS:* focal glomerulosclerosis; *F-B2MG:* familiar β-2 microglobulinuria; *CTA-I:* congenital tubular acidosis, type I; *ALP*: Alport syndrome.

proteins. After centrifugation and deproteinization, a portion (10 µl) of the clear supernatant was injected into the HPLC system. The product of AQ was measured at an excitation wavelength of 350 nm and an emission wavelength of 550 nm. AQ was assayed as previously described [7] with the exception of solvent A where a 0.1 M-sodium phosphate buffer (pH 2.1) was used instead of a 0.1 % aqueous TFA solution.

To assay the effect of urine volume on AQ liberation rate, each volume of urine (5, 10, 15, 20, 25, 30 µl) was mixed with 70 µl of substrate solution and a volume of distilled water (25, 20, 15, 10, 5, 0 µl) in order to maintain a final reaction volume of 100 µl (final substrate concentration : 28 µM). The mixtures were incubated at 37 °C for 1.5 h. Methanol (200µl) was added to stop the reaction and the mixtures were processed as described above.

For the time-course study on the liberation of AQ, incubation times of 1, 2, 3, 4, 5, and 6 h were used on one representative urine specimen (patient No. 2) and compared to boiled urine samples (tubes were immersed in a boiling water bath for 3 minutes). All the assays were run in duplicate and each run was repeated three times.

Biotinidase activity was calculated as follows: [AA = amount of AQ in 10 µl] (pmol) = [peak height (mm) of AQ in sample/peak height (mm) of 100 pmol AQ] x 100 (pmol). Enzyme activity ($pmol \cdot min^{-1} \cdot ml^{-1}$) = AA (pmol)x [0.3 (ml)/0.01 (ml)]/reaction time (min)/0.01 (ml) of urine. Specific activity was expressed as pmol AQ liberated $\cdot min^{-1} \cdot mg^{-1}$ of protein. If interference was detected, peak height of the blank sample (i.e. incubated without BAQ) was substracted from peak height of the sample incubated in the presence of BAQ prior to the calculation of enzyme activity as described above.

Protein content: Protein content was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.

RESULTS

A typical chromatogram from the patient's urine is shown in Fig.1, A. Interference was usually not detected in the chromatograms (Fig.1, B). Hydrolytic reaction product (AQ) appeared as a sharp peak (mean retention time: 1.6 min). The amount of AQ increased linearly with the volume of urine (5–30 μl) and the amount of urine added to the reaction mixture (Fig. 2). The hydrolysis of BAQ, as a function of time, by one urine sample (patient no. 2, Table I) is shown in Fig. 3. The hydrolytic reaction proceeded linearly for a minimum of 6 h while boiled urine did not hydrolyze the substrate at all. Mean intra- and inter-assay coefficients of variation were 1.2 % ($n = 6$) and 2.6% ($n = 6$), respectively.

The results of the application of the assay method to the urine samples of patients with renal disease are shown in Table I. Since the data showed a skewed distribution, the results are presented as median and range values. The patient samples exhibited biotinidase activity with the exception of six of the patients (median: 49.9 $pmol \cdot min^{-1} \cdot ml^{-1}$ of urine; range: 0 – 2498 $pmol \cdot min^{-1} \cdot ml^{-1}$). Median specific activity was 19.4 $pmol \cdot min^{-1} \cdot mg^{-1}$ of protein (range: 0 – 176 $pmol \cdot min^{-1} \cdot mg^{-1}$). On the other hand, none of the 40 control subjects urine specimens exhibited detectable BAQ-hydrolyzing activity, although incubation times of the reaction mixture were maintained for up to 18 h (data not shown).

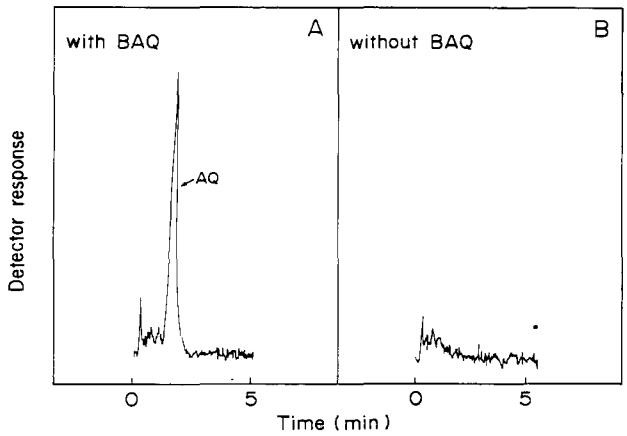

Fig. 1. Typical example of a chromatogram showing the hydrolysis of BAQ by one urine sample (patient no. 2, Table I). Panel A: urine, + BAQ; Panel B: urine, -BAQ (blank). Incubation time was 90 min. Conditions were as described in Materials and Methods.

DISCUSSION

A recently devised HPLC-fluorimetric assay, using BAQ as substrate, was applied to the determination of biotinidase activity in the urine. This assay method is relatively free from interference, due to prior separation by HPLC, and enables the detection of biotinidase activity in turbid specimens [6, 7]. The linear volume- and time- dependency of the hydrolytic reaction product (Figures 1 and 2) indicates that the urine from patients with proteinuria due to renal disease contained enzyme activity, i.e. BAQ hydrolase (biotinidase) activity.

In the patients with renal disease, median biotinidase activity was approximately 1/7 of the mean reported for human serum [5] and 2.6 times that reported for breast milk [6]. However, compared to the data

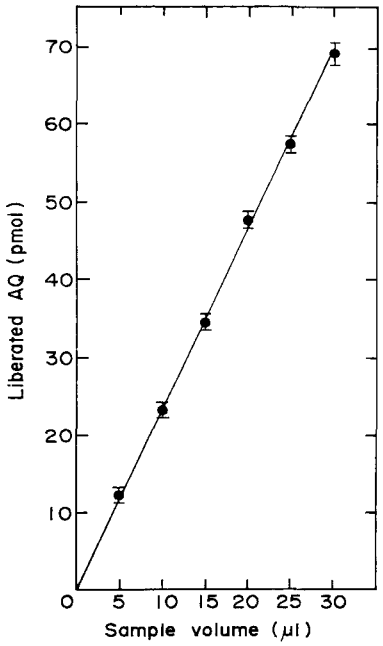

Fig. 2. Liberation of product (AQ), as a function of volume, for one urine sample (patient no. 2, Table I). Other conditions were as described in Materials and Methods. (Means ± SD; $n = 6$).

reported for serum and milk, enzyme activities in the urine were characterized by a wider dispersion. Moreover, despite the presence of proteinuria, biotinidase activity was not detected in six out of 25 (24 %) of the patient samples. Although the reason for the latter finding is not clear at this time, either considerably different etiologies of the renal disorders or varying degrees of glomerular/tubular damage may account for the reported skewness to the distribution. However, possible limits in the sensitivity of the assay or differences due to enzyme storage stability also need to be evaluated.

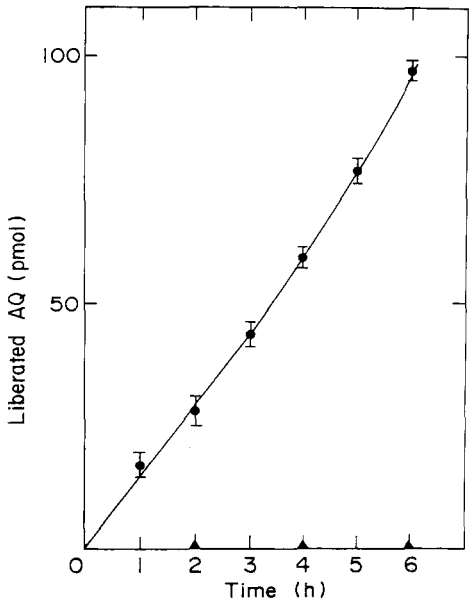

Fig. 3. Liberation of product (AQ), as a function of time, for one urine sample (patient no. 2, Table I). Liberated AQ per injection volume (10 µl) is indicated (circles: urine samples; triangles: boiled samples). Other conditions were as described in Materials and Methods . (Means ± SD; $n = 6$)

The actual origin of the biotinidase activity detectable in the urine of the majority (76%) of the urine samples from the renal patients remains to be established: although leakage of the serum enzyme protein through the glomerulus seems a plausible explanation, it remains to be tested.

Finally, our findings firstly indicates the urine from some patients with proteinuria as a novel potential source for purifying biotinidase.

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A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR QUANTIFICATION OF DIBOA, DIMBOA, AND MBOA FROM AQUEOUS EXTRACTS OF CORN AND WINTER CEREAL PLANTS

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ABSTRACT

A new HPLC method is described for the quantification of aglycones derived from cyclic hydroxamic acids: DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one), its degradation product MBOA (6-methoxybenzoxazolinone) and the demethoxy analogue DIBOA (2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one) in crude aqueous solutions from homogenized cereal tissues. After hydrolysis of ß-glycosides to aglycones, extracts of cereal samples were chromatographed on a PRP-1 column using a gradient of tris/citric acid and methanol/acetonitrile, and monitored at 288 nm. The three compounds were separated within 26 min. The minimum detection limit for all of them was 50 pmol. The recovery percentage was 100% for DIBOA, 87% for DIMBOA and 96% for MBOA. The levels of aglycones were determined in aqueous extracts from corn and winter cereal seedlings (20-100 mg), ranging from 3.80 to 11.50 mg DIBOA/g dry weight and 4.75 to 100.43 mg DIMBOA/g dry weight.

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INTRODUCTION

Hydroxamic acids have been associated with a broad range of functions in cereal plants, including disease and insect resistance, detoxification of herbicides, allelopathic effects, mineral metabolism and growth regulation (1,2).

Cyclic hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones) isolated from gramineous species are found as ß-glycosides (3). When plant tissues are damaged, ß-glycosides are enzymatically hydrolyzed to their corresponding aglycones (4). The main aglycone found in maize and wheat is DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-(4H)-one), while its demethoxy analogue, DIBOA, is mainly found in rye (3,5). The aglycones are converted to their corresponding benzoxazolinones MBOA and BOA when heated in aqueous solutions (6,7).

The naturally occurring glycosides showed little activity against insects (8), but the aglycones DIMBOA and DIBOA are known to mediate in the resistance of cereals to several insect pests (9). Furthermore, it has been suggested that these compounds could be used as a criterion of selection in cereal breeding programs to increase their pest and disease resistance, (10). Consequently, in order to screen large amounts of plant samples, a fast and accurate method to quantify these compounds is needed.

Several methods for qualitative and quantitative analysis of hydroxamic acids have been developed. Colorimetric methods based on quantification of a $FeCl_3$ complex are unspecific because they do not differentiate between the various hydroxamic acids species present in

an extract (11). More specific methods such as isotopic dilution (12), spectrofluorimetry (13), gas liquid chromatography (GLC) (14), high performance liquid chromatography (HPLC) (15) and thin layer chromatography (TLC) (16) measure hydroxamic acid concentrations after their conversion to benzoxazolinones, but the yields are not quantitative because this is not a stoichiometric reaction (17).

The quantitative GLC (18) and HPLC (19,20 and 21) methods previously reported are time consuming since they require complex sample extraction procedures and they are therefore not suitable to screen large amount of samples in breeding programs. On the other hand, another sample preparation methods (22,23) are fast and easy, but the HPLC quantification reported by these authors does not provide for the determination of the MBOA, the degradation product of DIMBOA, and the result is an underestimation of the total amount of DIMBOA.

Here we report on a simple and rapid quantitative extraction procedure and a sensitive HPLC analysis to quantify the most biologically active aglycones, DIMBOA and DIBOA, and the benzoxazolinone, MBOA, from corn and winter cereal aqueous extracts.

MATERIALS AND METHODS

Preparation of Standards

DIMBOA was isolated from 300 g of etiolated corn shoots of the hybrid EA2173 X Mo17 using Klun et al.'s method (24), MBOA was prepared by the Klun and Brindley method (12) using 500 g of fresh material. The identity of these compounds was confirmed by ultraviolet

and infrared spectra, as reported by Gutiérrez et al. (19). DIBOA was isolated from 800 g of etiolated rye seedlings (Cv. Petkus) by the Virtanen and Hietala method (8). The UV spectrum in absolute ethanol and the IR spectrum in a KBr pellet recorded in a Shimadzu 160 UV-VIS spectrophotometer and Perkin-Elmer IR 1420 spectrophotometer respectively, were in agreement with that obtained by Tipton et al. (25).

Sample Preparation

Seeds of *Zea mays* (Pioneer Hybrid 3138), *Triticum aestivum* (Cv. Chinese spring), *Triticum durum* (Cv. Camacho), *Secale cereale* (Cv. Elbon), *Triticosecale* (Cv. Cachirulo) and *Hordeum vulgare* (Cv. Logra) were grown in 5x5 cm pots (one seed/pot) containing a potting soil mixture ("Floragard", Germany) at 22±1°C, 75±5% HR, 204 $\mu Em^{-2}\ s^{-1}$ of photon flux and 16 h light-8h dark photoperiod. Seedlings were harvested at the G.S. 11 (26). Plant tissues (20-100 mg) were homogenized with distilled water (4x0.04 mL/mg) in a potter homogenizer. Aqueous extracts were incubated for 15 min at 25°C, to hydrolyze the aglycones from glycosides (27). Sample aliquots (1 mL) were centrifugated at 12500 rpm for 10 min, and filtered through 0,22 µm cellulose membrane filters (Millipore).

HPLC Conditions

Samples were quantitatively analyzed on a Beckman System Gold equipped with a gradient controller Model 126, diode array detector Model 168 and an auto-sampler Model 502, with a 20 µL sample loop. A

250x4.1 mm PRP-1 column (10µm, polystyrene-divinylbenzene, Hamilton) protected by a 25x2.3 mm PRP-1 guard was used. Elution was carried out with a linear gradient of solvents A (49 mM tris, 16 mM citric acid, pH= 7.0) and B (methanol 80%-acetonitrile 20%): 8-80%B from 0 to 30 min, 80-8%B from 30 to 31 min. Sample injections were at 40 min intervals to allow equilibration of the column. The flow rate was 1 mL/min. Detection was at 288 nm and 0.025 AUFS, and the scanning range was from 220 to 400 nm. Calibration curves were obtained by injecting 20 µL of standard solution mixtures with seven concentration levels of DIBOA, DIMBOA and MBOA, ranging from 0.05 to 1.0 nM. Two determinations were performed for each concentration level.

Identification and Quantitation of DIBOA, DIMBOA and MBOA in Aqueous Extracts

The identity of benzoxazinones and benzoxazolinones in the aqueous extracts was determined by co-chromatography with the appropriate standards, and by comparing the UV spectrum of unknown peaks and the standard peaks. Quantification was made by referring to the molar responses of standards from standard curves.

Percent Recovery

Five replicates with different quantities for each standard were added to 1mL of homogenated tissue from *T. aestivum* (Cv. Chinese Spring) prior to incubation, and processed as previously described. The recovery of DIBOA, DIMBOA and MBOA was calculated after HPLC separation.

RESULTS AND DISCUSSION

Method Development

The HPLC analysis was carried out on a poly(Styrene-divinylbenzene)resin because this stationary phase is more stable at any pH that the silica-based packing materials (28). Figure 1 shows the separation of a standard mixture containing DIBOA, DIMBOA and MBOA with retention times of 15.10, 17.46 and 25.07 min, respectively Retention times were highly reproducible with standard errors lower than 0.02 min in 10 replicated runs injected at 40 min intervals. Diode array detection was employed since it allows for the identification of the peaks by comparing of their retention times and UV spectrum with those of standards, and also permits peak purity to be verified.

Peaks I, II, III were enhanced when DIBOA, DIMBOA and MBOA standards respectively, were added to the sample. On this basis, peak I was identified as DIBOA, peak II as DIMBOA and Peak III as MBOA. Due to the differences in the UV absorption spectra of the three compounds, a wavelength of 288 nm was chosen to allow their joint determination with the best quantitative results.

Seven different levels of each standard were analyzed to obtain the calibration data. The correlation coefficients (r) for DIBOA, DIMBOA and MBOA were r=0.998, r=0.993 and r=0.993 respectively, indicating a good linearity in the calibration curves. The minimum detection limit for these compounds was 50 pmol which improves the sensibility of the methods previously reported by Gutiérrez et al. (19), Lyons et al. (20) and Xie et al. (21). The recovery of DIBOA, DIMBOA and MBOA was

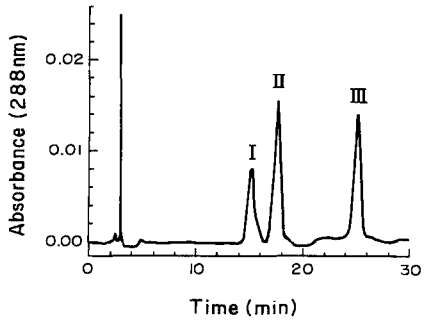

FIGURE 1. HPLC chromatogram of cyclic hydroxamic acids standards separated on a PRP-1 Column: I, DIBOA; II, DIMBOA; III, MBOA.

estimated to test the accuracy of the method. In order to avoid the differential distribution of hydroxamic acids in different tissues (27,29), five replicates of known amounts of DIBOA, DIMBOA and MBOA standards were added to 1 mL of homogenated seedlings of *T. aestivum* (Cv. Chinese Spring). Figure 2 shows the results obtained. The Y intersect values are the amounts of DIBOA, DIMBOA and MBOA present in the sample prior to the addition of standards. The percentages of recovery obtained by the slope of each regression were 100% for DIBOA, 87% for DIMBOA, and 96% for MBOA. These results point out the accuracy of this method in the quantification of DIBOA, DIMBOA and MBOA in plant extracts, and represent an improvement in respect to the previously reported DIMBOA recovery (20).

Sample Preparation

The hydroxamic acids naturally occur as glycosides, but in this work we quantified their aglycones for on the following reasons:

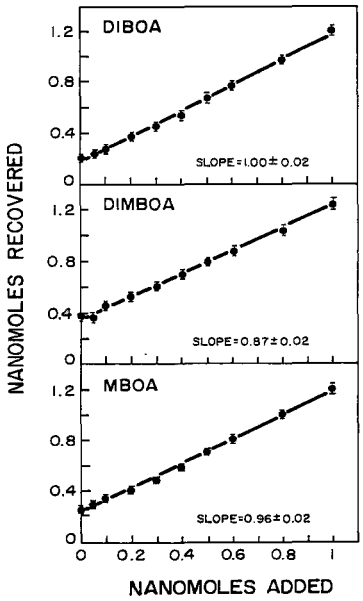

FIGURE 2. Recovery of DIBOA, DIMBOA and MBOA added to an aqueous extract from *T. aestivum* (Cv Chinese Spring). The slope of each regression show the amount of compound recovered. Values are means ± s.e. (5 replicates). Intersection represents concentrations obtained in vivo.

a) The isolation of the glycosides involves the inactivation of the hydrolytic enzymes present in the plant tissues before extraction since in damaged plant tissues a ß-glycosidase converts them into aglycons. Therefore the sample preparation for HPLC quantification of the glycosides is time consuming (20), and consequently it is not suitable for screening large amounts of samples in cereal breeding programs.

b) Corcuera et al. (8) showed that natural levels of glycosides in cereals were less active against insects than their corresponding

aglycones. Additionally, the incubation for 15 min at 25° C of aqueous cereal extracts hydrolyses their glycosides to aglycones (27) and the aqueous extraction of these compounds significantly reduces the sample preparation time when compared to their extraction with organic solvents as previously reported (19,20 and 21). Therefore we propose the quantification of these aglycones as a more suitable method to estimate the host-plant resistance.

Applications

To analyze the suitability of this method to screen samples in breeding programs, several species of cereal seedlings were processed under the conditions described above (see materials and methods).

The chromatograms obtained from the analysis of foliar corn seedling extracts and whole seedling extracts from other cereals are shown in Figure 3. DIBOA, DIMBOA and MBOA were detected in all cereals except barley. The presence of MBOA in the samples indicates that decomposition of DIMBOA took place during the extraction, pointing out the importance of MBOA identification in the samples to give an accurate quantification of DIMBOA. This identification is less sensible when samples are analyzed according to Niemeyer et al. (22) and Xie et al., (21); because these authors detected the compounds at 263 nm and 265 nm respectively, and under such conditions MBOA has a minimum of absorbance.

The DIMBOA level decreased when aqueous homogenates from *T. aestivum* (Cv. chinese Spring) were heated at 70°C. 45% DIMBOA was

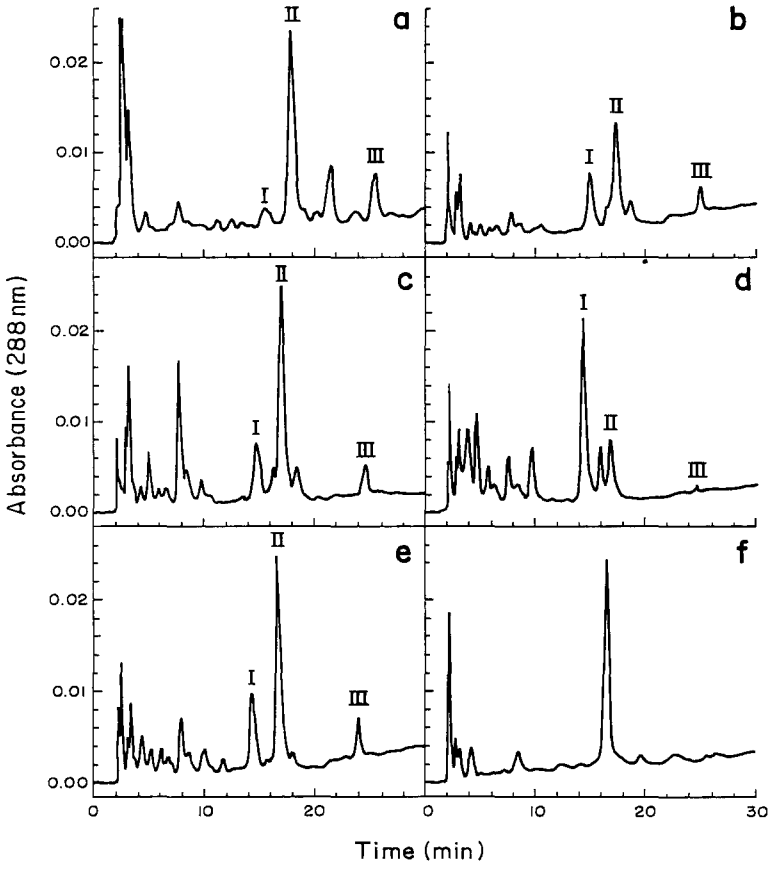

FIGURE 3. HPLC chromatograms of *Zea mays* leaf extracts (Pioneer hybrid 3138) (a) and seedlings from *Triticum aestivum* (Cv Chinese Spring) (b), *T. durum* (Cv Camacho) (c), *Secale cereale* (Cv Elbon) (d), *Triticosecale* (Cv Cachirulo) (e) and *Hordeum vulgare* (Cv Logra) (f); I, DIBOA; II, DIMBOA; III, MBOA.

degraded after 5 min and 65% after 10 min. Only 3% DIBOA was degraded when heated at 70°C for 10 min. Since DIBOA is more stable than DIMBOA (20), and no degradation to BOA was detected in our extraction conditions, we consider that its quantification should be unnecessary.

Table 1 shows our results from the quantification of DIMBOA and DIBOA in aqueous cereal extracts. The DIMBOA concentration was in most cases higher than DIBOA, and ranged from 4.75 to 100.43 mg/g dry weight, except for rye where the inverse situation was found. None of these aglycones were found in barley seedlings.

Under our experimental conditions, we have also found that the molar concentrations of DIBOA, DIMBOA and MBOA in standard methanolic solutions and cereal seedling aqueous extracts remained stable after several months of storage at -20°C.

We have described here a method for the quantification of the most biologically active hydroxamic acids: DIMBOA and DIBOA and the benzoxazolinone MBOA. This method improves other ones previously developed, since it provides a very simple and fast sample preparation procedure avoiding extraction with organic solvents. Additionally, the HPLC conditions described here give a good resolution of the sample components and a sensitive and accurate quantification of these compounds in aqueous extracts from winter cereals and grasses, which have lower concentrations of these chemical than corn.

Additionally, the great stability of these compounds in samples stored at -20°C will allow the harvesting, preparation and storage of

TABLE 1.

Levels of DIMBOA and DIBOA in Cereal Seedlings.

Species	Hydroxamic acids (mg/g dry wt.) *	
	DIBOA	DIMBOA
Zea mays (Hybrid Pioneer 3138)	10.70 ± 0.34	100.43 ± 1.83
Triticum aestivum (Cv Chinese spring)	3.80 ± 0.45	12.63 ± 0.10
Triticum durum (Cv Camacho)	4.86 ± 0.18	24.47 ± 0.34
Secale cereale (Cv Elbon)	11.50 ± 0.28	4.75 ± 0.19
Triticosecale (Cv Cachirulo)	4.92 ± 0.24	19.57 ± 0.51
Hordeum vulgare (Cv Logra)	0.00	0.00

* Means and s.e. (n=5) are represented.

large amounts of plant material and aqueous extracts prior to their HPLC analysis. Therefore, this method is suitable for screening samples in plant breeding programs dealing with pests and disease resistance that involve the analysis of a large number of genotypes.

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DETERMINATION OF DICAMBA BY REVERSE-PHASE HPLC

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ABSTRACT

Reverse-phase HPLC methodology was developed for resolution and quantitative analysis of dicamba (3,6-dichloro-2-methoxybenzoic acid) with a C-18 column and acetonitrile-phosphate buffer as the mobile phase under isocratic conditions. This solvent system was also suitable for mixtures containing dicamba, 2,4-dichlorophenoxyacetic acid (2,4-D), and 2-(2-methyl-4-chlorophenoxy)propionic acid (MCPP) and allowed the resolution of these herbicides with the respective retention times of 4.5, 7.1, and 9.5 min. UV-spectroscopy was unsuitable for the resolution of mixtures of dicamba, 2,4-D, and MCPP due to overlapping absorption spectra.

INTRODUCTION

Dicamba (3,6-dichloro-2-methoxybenzoic acid) is a commonly used pre- and post-emergence herbicide with major application in the lawn care industry. Dicamba is readily water-soluble and residues are relatively mobile in the

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environment. The degradation of this herbicide in soil is believed to be primarily mediated by microorganisms (1). Dicamba has been resolved and quantified by UV-spectroscopy, GC, and HPLC. TLC methodology is also available (2). Confirmation of dicamba by capillary SFC/MS and HPLC/MS techniques has been reported (3, 4). While UV-spectroscopy offers a quick and simple method for quantifying dicamba, its specificity is subject to interference from breakdown products. Traditionally, dicamba has been analyzed by GC (5-7). However, this approach is limited due to potential problems of thermal destruction associated with GC analysis. HPLC methodology alleviates this potential problem of thermal alteration or destruction of test compounds. Normal-phase HPLC has been used to resolve dicamba because the compound is relatively polar and does not generally adsorb strongly to a reverse-phase support (8). With tetrabutylammonium phosphate as an ion pair reagent, good resolution of dicamba has been achieved on a C-18 reverse-phase column (9, 10). An HPLC system utilizing a C-18 column and a gradient mobile phase was successfully used by Kim et al. (11) for dicamba and several phenoxyalkanoic herbicides. Previous studies have shown variable success with reverse-phase HPLC for dicamba and other herbicides depending on the solvent system (12).

In the present work, an analytical method based on isocratic HPLC was developed for dicamba that could also resolve it in mixture with the phenoxy-herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-(2-methyl-4-chlorophenoxy)propionic acid (MCPP).

EXPERIMENTAL

Materials and Reagents

Analytical-grade (>99.9% purity) and technical-grade (86.8% purity) dicamba were obtained courtesy of Sandoz Crop Protection (Des Plaines, IL). Analytical-grade 2,4-D (98.7% purity) and MCPP (98.0% purity) were obtained from Dow Chemical Company (Midland, MI). HPLC-grade distilled water and chemicals were used in the preparation of analytical standards and mobile phase.

Demineralized double distilled water was used for preparing the dicamba standard solutions for UV-spectroscopy.

HPLC Methodology

The HPLC system was composed of an Altex model 100A solvent delivery pump (Berkeley, CA) and a Hitachi 100-40 UV-detector (Danbury, CT) fitted with an Altex spectrophotometer flow cell set at 229 nm (range, 0.2; time constant, 0.3). The retention data and peak areas were recorded and analyzed by a Hewlett-Packard H3396 A integrator (Avondale, PA) which had the following settings: attenuation, 32; peak width, 0.2; threshold, 1.0; and area rejection 100. The HPLC column used was a commercially available Phenomenex ODS (4.6 mm x 150 mm) with Spherisorb packing consisting of a 5.0 μm pore size (Torrance, CA). The mobile phase was 40% acetonitrile-60% phosphate buffer (6.0 g K_2HPO_4 and 3.0 ml conc. H_3PO_4 l^{-1}, pH 3.0) (13). Stock solutions for HPLC analysis were prepared in 0.05 M NaOH and the working standards were diluted in 25 ml of 0.5 M NaOH containing 4.0 ml glacial acetic acid. All standards were filtered through a 0.45 μm Acrodisc LC25 filter (Gelman Sciences, Ann Arbor, MI) before injection (100 μl) into HPLC. Analyses were carried out under isocratic conditions at a flow rate of 1 ml min^{-1} and chart speed of 0.5 cm min^{-1}.

UV-Spectroscopy

The characteristic wavelengths of maximum absorption and the concentrations of dicamba, MCPP, and 2,4-D in aqueous solutions were determined by UV-spectroscopy (Varian 2200, Palo Alto, CA). Standard stock solutions of MCPP and 2,4-D were prepared in 0.05 M NaOH.

RESULTS AND DISCUSSION

The analytical-grade and technical-grade dicamba had a maximum absorbance at 274 nm and 276.5 nm, respectively (Figure 1). Standard curves for dicamba

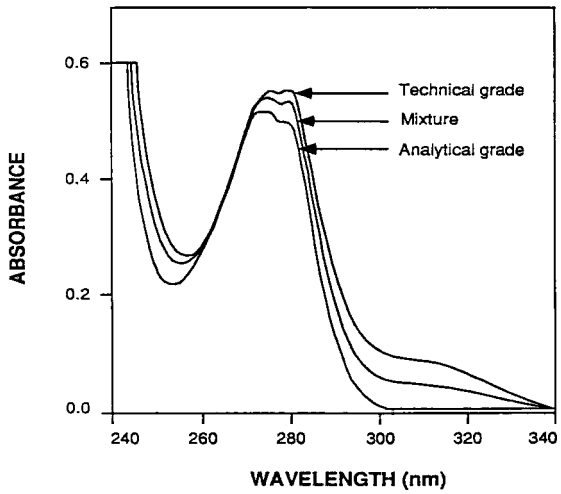

FIGURE 1. UV-spectral scans of standard aqueous solutions containing 250 mg l^{-1} of analytical-grade or technical-grade dicamba, and a mixture of 125 mg l^{-1} of both analytical-grade and technical-grade dicamba.

at these wavelengths displayed linearity between 1 and 1000 mg l^{-1}. The linear regression equations describing the standard curves for the technical- and analytical-grade dicamba were $y = 0.00239\ x + 0.0484$ ($r = 0.995$) and $y = 0.00242\ x + 0.017$ ($r = 0.998$), respectively ($y = A_{274}$, x = mg l^{-1}). Upon storage at 4°C and -20°C for four weeks, the losses of dicamba were 1% and 30%, respectively. Less than 0.1% dicamba was lost upon autoclaving at 121°C for 15 min.

MCPP and 2,4-D had peaks of maximum absorption at 279 and 283 nm, respectively (Figure 2A). UV-spectroscopic analysis of a mixture of dicamba, MCPP, and 2,4-D produced a broad peak in the 280 nm range in which the peaks overlapped (Figure 2B), and peaks of maximum absorption at either 274, 279, or 283 nm could not be discerned. Several other aromatic herbicides have wavelengths of maximum absorption in the same range which further limits the use of UV-spectroscopy in multiherbicide residue analysis.

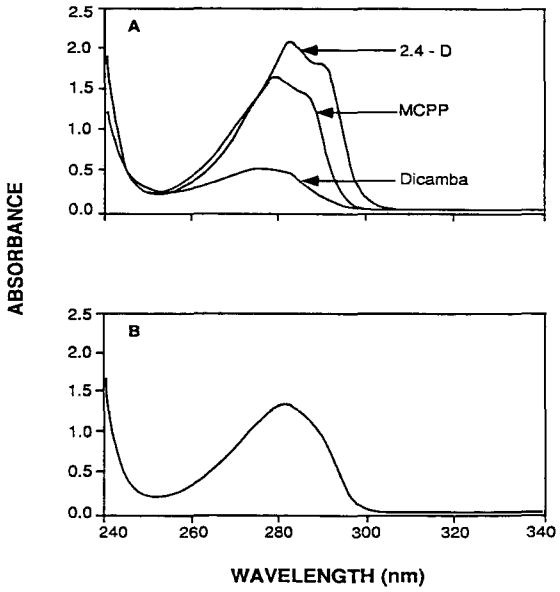

FIGURE 2. UV-spectral scans of standard aqueous solutions containing herbicides as follows. **A**, 250 mg l^{-1} of analytical-grade dicamba, 2,4-D, or MCPP; **B**, a mixture of analytical-grade dicamba, 2,4-D, and MCPP (83.3 mg l^{-1} each).

Reverse-phase HPLC, with a C-18 column and 40:60 acetonitrile-phosphate buffer as the mobile phase (flow rate 1 ml min^{-1}), was able to resolve analytical-grade dicamba at concentrations < 1 mg l^{-1}, with a single peak eluting at 4.5 min (Figure 3). This resolution under isocratic conditions compares favorably with results reported by Kim et al. (11) using a gradient mobile phase (Table 1). Thus, the data demonstrate that both isocratic and gradient analyses yield comparable results. This isocratic mobile phase has been previously used for resolution of 2,4-D and MCPP (13). The peak of technical-grade dicamba had identical retention time of 4.5 min, but a minor peak also eluted at 6.6 min. The identity of this impurity is unknown at this time. Standard curves based on

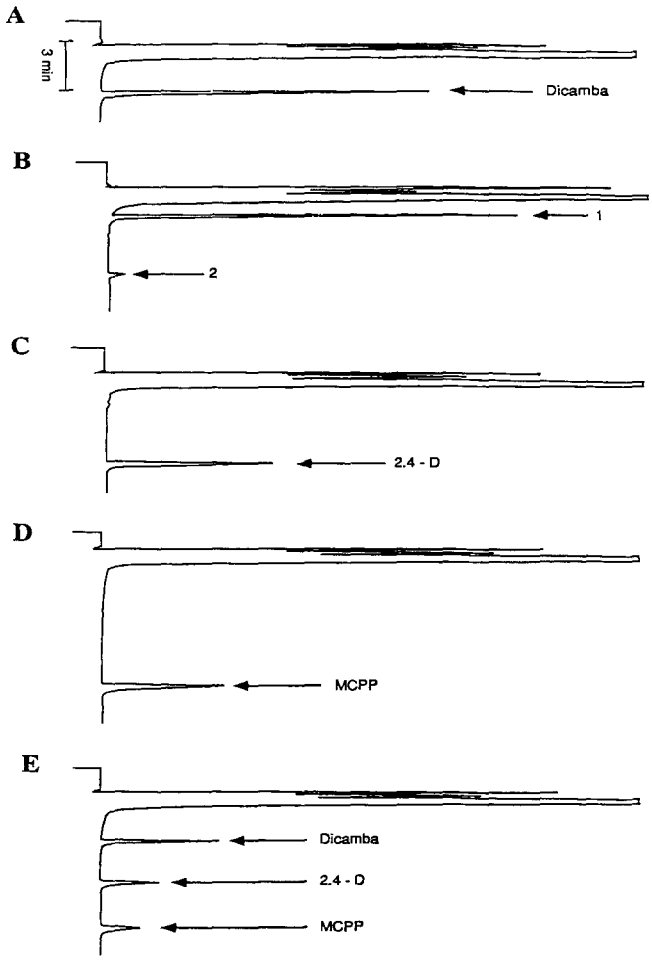

FIGURE 3. Reverse-phase HPLC chromatograms of (**A**) 10 mg l^{-1} of analytical-grade dicamba; (**B**) 10 mg l^{-1} of technical-grade dicamba; (**C**) 10 mg l^{-1} of 2,4-D; (**D**) 10 mg l^{-1} of MCPP; (**E**) a mixture of analytical-grade dicamba, 2,4-D, and MCPP (3.3 mg l^{-1} each). The retention times were 4.5 min for dicamba, 7.1 min for 2,4-D, and 9.5 min for MCPP.

TABLE 1. Comparison of HPLC retention times.

Analyte	Retention time (min)	
	Present work Isocratic; 40:60 Acetonitrile-phosphate buffer 15 cm column, 4.6 mm i.d.	Kim et al. (11) Gradient; Water:acetonitrile:acetic acid 10 cm column, 2.1 mm i.d.
Dicamba	4.5	4.5
2,4-D	7.1	7.4
MCPP	9.5	9.6

integrated peak areas displayed linearity at least up to 70 mg dicamba l^{-1}. The linear regression equations describing the standard curves for the technical- and analytical-grade dicamba were $y = 0.889 x + 1.736$ ($r = 0.997$) and $y = 1.29 x - 2.05$ ($r = 0.995$), respectively ($y = A_{229}$, x = mg l^{-1}).

A mixture of herbicides containing 3.3 mg each of dicamba, 2,4-D, and MCPP l^{-1} was successfully resolved by HPLC analysis using the same conditions as for dicamba alone (Figure 3). The retention times were 4.5, 7.1, and 9.5 min for dicamba, 2,4-D, and MCPP, respectively.

The HPLC system could thus be used to determine dicamba at concentrations of <1 mg l^{-1}. The resolution of dicamba from 2,4-D and MCPP at low concentrations was also accomplished using a acetonitrile-phosphate buffer solvent system that was compatible for all three compounds. Although dicamba, 2,4-D, and MCPP each have characteristic wavelengths of maximum absorption, UV-spectroscopy was unsuitable for the resolution of dicamba, 2,4-D, and MCPP in a mixture due to their overlapping absorption spectra.

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HPLC SEPARATION OF SELECTED CARDIOVASCULAR AGENTS ON UNDERIVATIZED SILICA USING AN AQUEOUS ORGANIC MOBILE PHASE

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ABSTRACT

High performance chromatographic separations of selected cardiovascular agents (propranolol, atenolol, metoprolol, verapamil, diltiazem, nifedipine, clonidine and prazosin) on underivatized silica using aqueous phosphate buffer - acetonitrile mobile phases were studied. Mobile phases differing in organic modifier concentration, ionic strength and buffer pH were prepared and tested for chromatographic separation of selected mixtures of the analytes grouped according to pharmacological activity. The best separations of all analytes were obtained using a mobile phase of 60:40 v/v aqueous pH 3 phosphate buffer - acetonitrile at a 1.0 mL/min flow rate. Substitution of methanol for acetonitrile gave increased retention of the analytes with some reduction in column efficiency measured as plate counts. Although ion-pairing appears to be the primary interactive force between the silica column and the analytes, other forces such as hydrogen bonding and hydrophobic interactions are also involved in the separation.

INTRODUCTION

Reversed-phase high performance liquid chromatography (RP-HPLC) on bonded stationary phases has developed into a major analytical tool for separation and quantitation of most pharmaceuticals. However, reports have appeared in the scientific literature describing the excellent separation of basic (1-8), acidic (9,10), and neutral drugs (11-13) on underivatized silica using aqueous organic mobile phases. These systems showed clear improvement in peak shape, plate counts and efficiency as compared to conventional bonded phase chromatography. The predominant retention mechanism for basic compounds was found to be cation exchange with silanol groups on the silica surface. A mixed retention mechanism of hydrogen bonding and quasi-reversed-phase retention was reported for acidic drugs. Neutral drugs were determined to be retained on silica by hydrogen bonding or other non-specific forces.

This study was designed to investigate the applicability of underivatized silica to the analysis of cardiovascular agents. Model compounds were selected from three classes, including β-adrenergic blockers, calcium channel blockers, and α-adrenergic blockers, which are medically important and widely used. Chromatographic separations for these cardiovascular drugs were developed on underivatized silica using an aqueous buffer- acetonitrile mobile phase.

EXPERIMENTAL

Reagents and Chemicals

The structural formulae of the compounds studied are shown in Fig 1. Propranolol HCl was a gift from Ayerst Laboratories Inc. (New York, New York, U.S.A.). Atenolol was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Metoprolol was a gift from CIBA-Geigy Pharmaceutical Company (Summit, NJ, U.S.A.). Nifedipine and Prazosin HCl were obtained from Pfizer Inc. (Brooklyn, NY, U.S.A.). Verapamil HCl was a gift from Knoll Pharmaceutical Company (Whippany, NJ, U.S.A.). Diltiazem HCl and Clonidine HCl were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acetonitrile and water were HPLC grade (J.T. Baker, Phillipsburg, NJ, U.S.A.) Monobasic sodium phosphate, sodium hydroxide and concentrated phosphoric acid were also obtained from Baker.

Instrumentation

Chromatography was performed on an HPLC system consisting of a Beckman Model 110B HPLC pump (Fullerton, CA, U.S.A.), a Rheodyne Model 7125 injector equipped with a 50-μL loop (Cotati, CA, U.S.A.), and a Micromeritics Model 787 variable UV/VIS detector (Norcross, GA, U.S.A.). The analytical wavelength was set at 254 nm. Data acquisition and reduction were performed on a Hewlett Packard Model HP-3395 integrator (Palo Alto, CA, U.S.A.) Separations were accomplished on a

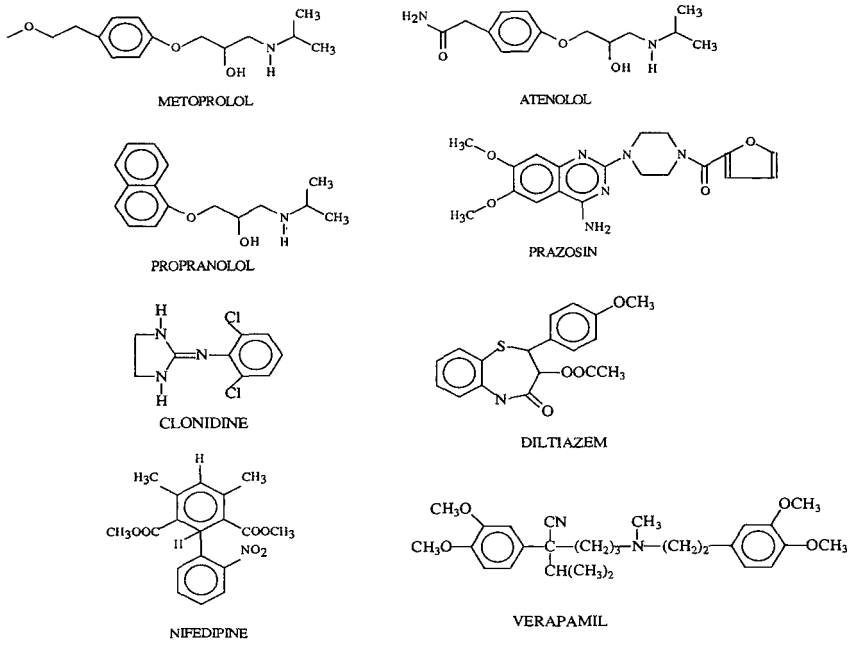

Figure 1 Chemical Structures of Selected Cardiovascular Agents

3-μm silica column (25 cm x 4.6 mm i.d., Phenomenex, Torrance, CA U.S.A.) The column was maintained at ambient temperature (23 ± 1°C).

Preparation of Mobile Phases:

Mobile phases containing aqueous phosphate buffers (pH 3, 4.5 and 6.0) and ionic strengths (3.125, 6.25 and 12.5 mM) combined with acetonitrile were prepared. The pH was adjusted with either 10%

phosphoric acid or 1N NaOH. All mobile phases were filtered through a 0.45 μm nylon-66 filter (MSI, Westborough, MA, U.S.A.) and degassed by sonication. The flow-rate was set at 1.0 mL/min.

Preparation of Stock Solutions

Individual stock solutions of propranolol, atenolol, and metoprolol at 25 μg/mL and verapamil, diltiazem, clonidine and prazosin at 50 μg/mL were prepared in aqueous acetonitrile mixtures containing the same proportion of acetonitrile as found in each mobile phase studied. Mixtures of the various stock solutions were prepared by using equal volumes of individual solutions. Triplicate injections were made into the liquid chromatograph.

RESULTS AND DISCUSSION

The purpose of this study was to demonstrate the applicability of using an underivatized silica stationary phase and organic-aqueous buffered eluents for the separation of cardiovascular agents of varied chemical structure. The model compounds were selected from a list of β-adrenergic blockers, calcium channel blockers, and α- adrenergic blockers. These drugs exhibit enough variation in chemical structure and functional group chemistry to provide a representative sample of cardiovascular compounds of pharmaceutical interest (see Fig. 1). Several mobile phases differing in organic modifier concentration, ionic

strength, and buffer pH were prepared and tested for the chromatographic separation of these analytes.

Aqueous 6.25 mM phosphate buffer pH 3.0 was mixed with varying acetonitrile concentrations to observe the effect of mobile phase composition on retention and column efficiency as measured by plate count (see Table 1). The mobile phase of 60:40 v/v phosphate buffer-acetonitrile gave the best overall separation within each class of cardiovascular agents. A decrease in the capacity factor (k') for the β-adrenergic blockers resulted from an increase in buffer concentration. This may be explained by other interactive forces, besides hydrogn bonding, which are more important for binding these drugs to the silica surface. These interactions may be dependent on nonpolar van der Waals

TABLE 1

Effect of Buffer and Organic Modifier Concentrations on Retention and Column Efficiency of Selected Cardiovascular Agents.

	40:60 v/v Buffer/CH_3CN		50:50 v/v Buffer/CH_3CN		60:40 v/v Buffer/CH_3CN	
Analyte	k'	N[a]	k'	N	k'	N
Propranolol	2.27	6670	2.23	7100	2.28	8717
Atenolol	2.75	8776	2.33	7421	2.02	7358
Metoprolol	2.63	8212	2.50	8084	2.48	7916
Verapamil	2.61	8254	2.91	9703	3.72	14923
Diltiazem	2.73	8643	2.80	9154	3.16	11494
Nifedipine	0.39	1498	0.41	1948	0.48	5852
Clonidine	2.71	34575	2.59	8169	2.49	9902
Prazosin	3.01	40879	3.02	10148	3.28	12030

[a] Calculated as $N = 5.54\ (tr/W0.5)^2$

forces between the analyte, the acetonitrile and silanol or siloxane groups (14).

As buffer concentration was increased, k' for the calcium channel blockers also increased (see Table 1). This indicates a dependence on hydrogen bonding between the silica surface and a calcium channel blocker. With increasing concentrations of water in the mobile phase, hydrogen bonding is more extensive and the retention time of the analyte was shown to increase (15). The more extensive hydrogen bonding lattice may also stabilize ion pairing which may occur between the basic analyte and surface silanols.

Each of the α-blockers showed a different type of behavior with an increase in buffer concentration (see Table 1). Clonidine showed a gradual decrease in k' with increases in buffer concentration, while the k' of prazosin increased with an increase in buffer concentration. This indicated that prazosin is dependent on the hydrogen bonding strengthened by higher concentrations of water, while clonidine is dependent on major retention forces other than hydrogen bonding to silanol groups.

The cardiovascular agents studied displayed a general trend of increase in column efficiency with an increase in the buffer concentration of the mobile phase. Exceptions to this were atenolol and metoprolol, which gave decreases in column efficiencies with increased buffer

concentration. This behavior is explained by these drugs showing a decreased k' with about the same peak width.

The chromatographic separation of the selected cardiovascular agents was further investigated by changing the pH of the buffer in the mobile phase. The mobile phase was held constant at 60:40 v/v 6.25 mM phosphate buffer-acetonitrile with pH varied in the range 3-6.0. Table 2 shows the effect of increasing the pH of the buffer on the separation of each analyte. Those compounds possessing ionizable amino functional groups may undergo ion-pairing with an undefined number of strongly acidic silanol groups and these interactions are controlled directly by pH (14). The pKa of silanols at the silica surface is approximately 6.8 ± 0.2 (16). At lower pH, there are more unionized silanol groups which permit greater ability of the silica to hydrogen bond with an analyte. This increased hydrogen bonding network yields decreased analyte retention, thus showing hydrogen bonding to be a secondary interaction force to cause retention. Increases in pH yield higher ionization of silanol groups, which contribute to increased retention through a greater occurence of ion-pairing. All of the drugs chromatographed in this study showed an increase in retention with an increase in pH. This may show that hydrogen bonding and other aromatic interactions with siloxane groups are secondary interactions which affect selectivity, while ion pairing is the primary means of retention.

TABLE 2

Effects of Buffer pH on Retention and Column Efficiency of Selected Cardiovascular Agents.

Analyte	pH 3.0 k'	N[a]	pH 4.5 k'	N	pH 6.0 k'	N
Propranolol	2.28	8717	3.03	10125	7.54	9796
Atenolol	2.02	7358	2.75	8754	7.14	8820
Metoprolol	2.48	7916	6.97	11469	8.71	9481
Verapamil	3.72	14923	4.84	9449	15.07	9035
Diltiazem	3.16	11494	4.18	16516	11.10	12584
Nifedipine	0.48	5852	0.51	5708	0.69	6070
Clonidine	2.49	9902	3.36	11697	8.49	12473
Prazosin	3.28	12030	4.26	17250	6.23	16805

[a] Calculated as $N = 5.54 (tr/W0.5)^2$

There was a general trend of increased column efficiency with increased pH (see Table 2). Two exceptions which did not follow this trend were metoprolol and verapamil. These drugs appeared to form stronger ion-pair interactions with silanol groups as the pH increased. This may be seen by a multifold increase in k' with increases in pH. Metoprolol and verapamil were affected more by band broadening even though the capacity factors are high. The stronger ion-pair interaction of these two drugs provided an explanation for the increased band width. Diltiazem gave a slight increase in column efficiency with an increase in buffer pH from 3.0 to 4.5, but a decline in column efficiency between pH 4.5 to 6.0. This behavior is not readily explained.

The use of different ionic strengths of the mobile phase was also investigated. In these studies, the mobile phase composition was again

TABLE 3

Effect of Changes in Ionic Strength of the Buffer on Retention and Column Efficiency of Selected Cardiovascular Agents.

Analyte	3.125 mM k'	3.125 mM N[a]	6.25 mM k'	6.25 mM N	12.5 mM k'	12.5 mM N
Propranolol	4.39	9719	2.28	8717	1.30	6102
Atenolol	4.01	11607	2.02	7358	1.16	13739
Metoprolol	4.76	8155	2.48	7916	1.40	5267
Verapamil	6.75	11858	3.72	14923	2.10	10940
Diltiazem	5.89	11910	3.16	11494	1.78	15906
Nifedipine	0.73	18959	0.48	5852	0.49	10184
Clonidine	4.79	13143	2.49	9902	1.43	5362
Prazosin	6.09	12623	3.28	12030	1.87	7448

[a] Calculated as $N = 5.54\ (tr/W0.5)^2$

set at 60:40 v/v aqueous phosphate buffer pH 3-acetonitrile. The molarity of the buffer was varied from 3.125 to 12.5 mM to observe the effect of ionic strength on analyte chromatographic parameters. Table 3 shows the effect of increasing the ionic strength of the buffer on the separation of the three classes of drugs. At pH 3.0, sodium ions in the mobile phase may compete with the positively charged analyte for ionized silanols; therefore, if more sodium ions are added, the analyte will be displaced from the silica surface thus decreasing its retention (17). In this study, all of the drugs analyzed in each class showed a decrease in retention with an increase in ionic strength of the mobile phase.

According to the data, there was no general trend for retention of these cardiovascular agents with respect to column efficiency vs ionic

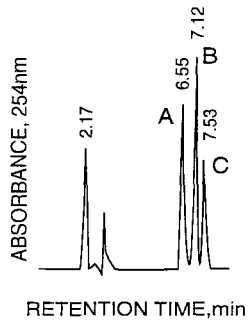

Figure 2 Typical HPLC Separation of Atenolol (A), Propranolol (B), and Metoprolol (C) on Underivatized Silica with 60:40 v/v pH 3 Phosphate Buffer - Acetonitrile at 1.0 mL/min

strength. For example, diltiazem showed a decrease in column efficiency from 3.125 - 6.25 mM, but an increase in column efficiency from 6.25 mM to 12.5 mM. This behavior is not easily explained by current knowledge of interactions of analytes with the silica surface.

The substitution of methanol as organic modifier in the mobile phase caused increased retention of the drugs as compared to equal amounts of acetonitrile. Diltiazem, verapamil, and prazosin showed the largest increases in the k' with methanol-containing mobile phases. This increased retention was accompanied by a decrease column efficiency as measured by N. Increased k' values for the various analytes may be attributed to the fact that methanol can participate in the hydrogen bonding lattice which retains analytes on silica gel. Acetonitrile is unable to form such bonds.

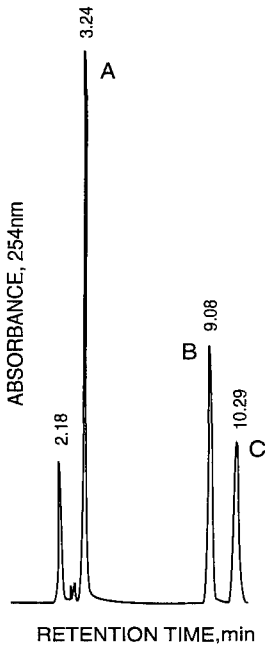

Figure 3 Typical HPLC Separation of Nifedipine (A), Diltiazem (B), and Verapamil (C) on Underivatized Silica with 60:40 v/v pH 3 Phosphate Buffer - Acetonitrile at 1.0 mL/min.

Typical HPLC separations of the selected cardiovascular agents using 60:40 v/v phosphate buffer pH 3.0-acetonitrile are shown in Figs. 2-4. Table 4 gives the analytical figures of merit for each drug in this HPLC system.

In summary, the HPLC separation of selected drug mixtures from three classes of cardiovascular agents were studied on underivatized

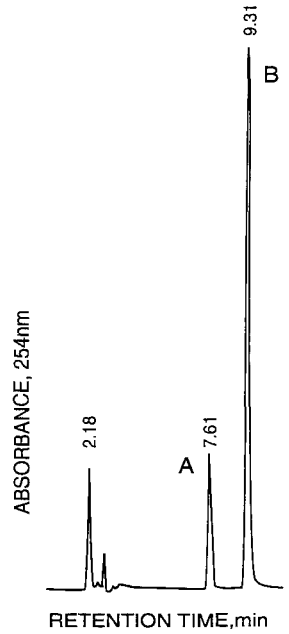

Figure 4 Typical HPLC Separation of Clonidine (A), and Prazosin (B) on Underivatized Silica with 60:40 v/v pH 3 Phosphate Buffer - Acetonitrile at 1.0 mL/min.

silica using aqueous acetonitrile mobile phases. The compounds were generally well-resolved with high column efficiencies. Although ion-pairing appears to be the primary interactive force between silanol groups and the analytes, other forces such as hydrogen bonding and hydrophobic interactions may also show effects on the chromatographic separation of these cardiovascular agents.

TABLE 4

Analytical Figures of Merit for Selected Cardiovascular Agents on Underivatized Silica with 60:40 v/v Phosphate Buffer pH 3.0-Acetonitrile.

Mix	Analyte	Rs	T_f[a]	N[b]	k'	Rt(min)	α
1.	Atenolol		1.19	7358	2.02	6.56	
		1.46					1.13
	Propranolol		1.00	8717	2.28	7.13	
		1.00					1.09
	Metoprolol		1.22	7916	2.48	7.54	
2.	Nifedipine		1.36	5852	0.48	3.25	
		13.93					6.58
	Diltiazem		0.95	11494	3.16	9.08	
		2.37					1.18
	Verapamil		1.33	14923	3.72	10.29	
3.	Clonidine		1.67	9902	2.49	7.61	
		3.11					1.32
	Prazosin		0.83	12030	3.28	9.32	

[a] Calculated at 5% peak height
[b] Calculated as $N = 5.54\ (tr/W0.5)^2$

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DIRECT DETERMINATION OF ANIONIC SURFACTANTS USING ION CHROMATOGRAPHY

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ABSTRACT

A method for the simultaneous qualitative and quantitative analysis of surfactants in solution via ion chromatography has been developed. This method has the capability of separating linear alkyl anionic surfactants which differ by as little as two carbon atoms in the hydrocarbon chain. In addition, the method can differentiate between surfactants of the same chain length which differ only in the chemical nature of the anionic head group. The ion chromatographic method is much simpler and more accurate than previous techniques of determining surfactant concentration which relied on detecting changes in secondary properties, such as surface tension. The method is also highly flexible and should be applicable to a wide variety of ionic and nonionic surfactants. Use of ion chromatography will greatly simplify studies of surfactant behavior.

INTRODUCTION

Surface active agents, or surfactants, are amphipathic compounds which are composed of a lyophobic portion which has little attraction for the solvent, and a lyophilic portion which has a strong attraction for the solvent. When dissolved in aqueous solution at low concentrations, it is energetically favorable for the surfactant to migrate to any available hydrophobic interface and orient itself with its hydrophobic portion toward the interface and its hydrophilic portion in the aqueous phase(1,2). Surfactants are widely used in many products and processes as indicated by the fact that although they are present at no more than a few percent by weight in any given

application, the total production of all types of surfactants in the United States was conservatively projected to be 3.3 million metric tons in 1992(3). Although they are typically associated with soaps and detergents, surfactants also play a crucial role in many other applications such as metal treatments, cosmetic and pharmaceutical formulations, pesticides, detergent motor oils, and sol-gel ceramic particle generation processes. Micellar surfactants have even been used as mobile phases in liquid chromatography(4). In all of these applications the effectiveness of the surfactants is determined by the extent to which they adsorb onto a given surface or interface, and the configuration in which they adsorb. This adsorption is controlled by many factors including the nature of the adsorbing surface, the structure of the surface active materials, properties of the bulk fluid phase, and the temperature.

In order to determine the extent of surface adsorption, the amount of surfactant adsorbed onto well characterized surfaces such as spherical polystyrene particles can be measured. Knowledge of the surfactant concentration in the fluid phase which is brought into contact with the surface allows the adsorbed surface concentration to be calculated if the bulk fluid phase concentration can be determined following adsorption equilibrium. When surfactant is added to an aqueous suspension of polymer particles, equilibrium is established between surfactant adsorbed at the air-liquid interface, free surfactant in the bulk solution phase, and surfactant adsorbed at the polymer-liquid interface. As more surfactant is added to the system, all of the interfaces become saturated with a monolayer of surfactant(2,5). When surfactant is added beyond this point, the free surfactant molecules become associated in organized aggregates called micelles. The concentration at which micellization occurs is called the critical micelle concentration (CMC), and is marked by abrupt changes in several physical properties, including surface tension(1,2,6). Maron *et al.*(5) developed a method for determining surface area and particle size of synthetic latexes via the soap titration technique. The titration endpoint can be used to calculate the total amount of surfactant adsorbed on the latex surface at saturation, but cannot distinguish between surfactant species. Use of the soap titration method to obtain

adsorption data at surfactant concentrations below saturation is also possible(7,8). However, the soap titration method is tedious, requiring nearly 30 minutes to obtain a single point on the titration curve. In addition, the titration endpoint is rarely well defined leading to large uncertainties in the results. It is much more efficient to determine these concentrations directly. It is also desirable that the analysis be capable of differentiating between multiple surfactants since most practical applications involve the use of two or more surface active species present in the same fluid phase. This investigation introduces one of the newest methods for direct determination of surfactant concentration, ion chromatography.

Ion chromatography is a term which encompasses many modes of the chromatographic separation of ions including conventional ion exchange chromatography and ion-pairing chromatography. Ion-pairing chromatography uses a neutral nonpolar stationary phase together with a mobile phase containing a lipophilic electrolyte called the ion interaction reagent(9), or the ion-pair reagent(10). The structure of the stationary phase dictates the type of ion-pair chromatography being used. Porous inert particles such as silica are used for size-exclusion chromatography which retains the shorter ion pairs longer due to the physical difficulties of moving through the porous regions of the stationary phase. Non-porous hydrophobic particles can also be used, in which case separation occurs based upon the hydrophobic character of the ion pair. Hydrophobic ion-pair chromatography is well-suited for surfactant systems because the ion pair includes the hydrophobic tail group of the surfactant ion, allowing for ideal separation conditions using the hydrophobic stationary phase while allowing control of the retention time and the peak width through the variation of the hydrophobic character of the mobile phase.

In this study, ion chromatography was proven capable of clearly separating closely related surfactant species according to both basic type and chain length, and quantitatively analyzing the relative amounts of the surfactant species present. Ion chromatography is the only technique, to date, which has demonstrated this ability, although capillary electrophoresis systems which may be able to accomplish this task have recently been introduced. The introduction of ion

chromatography for the separation and quantitative detection of surface active species offers a simplified and more accurate approach to the determination of adsorption isotherms. As a result, it is possible to accurately assess the concentration of various individual species on an adsorbing surface using a relatively simple analytical method.

EXPERIMENTAL

Instrumentation

The analysis of surfactants in the bulk fluid phase in equilibrium with a model surface was carried out using a Dionex series **4000i** ion chromatograph equipped with an advanced computer interface (ACI) for controlling the system from the **AI-450** version 3.1, Microsoft® **Windows**™ 3.0 compatible software (available from Dionex Corporation, Sunnyvale, California). The chromatograph was also equipped with an eluant gradient pump, an auto sampler with a 25 µL sample loop, the **AMMS-MPIC** micromembrane anion suppressor, and all teflon tubing and fittings. The **IonPac NS1-5µm** hydrophobic separator column was used in series with the **IonPac NG1-GUARD** guard column (all from Dionex). These columns separate analytes on the basis of hydrophobic character, and an eluant gradient was utilized to maximize the separation obtained while minimizing the total run-time required.

The method employed the use of three eluants which were combined in varying ratios through the gradient pump and mixed in the **GM-2** turbulent gradient mixer (Dionex). The mixed eluant stream was passed through the sample valve at a flow rate of 1 mL/minute, and into the guard and separator columns. The sample stream then continued through the suppressor column and into the conductivity cell for detection as shown schematically in Figure 1.

Eluants, Reagents, and Surfactants

Deionized (DI) water of not less than 17 Mohm-cm resistivity was obtained from a **Nanopure** high-purity deionization and filtration system (Barnstead/Thermolyne Corporation, Dubuque, Iowa), and used unaltered as one eluant (E1). Reagent grade acetonitrile and 30%

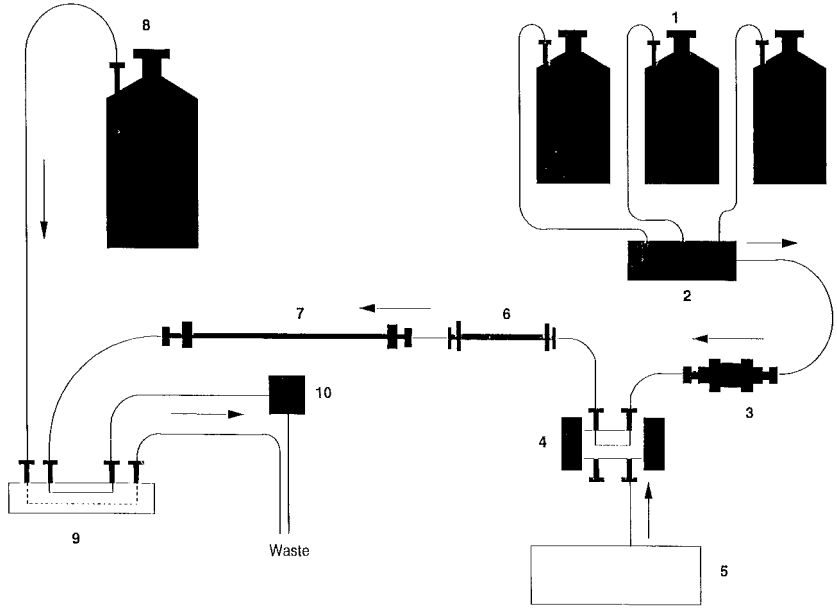

FIGURE 1: Schematic representation of the ion chromatograph system: 1 - eluant reservoirs, 2 - gradient pump, 3 - eluant mixer, 4 - sample valve, 5 - automated sampler, 6 - guard column, 7 - separator column, 8 - acid regenerant reservoir, 9 - suppressor column, 10 - conductivity cell.

ammonium hydroxide (both from J. T. Baker, Inc., Pittsburgh, Pennsylvania) diluted to 30% CH_3CN (v/v) and 10 mM NH_4OH with DI water was used as eluant two (E2) where the low concentration ammonium hydroxide was used as the ion-pair reagent. While most ion-pair applications utilize tetrabutylammonium hydroxide (TBAOH) as the ion-pair reagent, preliminary work indicated that the use of an interaction agent with a highly hydrophobic character resulted in greatly increased elution times for surfactant analytes. Ammonium hydroxide at a concentration of 10 mM offered the best separation and peak resolution within a reasonable analysis period. A 90% (v/v) solution of acetonitrile in DI water was used as the third eluant (E3). All eluants were degassed with helium for 20 minutes prior to use, and pressurized to 10 psi with helium during the analysis. A solution of approximately 5 mM sulfuric acid was used as the regenerant for the ion exchange membranes within the suppressor. The regenerant was pressurized to 10 psi with nitrogen during the analysis. A list of the surfactants used in this investigation and their level of purity is given in Table 1. A series of standard solutions of each surfactant was prepared at concentrations of 50, 100, 500, 1000, and 5000 mg/L in DI water (the saturation point of sodium lauryl sulfonate is around 3000 mg/L, therefore only 50 - 1000 mg/L solutions of SLSN were prepared).

Procedure

The chromatographic method involved a 15 minute gradient and analysis time beginning with a three minute initial purge period during which the mobile phase was maintained as a mixture of 33% eluant 1 (E1: DI water) and 67% eluant 2 (E2: 30% CH_3CN, 10 mM NH_4OH). The purpose of this initial phase was to purge any mobile contaminants from the column and to allow the baseline conductivity to recover following any previous run. Following the first phase, the 25 μL sample loop was introduced into the system for one minute to allow the sample to be completely flushed from the loop. The third phase involved a linear gradient, three minutes in duration, from the initial eluant mixture (33% E1, 67% E2) to a mixture of 83% eluant 2 and

TABLE 1. Surfactants used for ion chromatographic analysis

Surfactant	Acronym	n[a]	MW	Supplier	Purity
Sodium Octyl Sulfate	SOS	8	232.27	Aldrich	99%
Sodium Decyl Sulfate	SDS	10	260.32	Kodak	95%
Sodium Lauryl Sulfate	SLS	12	288.38	Aldrich	98%
Sodium Octyl Sulfonate	SOSN	8	216.28	Aldrich	98%
Sodium Decyl Sulfonate	SDSN	10	244.33	Aldrich	98%
Sodium Lauryl Sulfonate	SLSN	12	272.38	Pfaltz & Bauer	99%

[a] General chemical structure of linear alkyl sulfates: $CH_3(CH_2)_{n-1}OSO_3Na$
General chemical structure of linear alkyl sulfonates: $CH_3(CH_2)_{n-1}SO_3Na$

17% eluant 3 (E3: 90% CH_3CN). The effect of the change to a higher volume fraction of acetonitrile is to increase the hydrophobic nature of the mobile phase in order to elute the more hydrophobic species, long chain surfactants such as sodium lauryl sulfate (SLS), from the separator column more rapidly than would otherwise be possible. This has been found to significantly reduce the analysis time while simultaneously maintaining good peak resolution. This eluant mixture (83% E2, 17% E3) was maintained for six minutes, then a 30 second linear gradient returned the eluant mixture to the initial volumetric ratio (33% E1, 67% E2) where it was maintained for an additional 90 seconds.

The Dionex **4000i** ion chromatograph was calibrated using the concentration standard solutions prepared from the surfactant samples which were to be used for the adsorption experiments. The relationship between concentration and detector response (peak area) was determined to be linear over the defined concentration range of 0 to 5000 mg/L (0 to 1000 mg/L in the case of SLSN), with correlation coefficients of not less than 0.9977. The ion chromatograph required recalibration following any refilling or refreshing of any of the eluants. Slight variations in eluant concentration were found to have a significant effect on the reported concentration values (variations of up to 10% in concentration), as well as on the retention time

of a given component (variations of up to 20% in retention time). Column ageing was also found to affect calibration results, but only over periods of time on the order of several months.

The amount of surfactant adsorbed onto a model surface (in this case spherical polystyrene particles were prepared and characterized for this purpose) was determined by equilibrating a known quantity of a polymer latex sol with a known concentration of surfactant and analyzing the bulk fluid phase for surfactant concentration to obtain the difference. In this experiment, samples were prepared in 20 mL vials which contained approximately 1% (by volume) of the polymer particles and a known initial quantity of surfactant. The samples were allowed to reach equilibrium over a period of more than 24 hours at room temperature, the polymer and associated adsorbed surfactant was then removed by filtering the sample through a 0.05 μm polycarbonate microfiltration membrane (Nuclepore, Pleasanton, California). Blank sample analysis indicated that no appreciable amount of surfactant was lost on the membrane. Analysis of the filtrate by the ion chromatographic method described above yields the free surfactant concentration evaluated from the peak areas. If the amount adsorbed at the liquid-air interface is neglected(7), the adsorbed amount of surfactant can be determined from:

$$\Gamma = \frac{M_S}{M_L} \tag{1}$$

where Γ is the adsorbed amount reported as a mass fraction and M_L is the mass of latex present. M_S is the mass of adsorbed surfactant which is determined by difference from:

$$M_S = v(C_{S,i} - C_{S,f}) \tag{2}$$

where v is the liquid phase volume of the sample, $C_{S,i}$ is the initial added surfactant concentration (in mg/L), $C_{S,f}$ is the free surfactant concentration (in mg/L) in the filtered bulk solution, and M_S is reported as mg of surfactant. The resulting minimum detection limit was found to be about 5 to 10 mg/L adsorbed surfactant based on the liquid phase volume. In order to minimize the integration error caused by changes in the baseline conductivity due to the eluant gradient, a sample of DI water was run as a standard. The standard DI water baseline was then subtracted

from each analysis chromatogram to provide a more level baseline and consequently improve the accuracy of the peak integration by as much as 3% for those species which eluted during the gradient (e.g. SDS, SLS, and SLSN). An example of this process is shown in Figure 2. The reported free surfactant concentrations could then be converted to adsorbed amount and plotted against the bulk surfactant concentration to produce the adsorption isotherms.

RESULTS AND DISCUSSION

The Dionex series **4000i** ion chromatograph was calibrated for quantitative analysis using a six point linear calibration fit for each of the six anionic surfactants. Detector response (peak area) was recorded for the surfactant standard solutions following the baseline adjustment procedure outlined above, and was plotted against the concentration of the standard sample. A sample calibration curve is shown in Figure 3, and the calibration results for each surfactant are tabulated in Table 2. Each point on the calibration curve was calculated from three repeat samples which were found to be within ±10 mg/L (absolute standard deviation - ASD) of each other. Replicate calibration curve indicated no significant variation except when one or more of the eluants was replenished as previously noted. The intercept (b) on the linear fits could not be set to zero in the **AI-450** version 3.1 software, therefore the inherent weighting of the data points involved in the least squares calculation resulted in non-zero values for the intercept. Repeat analyses of the samples demonstrated that the instrumental precision was about ±5 mg/L (ASD), while the calibration was determined to be accurate to within approximately 10 mg/L (ASD). Forcing a zero intercept on the calibration curve resulted in slightly improved accuracy at low concentrations (±5 mg/L, ASD), while sacrificing some accuracy at concentrations above 1000 mg/L (±12 mg/L, ASD). However, these changes were relatively small and did not justify the amount of extra effort that would have been involved in external calibrations and concentration determinations. Newer versions of the **AI-450** software, which are now available, do offer the option to force a zero intercept for linear calibrations. All of the data collected in this experiment were based on calibrations with non-zero intercepts as shown in Table 2.

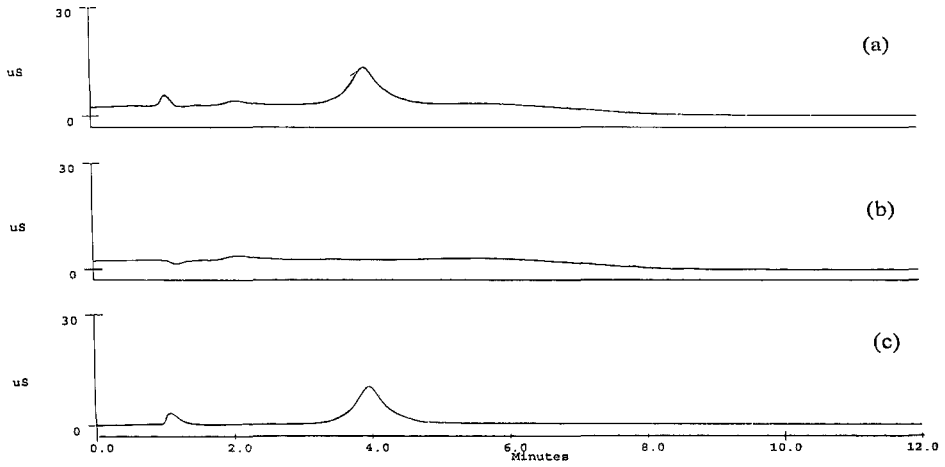

FIGURE 2: Example of the baseline adjustment process showing (a) the original chromatogram of sodium decyl sulfonate (SDSN), (b) the baseline chromatogram of deionized water, and (c) the resulting SDSN chromatogram obtained when the baseline chromatogram (b) is subtracted from the raw data chromatogram (a).

The retention times of the various surfactant species given in Table 2 are spaced in such a way that separation of all six species has been possible (although only at concentrations below 500 mg/L in the case of SOS and SOSN). This chromatographic technique can, therefore, differentiate between linear alkyl sulfates differing by only two carbon atoms in the alkyl chain, as well as distinguishing between sulfates and sulfonates of the same alkyl chain length. The separation of several closely related surfactants, as shown in Figure 4, is not only qualitative, but also quantitative in nature. Table 3 demonstrates that for the separation indicated in Figure 4 the ion chromatographic method accurately determines the concentration of each species to within 10 mg/L. This reliability is representative of the results acquired throughout the experimental investigation.

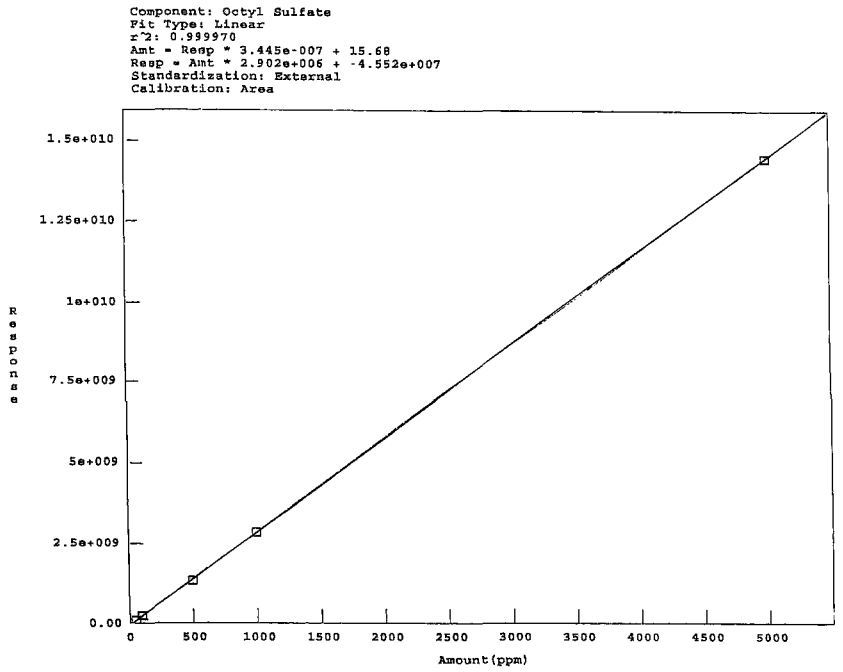

FIGURE 3: Ion chromatograph calibration curve of sodium octyl sulfate showing detector response versus concentration relationship and least squares fit results.

TABLE 2. Ion chromatograph example calibration results (linear fit)
Concentration = Response × *a* + *b*

Surfactant	Retention Time (min)	*a*	*b*	r^2
SOS	2.65	3.445×10^{-7}	15.68	1.0000
SDS	6.00	3.366×10^{-7}	-27.39	0.9977
SLS	7.95	3.766×10^{-7}	-6.03	0.9996
SOSN	2.40	2.573×10^{-7}	-39.89	0.9991
SDSN	4.25	2.794×10^{-7}	-23.49	0.9998
SLSN	7.35	3.984×10^{-7}	-6.15	0.9996

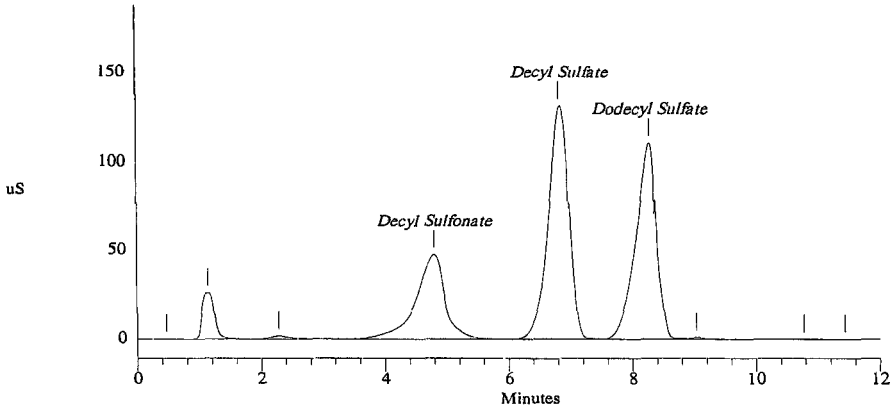

FIGURE 4: Separation of C_{10} and C_{12} alkyl sulfates and C_{10} alkyl sulfonate obtained using ion chromatography.

TABLE 3. Quantitative results of ion chromatographic separation shown in Figure 4.

Retention Time	Component Name	Sample Concentration	Observed Concentration
4.80	SDSN	500 mg/L	508 mg/L
6.83	SDS	1000 mg/L	993 mg/L
8.28	SLS	1000 mg/L	1002 mg/L

The separation and quantitative precision obtained using this technique is important for future investigations of surfactant adsorption behavior since most practical applications of surface active agents involve a multi-component system containing two or more unique surfactant species. In addition, minor modifications in the eluant gradient can be made which have been proven to allow extremely hydrophobic (i.e. strongly retained) species to elute in relatively short periods. While most of the work in this investigation was conducted using a method which involved a gradient from 20% (v:v) acetonitrile to 40% CH_3CN, a method using a 40% to 70% CH_3CN gradient was utilized to facilitate the detection of sodium lauryl benzene sulfonate (SLBSN,

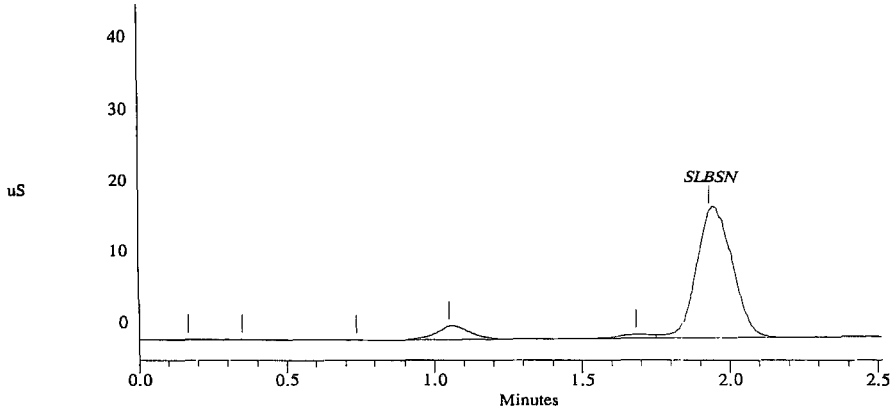

FIGURE 5: Chromatogram of sodium lauryl benzene sulfonate using modified eluant gradient 40-70 (%CH_3CN by volume).

Aldrich) with a minimal analysis time and without any loss of experimental accuracy. This analysis is shown in Figure 5 in which a 50 mg/L SLBSN standard solution was observed to contain 49.7 mg/L SLBSN. Work at this location has recently reported success in obtaining adsorption isotherms for single component systems of anionic surfactants(11). The analysis of multi-component systems of anionic surfactants and a comparison of these adsorption isotherms with those of the individual components is currently in progress. In addition, an investigation of the application of this technique to systems of nonionic and cationic surfactants as well as cosurfactant species such as linear alcohols is planned.

CONCLUSIONS

Today the chromatographic analysis of ionic materials is widely applied and rapidly expanding. The number of species which may be determined continues to grow, as does the number of areas of science and technology where ion chromatography plays an important role. This investigation has indicated that ion chromatography is capable of clearly separating closely

related anionic surfactants and reporting quantitatively accurate information regarding the concentration of each of these species. Techniques used for acquiring the same information in previous studies were tedious and inaccurate. In addition, the ion chromatographic technique is highly flexible and can be easily modified to be applicable to a wide variety of surface active materials. The introduction of an ion chromatographic method to directly determine the concentration of surfactant species in a bulk fluid phase greatly simplifies the analysis of surfactant adsorption behavior.

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INFLUENCE OF SAMPLE RECOVERY TECHNIQUES ON DETECTION OF BIOGENIC AMINES IN THE RAT HOOKWORM *NIPPOSTRONGYLUS BRASILIENSIS*

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ABSTRACT

Biogenic amines in *Nematoda* have previously been found by means of high-performance liquid chromatography (HPLC) equipped with various detectors. The methods of sample preparation have differed widely. In this study, we tested the influence of experimental conditions (temperature and duration of exposure to light, centrifugation or individual recovery by pipetting each worm, and saccharose gradient versus physiological saline) on catecholamines and indolamines content in *Nippostrongylus brasiliensis* at various developmental stages (larvae and adults). Amines were determined by liquid chromatography with electrochemical detection.

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To the best of our knowledge, octopamine, tyramin, N-acetylserotonin and N-acetyldopamine were found and determined for the first time in this nematode.

All experimental conditions listed above modify the endogenous amine levels in larval and adult *Nippostrongylus brasiliensis*. This should be taken into account in the design of future research protocols.

INTRODUCTION

Biogenic amines have been found in ***Nematoda*** by means of various techniques, including histochemistry [1], fluorescence labelling [2-8], gas chromatography [9], radioisotope enzyme assay [1], liquid chromatography (LC) with UV detection [10, 11] or electrochemical detection (ECD) [12-16].

Samples are usually heterogeneous in terms of the body parts analysed (whole worms or various organs), the population (mixed cultures of male and female adults or larvae), and the species (*Caenorhabditis elegans, Rhabditis pseudoelongata, Trichostrongylus colubriformis, Ascaris suum, Ascaris lumbricoides, Ascaridia galli, Nippostrongylus brasiliensis, Setaria cervi, Molinema dessetae, Acanthocheilonema vitae* and *Litomosoides carinii*). This heterogeneity has contributed to large differences between the published results concerning detection and analytical determination of amines. As a result, the published data cannot be directly compared.

Analytical methods are generally well described including the calibration of the detection system used (ECD). Nevertheless, the methods of sample preparation, particularly worm recovery, often are not.

We tested the recovering effect of the larvae and adults of *Nippostrongylus brasiliensis* under various experimental conditions (temperature, light, centrifugation or manual recovery, saccharose gradient or physiological saline) on biogenic amine levels.

Tryptophan (Trp), 5-hydroxytryptophan (5-HTP), serotonin (5-HT), 5-hydroxy-indol acetic acid (5-HIAA), N-acetylserotonin (Na5-HT), noradrenaline (NAd), tyramine (Tyr), octopamine (OA), dihydroxyphenyl acetic acid (DOPAC), dopamine (DA), N-acetyldopamine (NaDA), 3-methoxy-tyramine (3-MT), and homovanillic acid (HVA) were analysed simultaneously by HPLC with electrochemical detection, as described previously [16].

EXPERIMENTAL

Summary of the laboratory cycle

According to Luffau [17], third-stage larvae (L3) were collected from faecal cultures and inoculated into rats by skin puncture (2500 larvae per 250 g female rat). The larvae migrate to the lungs where they develop into fourth-stage larvae within 24 to 32 h. Following subsequent migration from the trachea to the oesophagus and onwards to the intestine, the larvae become adults. The first eggs appear in faeces five to six days after infestation under experimental conditions adopted.

Faecal culture and larval recovery.

Faeces from infected rats were collected between six and eight days after infestation. The droppings were macerated in water and mixed with an approximately equal volume of granular animal charcoal to form a paste. The mixture was spread on the center of a moist filter paper which was then placed on a wet sponge in a Petri dish and incubated at 25°C for 6-8 days [15,17]. Third-stage larvae were harvested by filling the Petri dish with water at 25°C, spontaneous sedimentation of the larvae recovered, then rinsed in distilled water.

They were divided into three groups and incubated in distilled water at 4°C, 25°C or 37°C for 4 hours. After incubation and spontaneous sedimentation, the supernatant was discarded and the worms were sponged with filter paper and stored at -20°C until analysis.

Harvest and preparation of adult worms

One week after infestation, rats were killed by ether asphyxiation and the small intestine was removed. The intestine was filled with 0.15 M NaCl at 37°C and opened longitudinally. The mucosa was excised and placed in a gauze bag, which was then placed in a beaker containing 0.15 M NaCl at 37°C. Under these conditions, adult worms settle to the bottom of the beaker, and a mixed population of male and female worms can be harvested after one hour of spontaneous sedimentation [17].

Four preparation methods were compared using nematodes from the same batch (See Table I) :

All samples were then sponged, weighed and stored at -20°C. Worm number and weight varied to different samples.

Sample preparation

Samples were placed in test tubes and homogenized (Ultraturrax, PolyLabo, Paris, France ; 30 sec.) in 0.4 N $HClO_4$ solution containing antioxidants (0.1% cysteine, 0.1% sodium metabisulphite and 0.1% sodium edetate) (1 mg of sample in 50 µl). Proteins were precipitated with perchloric acid and removed by centrifugation (Dupont Superspeed, Saint-Quentin en Yvelines, France ; 4°C, 20 min at 2 000 *g*) ; clear supernatants were used for the analysis.

Table I: Schema of the preparation of Nematodes samples before analysis

A pool of Nematodes is obtained by sedimentation and treated differently

Lot A : (about 75 % of adults)				**Lot B : about 25 % of adults**
Lot A_1 : Males and Females rinsed in NaCl 0.15 M	Lot $A_{1.1}$ analysed immediately	**Lot A_2** individually sexed Females were taken to test effects of light and temperature after exposure	Lot $A_{2.1}$ =1 hour	in sucrose (35 % in water) then centrifugation
	Lot $A_{1.2}$ analysed after light-temperature exposure during 3 hours		Lot $A_{2.2}$ =3 hours	
			Lot $A_{2.3}$ =5 hours	

Apparatus

Separation and determination of biogenic amines.

Samples were analysed by liquid chromatography with electrochemical detection according to Barreteau *et al* [16]. The chromatographic system consisted of a Beckman 112 pump (USA, Palo Alto, California) (constant flow rate of 1 ml/min); a Rheodyne injection valve (Touzart, Vitry, France) with a 20 µl loop ; a reverse-phase column (Ultrasphere ODS, 5 µm, 150 x 4 mm, Beckman) and a Metrohm 641 VA electrochemical detector (Roucaire, Vélizy, France) equipped with a glassy carbon electrode (set to a 0.85 V potential versus a KCl/AgCl reference electrode) [15].

A mixture of phosphate buffer [0.1 M KH_2PO_4, heptane sulphonic acid (5 mM)] and methanol (90/10, v/v) was used as the mobile phase ; pH was adjusted to 3.8 by using 3 M KOH.

Total elution was reached within 25 min. The system allowed the simultaneous detection of NAd, 5-HTP, OA, NaDA, DOPAC, DA, 5-HIAA, Na5-HT, HVA, Tyr, Trp, 3-MT and 5-HT (in the order of elution).

The perchloric acid medium formed a relatively large solvent front, hindering the detection of compounds eluted before Noradrenaline (*i.e.* during the first three minutes of each chromatographic analysis).

Chemicals

All reagents were of analytical grade ; methanol was from Merck, while Trp, 5-HTP, 5-HT, 5-HIAA, Na5-HT, NAd, Tyr, OA, DOPAC, DA, 3-MT, HVA and NaDA were from Sigma Co.

Statistical analysis

When sufficient repetitions were possible, analysis of variance (Fisher's test, ANOVA using Stat View software on a Macintosh computer) was used to

compare the different groups. However, a batch of worms obtained from one rat must be divided in several series to compare the effect of different sample recovery, and samples were often pooled to obtain quantities allowing assays; sometimes, the number of assays was insufficient and, in such a case, no statistical analysis was performed.

RESULTS

Two important facts are found. First, we have succeeded to detect some catechol and indol compounds for the first time in nematodes : octopamine (OA), tyramine (Tyr), N-acetylserotonin (Na5-HT) and N-acetyldopamine (NaDA) were found in larvae and adults.

Second, sample recovery including adaptative responses to environmental conditions greatly influenced the concentration of the indol and catechol compounds.

1- Effect of temperature on amine content of free third-stage larvae (fig.1)

Tryptophan was detected in all samples. Its concentration in larvae incubated at 4°C (15.4 ± 3 µg/g) was higher than in those incubated at either 25°C (5.6 ± 1.1 µg/g) or 37°C (6.7 ± 1.7 µg/g) ($p < 0.01$).

5-HTP was detected in all but two of the 11 samples (one at 4°C and one at 25°C) but accurate quantitative analysis was difficult because 5-HTP was generally eluted at the tail of the very broad solvent peak. When quantification was possible, 5-HTP concentrations were in the range of 170 to 195 ng/g.

Serotonin was detected (44 ng/g) in only one sample at 37°C.

5-HIAA was determined in all samples ; its concentration decreased with increasing incubation temperature (4°C : 353 ± 136 ng/g ; 25°C : 307 ± 246 ng/g ; 37°C : 87.5 ± 32 ng/g) ($p < 0.05$).

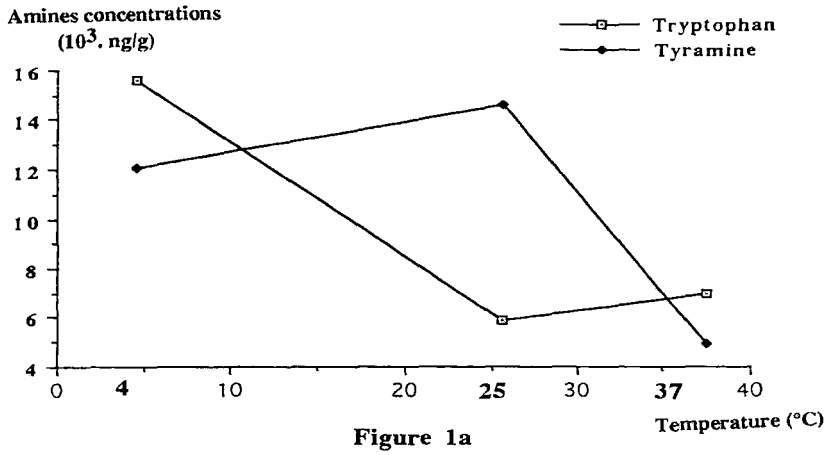

Figure 1a

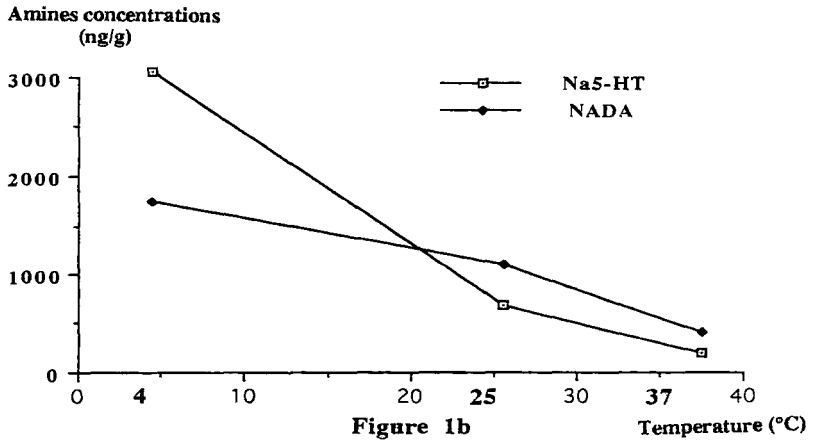

Figure 1b

Figure 1 : Effect of temperature on amine content of free third-stage larvae. Results are given only for some biogenic amines, for the others see text. Figure 1a : Tryptophan and Tyramine ; figure 1b : 5-HIAA, DA, DOPAC and 5-HT ; figure 1c : N-acetyl derivatives of 5-HT and DA.

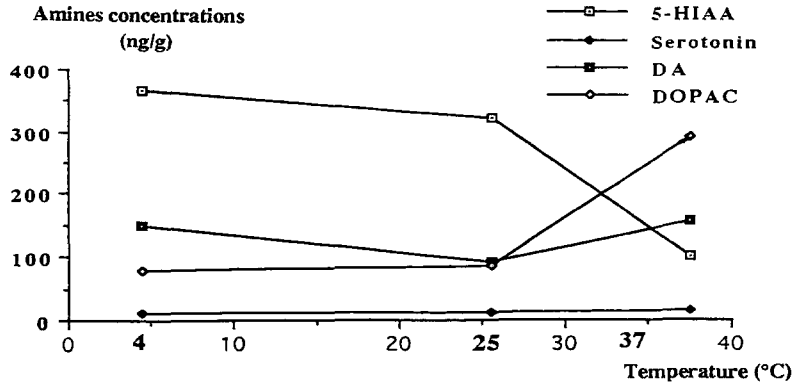

Figure 1c

Levels of Na5-HT were high at 4°C (2977 ng/g) in the three samples, but was either drastically lower or absent in samples incubated at 25°C (614 ng/g in one of four samples, mean : 153.5 ng/g) and 37°C (110 ng/g, 2 of 4 samples). Na5-HT was detected in all the samples, but levels were below the limit of accurate detection (2 ng/g).

Catecholamine concentrations also varied depending on incubation temperature.

Tyramine was detected in most samples but the quantitative determination was not always possible. It was determined in one of four samples at 4°C (35 µg/g), three of four at 25°C (mean : 14 ± 8.8 µg/g), and two of four at 37°C (9.1 µg/g, 9.34 µg/g). Thus, a tendency for concentration decrease with increasing temperature was observed.

Octopamine was detected in all samples at 4°C and 25°C, but in only 2 of 4 samples at 37°C. Accurate quantification was difficult, because octopamine eluted at the tail of the very broad solvent peak, OA was however determined at

4°C (32 µg/g, 1 out of 3 samples), 25°C (6.7 µg/g, 2 out of 4 samples), and 37°C (2 µg/g, 3 out of 4 samples).

Noradrenaline was present in all samples, often at higher concentrations than 3 µg/g. However, accurate determination was not always possible since Noradrenaline also eluted at the tail of the very broad solvent peak.

Dopamine was detected in 2 samples out of 3 at 4°C (137 ± 107 ng/g), 2 out of 4 at 25°C (78.8 ± 69 ng/g) and 3 out of 4 at 37°C (142 ± 61 ng/g).

DOPAC concentrations increased from 67.7 ± 48 ng/g at 4°C to 277 ± 125 ng/g at 37°C ($p < 0.05$).

Concentrations of NaDA decreased with increasing temperature (1659 ± 715 ng/g at 4°C ; 1026 ± 129 ng/g at 25°C ; and 338 ± 119 ng/g at 37°C ; $p < 0.05$).

2- " Temperature-illumination " effects in adults (Table II).

We observed marked differences inside and between groups (*i.e.* lot $A_{1.1}$ and lot $A_{1.2}$). Except for DOPAC, we found a general decrease in amine levels. However, statistical analysis showed that, OA, DOPAC, NaDA and tryptophan were unaffected by "temperature-illumination", whereas 5-HTP, dopamine, 5-HIAA and Na5-HT levels decreased.

3- Effect of temperature-illumination exposure time in female worms (Figure 2)

The analysis concerned the lots $A_{2.1}$, $A_{2.2}$ and $A_{2.3}$ (males and females in mixture).

Serotonin and tyramine were not detected, in any sample.

Tryptophan and 5-HIAA levels remained constant (21-26 µg/g and 120-148 µ/g, respectively).

There was a trend towards an increase in 5-HTP levels when the time of illumination increased from 1 to 5 hours (11 vs 32 ng/g, $p < 0.05$). Finally, Na5-

Table II : "Temperature-illumination" effects on amine contents in adults.

Content of biogenic amines (ng/g)	Physiological saline Illumination time 0 min	Physiological saline Illumination time 3 hours
NAd	74.26 ± 14	55.96 ± 10.59
5-HTP	62.05 ± 4.6*	20.33 ± 14.83 *
OA	11.22 ± 4.78 10^3	8.69 ± 4.27 10^3
DOPAC	38.53 ± 4.64	76.78 ± 42.15
NaDA	86.98 ± 12.57	40.99 ± 19.69
DA	38.59 ± 11.9 **	0.83 ± 0.18 **
5-HIAA	169.01 ± 6.78*	116.39 ± 27.5*
N-Ac-5-HT	285.56 ± 231.8**	2.3 ± 0.13**
Trp	18.35 ± 3,02.10^3	13.2 ± 3.44.10^3
Tyr	not detected	not detected
5-HT	not detected	not detected

* p = 0.05 ; ** p = 0.02. Each value reports mean ± S.E.M. for 3 samples.

HT was detected in all samples, but below the quantitative determination limit in one sample at 1, 3 and 5 hours. Mean levels decreased as the time of illumination increased.

There was a significant increase ($p < 0.05$) in noradrenaline (66 vs 222 ng/g), NaDA (140 vs 307 ng/g) and dopamine (0.92 vs 97 ng/g) levels when the time of illumination increased. In contrast, DOPAC levels decreased from 46 ng/g to about 29 ng/g after a 5-hour illumination period ($p < 0.05$). Finally, octopamine levels were not affected by illumination (13-17 µg/g).

4- Effect of centrifugation and sucrose gradient recovery on biogenic amines (Figure 3).

As a consequence of the large variability, no significant differences were observed between sucrose gradient (lot B) and saline (lot A) recovery groups (with the exception of tryptophan : 17.3 ± 1.64 vs 23.9 ± 2.57 µ/g, p = 0.04).

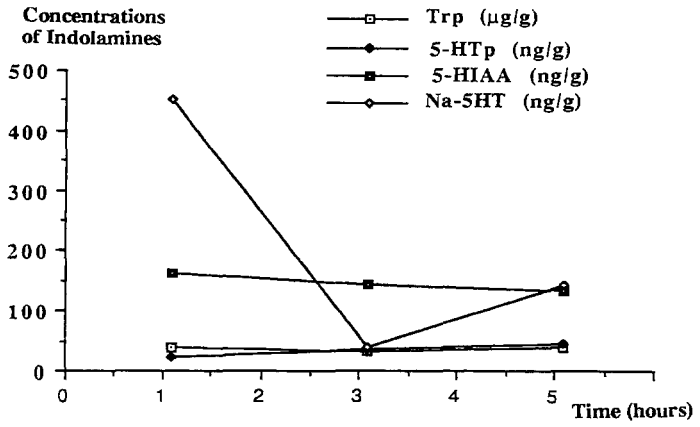

Figure 2a: indol compounds

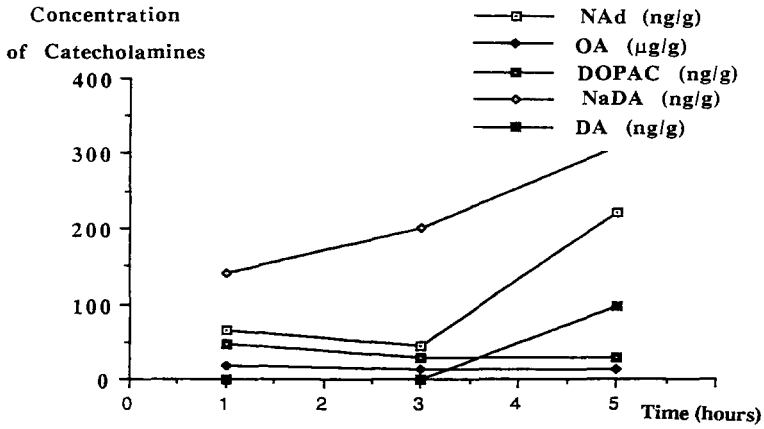

Figure 2b : catechol compounds

Figure 2 : Effect of the time of temperature-illumination and stress on biogenic amine content in females.

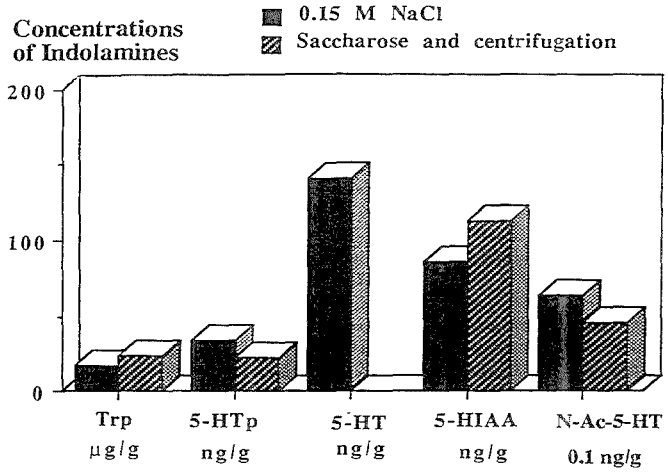

Figure 3a : indol compounds

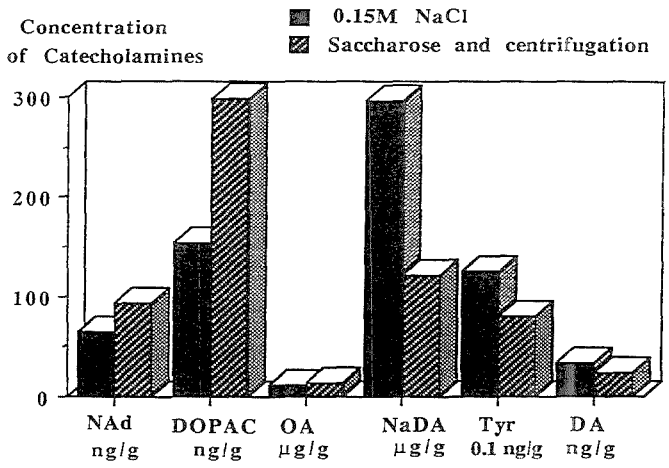

Figure 3b : catechol compounds

Figure 3 : Effect of worm recovery by a sucrose gradient and centrifugation on biogenic amines.

Tryptophan, 5-HTP, 5-HIAA and Na5-HT were detected in all samples (Fig. 3a). Serotonin was only determined in one saline-treated sample (142 ng/g). In saccharose-treated worms, 5-HTP and Na5-HT levels slightly decreased (21.24 ± 6.73 vs 34.07 ± 7.4 ng/g; 452.9 ± 127.4 vs 640.1 ± 160 ng/g respectively), 5-HIAA rose slightly (112.7 ± 26.6 vs 86.5 ± 25.5 ng/g) when compared to saline recovery group.

Tyramine, noradrenaline, DOPAC, dopamine, octopamine and NaDA were detected in all samples (Fig. 3b). In saccharose-treated worms, level decreases were registered for tyramine (800 ± 70 vs 1250 ± 600 ng/g), NaDA (121.25 ± 19.53 vs 295.7 ± 115 ng/g) and dopamine (24 ± 8.7 vs 33.3 ± 8.5 ng/g) while levels of octopamine (13.17 ± 1.62 vs 10.62 ± 1.69 µg/g), noradrenaline (93 ± 25.9 vs 65.5 ± 16.3 ng/g) and DOPAC (297.8 ± 69.45 vs 153.3 ± 42.2 ng/g) were enhanced, when compared to saline recovery group.

DISCUSSION AND CONCLUSION

A simultaneous determination of octopamine, tyramine, Na5-HT and NaDA was performed for the first time in *Nippostrongylus brasiliensis* by means of LC-ECD. In chromatographic separation followed with the electrochemical detection, behavior of these compounds was identical with standards.

Noradrenaline was detected in all samples, but both its high concentration and its elution in the tail of the solvent peak often precluded a quantitative analysis. For the same reason, concentrations of 5-HTP and octopamine were accurately not determined in some samples .

The presence of serotonin in nematodes has been reported by several authors [10, 11, 13-16], however this compound was hardly detectable in most of

our samples (even in presence of serotonin derivatives). This inconsistency with previous reports [15, 16] may be accounted for by the detection limit of our method, since in the present study we used 1 mg of worms per 50 µl of buffer, whereas Barreteau [15] used 1 mg per 10 µl.

3-MT and HVA were never detected, confirming the previous reports.

In addition to the detection of various indol and catechol compounds in nematodes, our study clearly shows that endogenous levels of bioamines depend not only on analysis method but mainly on worm recovery conditions.

Cooling of larvae increased the concentration of all the biogenic amines determined, with the DOPAC and tyramine exception, which remained constant. The stress induced by modifying temperature conditions could be evidenced by an acceleration of arylalkylamines, indolamines pathways, contrarily to dopaminergic pathways.

We observed the differences in biogenic amine contents at 25°C (typical temperature of free larval cultures) and 37°C (host temperature). Concentration of serotonin derivatives (tyramine, octopamine and NaDA) decreased with increasing temperature, while those of dopamine and DOPAC increased. A general decrease in N-acetyl derivatives was observed when temperature increased.

In mixed male and female adult worms, exposure to light and high temperatures reduced the biogenic amine content. If it is likely that enzyme activities are affected by temperature changes, especially above 37°C, these data are difficult to interpret, because they are a result of the combined influence of light and temperature. In most cases, the registered effect was negative (DOPAC was the only increasing biogenic amine which increased). However increasing times of illumination resulted in females by a significant increase in NaDA and

noradrenaline levels and a decrease in Na5-HT levels, other amines being unaffected .

To estimate the effects of handling (*i.e.* manipulation of worms under a microscope lamp for sexing), two different populations were used (handled females and a mixture of both sexes) and the results therefore cannot be directly compared. Indeed, it has been recently demonstrated that, biogenic amine contents in males and females are minimal after 7 days post-infestation [18]. Despite this restriction, manipulation of the worms seemed to induce modifications, with levels varying in the opposite way due to temperature-illumination exposure variations.

Centrifugation and osmotic pressure stress in sucrose solution induced variations of the level of most biogenic amines in worms.

Temperature, light and handling affect the physiology of living organisms, as indicated in this study by changes in biogenic amine contents. Recovery and buffer washing, followed by spontaneous sedimentation or centrifugation methods resulted also in adaptative responses, but to a lesser extent. We could not determine where, precisely, modifications took place, because this was a whole worm assay.

Our findings suggest that sample recovery techniques should be taken into account when measuring biogenic amines, and that experimental culture and recovery conditions should be defined clearly. Combined with information on the physiological state of the population (stage, sex, reproductive period, etc..) and the recovery of samples, LC-ECD is likely to be fruitful for simultaneous studies of biogenic amines in nematodes.

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THE DETERMINATION OF SCOPOLETIN IN ENVIRONMENTAL TOBACCO SMOKE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method was developed for the quantification of scopoletin in environmental tobacco smoke (ETS) for use as an indicator of tobacco smoke in indoor air. Samples were collected on 1 μm pore size Fluoropore membrane filters and extracted with methanol (MeOH). This single extract was used for four analyses including scopoletin after dilution with 1% aqueous acetic acid (HAc). Scopoletin was determined on an octadecyl silane, silica-based column with fluorescence detection. A mobile phase gradient of 1% HAc and 99% acetonitrile + 1% glacial acetic acid was used. The method was reproducible with a relative standard deviation of 6.4%. Percent recovery was 98%. Analyses of smoke from oak leaves indicated the presence of scopoletin, but it was not detected in fireplace smoke. A short sampling time of one h was required and the procedure was capable of detecting < 3 ng m^{-3}.

INTRODUCTION

Scopoletin, a known constituent of tobacco, has been determined by using liquid chromatographic procedures (1,2). These procedures use semi-selective

absorbance detection as the endpoint to determine scopoletin in an aqueous methanol extract of tobacco. Previous work in our laboratory indicated that scopoletin is strongly fluorescing and is present in tobacco smoke (3). Although the reported technique was valid for other phenolic compounds in smoke, scopoletin suffered from a poor recovery from the collection device, a mixed cellulose ester membrane (MCEM).

The procedure reported here is based on the MeOH extract of the Fluoropore membrane used to determine respirable suspended particles (RSP) in indoor air (4). This same sample extract is being used for three other routine analyses in our laboratory: 1) ultraviolet particulate matter (UVPM) (4), 2) fluorescence particulate matter (FPM) (5) and 3) solanesol (6). This reduces the number of separate samples required for these analyses, an essential attribute when conducting field studies.

The method described in this work alleviates the recovery problem of scopoletin associated with earlier techniques (3) and validates its use as a particulate marker for ETS. This is the first reported, reliable procedure using selective fluorescence detection for the determination of scopoletin and the first application of the procedure for the determination of scopoletin levels in indoor air.

EXPERIMENTAL

Chemicals

Scopoletin was obtained from Aldrich (Milwaukee, WI, USA). Acetonitrile (ACN) and methanol (MeOH), "distilled in glass", were obtained

from Burdick and Jackson (Muskegon, MI, USA). Acetic acid (HAc) was reagent grade (Reagents, Inc., Charlotte, NC, USA). Water was obtained from a Nanopure system, which consisted of a carbon cartridge, two high-capacity mixed ion exchange cartridges and a 0.45-μm filter (Barnstead Co., Div. of Sybron Corp., Dubuque, IA, USA).

Equipment

HPLC

The HPLC system consisted of two ABI Spectroflow 400 pumps (Applied Biosystems, Inc., Foster City, CA, USA), an 878A autosampler fitted with a 200-μL sample loop and a Perkin-Elmer LS-4 Fluorescence Spectrometer (Perkin-Elmer Corp., Norwalk, CT, USA). The pumps and the autosampler were controlled by a DS-650 Data System and data were acquired on VAX MULTICHROM (VG Instruments, Danvers, MA, USA). Separations were accomplished on a Beckman Ultrasphere ODS, 4.6 X 150 mm, 5-μm particle size column (Beckman, San Ramon, CA, USA). A 30 X 4.6 mm Brownlee RP-18 Spheri-5 MPLC guard refill in a Brownlee 3-cm MPLC holder (Brownlee Labs, Inc., Santa Clara, CA, USA) was placed directly before the analytical column.

Sampling

The sampling device was a Fluoropore, 1-μm pore size, 37-mm membrane filter (Millipore Corp., Bedford, MA, USA) contained in a cassette (SKC Inc., Eighty Four, PA, USA) sealed with a 37-mm gasket (Sloan Valve Co., Franklin Park, IL, USA), connected by a nylon adaptor (SKC Inc., Eighty Four, PA, USA) and a 4-cm length of 0.635 cm I.D. tubing to the pump manifold. A

Dawson High Volume Air Sampler (Dawson Assoc., Inc., Lawrenceville, GA, USA) was used to draw air through the membrane.

Preparation of Standard Solutions

A stock standard was prepared by dissolving scopoletin in MeOH and diluting to the appropriate concentration with 8:2 (V/V), 1% HAc:MeOH.

Procedure

Chromatographic

All chromatographic separations were performed at room temperature with a mobile phase gradient of 1% HAc (A) and 99% ACN plus 1% glacial acetic acid (B) at a flow rate of 2.0 mL min^{-1}. Initial conditions were 100 % A followed by a linear change in B to 35 % over 10 min. A four min column wash of 100 % B and six min equilibrium delay of 100 % A prior to the next injection were included in the gradient program. The excitation wavelength was 342 nm and the emission wavelength was 464 nm. Quantitative results were obtained by means of an external standard procedure and a complete analysis took 20 min.

Sampling and Sample Preparation

The air sample collection device was precalibrated with a soap film flow meter (The Gilibrator, Gilian Instrument Corp., Wayne, NJ, USA) at about 2 L min^{-1} prior to sample collection. After sampling was complete (1h) the flow was rechecked. Following use, the cassette was disassembled and the membrane transferred to a 4-mL autosampler vial. MeOH (4 mL) was added and the membrane extracted for 30 min using a wrist-action shaker. One part of the MeOH extract was diluted with four parts 1% HAc prior to analysis.

RESULTS AND DISCUSSION

Recovery Re-evaluation

Since poor recovery (< 50%) was obtained in previous work when adding scopoletin to an MCEM (37 mm, 0.8 μm pore size, SKC Inc., Eighty Four, PA, USA) prior to sampling, it was decided to add scopoletin after sampling (3). The recovery results were still poor and even a blank MCEM (not used for sampling) yielded recoveries of only 38.6$\pm$7.2% (n=2).

The method of extraction, sonication for 10 min, was evaluated against wrist-action shaking for 30 min. The recovery from the MCEM did not improve.

A Teflon membrane (Fluoropore, described in the Experimental section) was then evaluated for collection of scopoletin. [The Teflon membrane had been evaluated previously versus the MCEM but was found not as efficient for the collection of catechol, a main component of interest under study (3).] This work showed that the Teflon membrane yielded more scopoletin than the MCEM under the same sampling conditions. It was decided to evaluate the recovery of scopoletin from the Teflon membrane.

A blank Teflon membrane, to which scopoletin was added, gave 103.4$\pm$0.0% (n=2) recovery, unlike the MCEM (see above). It can be concluded from these results that scopoletin either reacts with the MCEM or is irreversibly retained and cannot be completely extracted. The Teflon membrane has a more inert surface which enables quantitative extraction. Table 1 shows that the recoveries are acceptable and the sample results found by external standard quantitation are in agreement with those obtained by standard addition. The Teflon membrane was chosen as the device on which to collect scopoletin.

TABLE 1. Recovery/Standard Addition of Scopoletin Added to the Fluoropore Membrane[a]

Amount Added (ng)	Amount Found (ng total)	% Recovery
108	338	96.2
216	438	94.4
540	786	102.2
		$\bar{x}$ = 97.6
ng m^{-3}: standard addition = 1700, external standard = 1892		

[a] - amount added in MeOH after sampling the smoke of two cigarettes in an 18-m^3 chamber with no air exchange, sampled for one h at 2 L min^{-1}, n=2

Dilution of Sample Extract versus Decrease in Injection Volume

An objective of this work was to conduct different analyses from a single sample. The MeOH extract of the Teflon membrane used for the determination of RSP (4) was currently being used in our laboratory for UVPM (4), FPM (5) and solanesol (6) analyses. The determination of scopoletin from the same MeOH extract would eliminate the need for a separate sample.

It was found that the injection volume of the MeOH extract had to be reduced from 200 μL used in previous work (3) to 20 μL so that the peak shape of scopoletin remained symmetrical. This was due to the sample extract being in solvent (MeOH) which was much stronger than the initial mobile phase (1% HAc). Rather than reduce the injection volume, it was decided to dilute the MeOH initial extract with 1 % HAc in order to reduce the solvent strength. A 1:4, MeOH:1 % HAC, dilution resulted in a symmetrical peak for scopoletin when a 200 μL injection was used. The sensitivity using the 1 % HAc dilution

of the MeOH extract would amount to a 5-fold decrease versus a 10-fold decrease if reduction of the injection volume was used to improve the peak shape. Therefore, it was decided to dilute the initial MeOH extract of the Fluoropore filter. This dilution presented no problems since scopoletin in the samples was of sufficient quantity for detection.

Chromatographic Conditions

A previous method used for the analysis of six other phenolic compounds excluding scopoletin, required a two-step gradient using a 25 cm column and the analysis time was 1 h (3). A change from the 25 cm column to a 15 cm column reduced the retention time of scopoletin from 22 to 18 min, but it was still desired to reduce the runtime still further. Isocratic conditions were evaluated using the 15 cm column, but did not give a greater height response for scopoletin over that obtained using the original gradient when identical standards were evaluated (3). A gradient to 25% ACN+1% glacial acetic acid over 10 min gave increased response, so an increase in the amount of ACN+1% glacial acetic acid to 35% over 10 min was evaluated. This gave even greater response for scopoletin due to its elution in less mobile phase and thus, is the gradient used for analysis. Identical samples evaluated using these conditions yielded similar results, 566 ± 25ng (n=2), to those obtained under previous conditions (3), indicating no coelution of other compounds with scopoletin when using the 15 cm column and 10 min gradient to 35% ACN+1% glacial acetic acid.

Extractant and Amounts

Table 2 shows that MeOH was as efficient as 1% HAc for removal of scopoletin from the Teflon membrane. A 2.5-fold increase in MeOH, 10 mL, did

TABLE 2. Extractant and Amount of Scopoletin Extracted[a]

Extractant (n)	mL	ng±SD
1% HAc (4)	4	256±5
MeOH (4)	4	251±9
MeOH (2)	10	245±11

[a]- two cigarettes smoked in an 18-m^3 chamber, no air exchange, sampled for one h at 2 L min^{-1}

not remove more scopoletin and, therefore, 4 mL MeOH was deemed to be sufficient for quantitative extraction.

Incremental Cigarette Smoking

Table 3 shows that the amount of scopoletin collected was essentially a linear function of the number of cigarettes smoked. It also shows the effect of dilution air when an air exchange rate of 2.5 air exchanges h^{-1} was used during sampling.

Precision

The instrument and overall precision were both found to be acceptable as seen in Table 4. Figure 1 is a typical chromatogram obtained in this overall precision study. The minimum detectable quantity was < 3 ng m^{-3} with an air volume of 0.12 m^3.

Linearity

The response for scopoletin was found to be linear over a 50-fold concentration range, $R^2 = 0.9999$, Table 5. The y-intercept was < 0.2 ng mL^{-1}.

TABLE 3. Environmental Tobacco Smoke Scopoletin from Two, Four and 16 Cigarettes[a]

No. Cigt. (n)	No. of Air Exchanges h^{-1}	ng Collected±SD	ng $cigt^{-1}$
2[b] (6)	0	212±12	106
4[b] (6)	0	496±16	124
16[c] (2)	2.5	654±31	102[d]

[a]- smoked in 18-m^3 chamber
[b]- sampled for one h at 2 L min^{-1}
[c]- sampled for four h at 2 L min^{-1}
[d]- corrected for air exchange

TABLE 4. Overall Precision of Scopoletin Collection (n = 6)

	Instrument[a] (mV)	Overall[b] (ng m^{-3})
	1036	1920
	1033	1578
	1032	1712
	1039	1789
	1039	1782
	1028	1750
$\bar{x}$	1034	1755
SD	4.3	112
% RSD	0.4	6.4
MDQ[c]	----------	2.8

[a]- 10.8 ng mL^{-1}, 200 μL injection volume
[b]- two cigarettes smoked in 18-m^3 chamber, no air exchange, sampled for 1 h at 2 L min^{-1}, Teflon membrane extracted with 4 mL MeOH, 1 mL MeOH extract diluted to 5 mL with 1% HAc
[c]- MDQ = minimum detectable quantity, at twice the signal-to-noise ratio

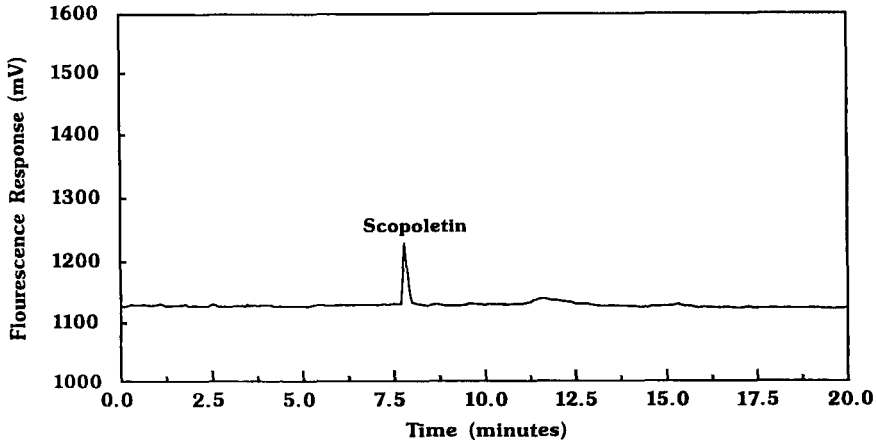

FIGURE 1. Chromatogram from overall precision study. Conditions: see text.

TABLE 5. Linearity of Scopoletin Standard Response

Concentration range (ng mL^{-1})	Height (mV), Fresh	Height (mV), Aged One Mo
1.08	117	140
5.40	536	624
10.80	1065	1253
16.20	1574	1852
32.40	3136	3688
43.20	4118	4810
54.00	5240	6126
	R^2 = 0.9999	R^2 = 0.9998
	Y - Intercept = 16 mV (0.14 ng mL^{-1})	Y - Intercept = 25 mV (0.19 ng mL^{-1})

A re-evaluation of the same set of standards after one month left in the laboratory in the light at room temperature showed $R^2 = 0.9998$ and the y-intercept again < 0.2 ng mL^{-1}. A peculiar difference between these fresh standards versus those that were aged one month was that the aged standards gave a response about 20% higher than the fresh standards. This was not understood but appeared to have no effect on sample results (see below, Storage).

Flow Dependence and Breakthrough

Table 6 gives the results for scopoletin after smoking 16 cigarettes and sampling at four different flow rates. As can be seen, the results in ng m^{-3} are equivalent. The apparent breakthrough at 1.0 L min^{-1} was probably caused by a small leak around the membrane in the first cassette. From these results, one Fluoropore membrane is sufficient for sample collection.

Storage

There was no problem with the storage of the MeOH extract in the presence or absence of the Fluoropore membrane either at 24°C or -2°C. The 1% HAc dilution of the MeOH extract was also stable at these temperatures for two weeks as seen in Table 7. Table 7 also gives the percentage change of scopoletin from samples when stored on the Fluoropore membrane contained in the cassette. There was a significant loss at 24°C after being stored in the laboratory. This loss may be the effect of light, since there was less loss when stored at -2°C in a dark freezer. As a result, samples should be stored in a freezer immediately after collection.

TABLE 6. Flow Dependence and Breakthrough of Scopoletin Collection[a]

Flow rate, L min^{-1}	Amount on 1st membrane, ng	Amount on 2nd membrane, ng	% on 2nd membrane	ng m^{-3}
0.5	156	BDL	------	1300
1.0	323	48	14.8	1346
2.0	649	BDL	------	1352
3.0	1002	BDL	------	1392

[a]- 16 cigarettes smoked in 18-m^3 chamber, sampled for four h, 2.5 air changes h^{-1}, Teflon membrane extracted in 4 mL MeOH, 1 mL MeOH extract diluted to 5 mL with 1% HAc, n=2
BDL = below detection limits

TABLE 7. Percentage Change of Amount of Scopoletin After Two Weeks Storage of Extract and Fluoropore Membrane[a]

	Condition	
Sample (n)	24°C, % Change±SD	-2°C, % Change±SD
MeOH Extract[b,c] (2)	-2.0±2.2	+1.4±1.4
MeOH Extract[c] (2)	+1.4±0.6	+4.4±3.6
MeOH Dilution[c] (4)	+1.4±1.0	+1.1±1.8
Fluoropore Membrane[c] (3)	-35.8±17.2	-10.2±4.7

[a]-two cigarettes smoked in 18-m^3 chamber, no air exchange, sampled for 1 h at 2 L min^{-1}
[b]-Fluoropore membrane left in extract
[c]-1 mL of 4 mL MeOH extract diluted to 5 mL with 1 % HAc

TABLE 8. Scopoletin in Smoke from Other Sources

Source (n)	Volume of Air Sampled (L)	ng m^{-3}±SD
Two Cigarettes[a] (6)	120	1755±112
Oak Leaves[a,b] (8)	120	527±20
Fireplace[c] (4)	120	BDL

[a]- smoked in 18-m^3 chamber, no air exchange, sampled for two h at 2 L min^{-1}, Teflon membrane extracted with 4 mL MeOH, 1 mL MeOH diluted to 5 mL with 1% HAc

[b]- 2 g burned

[c]- three feet from fireplace, Teflon membrane extracted with 3 mL MeOH, 1 mL MeOH diluted to 5 mL with 1% HAc

BDL = below detection limits, < 2.8 ng m^{-3}

Scopoletin from Other Sources

Besides tobacco smoke from a tobacco blend containing flue cured tobacco, a dominant source of polyphenols (1,2), smoke from oak leaves and a fireplace were also sampled for scopoletin (Table 8). Scopoletin has been reported in oak leaves (7) and was found in their smoke. Scopoletin was below detection limits in fireplace smoke.

CONCLUSIONS

A method has been developed for the determination of scopoletin in environmental tobacco smoke from a single MeOH extract which can be used for other analyses. Since amounts above 3 ng m^{-3} can be detected, the procedure is very sensitive to the presence of scopoletin.

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SIMULTANEOUS DETERMINATION OF ε-CAPROLACTAM AND ε-AMINOCAPROIC ACID BY PLANAR CHROMATOGRAPHY

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ABSTRACT

This paper describes the qualitative and quantitative determination of ε-caprolactam and ε-aminocaproic acid, which are potential contaminants of polyamid 6, by instrumental thin-layer chromatography (planar chromatography). A validation of the method is proposed. Detection was performed by photodensitometry in UV range at 200 nm for ε-caprolactam, and at 588 nm after derivatization by ninhydrin reagent for ε-aminocaproic acid. Correlation coefficients for calibration were found about 0.996 - 0.999. Repeatability results were included between 2.4% and 5.5%. Derivatization by Overpressured Derivatization (OPD) technique gave similar results as direct UV detection.

INTRODUCTION

Polyamid 6 (Nylon 6) is a widely used plastic material. The application fields of this polymer are multiple: car manufacture, textile, medical... The monomer of this material is ε-caprolactam. The synthesis way is well known : ε-caprolactam is first synthetized from hydroxylamine. In a further step, ε-caprolactam - cyclic molecule - is hydrolyzed in ε-aminocaproic acid, which is an aliphatic aminoacid. ε-aminocaproic acid is then polycondensed at 120° C, with elimination of water, to produce polyamid 6 (1). Thus, the two main potential impurities or degradation products of polyamid 6 are ε-caprolactam and ε-aminocaproic acid.

Polyamid 6 is described in a monograph of the French Pharmacopoeia X° edition. The limit level of ε-caprolactam required is 1% in the raw material. The assay method is gas-chromatography. However, all the studies performed in our laboratories were negative concerning the GC separation of ε-caprolactam and ε-aminocaproic acid. The method described in the pharmacopoeia's monograph allows only to quantify both products. The difficulty in the separation of the two components may be due to the possible condensation of ε-aminocaproic acid in ε-caprolactam in the injector or in the column (cf synthesis process above) due to the temperature.

The aim of this work is the simultaneous determination of ε-caprolactam and ε-aminocaproic acid separately, as potential contaminants of polyamid 6 used in the packaging of drugs in pharmaceutical industry, or in medical device. Indeed, the migration of ε-caprolactam into parenteral solutions packaged with polyamid has already been described. ULSAKER and TEIEN (2) showed the migration of ε-caprolactam from the envelope through the PVC barrier of an overwrapped PVC bag.

The technique tested is instrumental thin layer chromatography (planar chromatography).

EXPERIMENTAL

The tested sample was a methanolic solution containing 2 mg/ml of ε-caprolactam and 2 mg/ml of ε-aminocaproic acid. The stationnary phase was HPTLC silicagel without fluorescence indicator (Merck - Darmstadt-Germany).

The mobile phase was methanol/chloroform (50/50) in a saturated classical tank. A preliminary washing of the plate was carried out by development in the mobile phase; the aim was, on one hand to reduce the saturation time of the tank, on another hand to obtain a minimal solvent front after development.

Increasing amounts (2-10 mg) of ε-caprolactam and ε-aminocaproic acid were automatically streaked on 3 mm with a TLC-applicator AS30 (Desaga-Heidelberg-Germany).

The development distance was 50 mm from the line of streaks. The duration of development was about 15 min.

The post-chromatographic derivatization step was performed with ninhydrin/collidin reagent (3), by OverPressured Derivatization (OPD) technique (4,5), using a Derivabox° (Europlanaire-Châtenay-Malabry-France). This technique implies the use of a polymer foam which is first impregnated with the reagent, and secondly applied with pressure on the plate. The physical properties of the foam allow to reabsorb the excess of reagent at the pressure release.

The detection was carried out before and after derivatization, using a photodensitometer scanner CD60 (Desaga-Heidelberg-Germany), at a wavelength of 200 nm for ε-caprolactam (before derivatization) and 558 nm for ε-aminocaproic acid (after derivatization). The chromatograms were evaluated by integration of peak areas.

RESULTS AND DISCUSSION

Detection

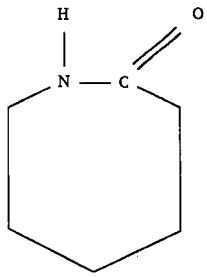
At 200 nm in direct UV detection :

Only ε-caprolactam was detected at 200 nm, because of the structure of this compound compared to ε-aminocaproic acid (fig 1) : the cycle and the amide group induce a light electronic delocalization, responsible for the absorption in the low wavelengths zone (fig 2).

On the contrary, the linear structure of ε-aminocaproic acid does not allow any UV absorption properties.

At 588 nm after OPD by ninhydrin/collidin reagent :

Ninhydrin/collidin reagent reacts with only primary amines, giving blue-violet coloured spots. Thus, it reacts with ε-aminocaproic acid,



E - CAPROLACTAM

E-AMINOCAPROIC ACID

H_2N —— CH_2 —— CH_2 —— CH_2 —— CH_2 —— CH_2 —— COOH

FIGURE 1: Chemical formulas of ε-caprolactam and ε-aminocaproic acid.

which has presents primary amine structure, and not with ε-caprolactam, because of the secondary amine group. The spots of ε-aminocaproic acid gave a maximum at wavelength 588 nm.
Detection at 200 nm is the first mean to differentiate the 2 components.
Detection at 588 nm (visible) is the second suitable way of differentiation.

Chromatographic separation

The Rf determined for the 2 components were about 0,1 (5 mm of migration) for ε-aminocaproic acid, very polar, and about 0,93 for ε-caprolactam (fig 3). The separation is completely realized.
The suitable chromatographic separation is the third mean of differentiation of the 2 components.

Quantitation

Densitometric evaluation was performed laterally, thus only 1 chromatogram was registered to measure all the spots of each

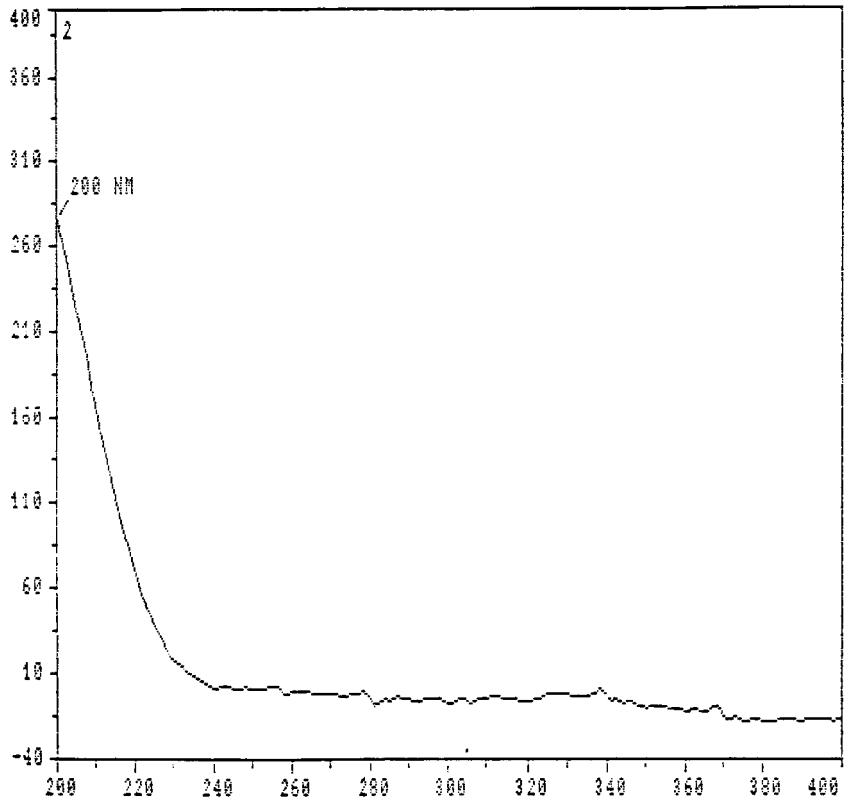


FIGURE 2: UV spectrum of ε-caprolactam.

component (fig 4). The main advantage of this method was the simultaneous integration of all the chromatograms, producing better results (same baseline). On another hand, the possible inaccuracy of the localization of streaks automatically applied is avoided. Indeed, even a little variation of distance between the spots, due to the application device, the detector, or the plate positioning can produce a significant difference.

Example : a variation of 0,1 mm of the distance between the spots at the application step induces a localization error of 1 mm at the detection of the 10^{th} spot.

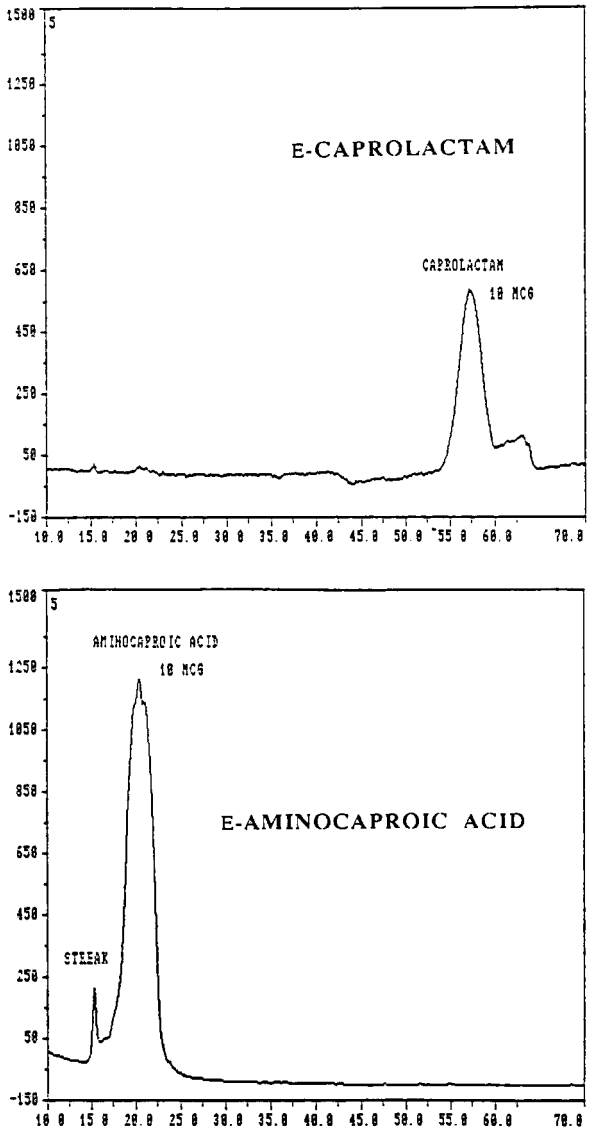


FIGURE 3: Chromatograms of ε-caprolactam and ε-aminocaproic acid.

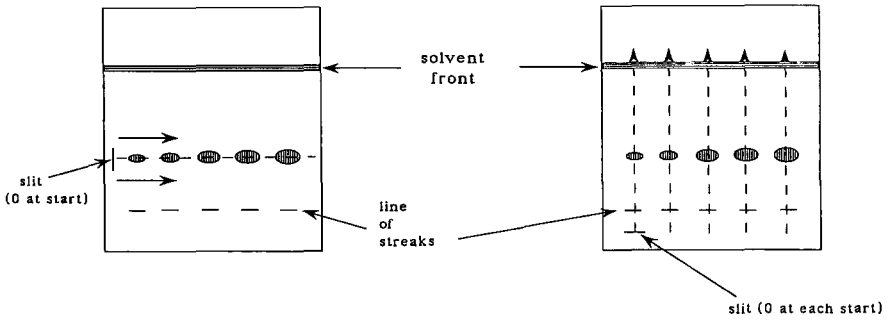


FIGURE 4: Detection modes.

However, before the "lateral" way of detection, it was necessary to perform a previous detection in the "normal" way, to evaluate the low Rf variations between the spots, and the size of the largest one (the highest calibration point), for the choice of the size and the co-ordinates of the slit position.

Calibration

The linearity in the calibration ranges of ε-caprolactam and ε-aminocaproic acid was checked. The chromatograms obtained for the two components are presented in the figure 5. The linear regression correlation coefficients (R^2) were 0,996 for caprolactam and 0,999 for aminocaproic acid (fig 6).

Detection limit

The detection limit for caprolactam was about 0,2 mg, and 0,02 mg for aminocaproic acid (fig 7). The effect of overpressured derivatization by ninhydrin-collidin reagent was to enhance the sensitivity of the detection, compared to direct UV detection of caprolactam.

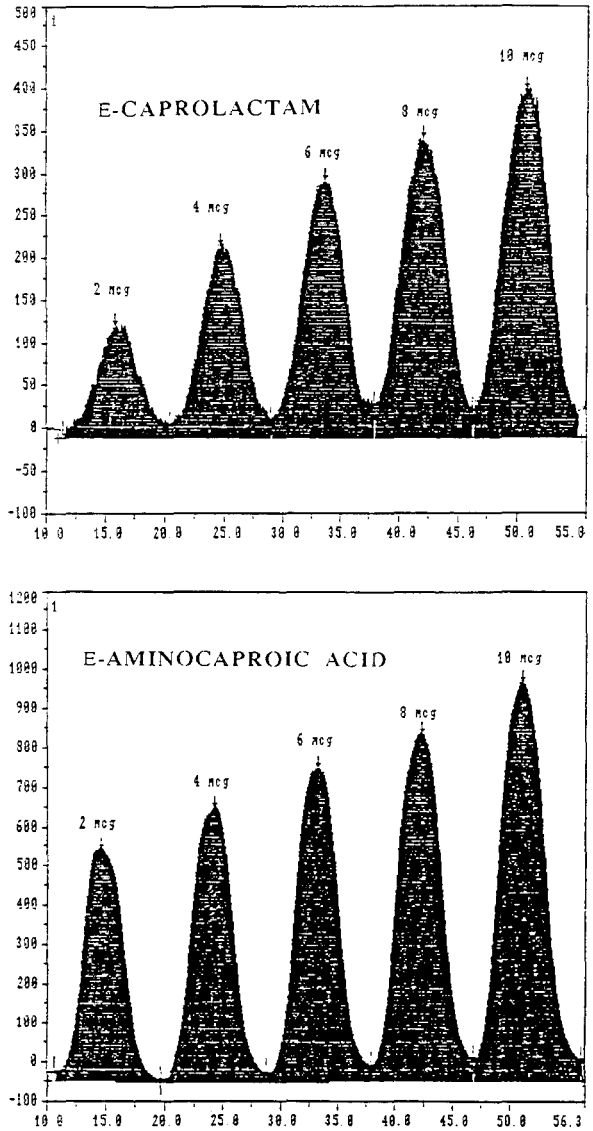


FIGURE 5: Chromatograms of calibration ranges of ε-caprolactam and ε-aminocaproic acid.

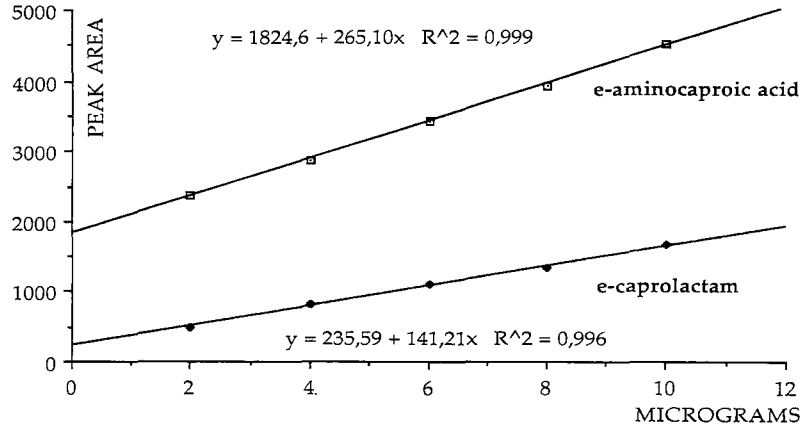


FIGURE 6: Calibration curves of ε-caprolactam and ε-aminocaproic acid.

Overpressured derivatization

OPD with Derivabox° apparatus was very easy-to-use, and produced good results (linearity, detection limit). After derivatization, it was necessary to keep the box air-tighted to avoid the drying of the foam, and to spare the reagent. Indeed, in case of drying, the homogeneity of the distribution of the reagent was not respected when impregnated again : so it was necessary to replace the foam. The manifestations of this "overloading" of the foam were founded on a plate containing 8 spots of the same solution, same volume (6 mg of ε-aminocaproic acid and ε-caprolactam), after development in the same conditions described above. We observed :
- increase of the mean coloration of the spots,
- inhomogeneity of the coloration of the spots : we observed the presence of yellow trails on the plate; the spots localized in these zones were much more coloured than the others.
- fringed outline of the spots.

The figure 8 shows the chromatograms of ε-aminocaproic acid in the repeatability study. The laterally detection mode was used. The chromatographic profile obtained with an "overloaded" foam is

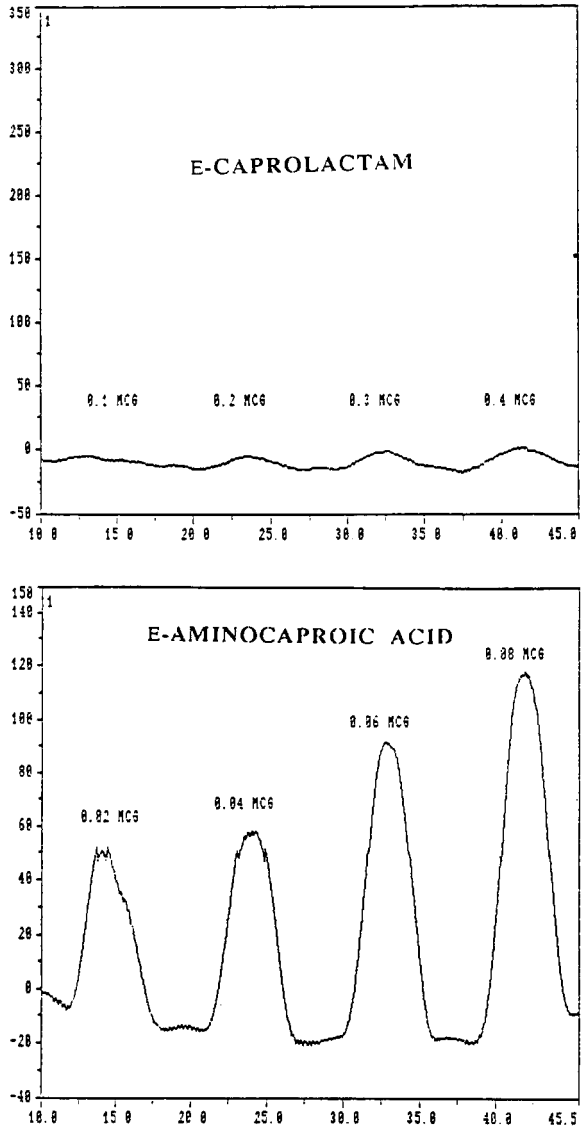


FIGURE 7: Detection limit of ε-caprolactam and ε-aminocaproic acid.

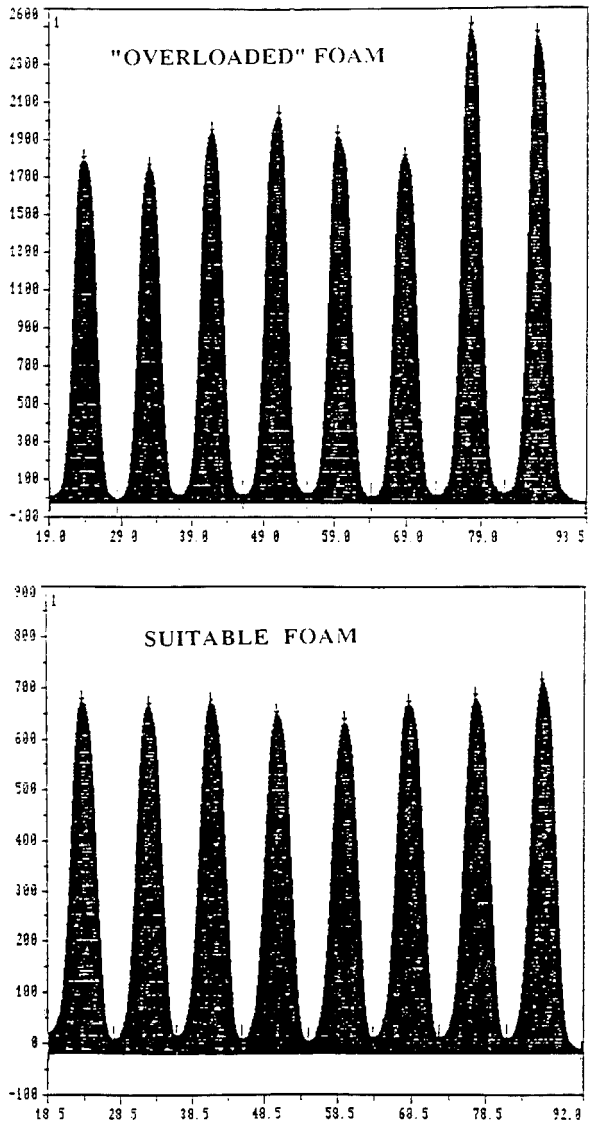


FIGURE 8: Compared chromatograms of ε-aminocaproic acid derivatized with "overloaded" foam or suitable foam.

TABLE 1: Compared repeatability results between UV detection, "overloaded" foam, and suitable foam.

n=8	caprolactam (200 nm)		aminocaproic acid "overloaded" foam	aminocaproic acid suitable foam
Mean of peak areas	995,0	950,9	7206	2856
RSD of peak areas	2,4%	5,5%	10,8%	3,7%

presented, compared to another plate in the same conditions using a new foam: the two spots on the left of the chromatogram obtained after derivatization with the "overloaded" foam were included in a yellow trail on the plate; the signal is therefore very higher.
The results of the absorbance and RSD values are presented table 1: these results show the importance of the quality of the foam in OPD. The RSD obtained with OPD performed in suitable conditions were similar than those obtained in direct UV detection.

In conclusion, the foams used in OPD must be removed and replaced in case of drying. Another case of replacement is the expiry date of the impregnating reagent.

CONCLUSION

Important improvements occured in planar chromatography during the last 10 years, and the field of applications grew rapidly.

The separated determination of ε-caprolactam and ε-aminocaproic acid is particularly interesting, in regard to their simultaneous presence as impurities of polyamid 6, and their difference of toxicity.

Gas chromatography is not suitable in this aim. Planar chromatography allowed to perform this dosage, and the method proposed was validated.

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3 JORK H., FUNK W., FISHER W. and WIMMER H. in : Thin-layer chromatography - Reagents and detection methods - VCH, Weinheim (FRG), Vol 1a., 1990.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 17(12), 2751-2757 (1994)

LIQUID CHROMATOGRAPHY CALENDAR

1994

JULY 31 - AUGUST 5: American Society of Pharmacognosy Annual Meeting, International Research Congress on Natural Products, joint meeting with the Assoc. Français pour l'Enseignement et la Recherche en Pharmacognosie, and the Gesellschaft fur Arzneipflanzenforschung, and the Phytochemical Society of Europe, Halifax, Nova Scotia, Canada. Contact: D. J. Slatkin, P. O. Box 13145, Pittsburgh, PA 15243, USA.

AUGUST 14 - 17: Summer National Meeting & Particle Technology Forum, AIChE, Denver, Colorado. Contact: AIChE Express Service Center, 345 East 47 Street, New York, NY 10017, USA.

AUGUST 21 - 23: Australasian Plastics & Rubber Inst. 7th Technology Convention, Melbourne, Australia. Contact: APRI, P. O. Box 241, Mont Albert 3127, Australia.

AUGUST 21 - 26: 208th ACS National Meeting, Washington, DC. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 29 - SEPTEMBER 2: Synthetic Membranes in Science & Industry, University of Tubingen, Germany. Contact: Dechema e.V., Exhibitions & Congresses, Theodor-Heuss-Allee 25, P. O. Box 150104, D-60486 Frankfurt am Main, Germany.

SEPTEMBER 4 - 9: 4th European Rheology Conference, Seville, Spain. Contact: C. Gallegos, Dept. de Ingenieria Quimica, Universidad de Seville, 41071 Seville, Spain.

SEPTEMBER 5 - 7: 25th International Symposium on Essential Oils, Grasse, France. Contact: Assoc. des Ingenieurs et Techniques de la Parfumerie (A.I.T.P.), 48 ave. Riou-Blanquet, 06130 Grasse, France.

SEPTEMBER 8 - 10: Surfaces in Biomaterials 1994, Scottsdale, Arizona. Contact: Surfaces in Biomaterials Foundation, c/o Ardel Management, P. O. Box 26111, Minneapolis, Minn, USA.

SEPTEMBER 12 - 15: 3rd International Conference on Separations in Biotechnology, University of Reading, U.K. Contact: SCI, Conference Office, 14/15 Belgrave Square, London SW1X 8PS, U.K.

SEPTEMBER 25 - 28: Future of Spectroscopy: From Astronomy to Biology, Ste.-Adele, Que., Canada. Contact: Doris Ruest, Conference Mgr., NRC Canada, Ottawa, Ont., Canada K1A 0R6.

SEPTEMBER 27 - 29: CCPC International Conference on Chemical Process Automation, Houston, Texas. Contact: AIChE Express Service Center, 345 East 47th Street, New York, NY 10017, USA.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

1995

MARCH 6 - 10: PittCon'95: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, New Orleans, Louisiana. Contact: Pittsburgh

Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

1996

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

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

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

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

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

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

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

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

1997

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

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

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

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

1999

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

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

2000

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

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

2001

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

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

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

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

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