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SEPARATION BEHAVIOR OF COMMON FULLERENES IN CYCLODEXTRIN-HPLC BASED ON COMPUTATIONALLY-DERIVED INTERACTION ENERGIES

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ABSTRACT

Molecular modeling and basic thermodynamic considerations are used to explain previously reported HPLC retention behavior of C_{60} and C_{70} using a γ-cyclodextrin stationary phase. Evidence thatinclusion complex formation between the fullerene and γ-cyclodextrin is not essential to resolve C_{60} and C_{70} is presented. Computationally-derived interaction energies alone do not correlate with the observed HPLC retention behavior. However, interaction energy values combined with mobile phase solubility data, non-inclusion interactions, and a discussion of entropy changes due to phase transfer provide a more thorough explanation of retention in this separation system.

INTRODUCTION

Fullerenes have unique chemical and physical properties Recently, this has stimulated considerable research in chemistry

and physics [1]. For further advancement in fullerene research, methods of creating and isolating the various fullerene compounds are essential. The generation of fullerenes is well documented but the separation of the less common fullerenes (higher than C_{60}) from C_{60} is still being developed [2, 3].

Numerous reports of the separation of C_{60} and C_{70} have appeared [4-11]. These include HPLC separations using a C_{18} bonded phase with an n-hexane mobile phase [4], gel permeation columns with a dichloromethane/cyclohexane mobile phase [5], monomeric and polymeric C_{18} bonded phases with toluene/methanol or toluene/acetonitrile mobile phases [6], and γ-cyclodextrin (γ-CD) chemically bonded to silica with an n-hexane/toluene mobile phase [7]. Electrochromatography using a 50 μm inner diameter capillary column packed with 3 μm C_{18} particles has also provided efficient separation of C_{60} and C_{70} [8].

Cyclodextrins (CDs) have proven to be a powerful separation selector when bonded to a stationary phase or as free-flowing mobile phase additives [12-14]. CDs are cylindrically-shaped macrocyclic sugar molecules that possess an axial hydrophobic cavity and an outer hydrophilic surface. The most common CDs are comprised of six (α-CD), seven (β-CD), or eight (γ-CD) glucopyranose units [15]. These molecules provide discrimination based on solute size, shape, hydrophobicity, or can even act as chiral selectors. Cabrera and coworkers [7] and Armstrong and Gasper [16] have reported the use of cyclodextrins in the separation of fullerenes. However, these authors present no or only general explanations of the interaction between the fullerene solutes and the cyclodextrin phase. A more thorough understanding of the molecular interactions

responsible for the observed separation behavior should be possible using interaction energy calculations obtained by way of molecular modeling.

Recently, we have reported correlations between computationally-derived interaction energies and the CD-modified micellar electrokinetic capillary chromatography (MECC) performance of numerous benzo(a)pyrene isomers [17]. We have also successfully compared retention behavior of several derivatized amino acid enantiomers in CD-modified capillary zone electrophoresis (CZE) to interaction energies derived by molecular modeling [18]. In these reports, correct elution order for geometrical and optical isomers was predicted based on molecular modeling studies.

Correlations between molecular modeling data and separation performance in HPLC have also been reported [19, 20]. In relation to this article, Arnold et al. compared the retention times of several solutes using a β-CD bonded column to computationally-derived interaction energies of the corresponding β-CD inclusion complexes. They were able to correlate interaction energies and HPLC retention times within a given series of similarly substituted benzenes.

In this paper we present molecular modeling studies of C_{60} and C_{70} with γ-CD in order to further explain the HPLC retention behavior of these compounds reported by Cabrera et al. [7]. Our data indicates that interaction with the CD phase is not the most important factor in the resolution of C_{60} from C_{70}. Instead, it is differences in the interactions of the fullerenes with the "weakly solubilizing" mobile phases that are employed that leads to the observed separation behavior. Other evidence that supports this

conclusion arises from molecular modeling studies of C_{60} and C_{70} with the smaller, β-CD, as well as HPLC separations using a β-CD column and solubility studies.

EXPERIMENTAL

Molecular Modeling Studies:

Apparatus

An Evans & Sutherland (Salt Lake City, UT, USA) workstation was used to run the SYBYL 6.0 molecular modeling software developed by Tripos Associates, Inc. (St. Louis, MO, USA). The structure of C_{60} was imported from the Computational Chemistry List Archives, while C_{70} was constructed using the BUILD routine of the software. Both were minimized using Gasteiger-Huckel charges with a convergence limit of 0.05 kcal/mol [17, 21]. These minimized structures were used in all subsequent operations. β- and γ-CD structures were generated using crystallographic coordinates imported from the Cambridge Crystallographic Database (Cambridge, England). Centroids were defined for fullerenes and CDs as the point marking the center of mass of the molecule.

Procedure

SYBYL programming language (SPL) was used to generate a routine that permitted operator control of the relative positioning of the fullerene and CD molecules [21]. Specifically, the SPL routine allowed the fullerene's position to be systematically altered relative to the CD cavity. The initial and final positions of the fullerene relative to the CD (as defined by the distance between the centroids of the molecules), translational increments, and rotational increments could all be specified within the SPL routine (see Table I

notes for specific values). Energies of the complex were computed using the Tripos force field of SYBYL, which includes considerations of van der Waals, electrostatic, and hydrogen bonding forces between guest (fullerene) and host (CD). In some instances, the MINIMIZATION function of SYBYL was employed starting at selected of these positions, with the maximum number of iterations defined as 100,000 to insure complete minimization.

The energies of the minimized, free fullerene and CD were subtracted from the computed energy of the complex to give an interaction energy at each translational/rotational position. In this manner, an interaction energy matrix was generated. As stated above, some matrices were created using complexes that were minimized using the MINIMIZATION routine (although this was a very computationally intensive procedure). The matrices were then treated in different manners; e.g., a simple statistical mechanical partition function (Z) calculated by summing over all the values in the matrix using the equation:

$$Z = \Sigma\, e^{-E / kT} \qquad (1)$$

where E is the interaction energy, k is the Boltzmann constant, and T is defined as 298 K.

HPLC Separations :

Chemicals

HPLC grade solvents were used in all work and were purchased from Baxter Scientific (McGaw Park, IL, USA). Fullerene samples (pure C_{60} and the C_{60}/C_{70} mixture) were purchased from Fluka (Ronkonkoma, NY, USA).

Apparatus

Chromatographic separations were performed with an Astec Cyclobond I column (250 mm X 4.6 mm, 5μm d_p) (Advance Separations Technologies Inc., Whippany, NJ, USA). The chromatographic system consisted of a Millipore Model 510 HPLC pump (Millipore, Waters Chromatography Div., Milford, MA, USA) with a 20 μL injection loop and a Spectroflow 757 absorbance detector, operated at 334 nm, (Kratos, Ramsey, NJ, USA). Data acquisition was handled with Peak Simple software (SRI Instruments, Torrance, CA, USA).

Procedure

A mobile phase that was 70:30 (n-hexane:toluene) was flowed at a rate of 1.0 mL/min. The C_{60}/C_{70} fullerene mixture (approximately 10:1 ratio in concentration) was dissolved in toluene and injected. A separate solution of pure C_{60} was used for peak identification.

Solubility determination:

Apparatus

Absorbance measurements were made using an HP Model 8452A (Palo Alto, CA, USA) photodiode array spectrophotometer.

Procedure

Saturated solutions of C_{60} and the C_{60}/C_{70} mixture were prepared by placing the respective solids in 2 mL of hexane and sonicating for 2 hrs. After allowing the solutions to cool to room temperature, they were centrifuged and the supernate was transferred to a cuvette to perform absorbance measurements. These measurements were made in triplicate and the average of the three was used in concentration calculations.

RESULTS AND DISCUSSION

Cabrera et al. present a separation of C_{60} and C_{70} (C_{70} eluting second) using a γ-CD bonded silica column and an n-hexane mobile phase with various concentrations of toluene present [7]. They achieved the best efficiency, in the shortest amount of time, employing a 70:30 (v/v) n-hexane/toluene mobile phase. In that report, they speculate that the resolution of C_{60} and C_{70} is due to the size and shape of C_{70} being more favorable for inclusion complex formation with γ-CD. Based upon interaction energies calculated by molecular modeling (see below), the following discussion presents a more detailed, and quite different, explanation for the observed chromatographic behavior.

In our molecular modeling studies, each fullerene was systematically translated and rotated with respect to the cavity of the CD. An interaction energy was calculated at each of these positions. The lowest (most favorable) interaction energies resulted from the inclusion complex structures depicted in Figure 1. The smaller cavity of β-CD allows for minimal inclusion of C_{60} (Figure 1a), while the larger γ-CD includes the guest molecule to a greater extent (Figure 1b). γ-CD can also act as a host for the C_{70} fullerene if it is oriented in the manner shown in Figure 1c. These structures show that both C_{60} and C_{70} fit into the γ-CD cavity. The ability of a solute molecule to occupy the cavity of a CD is the primary basis of Cabrera et al., and many other researchers' explanations of CD-aided separation behavior. While this is an obvious starting point in describing CD separation systems, it is probably more accurate to consider differences in interaction energies of the individual solutes with the CD. For it is the strength

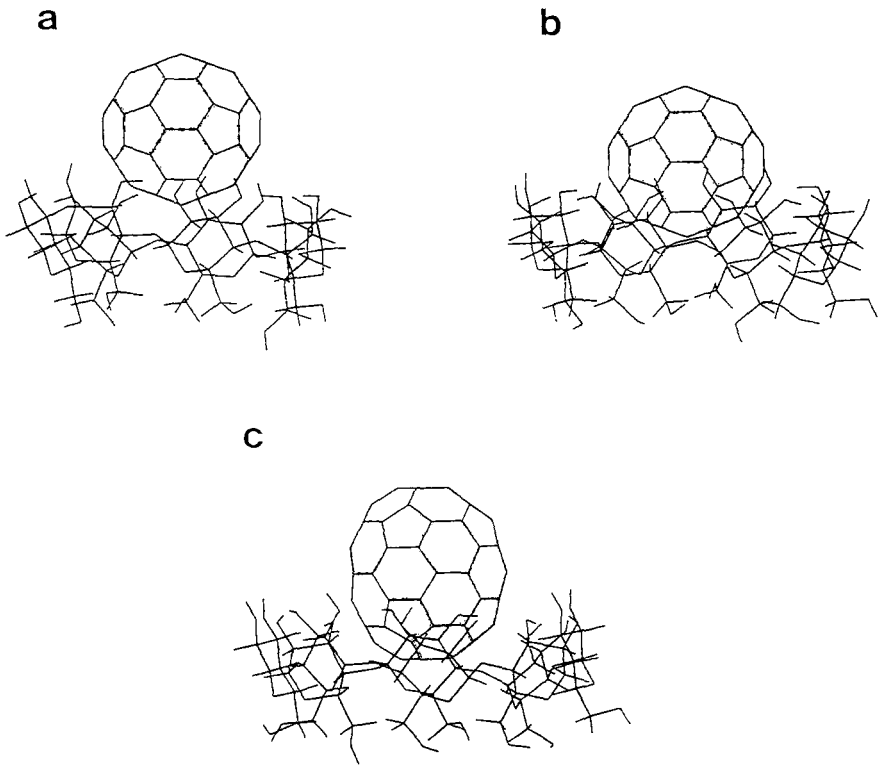

FIGURE 1. Depictions of the lowest energy (most favorable) inclusion complexes. a) C_{60} and β-CD, b) C_{60} and γ-CD, and c) C_{70} and γ-CD.

of a solutes interaction (by inclusion complex formation or non-inclusive association) with the CD that is important. However, in the case of C_{60}/C_{70} separation using γ-CD, our studies reveal that neither molecular fit nor interaction energy differences are major contributing factors (see below).

Interaction energies were calculated using several different methods which were successful in predicting elution order in our

TABLE 1
Molecular Modeling Data

	Z [a]		Z w/min		5 low[d]		5 low[e] w/min	
	γ	β	γ [b]	β [c]	γ	β	γ	β
C_{60}	2.5E20	6.0E16	1.3E22	8.0E22	-25.8	-21.7	-30.3	-30.4
C_{70}	5.1E19	5.8E16	1.5E21	2.1E19	-25.6	-21.5	-27.5	-25.1
C_{70} [f] (side)	6.6E18	5.0E18	5.2E20	1.1E22	-24.4	-24.3	-27.1	-28.8

a) Partition function, calculated using equation (1), over the following translational/rotational space:
Translation: beginning, -7.0 Å, ending, +2.0 Å, increment, 0.25 Å
Rotation: beginning, 0^0 , ending, 360^0, increment, 15^0

b) Partition function, calculated using equation (1), with minimization at each postion:
Translation: beginning, -5.5 Å, ending, -4.0 Å, increment, 0.5 Å
Rotation: beginning, 0^0, ending, 90^0, increment, 5^0

c) Same as b) but with the following matrix:
Translation: beginning, -6.5 Å, ending, -4.5 Å, increment, 0. 5 Å
Rotation: beginning, 0^0, ending, 90^0, increment, 5^0

d) Average of the 5 lowest interaction energies (kcal/mol) resulting from the following matrix:
Translation: beginning, -10.0 Å, ending, +5.0 Å, increment, 0.25 Å
Rotation: beginning, 0^0, ending, 360^0, increment, 15^0

e) Average of the 5 lowest interaction energies (kcal/mol) with minimization at each position:
Translation: beginning, -6.5 Å, ending, -4.0 Å, increment, 0.5 Å
Rotation: beginning, 0^0, ending, 90^0, increment, 5^0

f) This row of data was obtained with the C_{70} molecule oriented such that its longer dimension is parallel to the lip of the CD's cavity.

with β- and γ-CD are presented in Table 1. The partition function and the averages of the five lowest interaction energy values within the energy matrix consistently indicate a slightly more favorable interaction for C_{60} with γ-CD. From this trend, it seems that C_{60} should actually interact more strongly with the γ-CD stationary phase and thus elute slightly after C_{70}. This contradiction between calculated interaction energies and the separation behavior presented in reference 7 is evidence that the enthalpic interaction strength of these common fullerenes and γ-CD is not the sole factor that dictates their separation.

Other factors that must be considered when describing the mechanism by which this fullerene separation occurs include: 1) the entropy change associated with phase transfer (mobile phase:CD phase) of the fullerene, 2) non-inclusion interactions between the fullerene and the CD stationary phase, 3) the enthalpy (as estimated in this report by solubility) of the fullerenes in the mobile phase. These factors can be explained in terms of the fundamental thermodynamic relationship:

$$\Delta G = \Delta H - T\Delta S = - RT \ln K_p \qquad (2)$$

where ΔG, ΔH, and ΔS are the differences in Gibbs free energy, enthalpy, and entropy, respectively, which are associated with the transfer of solute from one phase to the other, R is the universal gas constant, T is the temperature, and K_p is the partition coefficient of the solute. The computations used to generate data in Table 1 consider only the enthalpy of each fullerene when associated with γ-CD. Mobile phase enthalpies are estimated by solubility studies (see below).

In our previous work, we ignored entropy considerations since the solute molecules that we were studying were all non-symmetric and structurally similar [17, 18]. However, in the case of C_{60} and C_{70}, individual entropy values in solution are expected to be different due to a greater degree of symmetry in the C_{60} molecule. Upon interaction (inclusion complex formation) with γ-CD, the entropy of each of these solutes will decrease (negative ΔS). Moreover, it is logical to expect that, due to a greater reduction of rotational freedom, the decrease for C_{60} will be larger. For this

reason, consideration of entropy should better provide a correlation with the observed separation behavior since it would serve to diminish ΔG to a greater extent for C_{60}. Although Cabrera et al. carried out C_{60}/C_{70} separations at more than one temperature, insufficient retention data was provided in their report to generate a van't Hoff plot to compute ΔS values.

Another event that is important in the study of this separation system is non-inclusion interaction between the fullerene and γ-CD. It has been shown that guest molecules can interact with the lip of the CD's cavity to an appreciable extent [22]. This type of interaction could be contributing to the separation of C_{60} and C_{70} using γ-CD. Non-inclusion interactions are predicted by our molecular modeling studies. Specifically, the interaction energy values become increasingly negative (i.e. favorable) as the fullerene is moved closer to the cavity of the CD. However, just prior to inclusion of the fullerene into the CD, these values are only slightly less favorable (2-3 kcal/mol) than those at the lowest energy (inclusion complex) positions.

Other evidence of non-inclusion interactions is seen by the magnitude of the interaction energies for the sideways insertion of C_{70}. In this orientation, the C_{70} molecule cannot form an inclusion complex with γ-CD. However, the average of the 5 lowest interaction energy values is only one kcal/mol greater (less favorable) than those associated with inclusion (see Table 1). Furthermore, neither C_{60} nor C_{70} fit into the β-CD cavity but the interaction energies calculated for these systems are similar to those for γ-CD inclusion.

The discussion above indicates that inclusion complex

formation is not the only possible mechanism for the separation of C_{60} and C_{70} using γ-CD. In fact, it appears that the γ-CD stationary phase functions in a manner that is similar to other organic stationary phases that have been successful in separating these fullerenes. HPLC separations employing monomeric and polymeric C_{18} stationary phases and electrochromatography using C_{18} particles provide resolution of C_{60} and C_{70} (with a k' ratio of 2.0 (C_{70}:C_{60}) in the later case) [6, 8].

Our own HPLC experimental results provide further evidence that inclusion complex formation is not essential in separating C_{60} and C_{70}. Even though significant inclusion of C_{60} or C_{70} into β-CD is not possible (see Figure 1), these compounds were resolved using a β-CD column. We obtained a k' ratio (C_{70}:C_{60}) of 1.9, using the same chromatographic conditions as Cabrera et al.; who obtained a k' ratio of 2.6 with a γ-CD column.

A solubility study was performed in order to roughly estimate the relative enthalpies of these fullerenes in the HPLC mobile phase. We measured the absorbance of saturated solutions of C_{60} and the C_{60}/C_{70} mixture using hexane as a solvent; as this is the mobile phase used by Cabrera et al. The absorbance of the C_{60} solution was subtracted from that of the mixture to obtain the absorbance of C_{70}. Previously reported extinction coefficients of these fullerenes (in toluene) and absorbance values at 406 nm and 474 nm (the wavelengths of major peaks for C_{60} and C_{70} respectively) were used to calculate an approximate concentration of these fullerenes in hexane [23]. The concentration determined for C_{60} (5.9×10^{-5} M) is nearly twice that of C_{70} (3.4×10^{-5} M). It is worth noting that the HPLC separations reported herein and by Cabrera et al. were

performed near the solubility limit of these fullerenes in the hexane mobile phase employed. The greater solubility of C_{60} corresponds to the HPLC retention behavior (C_{60} eluting prior to C_{70}) observed by Cabrera et al. Although this is a very rough method of determining solubilities, the appreciable difference in the calculated values supports the idea that differences in interactions with the mobile phase, not inclusion complex formation, are primarily responsible for the C_{60}/C_{70} separation observed by Cabrera et al.

CDs have been successfully employed in the separation of higher fullerenes. However, in explaining the mechanism by which separation occurs, molecular fit is not the only factor to be considered. It has been shown that mobile phase interactions, non-inclusion interactions, and possibly entropy changes due to phase transfer are also important.

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COMPUTATIONAL CHEMICAL ANALYSIS OF THE CHIRAL RECOGNITION OF BINUCLEAR COPPER (II) OF N-SALICYLIDENE (R)-2-AMINO-1,2-BIS(2-BUTOXY-5-*tert*.BUTYLPHENYL)-3-PHENYL-1-PROPANOL IN LIQUID CHROMATOGRAPHY

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SUMMARY

The chiral recognition of a binuclear copper (II) complex of N-salicylidene-(R)-2-amino-1,2-bis(2-butoxy-5-tert.-butylphenetyl)-3-phenyl-1-propanol was analyzed by computational chemical calculation. The difference in the final energy calculated by molecular mechanics indicated the elution order of enantiomers on this chiral selective molecule.

INTRODUCTION

Chiral recognition is an important means of controlling normal metabolism. The required chiral form is usually the L-form for amino acids and D-form for saccharides, and that of drugs depends on the situation. The prediction of chiral selectivity is therefore very important for drug development. However, such a system is under development. Enantiomers can be separated chromatographically by the selection of an appropriate column and eluent. Such selection is however tedious, and the development of a basic rule is required. On the other hand, differences in molecular interactions have been identified as an energy value difference by computational chemical analysis. The method was applied to study the enantiomer selection of the chiral phase of chromatography.

Chiral recognition mechanism indicated by means of a comparison of the chromatographic behavior of modified chiral phases [1]. The analysis of chiral complexes by NMR and IR indicated that hydrogen bonding is important [2]. Furthermore, hydrogen bonding formation was analyzed by X-ray crystallography [3]. First the conformation of chiral phases derived from N-(3,5-dinitrobenzoyl)amino acids was theoretically studied [4], followed by the chiral recognition of (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol [5]. Further analysis of chiral phases with different analytes indicated that hydrogen bonding was important role for chiral recognition [6]. Complexes of (S)-methyl N-(2-naphtyl)alaninate with N-(3,5-dinitrobenzoyl) leucine n-propylamide were studied from the perspective of the interaction energy difference calculated by AM1, and through-space field effects was important for chiral recognition [7]. The chiral selectivity of (S)- and (R)-(1-naphthylethyl)-carbamoylated β-cyclodextrin bonded phases was studied using experimentally obtained free energy. The feasibility of predicting enantiomer separation was proposed [8].

The chiral recognition of N-butylylvaline-tert.-butylamide for (R)- and (S)-4-nitrobenzoyl amino acids was investigated using molecular mechanics calculation of CAChe™ [9]. Chiral recognition has been studied based upon the ligand exchange mechanism of binuclear copper (II) complex of N-salicylidene-(R)-2-amino-1,1-bis(2-butoxy-5-tert.-butyl-phenyl)-3-phenyl-1-propanol [10].

EXPERIMENTAL

The computer used for the calculations was a Macintosh IIfx, and the software for the computational chemical calculations was CAChe™ from Sony-Tektronix (Tokyo, Japan).

RESULTS AND DISCUSSION

A chiral recognition molecule, binuclear copper (II) complex of N-salicylidene-(R)-2-amino-1,2-bis(2-butoxy-5-tert.-butylphenetyl)-3-phenyl-1-propanol and its analytes were constructed using the molecular editor program and their structure was optimized by molecular mechanics (MM2). The optimization was performed as the energy change was less than 10^{-6} Kcal/mol. The molecular weight of the chiral phase was 1449, and the final and van der Waals energies were 77.04 and -8.77 Kcal/mol, respectively. Three dimensional analysis suggested that the binding site is at the front of the structure shown in Fig. 1, due to the wide open space compared with the other side, where the site of the copper atom was very narrow. The nitrogen atom of the amino group or oxygen ion of carboxy group of analytes was bound with one copper atom which was more visible than the other. The water molecule, however was not bound to the copper atom which forms a hexadentate complex, because it did not geometrically interrupt the complex formation and the location should be opposite that of the analyte.

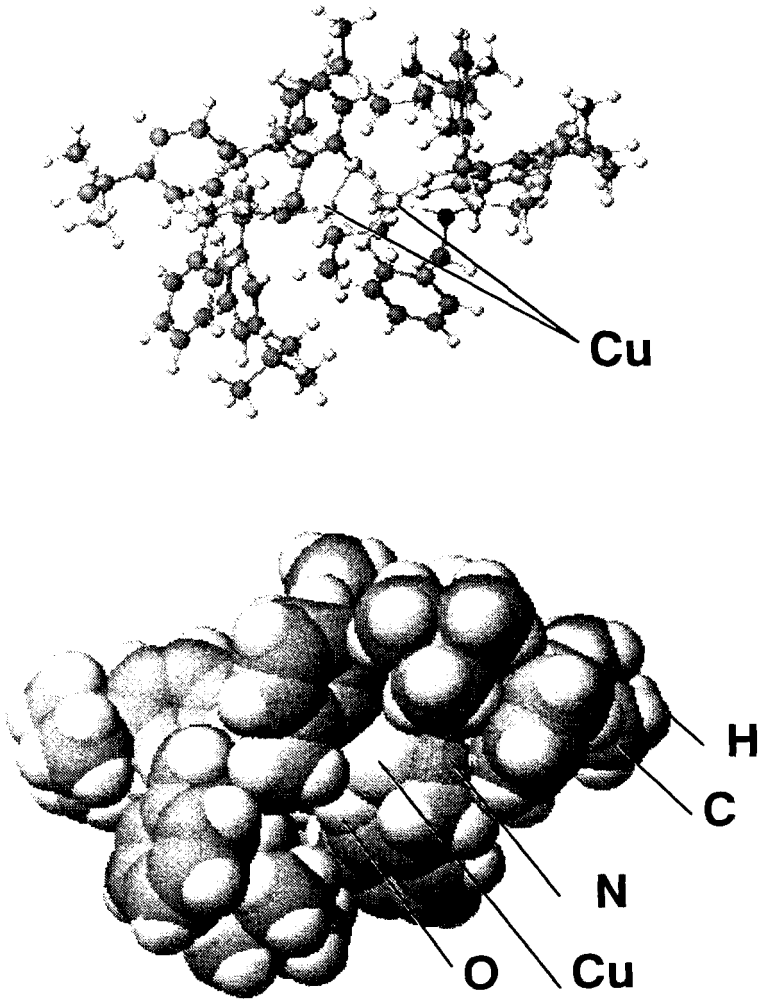

Fig. 1 Molecular structure of N-salicylidene-(R)-2-amino-1,2-bis(2-butoxy-5-tert.-butylphenetyl)-3-phenyl-1-propanol drawn at different atomic sizes (100 and 20%)

The calculated final and van der Waals energies are summarized in Table 1 with the values of the separation factor (α) taken from the literature [11]. The difference in the final energy value calculated from the molecular mechanics indicated chiral selectivity. The higher energy value of the complexes means that the elution is faster. The more stable the complex form is, the lower are the energy values. The energy difference roughly indicated the a values, however the correlation was not good enough for predictive accuracy. This may be due to the geometrical difference. The calculation was achieved in unlimited space, and the separation was performed in a very limited space if the packing material was well coated. The separation factor was also affected by the selectivity of the eluent.

The values of the van der Waals energy did not indicate the elution order, however, they were usually low for stable complexes. An example of complexes with (R)- and (S)-tyrosines is shown in Fig. 2a-d. Figure 2a and b shows front views of complexes with (R)- and (S)-tyrosines, and Fig.2c and d show upper views of complexes with (R)- and (S)-tyrosines.

The final energy values of the (R)- and (S)-tyrosine complexes are 77.5 and 68.3 Kcal/mol, respectively. These values are greater than the sum of the chiral phase (77.0 Kcal/mol) and tyrosine (-13.3 Kcal/mol). When the complex was formed at the opposite side of chiral phase, the final energy of the (S)-tyrosine complex was 153.1 Kcal/mol and that of the (R)-tyrosine complex was 141.0 kcal/mol. The energy difference indicted enantiomer separation, however the energy values were too big, and such complex formation is doubtful. The final energy of other complexes on the opposite side such as those of octamine, phenylglycinol, phenylglysine, 2-amino-1-phenylethanol and 1,2-diphenylethylamine, was also about 150 Kcal/mol. Complex formation on this side is undesirable.

Furthermore, chiral selectivity was studied by means of the molecular interaction model used in the analysis of enanti-

Table 1 Physical properties and separation factor (α) of enantiomers

Compounds	α	1*1	e*2	R-form FE*3	R-form VE*4	S-form FE*3	S-form VE*4
Amino alcohols							
2-Amino-1-phenylethanol	1.19		A	72.63	-5.41	70.24	-5.30
Atenolol	1.07		A	92.13	-1.98	94.99	-6.54
p-Hydroxynorephedrine (1R2S & 1S2R)	1.13		A	77.73	-3.40	79.01	-0.02
Norphedrine(1R2S & 1S2R)	1.11		A	80.26	-3.12	81.67	1.48
Normetanephrine	1.15		A	71.10	-5.28	71.66	-6.46
Norphenylephrine	1.23		A	69.96	-5.45	70.60	-6.14
Phenylalaninol	2.04		A	86.58	0.35	81.61	-2.30
Phenylglycinol	1.35		A	83.20	-6.50	74.55	-5.94
Propranolol	1.06		B	186.49	43.34	166.13	29.14
Amines							
α-Amino-e-caprolactam	1.91		A	94.38	-4.47	85.26	-6.17
Homocysteine thiolactone	1.19		A	110.58	-10.26	104.36	-8.55
Octopamine	1.30		A	69.19	-7.28	67.89	-5.47
Ketamine	1.26		B	173.54	32.55	188.35	34.80
1,2-Diphenylethylamine	1.64		B	68.41	-2.69	65.97	-6.38
1-Phenyl-2-(p-tolyl)ethylamine	1.62		C	68.38	-1.59	62.01	-7.60
Amino acids							
Aspartic acid	1.11	S	A	73.85	-4.21	77.23	-3.62
Histidine	1.18	R	A	96.72	-8.62	89.58	-10.66
Isoleucine	1.15	S	A	97.24	-0.91	99.47	-4.12
Leucine	1.09	S	A	90.96	-6.82	96.14	-6.93
t.-Leucine	1.34	S	A	97.36	0.43	109.10	3.51
Methionine	1.30	R	A	69.19	-7.28	67.89	-5.47
Phenylglycine	1.24	R	A	79.49	-1.66	71.86	-5.01
Proline	1.22	S	A	84.79	-8.34	91.32	-7.95
Serine	1.19	R	A	89.40	-5.87	86.51	-4.27
Tyrosine	2.06	R	A	77.50	-5.28	68.31	-6.97
Valine	1.29	S	A	89.24	-4.93	95.32	-2.83
3-Aminobutyric acid	1.20		A	88.01	-3.05	83.14	-3.37
3-Amino-2-methylpropionic acid	1.08		A	81.79	-6.92	80.23	-3.76
Phenylalanine	1.74	R	B	79.67	-5.48	72.36	-6.92
Tryprophan	2.05	R	B	83.61	-4.32	76.55	-8.52
Hydroxy acids							
Glyceric acid	1.13		B	84.55	-7.63	87.22	-8.25
2-Hydroxybutylic acid	2.19		B	94.71	-4.76	88.95	-9.12
3-Hydroxybutylic acid	1.16		B	76.81	-9.72	76.51	-8.97
Lactic acid	1.56		B	80.10	-7.56	86.29	-8.01

1: first eluted compound, 2: eluent A: 1mM copper(II) sulphate in water, B: 2mM copper (II) sulphate in 15% aq. acetonitrile, C: 2mM copper (II) sulphate in 20% aq. acetonitrile , 3: final energy (Kcal/mol), 4: van der Waals energy (Kcal/mol).

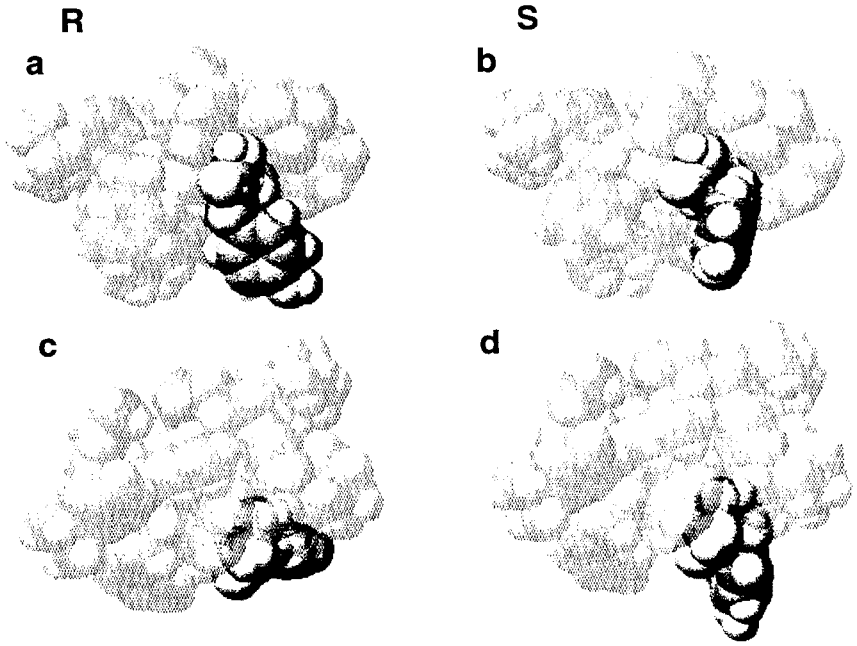

Fig. 2 (R)- and (S)-tyrosine complexes with N-salicylidene-(R)-2-amino-1,2-bis(2-butoxy-5-tert.-butylphenetyl)-3-phenyl-1-propanol. a and b : front view, c and d : upper view, respectively

omer selectivity in normal phase liquid chromatography [9]. The molecular mechanics calculation was performed after the amino group of an analyte was placed near the copper atom, but not bound to it. The calculated energy was low compared that of bound compounds, and the values of (R)- and (S)-form complexes were nearly equal, which did not indicate chiral recognition. The final energy values of the molecular interaction for (R)- and (S)-phenylglycine with this chiral phase were 58.4 and 60.9 Kcal/mol. Those for (R)- and (S)-1,2-diphenylethylamine, (R)- and (S)-octamine were 57.3, 58.2, 59.8 and 59.8 Kcal/mol, respectively. Further study of other

chiral recognition models will result in a method of column selection, and help to design new chiral phases.

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SALT CONCENTRATION EFFECTS IN HIGH-PERFORMANCE HYDROPHOBIC-INTERACTION CHROMATOGRAPHY IN COMPARISON WITH NMR OF PROTEINS IN SOLUTION

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ABSTRACT

The effects of salt concentration on the chromatographic behavior of cytochrome C, ribonuclease A, and α-chymotrypsinogen A in hydrophobic interaction chromatography (HIC) has been examined by isocratic elutions on a Bio-Gel TSK Phenyl 5 PW column. In some cases, conformational variations were manifest chromatographically by reproducible changes in peak shape and appearance of multiple peaks as a function of sodium sulfate concentration in the mobile phase. A parallel study by proton nuclear magnetic resonance (NMR) spectroscopy on the salt concentration dependence of the spectral property of these proteins is in agreement with the possible contribution of the mobile phase composition to the observed chromatographic behavior.

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INTRODUCTION

HPLC has found in the last ten years extensive application in the high-resolution analysis and purification of many proteins and other biological macromolecules, as a result of the development of highly efficient columns.

However, proteins are sometimes eluted from HPLC columns as multiple or/and irregular-shaped peaks. This has been ascribed to several causes, including heterogeneity in the interactive sites of the stationary phase (1), aggregation (2), gradient artefacts (3), and denaturation (4). Proteins are stabilized by a combination of hydrogen bonding, electrostatic interactions, and hydrophobic interactions. The same forces are also involved in the chromatographic processes, thus conformational changes may occur induced by mobile or stationary phase or both, leading to loss of bioactivity and denaturation. The native and denaturated forms can be resolved if the kinetic process of conformational change is slow relative to the time scale of migration through the column, otherwise, a single broad peak will be obtained, which is the weighted average of the forms in equilibrium.

Hydrophobic-interaction chromatography (HIC) with weakly hydrophobic stationary phases and mostly with the use of decreasing gradients of stabilizing salts (e.g., ammonium and sodium sulfate) (5) has gained wide acceptance as a mild non-denaturating HPLC technique (6-11). Nevertheless, also in HIC proteins can yield broad or multiple peaks, depending on the chromatographic conditions and lability of the protein (12-14).

Biological activity (15), second-derivative UV spectroscopy (12), fluorescence spectroscopy (16), and circular dichroism spectroscopy (13,15,17) have been used to detect conformational variations induced by the chromatographic process.

In the last decade, nuclear magnetic resonance (NMR) spectroscopy has emerged as a powerful and versatile tool for the structural study of proteins in solution (18). Proton NMR is capable of distinguish conformational variations of proteins either by determining the whole structure through complex two dimensional methodology for complete resolution and assignment of resonances to the amino acids in the sequence

0.45-μm membrane filter (Millipore, Bedford, MA, USA) and degassed by sparging with helium before use as eluents. After each change of mobile phase composition a period of 30 min (± 0.5 min) was allowed for equilib(19), or more simply by comparing the line width of resonances in different conditions, such as pH, temperature, buffer or protein concentration (18,20). In particular, conformational variations of proteins as a function of ionic strength have been investigated by ^1H NMR (21-22).

In this paper, we examine the salt concentration dependence of the HIC behavior of ribonuclease A, α-chymotrypsinogen A, and cytochrome C and the variations occurring in the ^1H NMR spectra of these proteins in solutions containing sodium sulfate at the same salt concentrations used to perform the chromatographic experiments.

EXPERIMENTAL

Materials

The proteins ribonuclease A, α-chymotrypsinogen A, and cytochrome C were purchased from Sigma (St.Louis, MO, USA). Reagent-grade monobasic sodium phosphate, dibasic sodium phosphate, sodium sulphate, sodium hydroxide, as well as HPLC-grade water were obtained from Carlo Erba (Milan, Italy). Deuterated water (99.8%) was purchased from Stholer Isotope Chemicals (Rutherford, NJ, USA), and 2,2-dimethyl-2-silapentane-5-sulfonate was supplied by Aldrich (Milwaukee, WI, USA).

Chromatography

The experiments were performed with a Beckman (Fullerton, CA, USA) Model 342 Liquid Chromatograph, consisting of two Model 114 M solvent delivery pumps, a Model 420 system controller, a Model 340 dynamically-stirred high-pressure mixer, and a Model 163 variable wavelength UV detector. Samples were injected with a Rheodyne (Cotati, CA, USA) Model 7125 sample valve equipped with a 20 microliter sample loop. Chromatograms were obtained with a Model 5117 Omniscribe (Houston Instrument, Gistel, Belgium) strip chart recorder, or with a Shimadzu (Kyoto, Japan) Model C-R5A Chromatopac Integrator. A Bio-Gel

TSK Phenyl 5 PW column (75 x 7.5 mm) was supplied by Bio-Rad Labs. (Richmond, CA, USA). The chromatographic experiments consisted of isocratic elution at several salt concentration in the range from 0 to 1.0 M sodium sulfate. The background electrolyte was 50 mM phosphate buffer, prepared by mixing 25 mM monobasic sodium phosphate with 25 mM dibasic sodium phosphate. All solutions were filtered through a type HAration. Protein solutions (0.1 mM) were freshly made up in HPLC-grade water. A 20-μl sample size was injected. Proteins were detected by monitoring the column effluent at 280 nm.

NMR

The ^1H NMR spectra were obtained at 200.13 MHz on a Bruker (Fallanden, Switzerland) Model AC 200 Spectrometer. The spectra were run in D_2O at 294 k. Residual water signal was suppressed by pre-saturation (23), and its absorption frequency was used as internal standard and referred to external 2,2-dimethyl-2-silapentane-5-sulfonate. The stability of the water chemical shift within 2 Hz was verified after each experiment. The peaks were assigned according to Bradbury et al. (24). For each protein, either a 0.1 or a 0.05 mM solution was prepared in 50 mM D_2O sodium phosphate buffer, pH 6.9 (low-salt conditions). Part of this solution was then added with sodium sulfate up to 1.0 M concentration (high-salt conditions). Samples at 0.25, 0.50, and 0.75 sodium sulfate were obtained by mixing appropriate volumes of the low-salt solution with the high-salt solutions.

RESULTS AND DISCUSSION

The effect of salt concentration on the chromatographic behavior of ribonuclease A, cytochrome C, and α-chymotrypsinogen A was investigated by eluting these proteins under isocratic conditions with mobile phases having different salt concentrations.

Figure 1 presents the isocratic elutions of ribonuclease A with mobile phases at several salt concentrations. Different peak shapes are observed in the salt concentration range from 0.25 to 0.75 M. With the mobile phase containing up to 0.25 M sodium sulfate, ribonuclease A was eluted as a

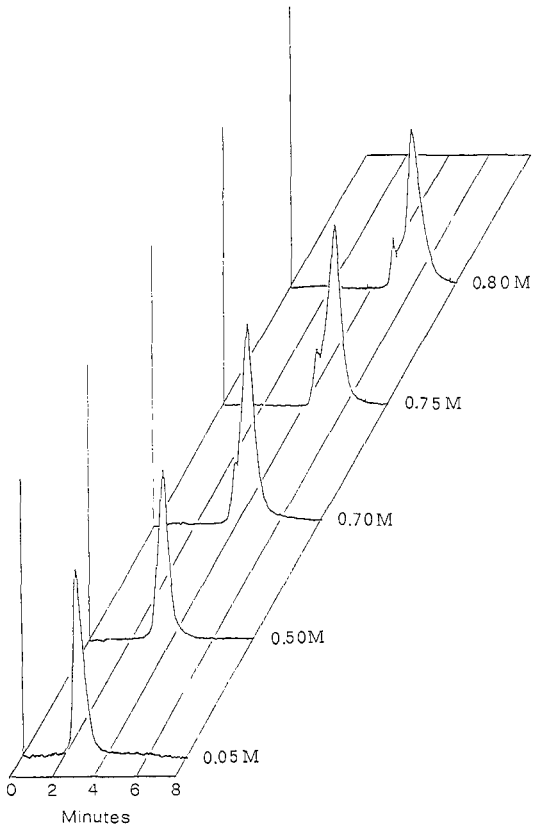

FIGURE 1. Effect of sodium sulfate concentration on the elution profiles of ribonuclease A on the Bio-Gel TSK Phenyl 5 PW column (75 x 7.5 mm I.D.). Background electrolyte, 50 mM phosphate buffer (pH 6.9); flow rate, 1.0 ml/min; detection, 280 nm, 0.05 a.u.f.s.; temperature, 21°C.

relatively sharp peak. As the salt concentration was raised, a shoulder on the upslope of the chromatographic peak was observed, and a peak splitting occurred at higher salt concentration. The peak splitting and band broadening increased with increasing the salt concentration in the mobile phase. Fractions were collected from each peak and individually rechromatographed under isocratic conditions with the mobile phase containing 0.8 M sodium

sulfate. A chromatogram similar to that reported in Figure 1 was observed. The area ratio of the two peaks were the same for both the parent protein sample and the rechromatographed peak sample. This result leads to the conclusion that the observed second peak arises from ribonuclease A itself and not from impurities. Several examples of similar two-peaks separations have been previously observed in HIC of proteins and in most cases has been ascribed to conformational changes occurring on the column (14-16). The main factors found to effect the conformational variations of proteins in HIC are the stationary phase hydrophobicity (14,16), the contact time between the protein and the stationary phase (14, 16), the temperature (12-14,16), the pH (14), and the mobile phase composition (14).

The effect of salt on the conformational stability of proteins in aqueous solution is a function of both the ionic species present and their concentration. Proton NMR spectroscopy is well suited to investigate aspects of protein structure in solution which may be affected by solvent composition.

Figure 2 presents the ^1H NMR spectra of ribonu-clease A in solution at various salt concentration ranging from 0 to 1.0 M sodium sulfate. By increasing the salt concentration, two variations are observable in the NMR spectra, both involving the broadening of related lines. The broadening of signals due to aromatic residues (6.5-7.5 ppm), i.e. aromatic residues are less exposed to the solvent by increasing the salt concentration. In the meantime resonances at 0.8 ppm, due to methyl groups in the proximity of aromatic residues undergo the same broadening. This indicates that hydrophobic portions of the protein stiffen by increasing the salt concentration. Broadening of signals due to the methyl groups of serine residues indicates that also these hydrophilic residues are involved in the stiffening, whereas the polar arginine and lysine residues (signals at about 3 ppm) do not show any variation.

The salt concentration dependence of the retention behavior of α-chymotrypsinogen A and cytocrome C on the HIC column under isocratic conditions is illustrated in Figure 3 as plots of logarithmic retention factor against the salt concentration in the mobile phase. At salt concentration higher than 0.5 M, α-chymotrypsinogen A was strongly retained and did not elute from the HIC column. The plot of the logarithmic retention factor as a

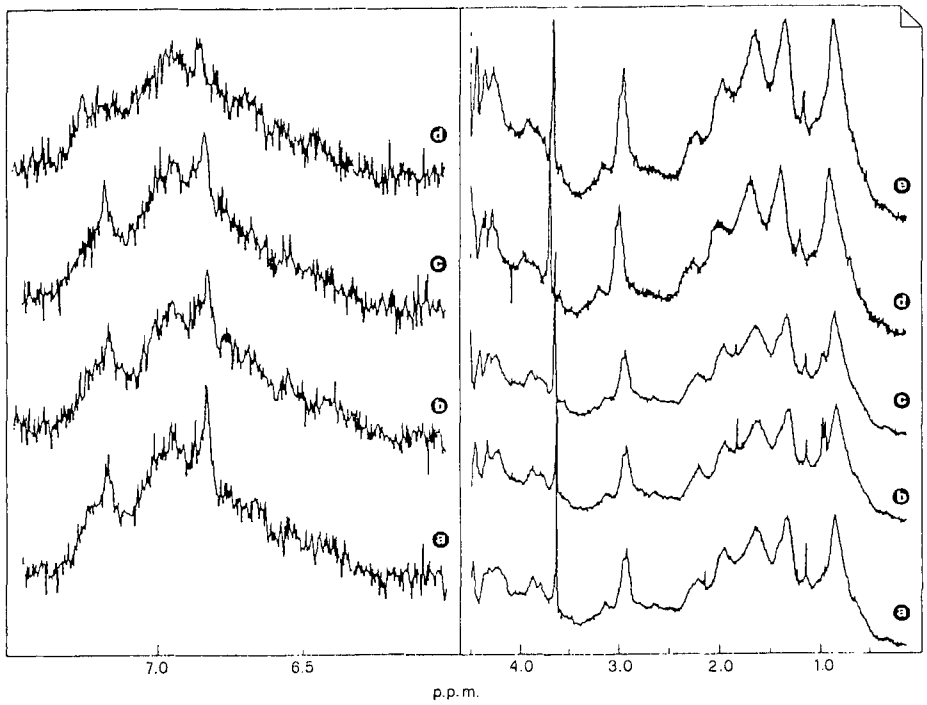

FIGURE 2. ^1H NMR spectra at 200.13 MHz (1600 scans) of a 0.1 mM solution of ribonuclease A in 50 mM D_2O sodium phosphate buffer (pH 6.9), containing no salt (a), and 0.25 M (b), 0.50 M (c), 0.75 M (d), and 1.0 M (e) sodium sulfate.

function of salt concentration in the range from 0.2 to 0.5 M yields a straight line having a positive slope, the magnitude of which, according to the theory (25-27), is expected to be proportional to the exposed hydrophobic surface area of the protein. Thus, the slope of the plots reported in Figure 3 may provide a scale of the hydrophobic character of proteins (27).

The ^1H NMR spectra of α-chymotrypsinogen A in solutions containing salt concentrations in the range from 0 to 0.75 M sodium sulfate are reported in Figure 4. ^1H NMR signals, already broad at low salt concentration, show a further broadening by increasing salt concentration.

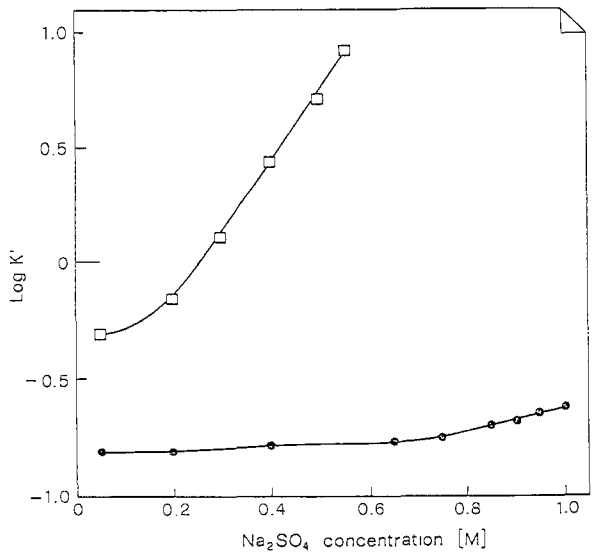

FIGURE 3. Plots of logarithmic retention factor of cytochrome C (•) and a-chymotrypsinogen A (□) against sodium sulfate concentration in the mobile phase. All conditions as in Figure 1.

The presence of large unresolved peaks does not allow any precise NMR assignment. A possible interpretation of this behavior is the formation of aggregates. The self-association characteristics of α-chymotrypsinogen A in aqueous solutions have been reported by many authors and have been related to changes in pH, ionic strength, temperature, or specific nature of the medium (28-31). Furthermore, aggregation would be consistent with the observed appearance of an opalescence in both 0.1 and 0.05 mM protein solutions containing sodium sulfate at concentration higher than 0.75 M, which did not allow NMR measurement, and with the highly hydrophobic character of α-chymotrypsinogen A, as it can be inferred by the retention behavior in HIC depicted in Figure 3.

Cytochrome C is the less hydrophobic protein among the other investigated here and was weakly retained on the phenyl column. Under

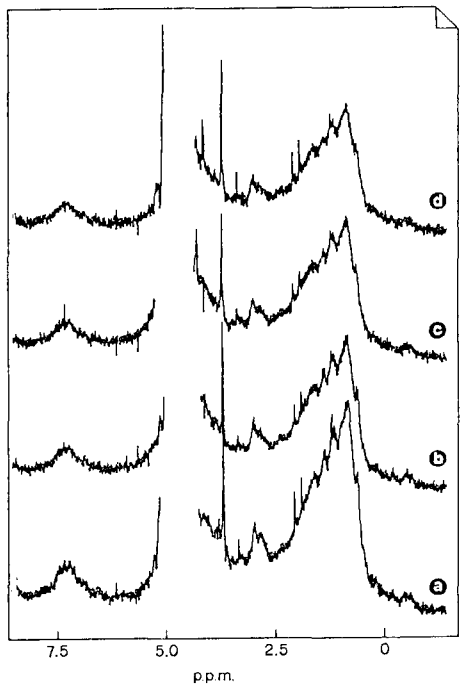

FIGURE 4. ^1H NMR spectra at 200.13 MHz (3200 scans) of a 0.05 mM solution of a-chymotrypsinogen A in 50 mM D_2O sodium phosphate buffer (pH 6.9), containing no salt (a), and 0.25 M (b), 0.50 M (c), and 0.75 M (d) sodium sulfate.

isocratic conditions, an increase in salt concentration lead to a small increase in retention time (see Figure 3), and no noticeable variations in peak shape. The ^1H NMR spectra of cytochrome C were also little effected by the salt concentration in the solutions. Figure 5 shows that besides the broadening of the signals in the range from 6 to 8 ppm, relative to aromatic residues, there are no significant variations of the NMR spectra of cytochrome C with increasing salt concentration.

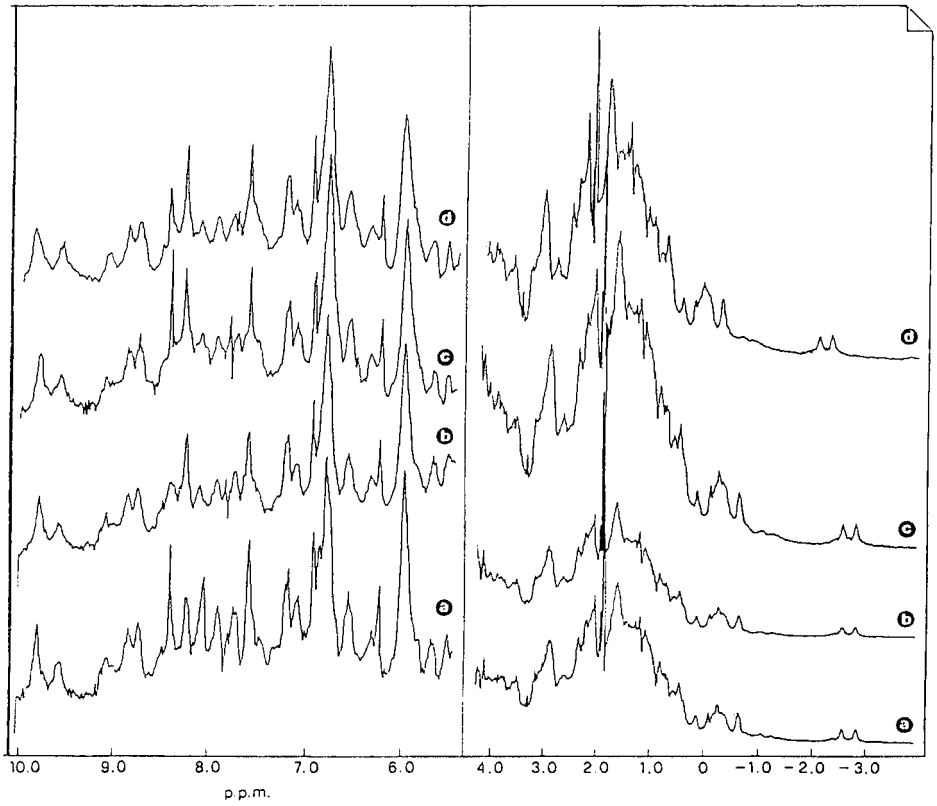

FIGURE 5. ^1H NMR spectra at 200.13 MHz (1600 scans) of a 0.1 mM cytochrome C solution. Conditions as in Figure 4.

CONCLUSIONS

This paper represents an attempt to correlate the salt concentration dependence of the ^1H NMR spectra of proteins in aqueous solutions to the possible contribution of the mobile phase effects on the chromatographic behavior of these biopolymers in HIC. Although it is restricted to a limited number of experiments and proteins, it shows that ^1H NMR may represent a

strategy to investigate variations in the protein structure due to solvent composition, which can be related to the mobile phase mediated chromatographic behavior of these biopolymers.

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COMPARATIVE STUDY OF DIFFERENT PREDICTIVE METHODS OF PEPTIDES RETENTION TIME ON CHROMATOGRAPHIC REVERSED-PHASE COLUMNS

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ABSTRACT

Two peptides samples were studied in order to compare the validity of some predictive retention times. We examined the predictive retention times stated by Guo and based on the relative hydrophobic contribution of each amino acid residue, by Mant who takes into account the length of the peptides and by Chabanet who discriminates three groups of amino acids. Although Chabanet's model provided good results, we found that a more general correlation was obtained with Mant's retention prediction especially in the case of peptides containing a high fraction of basic residues.

INTRODUCTION

Peptide mapping is a commonly used technique to analyse the structure of a protein. This technique involves the enzymatic hydrolysis of the protein, followed by a reversed-phase high performance liquid chromatography (RP-HPLC) of the digest. RP-HPLC allows a good separation of a large range of peptides on the basis of small changes in polarity and length when a A-B gradient eluent is applied (A : H_2O ; 0,1% trifluoro-acetic acid (T.F.A) / B : acetonitrile (CH_3CN) ; 0,1 % T.F.A).

Furthermore, RP-HPLC provides information about the amino-acid composition of peptides : thus in 1981, Meek [1, 2] could predict the retention times of small peptides by summing the relative hydrophobic contribution (Rc) of every constitutive amino-acid residues and terminal groups (terminal amino group and carboxylic function).

$$T_{retention} = \Sigma Rc \qquad (1)$$

Since then, several researchers [3-5] have determined different values of Rc, for specific chromatographic systems. All of them were stated on experimental results : retention times of peptides of known composition were treated by a multi-regression computer program. Later, Guo [6, 7] employed synthetic octapeptides as models to determine Rc values which are commonly used.

However, experimental retention times of peptides larger than 9 or 10 amino-acid residues are lower than the ones calculated from equation 1 [8,9]. Mant et al. [10, 11] evidenced that the difference between experimental and calculated retention times varied with the length and hydrophobicity of the residues. Consequently these authors suggested to use a linear relationship in which the cross-product (Σ Rc ln N) appears :

$$\Sigma Rc - T_{retention} = A \Sigma Rc \, Ln \, N + B \qquad (2)$$

A and B are constants, N is the total number of amino-acid residues.

Nevertheless, this modified expression of the predicted elution times of peptides is not sufficient to explain deviations observed for large peptides.

Recently Chabanet [13] developed a new prediction model based on Chothia's conclusions and equation 1. Chothia [12] pointed out that 50% of the surface of the charged residues in folded proteins remains accessible . Chabanet divided amino-acid residues into three groups : charged, polar and non polar and assigned a pair of constants (k, f) to each

group. The k values depend on amino-acids residues accessibility in proteins, the f and rc_i values are determined by multilinear regression. The contribution of each residue to peptide retention is a decreasing function ($R'c_i$) of the peptide length, thus it appears that the $R'c_i$ of these three distinct groups are differently affected by the peptide length according to :

$$T_{retention} = \Sigma\, n_i\, R'c_i + b' \qquad (3)$$

$$R'c_i(rc_i,N) = (rc_i - rc_i/k)\exp(-fN^2) + rc_i/k$$

$$\lim_{N\to 0} R'c_i = rc_i$$

To each amino-acid residue corresponds a constant rc_i, b' represents the retention contribution of the terminal amino group and carboxylic function, N is the total number of amino-acid residues.

Yet, these empirical models are not sufficient to predict and to explain the retention of peptides, of any length. In many examples peptides with the same residue composition have different retention times [14, 15] because of non similar residue sequences. Other factors could indeed influence the peptide retention. Conformational and nearest neighbour effects can play a important role. Zhou [16] showed that the existence of amphipatic α-helices could explain the deviations observed in comparison with the predicted retention times. Sereda et al [17] evidenced that the influence of a α amino group on the hydrophobicity of the N terminal residue depends of its nature.

In order to appreciate their validity for peaks identification peptide maps we compared three prediction methods : Meek's (with Guo's Rc), Mant's and Chabanet's ones.

Consequently, we studied here on one hand a serie of synthetic peptide containing 9 or 10 amino-acid residues and on the other hand an enzymatic digest of r-hu IFNγ (Scheme 1) containing peptide of 4 to 31 residues.

These two sets of experiments were carried out on two different columns, with different gradient shapes, so we first examined the influence of these factors on peptide retention.

SCHEME 1: Primary structure of r-hu. IFN γ (144 amino-acids)

```
0                                                  10
MET GLN ASP PRO TYR VAL LYS GLU ALA GLU ASN LEU LYS LYS TYR PHE ASN ALA GLY
      20                                                  30
HIS SER ASP VAL ALA ASP ASN GLY THR LEU PHE LEU GLY ILE LEU LYS ASN TRP LYS GLU
      40                                                  50
GLU SER ASP ARG LYS ILE MET GLN SER GLN ILE VAL SER PHE TYR PHE LYS LEU PHE LYS
      60                                                  70
ASN PHE LYS ASP ASP GLN SER ILE GLN LYS SER VAL GLU THR ILE LYS GLU ASP MET ASN
      80                                                  90
VAL LYS PHE PHE ASN SER ASN LYS LYS LYS ARG ASP ASP PHE GLU LYS LEU THR ASN
          100                                                 110
TYR SER VAL THR ASP LEU ASN VAL GLN ARG LYS ALA ILE HIS GLU LEU ILE GLN VAL
               120                                                 130
MET ALA GLU LEU SER PRO ALA ALA LYS THR GLY LYS ARG LYS ARG SER GLN MET LEU
                    140
PHE ARG GLY ARG ARG ALA SER GLN
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EXPERIMENTAL SECTION

Materials:

Synthetic peptides tested are reported in table I. Peptides 1 and 2 were obtained from Neosystem (Strasbourg, France), peptides 3 to 15 were from Interchim (Montluçon, France). Recombinant human interferon gamma (r-hu. IFNγ) is extracted from E. Coli, into which a plasmid coding for this protein has been transfected.

r-hu. IFNγ contains the sequence of 143 amino-acid residues as for the natural protein, plus an additional N-terminal methionine. This recombinant protein is not glycosylated.

r-hu. IFNγ (1mg/ml) was incubated in 50mM tris-HCl buffer (pH=7.0) during 15 hours at 37°C at a 2% ratio (weight:weight) with endoprotease from Staphylococcus Aureus strain V8 (Böhringer. Mannheim).

Chromatographic measurements

The analysis of the synthetic peptides and of the r-hu IFNγ hydrolysate was performed on the HPLC system 1 : two pumps (model 420, Kontron, Massachussetts, U.S.A.) connected to a Rheodyne sample injector (model 7125, Berkeley, CA, U.S.A.) equipped with a 20μl sample loop. A Spectra-Physic UV detector (model 100) was used at 220nm or 280nm. The column containing RP C18 (5μm spherical particles, 130 x 4.6mm I.D.) was obtained from Brownlee-Labs (Santa-Clara, C.A., U.S.A.).

TABLE I : Synthetic peptides composition and retention times on chromatographic system 1

n°	SYNTHETIC PEPTIDES	observed retention times($\%CH_3CN$)
1	ALA ALA ALA LYS LYS LYS ARG ALA ALA ALA	13.5
2	ALA LYS ALA ALA LYS ALA ARG ALA LYS ALA	14.3
3*	pGLU ALA LYS SER GLN GLY GLY SER ASN	9.6
4	TYR SER ARG VAL SER ARG ARG SER ARG	17.1
5	ARG ARG LYS ALA SER GLY PRO PRO VAL	20.2
6	TRP ALA GLY GLY ASP ALA SER GLY GLU	17.7
7	ALA PRO LEU LYS PRO ALA LYS SER ALA	17.5
8	TYR GLY ALA VAL GLY VAL GLY LYS SER	19
9	MET GLN MET LYS LYS VAL LEU ASP SER	24
10	GLU LEU ALA GLY ALA PRO PRO GLU PRO ALA	20.9
11	TYR GLY GLY PHE LEU ARG LYS TYR PRO	29.2
12	pGLU TRP PRO ARG PRO GLN ILE PRO PRO	29.6
13	TYR GLY GLY PHE LEU ARG ARG ILE ARG	27.6
14	SER PHE PRO TRP MET GLN SER ASP VAL THR	33.1
15	ALA PRO ARG LEU ARG PHE TYR SER LEU	33.4

* according to Snider's [19] results, we made the assumption that the Rc for pGLU is the same as that for GLN

Gradient linear elution was carried out with eluent A (0.1% T.F.A.. H_20) and eluent B (CH_3CN-H_20 (60 : 40) (v : v), 0.1% T.F.A.). The gradient slope was 1% CH_3CN per minute and the flow-rate 1ml/min.

The peptides of the r-hu. IFNγ hydrolysate were separated on the HPLC system 2 : a Waters (Millipore Corp., model 600) low pressure gradient HPLC pump system, a sample injector (model 7010/ 7012, Rheodyne, Berkeley, C.A., U.S.A) a Beckman ultrasphere C-18 column (750 x 4mm I.D., 3μm spherical particles), a Waters

990+ diode array detector system (the detector was equipped with a special high-pressure-resistant cell in order to enable the on line coupling with a thermospray mass spectrometer). 200μg of the hydrolysed protein were injected directly onto the HPLC column.

The peptides were eluted with a A-B gradient (A : H_20; 0,1%T.F.A.) (B : CH_3CN; 0,1% T.F.A.) as follows :

0 - 40min : linear gradient 0% to 20% (v:v) B in A

40 -60min : linear gradient 20% to 40% (v:v) B in A

60 - 65min : linear gradient 40% to 50% (v:v) B in A

65 - 70min : linear gradient 50% to 0% (v:v) B in A

Flow-rate = 1.2ml/min

U.V. spectra of the eluate were recorded at given intervals (about 1s) at a wavelength range between 220nm and 290nm.

Retention times were expressed as the CH_3CN concentration in the solvent at the time of elution. This was calculated by subtracting the gradient elapsed time (as defined by Guo [6]) from the peak elution time and multiplying the result by the percentage of CH_3CN per minute in the linear gradient.

All chemicals were of analytical purity and special chromatographic grade.

RESULTS AND DISCUSSION

Comparison of peptide retention times predicted and observed on the two chromatographic systems.

The two sets of peptides were analysed with two different chromatographic systems which differed mainly by the chromatographic columns and the flow-rate chosen.

In order to compare the results obtained for the two sets of peptides, we studied the retention of 6 well identified IFNγ hydrolysate peptides (Table II) on both chromatographic systems. We observed that peptide retention times, obtained on each one, differed only by a shift (10% CH_3CN) (Fig. 1). The relationship between the retention times on the two systems was linear with a correlation coefficient of 0.997.

TABLE II : r-hu I.F.N. γ hydrolysate peptides and their retention times on chromatographic system 1 and 2

peptides	Observed retention(%CH_3CN) system 1	system 2
[103-112]	21	11.3
[0-7]	22	11.7
[94-102]	22	12.1
[76-93]	29	18.4
[76-102]	31	22.0
[25-39]	39	30.2

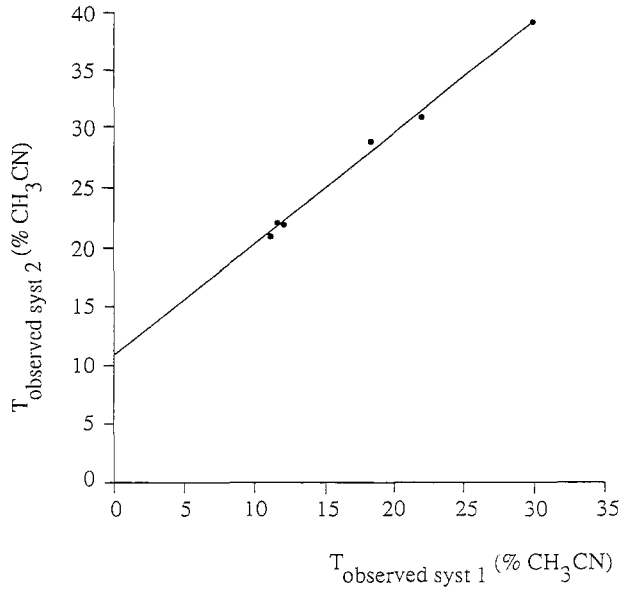

Figure 1 : Correlation between observed retention times obtained on system 1 and system 2 for 6 peptides (•) of the I.F.N. γ hydrolysate.
$T_{system\ 1} = 0.94\ T_{system\ 2} + 10.8$, the correlation coefficient is 0.997

This confirms the results obtained by Guo [6, 7] : he observed that peptides retention times obtained on various R.P. chromatographic columns exhibit a constant time difference and he showed that the influence of the flow-rate (in the range we used) and the support was negligible.

Consequently, we calculated the theoretical retention times of those 6 peptides according to Guo, Mant and Chabanet's rules. The correlation coefficients between these predicted retention times and the experimental ones (observed on each chromatographic system) are reported on table III. These linear functions were similar with a shift of 10% CH_3CN.

Analysis of sample 1 containing peptides of 9 or 10 amino acid residues.

Meek's model :

We compared the experimental peptide retention times (Table I) to the ones calculated by summing Guo's Rc according to the equation n° 1. and obtained with experimental conditions [6] similar to ours. The results in figure 2 show a linear correlation with a correlation coefficient of 0.94. However the slope is less than unity. The difference in length between Guo's octapeptides and the peptides studied here may already affect the retention prediction times.

Mant's model :

On figure 3, we also fitted the results according to equation n°2 as reported by Mant [10].

A linear correlation was observed and allowed us to obtain the A and B values of equation n°2 (A=0.15; B=-15.9). With these values, we calculated the theoretical retention times for comparison with the others models the correlation coefficient obtained between the predicted and the observed retention times was found equal to 0.94 (fig 4).

Chabanet's model :

Chabanet's prediction rules were applied to this sample of peptides. The comparison between the predicted and the observed retention times is presented on figure 5. The

TABLE III : Comparison of the results obtained on both systems for 6 peptides

Theoretical relationships of peptide retention times	Correlation coefficients (r) obtained on system 1	on system 2
$T = \Sigma Rc$ (1)	$Tobs(\%CH_3CN) = 0.50 \Sigma Rc + 17.6$ $r = 0.94$	$Tobs(\%CH3CN) = 0.55\Sigma Rc + 7.0$ $r = 0.96$
$\Sigma Rc - T = A\Sigma RcLnN + B$ (2)	$A = -16,39$ $B = 0.16$ $r = 0.97$	$A = -6.01$ $B = 0.15$ $r = 0.97$
$T = \Sigma n_i R'c_i(rc_i, N) + b'$ (3)	$Tobs(\%CH3CN) = 0.81\Sigma n_i R'c_i + 14.4$ $r = 0.99$	$Tobs(\%CH3CN) = 0.86\Sigma n_i R'c_i + 3.9$ $r = 0.98$

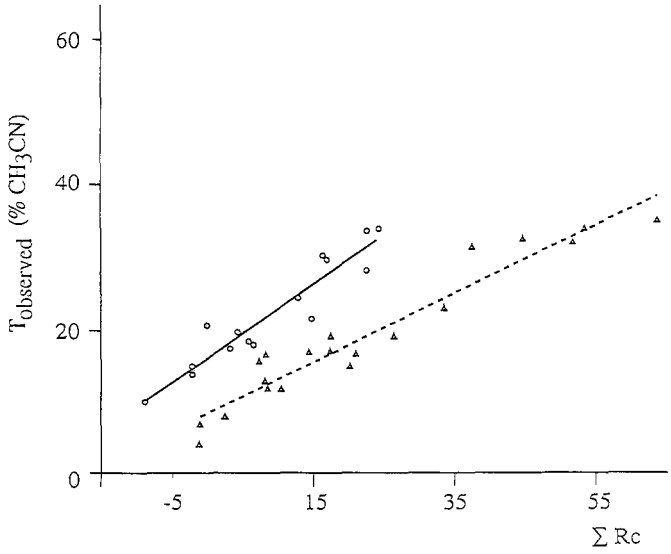

Figure 2 : Correlation between peptide retention times and the sum of residue contributions according to Guo's results (6).
-⊖- linear regression obtained with the first peptide sample obtained on chromatographic syst. 1
$T_{real} = 0.68\Sigma n_i Rc_i + 15.9$, the correlation coefficient is 0.94
- -Δ - linear regression obtained with the second peptide sample obtained on the chromatographic syst. 2
$T_{real} = 0.45\Sigma n_i Rc_i + 8.1$, the correlation coefficient is 0.96

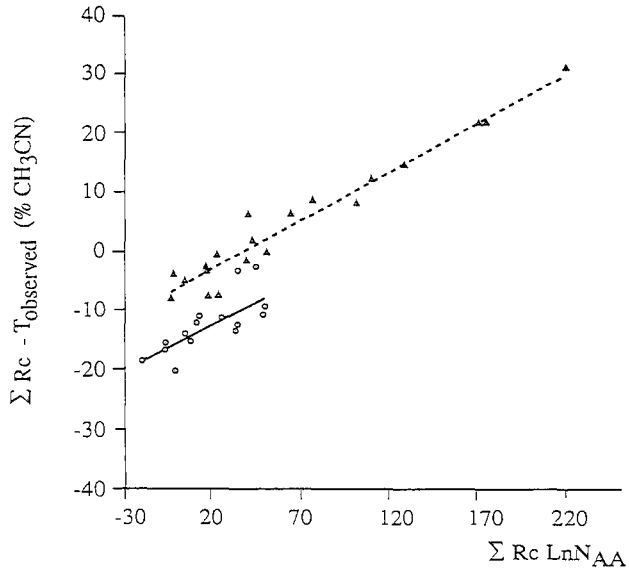

Figure 3 : Determination of A and B values of from the correlation between the observed retention time, length and hydrophoby of peptides according to equation n° 2.
-o- linear regression obtained with the first peptide sample obtained on the chromatographic syst. 1
A = 0.15, B = -15.9, the correlation coefficient is 0.80
- -Δ- linear regression obtained with the second peptide sample obtained on the chromatographic syst. 2
A = 0.16, B = -6.6, the correlation coefficient is 0.98

correlation factor between predicted and actual retention times was 0.95 but the slope remained different from unity (0.73). However if the linear regression is carried out without taking into account any of the three peptides with more than four positives charges, then the slope increases (0.85) and the correlation factor is improved (r = 0.97). It means that Chabanet's model may not be sufficient to provide accurate retention prediction for very basic peptides of small length.

Analysis of sample 2 : a r-hu IFNγ peptide map

We applied the same three prediction methods to calculate the retention of 20 peptides of a r-hu. IFNγ hydrolysate (table IV). The peptide map of this protein has been

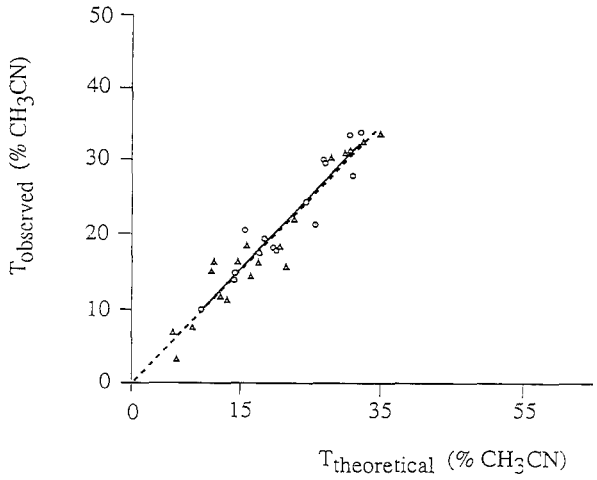

Figure 4 : Correlation between the calculated retention times according to the A and B values given on figure 3.
-o- linear regression obtained with the first peptide sample obtained on the chromatographic syst. 1
$T_{real} = T_{theoretical}$, the correlation coefficient is 0.94
- -Δ- linear regression obtained with the second peptide sample obtained on the chromatographic syst. 2
$T_{real} = 0.98T_{theoretical} + 0.39$, the correlation coefficient is 0.96

characterised by different techniques involving diode array absorbance detection, mass spectrometry and chemical sequencing [20].

In the chromatographic map of these peptides obtained with system 2, a few peaks could not be identified. For this study, we analysed only on the peaks, the nature of which was certain after identification with thermospray mass spectrometry.

Meek's model :

Peptide retention times were calculated by summing Guo's Rc. The relationship between these theoretical values and the experimental ones was linear as shown on figure n°2 with a correlation coefficient of 0.958. Nevertheless the slope was even lower than the one obtained for the previous peptides sample. It should be noted that most of the peptides of the hydrolysate contained a higher number of amino-acid residues than the synthetic

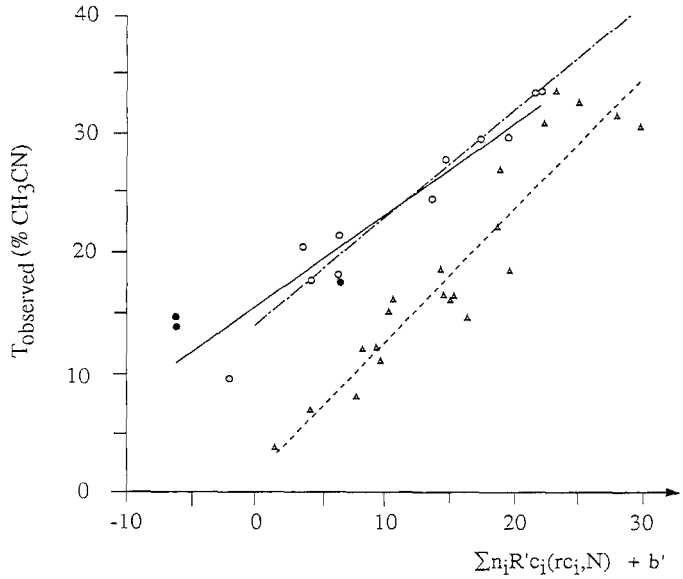

Figure 5 : Correlation between the observed and predicted retention times, calculated with equation n°3.
-o-●- linear regression obtained with the first peptide sample obtained on the chromatographic syst. 1
T_{real} = 0.73 $\Sigma n_i R'c_i(rc_i,N)$ + 15.5, the correlation coefficient is 0.95
-o- · — linear regression obtained with the first peptide sample without taking into account the three peptides (●) with more than four positive charges
T_{real} = 0.85 $\Sigma n_i R'c_i(rc_i,N)$ + 14, the correlation coefficient is 0.97
- -Δ- linear regression obtained with the second peptide sample obtained on the chromatographic system 2
T_{real} = 1.1$\Sigma n_i R'c_i(rc_i,N)$ + 1.5, the correlation coefficient is 0.94

peptides we studied. As noticed by various authors [8-13], equation 1 overestimates the retention of large peptides. The formation of stabilised secondary and tertiary structure may perturb the interaction of certain residues with the hydrophobic stationary phase.

Mant's model :

For each peptide, we correlated the difference between the sum of the retention coefficients and the observed retention time to the cross-product ΣRcLnN (fig.3). We obtained a correlation coefficient of 0.98 for a linear relationship. The value of the slope A

TABLE IV : r-hu I.F.N. γ hydrolysate peptides and their retention times on chromatographic system 2

PEPTIDES	observed retention times (%CH3CN)
[0-7]	11.7
[94-102]	12.1
[120-143]	14.3
[10-21]	15.0
[72-75]	3.5
[120-133]	6.9
[40-46]	7.7
[103-112]	11.3
[113-119]	15.7
[92-102]	16.2
[76-91]	16.2
[10-24]	16.3
[94-112)	18.2
[76-93]	18.4
[76-102]	22.0
[25-39]	30.2
[10.39]	30.7
[22.39]	31.0
[47-71)	32.3
[40-71]	33.4

(A=0.16) was similar to the one we got for the other sample of peptides. Furthermore, the shift of peptide retention times observed between the two chromatographic systems corresponds to the difference between the B values obtained for each sample of peptides ($\Delta B = 10\% CH_3CN$). Therefore we conclude that Mant's model is valuable whatever the length or composition of peptides. The knowledge of A and B values allowed us to calculate the theoretical retention times of the peptides of sample 2. The correlation coefficient was found equal to 0.96 (Fig.4). This peptide sample contains more items than the one previously studied, what can explain why the correlation coefficient is higher.

Chabanet's model :

The figure 5 represents the correlation between the predicted and the observed retention times. The correlation coefficient is 0.94. Predicted and observed retention times differ only by a shift ($1.5\% CH_3CN$) due to the difference between our column and the one used by Chabanet.

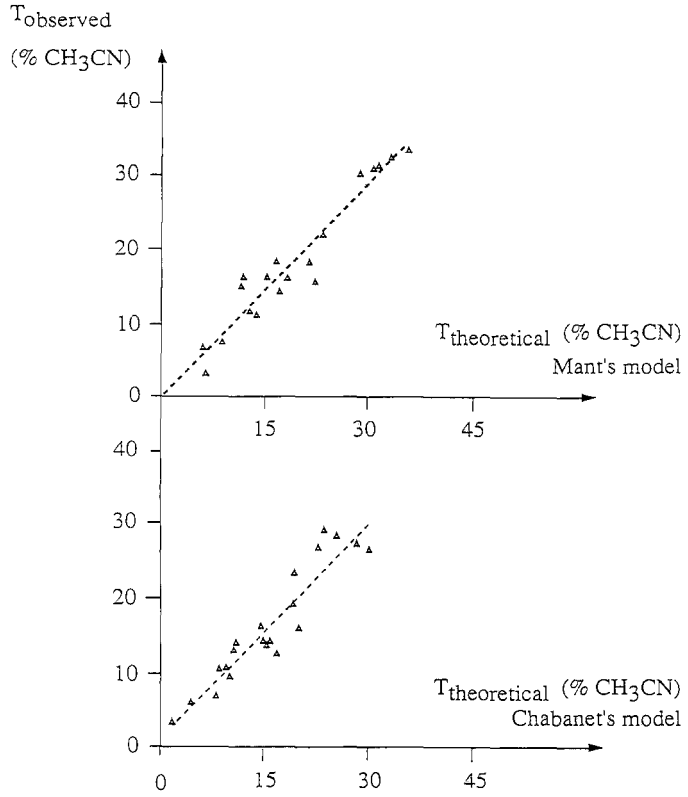

Figure 6 : Correlation between the observed and calculated retention times with the second peptide sample obtained on chromatographic system 2 with Mant's model, r=0.96 ; with Chabanet's model, r=0.94

Comparison between Mant's and Chabanet's models :

We compared on the figure 6 the predicted retention times obtained with Mant's and Chabanet's models. Both ones can be used indifferently.

CONCLUSION

For the comparison of three models of prediction of peptide retention we used two peptide samples:

a/ A sample of synthetic peptides of identical length but differing in composition (mainly basic and neutral amino acids).

b/ A hydrolysate of r-hu IFNγ : this hydrolysate was obtained using S. Aureus Strain V8 endoprotease. Thus it consists mainly of neutral and basic peptides

The application of Meek's model confirms the observation that the differences in peptide length neglected by Meek influence the retention of the peptide on a RP HPLC.

Mant's model fit's well the retention times of the peptides in RP HPLC. We have shown that it could be applied to a peptide map containing mainly neutral and basic peptides. We were able to compared separation obtained on different columns.

The validity of Chabanet's model is confirmed by the analysis of a peptide map containing essentially peptides of various length. However, we pointed out that for extremely very basic peptides (over 50 % basic amino acids) then this model is not valid. Further it should be noted that R. Cowan and R.G. Whittaker[18] have outlined the fact that different scales of hydrophobicity of amino-acid residues were stated but they differed widely particularly in the values of the polar ones.

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THE ROLE OF pH OF THE MOBILE-PHASE IN ION-INTERACTION RP-HPLC

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ABSTRACT

The dependence of retention on pH of the mobile phase is investigated in reversed-phase ion interaction chromatography, being the mobile phase an aqueous solution of 5.0 mM octylammonium and ortho-phosphoric acid at different pH values.

The analytes considered were amines, diamines, amides, and species characterized by different functionalities. The behaviour of retention as a function of pH is discussed with comparison with literature data and correlation with pk_a values is shown.

The optimization of separation and resolution in a mixture of acids and amines through pH variations is also presented.

INTRODUCTION

The dependence of retention on *pH* of the mobile phase has been studied in reversed-phase chromatography (1-8) and models able to

predict retention as a function of *pH* have been described (2-8). Some examples are also found in ion-pair chromatography, in which the mobile phase is an hydroorganic mixture which contains a ion-pairing agent (9-14). At our knowledge, only one study from this laboratory concerns the dependence of anions in ion-interaction chromatography, which makes use, as the mobile phase, of an aqueous solution of the interaction reagent (15). In reversed-phase ion-interaction chromatography retention greatly depends on the *pH* of the mobile-phase, since *pH* variations can affect the reaction equilibria of both the analytes and the components of the ion-interaction reagent. Dissociation equilibria as well as of ion-pair formation must be considered.

According to the interaction model which better fit our experimental data, the interaction reagent flowing in the mobile-phase is dynamically adsorbed as ion-pair onto the surface of the reversed-phase material packing, whose originary interaction properties are therefore modified (15-19). In these conditions, the *pH* of the mobile phase not only can influence the capability of the analytes to be retained but can also affect the amount and the interaction properties of the moiety adsorbed onto the surface of the stationary phase. As a function of the mobile-phase *pH*, the properties and the capacity of the modified stationary phase can therefore vary.

As mentioned, a previous study from this laboratory dealt with the retention of anions as a function of the *pH* of the mobile phase, when the interaction reagent was octylammonium phosphate (15). Different behaviours were observed for anions of strong and weak acids and the results obtained were explained by considering different

effects. In particular, a different capacity of the stationary-phase for different *pH* values was suggested, on the basis of the stability of the different ion-pair species which can be formed between octylammonium and o-phosphate.

This paper studies the *pH*-dependence of amines, diamines, amides and of species, like *4*-aminobenzoic, nicotinic and orotic acids, which contain in their molecule two different functionalities.

It is also shown how separation and resolution of components of a mixtures can be optimized as a function of mobile-phase *pH*.

MATERIALS

Apparatus

Analyses were carried out with a Merck-Hitachi Lichrograph chromatograph Model L-6200, equipped with a two-channel Merck-Hitachi model D-2500 Chromato-integrator, interfaced with a UV-vis detector model L-4200 and a L-3720 conductivity detector with temperature control, of the same firm.

A Metrohom 654 *pH*-meter equipped with a combined glass-calomel electrode was employed for *pH* measurements and a Hitachi mod.150-20 spectrophotometer for absorbance measurements.

Chemicals and Reagents

Ultrapure water from Millipore Milli-Q was used for the preparation of all solutions. Sodium iodide, nicotinic acid, benzylamine,

nicotinamide were Merck analytical grade reagents. Octylamine, sodium azide, sodium bromate, sodium nitrate, *1,2*-phenylenediamine, *1,3*-phenylenediamine, phenethylamine, orotic acid, creatinine, *4*-aminobenzoic acid, aniline, ortho-phosphoric acid were Fluka analytical grade chemicals. Potassium thiocyanate and potassium chromate were C.Erba analytical grade chemicals.

METHODS

A 5 μm ODS-2 Spherisorb Phase Separation column fully end-capped 250.0 x 4.6 mm with a carbon load of 12% (0.5 mM/g), together with a 15.0 x 4.6 mm Lichrospher RP-18, 5 μm guard pre-column.

The solutions to be used as mobile phase were prepared by adding to the amount of octylamine weighed to prepare a 5.0 mM solution the required amount of ortho-phosphoric acid up to obtain the desired *pH* value. The solutions prepared at the different *pH* values contained therefore the same analytical concentration of octylamine (5.0 mM) and different analytical concentrations of the acid. With this procedure the presence in the mobile phase of any other component different from octylamine and o-phosphoric acid was avoided. A *pH* range within 2.5 and 8.0 was explored.

The chromatographic system was conditioned by passing the eluent through the column until a stable baseline signal was obtained; a minimum of 1 hour was necessary. This procedure was always followed when a new mobile phase was used. After use, the column

was washed and regenerated by flowing a 50/50 v/v water/methanol mixture (0.5 mL/min for 1 hour).

No particular degradation of the column was observed with *pH* variations.

The dead time was evaluated through injection of $NaNO_3$ (20 ppm) and conductometric detection of the unretained Na^+ ion. It was shown that the dead time does not significantly depend on the *pH* of the mobile phase and, at the operating conditions of flow-rate (0.7 mL/min), the average measured was 3.56 min.

RESULTS AND DISCUSSION

Table 1 lists the retention times (as the average of at least three experiments) obtained as a function of *pH* for the analytes studied. Analytes containing aminic functionality as well as aminic- carboxylic functionalities were chosen: the structures are reported in Figure 1 (A, B). 5.0 mM octylammonium phosphate was the interaction reagent and spectrophotometric detection at 230 nm was employed. The range of *pH* investigated (between 2.5 and 8.0) was imposed by the use the of silica-based stationary phase, since it was not possible (15) to obtain a reproducible and good extent of surface modification for a reversed-phase C-18 polymer-based material packing, that would have allowed to investigate a larger *pH* range.

Previous results, based on the calculation of the distribution, as a function of *pH*, of all the species formed between octylammonium and o-phosphate, suggested that the greater capacity of the stationary phase

TABLE 1

Retention times t_R (minutes) at different pH values for the Analytes investigated. The values are the average of at least three experiments. Stationary phase: Spherisorb ODS-2, 5 μm, fully endcapped, 250 x 4.6 mm; Ion Interaction Reagent: Octylammonium 5.0 mM and ortho-phosphoric acid; Flow Rate=0.7 mL/min; Spectrophotometric Detection at 230nm.

	pk_a (25°C, 0)	t_R (min) pH 2.5	t_R (min) pH 3.0	t_R (min) pH 4.5	t_R (min) pH 5.0	t_R (min) pH 6.4	t_R (min) pH 7.0	t_R (min) pH 8.0
aniline	4.63	3.76	4.50	22.05	27.96	29.00	29.79	21.30
1,2-phenylenediamine	4.63*	n.r.	4.02	8.00	15.20	17.40	15.20	13.40
1,3-phenylenediamine	2.50° 5.11°	n.r.	n.r.	6.93	8.37	9.57	8.70	8.20
creatinine	3.55	n.r.	n.r.	4.93	5.04	6.12	5.77	5.89
nicotinamide	3.35	7.19	14.20	25.17	25.11	17.27	18.13	12.40
benzylamine	9.35	n.r.	n.r.	4.00	4.11	6.30	8.36	15.80
phenethylamine	9.97*	4.21	4.16	4.97	5.15	8.60	11.16	19.50
4-aminobenzoic acid	2.08 4.96	14.89	22.80	41.45	41.93	22.50	21.50	15.00
nicotinic acid	2.05 4.81	7.95	13.69	94.68	105.43	56.68	52.00	39.38
orotic acid	4.05 8.78	42.80	52.47	47.65	44.00	20.40	20.50	17.20

* 25°C, 0.1 ° 20°C, 0 n.r.= not retained

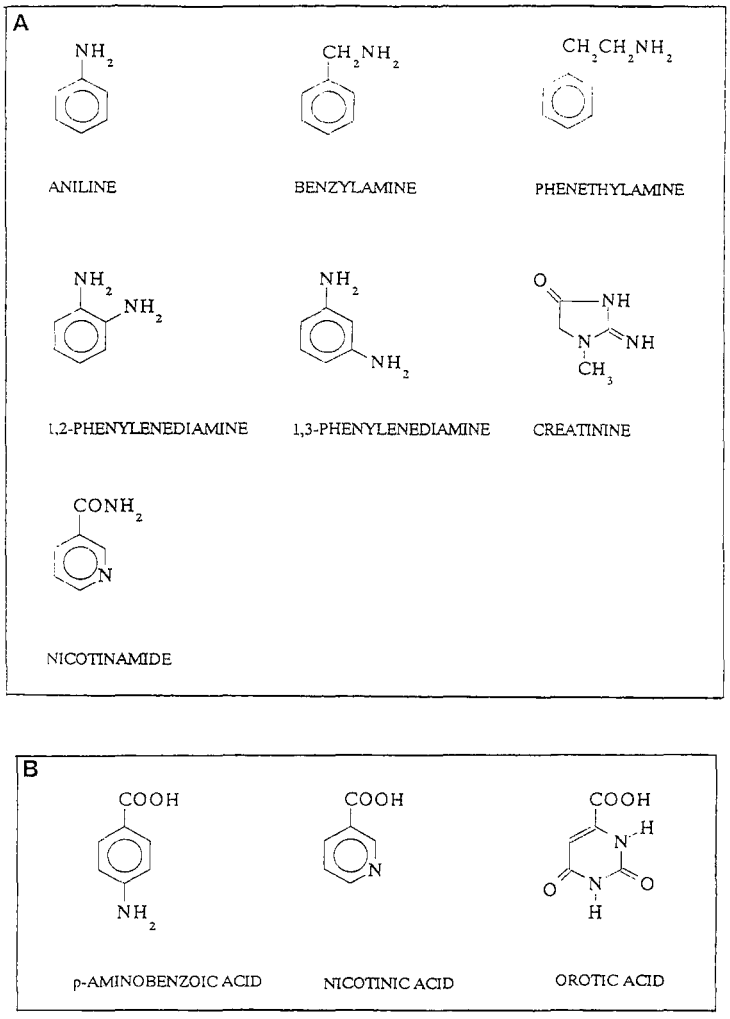

FIGURE 1 (A, B).
Analyte structures.

(expressed as the maximum number of active sites) can be hypothized at lower *pH* values. In agreement, anions of strong acids showed at lower *pH* values higher retention values. For the anions of weak acids, retention depends on the dissociation constant k_a and a maximum of retention was shown for *pH* values close to pk_a values.

Figures 2 and 3 show the behaviour of capacity factor k' ($k' = \frac{t_R - t_o}{t_o}$, where t_R is the retention time and t_o the dead time) for the amines, diamines and amides studied. Two kind of behaviours can be envisaged. Aniline, creatinine, nicotinamide, *1,2-* and *1,3-* phenylenediamine (Figure 2) show a maximum of retention in the *pH* range of about 4-6, while benzylamine and phenylethylamine (Figure 3) show a progressive increase of retention for the whole *pH* range investigated. It can be observed that the amines characterized by a maximum of retention are characterized by pk_a values around 4-5 (see Table 1) while benzylamine and phenethylamine show pk_a values around 9.5 (see Table 1).These results are in agreement with literature results (12) obtained for adenine (pk_a = 4.12) in ion-pair chromatography (sodium octylsulphonate as the ion-pairing and 10% v/v of methanol in the mobile phase).

The behaviours observed in Figure 2 and 3 can be explained as follows. The results previously obtained for the same chromatographic system (15) indicated a greater capacity of the stationary phase for lower *pH* values. In addition, at lower *pH*, amines are preferentially present in their protonated form, under which the formation of ion-pairs with o-phosphate should be favoured. But, as lower is the *pH* and more protonated the amines, as stronger are the repulsion electrostatic forces

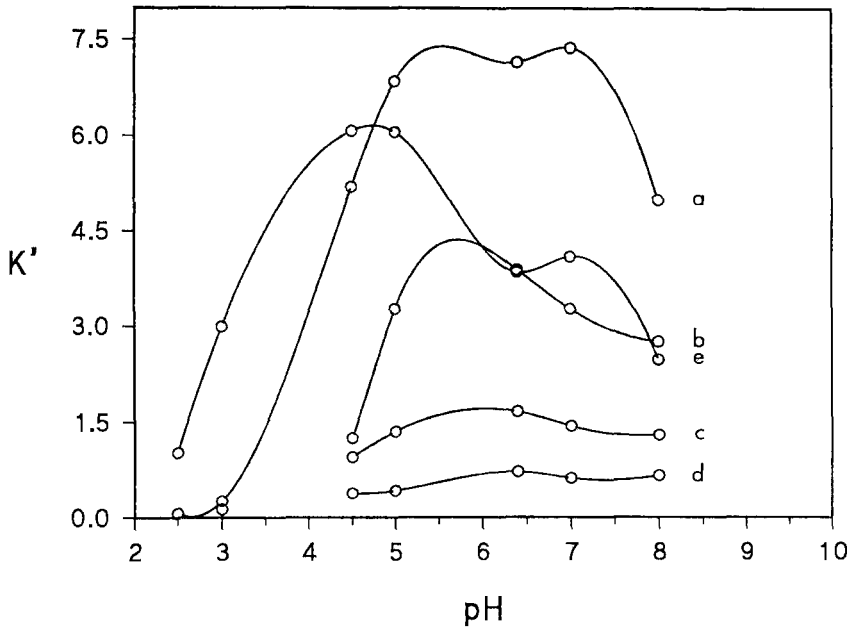

FIGURE 2.
Capacity factor k' as a function of pH. Operating conditions: Stationary phase: Phase Separation Spherisorb ODS-2, 5 μm, fully endcapped, 250 x 4.6 mm; Ion interaction reagent: 5.0 mM octylammonium and ortho phosphoric acid; Flow rate: 0.70 mL/min; Injection volume: 100 μL; Spectrophotomectric detection at 230 nm.
Analytes: a= aniline, b= 1,2-phenylenediamine, c= 1,3-phenylenediamine, d= creatinine e= nicotinamide.

aging between the adsorbed octylammonium and the analytes and the final result is a lower retention.

The retention decrease observed (Figure 2) for higher *pH* values can be ascribed to the combined effect of the lower retention capacity of the stationary phase together with the always lower molar fraction of the analyte which is present in the protonated form.

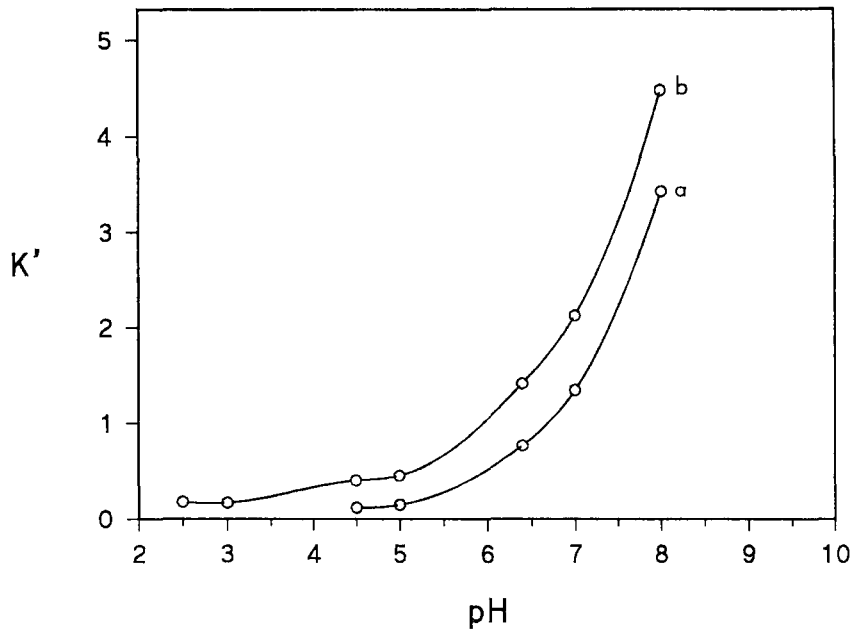

FIGURE 3.
Capacity factor k' as a function of pH. Experimental conditions as in Figure 2.
Analytes: a= benzylamine, b= phenethylamine.

These considerations do not hold for benzylamine and phenethylamine, which are characterized by very higher pK_a, so that at *pH* 8 the molar fraction a of the protonated form is still sufficiently high ($\alpha > 0.95$) to give easily rise to the formation of ion-pairs.

Figure 4 shows the behaviour of retention as a function of *pH* for the following acids: *4*-aminobenzoic, nicotinic and orotic acids which (see Figure 1 B) show in their molecule both carboxylic and nitrogen containing functionalities. The dependence shows in every case a

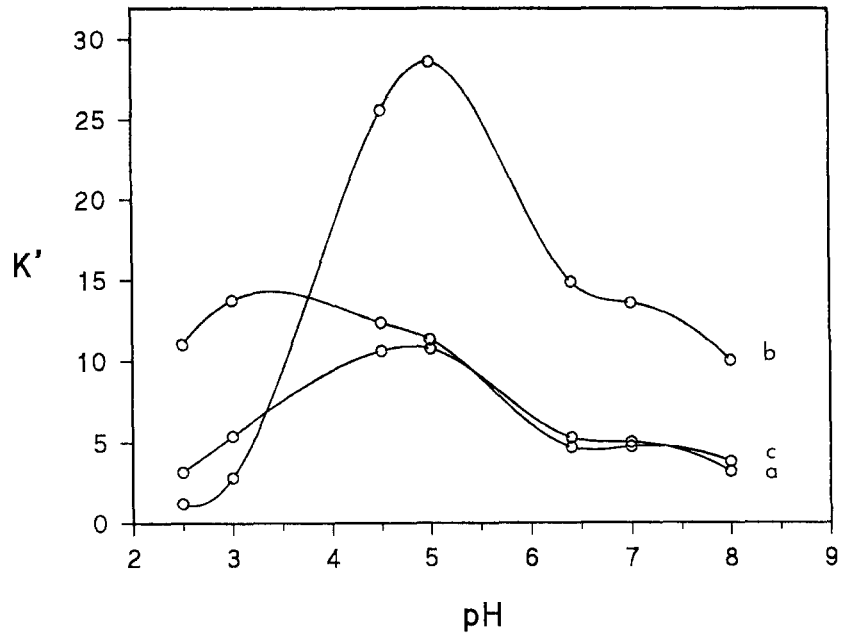

FIGURE 4.
Capacity factor k' as a function of pH. Experimental conditions as in Figure 2.
Analytes: a= 4-aminobenzoic acid, b= nicotinic acid, c= orotic acid.

maximum, around *pH* 5 for nicotinic and 4-aminobenzoic acids and around 3.0 - 3.5 for orotic acid.

It would be of interest to understand which of the two functional groups present in the molecule is participating in the retention.

On the basis of the results collected up to now for anions (15) and amines, it was always shown that the maximum of retention nearly corresponds to the pK_a value of the analyte. We can therefore propose that the carboxylic group is always responsible for retention for the

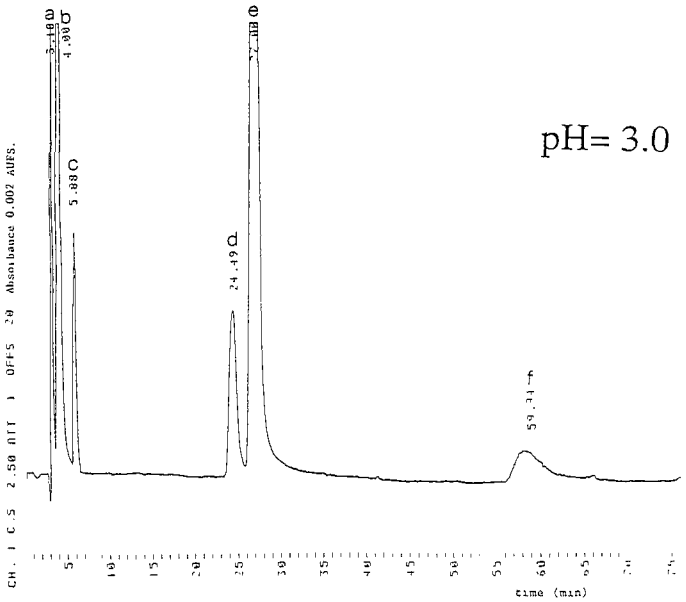

FIGURE 5.
Separations at pH=3.0 (A), pH=6.4 (B), pH=8.0 (C) of a mixture containing: a= 1,3-phenylenediamine (0.50 ppm), b=1,2-phenylenediamine (0.50 ppm), c= sodium azide (0.50 ppm), d= nitrate (0.50 ppm) e= iodide (0.50 ppm), f= thiocyanate (0.50 ppm) and g= chromate (1.00 ppm). Operating conditions as in Figure 2.

three compounds investigated, with respect to the nitrogen-containing group.

From a practical point of view, the different *pH* dependence shown by the retention of different analytes can helpfully assist in solving problems of identification and resolution between the components of a mixture. In Figure 5 typical chromatograms recorded at three different *pH* values (*pH* = 3.0, 6.4 and 8.0, 5.0 mM

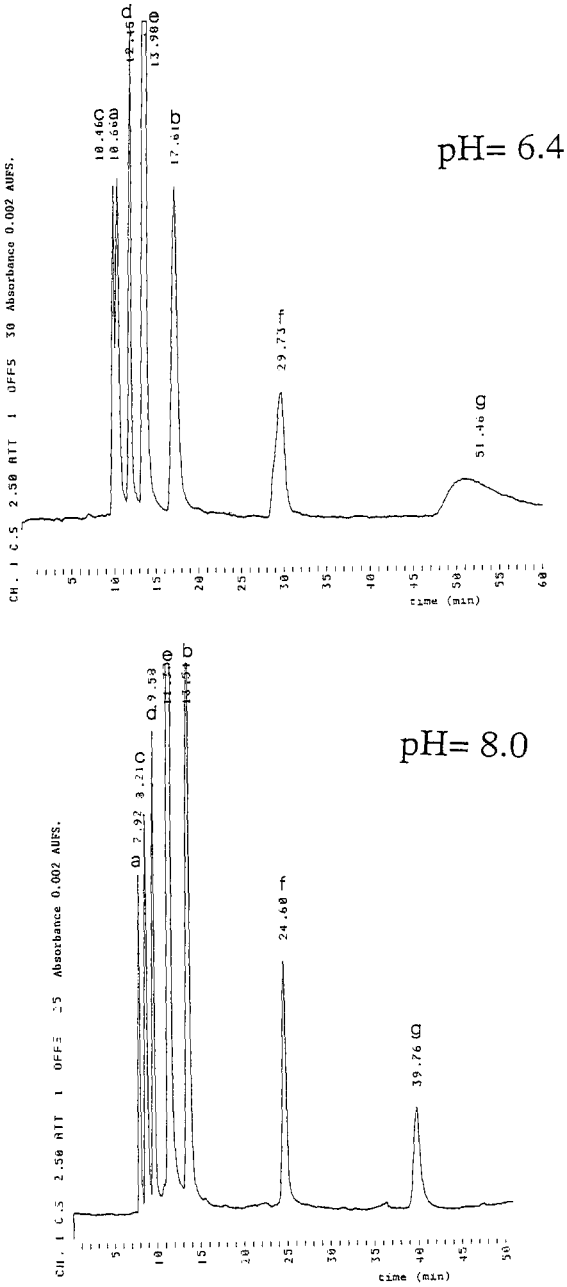

FIGURE 5 (continued)

octylammonium o-phosphate as the ion-interaction reagent and spectrophotometric detection at 230 nm) are reported for a mixture which contains anions and amines, namely: *1,3*-phenylenediamine (0.50 ppm), *1,2*-phenylenediamine (0.50 ppm), hydrazoic acid (1.00 ppm), nitrate (0.50 ppm), iodide (0.50 ppm), chromate (1.00 ppm) and thiocyanate (0.50 ppm).

It can be observed that not only sensitivity and resolution can vary with pH but also the elution sequence order as it can be for example observed for hydrazoic acid, nitrate and iodide and *1,2*-phenylenediamine at *pH* 3.0 and 6.4 and for *1,3*-phenylenediamine and hydrazoic acid at *pH* 6.4 and 8.0.

Figure 5 also shows the remarkable improvement which can be obtained in sensitivity and resolution when working with a *pH* of the mobile phase equal to 8.0.

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DETERMINATION OF Fe(III) IN FERMENTATION BROTH BY ION-INTERACTION CHROMATOGRAPHY

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INTRODUCTION

Transition metals are essential in microbial metabolism. Iron, for example, is commonly found in iron-sulfur proteins and electron-transport proteins. Manipulation of transition metal concentration in fermentation media is often employed to retard microbial growth or influence secondary metabolism. For example, iron, calcium, magnesium, and zinc were found to stimulate growth of *Legionella pneumophilia*[1].

The chromatographic determination of Fe(III) has been accomplished by ion chromatography/post column derivatization[2,3], chelation ion chromatography[4-8], and ion-interaction[9-13] chromatography. In general, these methods were applied to relatively clean samples, such as river water. Ion-interaction chromatography is based upon pre-column derivatization with ethylenediaminetetraacetic acid, EDTA, to form Fe(III)EDTA$^-$. The resultant complex is eluted as an ion pair with tetrabutylammonium ion at a low pH value. In this work, ion-

interaction chromatography is applied to the rapid determination of Fe(III) in two common growth media, Mueller-Hinton and tryptic soy broths.

EXPERIMENTAL

Chemicals

Ethylenediaminetetraacetic acid (99+%), 40% tetrabutylammonium hydroxide, and sodium acetate were obtained from Aldrich Chemical Company (Milwaukee, WI) and used without further purification. Glacial acetic acid was purchased from Fisher Chemical Company (Fairlawn, NJ). Mueller-Hinton and tryptic soy broths were obtained from Sigma Chemical Company (St. Louis, MO). Acetonitrile and water used in the preparation of mobile phases were of HPLC grade and obtained from Fisher Chemical Company. Reference standard Fe(III) was purchased from Alltech Associates (Deerfield, IL) as a standard solution.

Instrumental

Ion-Interaction Chromatography A Dionex model DX300 liquid chromatograph (Dionex Corp., Sunnyvale, CA) interfaced with a Spectra-Physics model AS3500 autosampler (Spectra-Physics, Freemont, CA) was used. A Dionex spectral array UV-vis absorbance detector was used and data collected and analyzed with Dionex AI450 software. Unless otherwise specified, a Vydac 218TP54 column (Vydac Associates, Hesperia, CA) of dimensions 25 cm X 4.6 mm was used throughout method development. The pore diameter and particle size were 300Å and 5 μm, respectively.

Ion Chromatography with Post-Column Reaction Detection A Scientific Systems, Inc. model 350 pump was used to deliver the reagent solution to the above HPLC system. Post-column mixing of

reagent with mobile phase was accomplished using 10′ of 0.010" Teflon tubing in a FIAtron FH40 heater set at 40°C.

Chromatographic Parameters

Ion-Interaction Chromatograhy The mobile phase was prepared by mixing 4.1 g sodium acetate, 2.75 mL of 40% tetrabutylammonium hydroxide, 3.75 g ethylenediaminetetraacetic acid (EDTA), and 5.00 mL glacial acetic acid per liter of HPLC grade water. The contents were thoroughly dissolved and filtered to 0.45 μm. The mobile phase was pumped at a rate of 1.00 mL/min, resulting in a back-pressure of about 1900 p.s.i. Unless otherwise specified, the injection volume and detection wavelength parameters were 20 μL and 320 nm, respectively. The column was periodically cleaned with a mobile phase consisting of a 70:29.9:0.1 mixture of acetonitrile, water, and trifluoroacetic acid. Mobile phases were filtered to 0.45 μm prior to use.

Ion Chromatography The mobile phase was prepared as described in reference (2) and consisted of 6 mM pyridine-2,6-dicarboxylic acid, 50 mM acetic acid, and and 50 mM sodium acetate. The post column reagent consisted of a 1% solution of EDTA in water. Mobile phase and reagent were filtered to 0.45 μm prior to use. Dionex HPIC-CG5 and -CS5 guard and analytical columns were used. The mobile phase and reagent flow rates were 1.0 and 0.5 mL/min, respectively. The detector was set at 520 nm.

Sample Preparation

Mueller-Hinton and tryptic soy broths were prepared per label instruction. Samples to be analyzed by ion-interaction chromatography were diluted with mobile phase 1:4 and filtered to 0.45 μm prior to injection. Samples to be analyzed by ion chromatography were diluted 1:4 with 0.05N HCl to achieve a final

composition of 0.01N HCl. These procedures appeared to stabilize iron in solution and improved peak shape. Prior to injection, samples were filtered with a syringe filter to 0.45 μm. Standards were prepared in HPLC grade water and diluted identically to the samples to minimize calibration bias.

RESULTS AND DISCUSSION

Figure 1 depicts chromatograms typical of fermentation broth. The Fe(III)EDTA$^-$ complex eluted as a symmetrical peak with a k′ value of about 0.8. The values of A_s and N were 1.2 and 1000 m^{-1}, respectively. Others have demonstrated that the retention of Fe(III)EDTA$^-$ in ion-interaction chromatography does not vary significantly with the concentration of organic

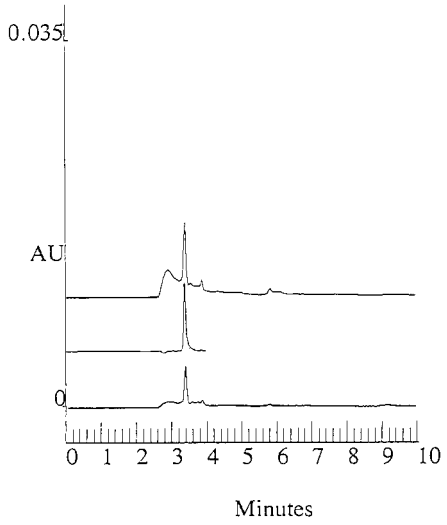

Figure 1. Typical chromatograms of Fe(III)EDTA$^-$ (top) Mueller-Hinton broth, (middle) 2.0 μg/mL standard, (bottom) tryptic soy broth.

modifier[9,13]. For this reason, no organic modifier was used in the mobile phase. As a result, few matrix components interfered with the analyte peak. Occasionally, the column needed to be stripped of absorbed matrix components with a mobile phase of stronger elutropic strength. This procedure was performed after approximately 50 sample injections.

The relationship of chromatographic response to the concentration of EDTA in the diluent was evaluated by diluting a concentrated solution of tryptic soy broth with mobile phase and water to achieve varying concentrations of EDTA at constant Fe(III) concentration. The results are shown in Figure 2. Below

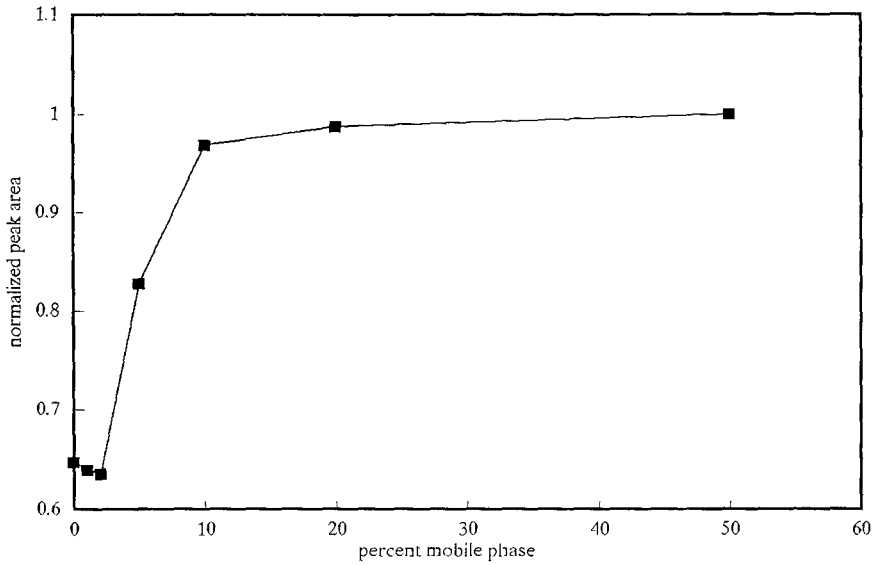

Figure 2. Plot of peak area of Fe(III)EDTA$^-$ complex (arbitrary units) as a function of mobile phase added to tryptic soy broth. The concentration of tryptic soy broth was held constant. Details of sample preparation are in text.

10% mobile phase (1.0 mM EDTA) the peak area is significantly affected by EDTA concentration. The sigmoidal response is suggestive of a competitive equilibrium of EDTA with other chelating agents in the medium. Dilution of the sample with mobile phase to a final composition of 20% mobile phase (2.0 mM EDTA) appears to be sufficient to minimize other interactions and provide a consistent response.

The precision of the assay is determined by the precision of the autosampler injection volume and reproducibility of sample preparation. The former value was evaluated by repetitive injection of the same solution of Fe(III) standard, approximately 1 μg/mL in concentration. Typically, the relative standard deviation of peak area measurement was below 2%. The precision of sample preparation was determined by repetitive preparation of a fermentation broth sample. The relative standard deviation (N=5) was approximately 4%.

The $Fe(III)EDTA^-$ peak area was linear over the concentration domain 0.2 to 12.0 μg/mL Fe(III), as shown in the lower trace in Figure 3. The value of r^2 was 0.9999, demonstrating excellent linear fit. The value of the concentration equivalent of the intercept was -0.02 μg/mL, indicating that Fe(III) was not leached from extraneous sources. Fe(III) was spiked into tryptic soy broth over the concentration domain 0.0 to 12.0 μg/mL. The upper trace of Figure 3 depicts the detector response as a function of the concentration of Fe(III) added. The value of r^2 was 0.9999, indicating excellent fit to a linear model. The concentration equivalent of the intercept was 0.73 μg/mL. The values of the slopes for standards and samples were 19601 and 19459 arbitrary units, respectively. The essentially equivalent slopes indicate the absence of matrix interactions with the analyte by the addition of EDTA to the sample.

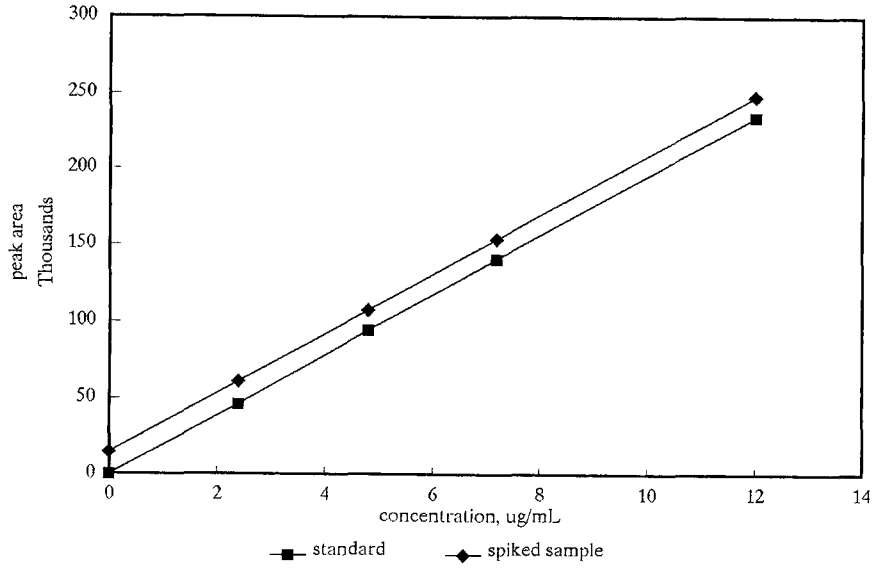

Figure 3. Plot of peak area for standard (bottom) and spiked tryptic soy broth (top). Equations of lines are given in text.

The purity of the Fe(III)EDTA$^-$ peak was evaluated by varying the detector wavelength with a constant sample of tryptic soy broth. The detector wavelengths used were 280, 320, and 350 nm. The apparent concentrations of Fe(III) were 8.41, 0.78, and 0.76 μg/mL, respectively. The values obtained at 320 nm and 350 nm are statistically equivalent, given a relative standard deviation of about 4% for each measurement. The high result obtained at 280 nm may indicate a proteinaceous interference, the effect of which is minimized at higher detector wavelengths. The relative peak areas for a standard at 280, 320, and 350 nm were 1.00, 0.51, and 0.20, respectively. These values are in excellent agreement with published spectra of the Fe(III)EDTA$^-$ complex[9,10].

For routine use, 320 nm was selected as a compromise in sensitivity in favor of selectivity.

The storage stability of fermentation broth samples was investigated by preparing samples of tryptic soy broth in 0.01N HCl and 20% mobile phase and analyzing the solutions periodically. The samples and a control, dissolved in water, were incubated at room temperature. Figure 4 shows the normalized peak area as a function of time. Significant degradation of the control and 0.01N HCl sample occured after 24 hours of storage. With increased storage time, adventitious microbial growth was evident. In contrast, the sample dissolved in 20% mobile phase was stable at least a week. No microbial

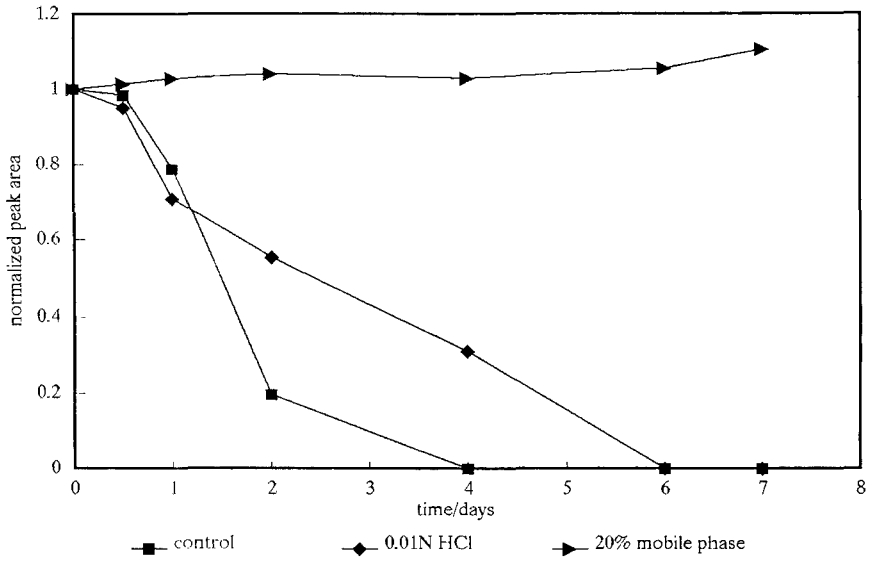

Figure 4. Plot of normalized peak area of Fe(III)EDTA complex in tryptic soy broth as a function of time. Broth concentrate was dissolved in ion-interaction chromatography mobile phase (top), 0.1N Cl (middle), and water (bottom) as a control.

infestation was evident. These observations are consistent with inhibition of microbial growth by metal chelating agents[1].

The accuracy of ion-interaction chromatography for the determination of Fe(III) in fermentation broth was evaluated by comparison of results obtained by ion-interaction chromatography to those obtained by ion chromatography for identical samples of fermentation broth. The results are compared in a Youden plot[14], given in Figure 5. Linear regression of the data gave the equation

$$IIC = 0.988*IC + 0.11 \qquad r^2 = 0.998$$

where IIC and IC represent ion-interaction chromatography and ion chromatography, respectively. The values of the slope and intercept agree with the theoretical values of 1.00 and 0.00,

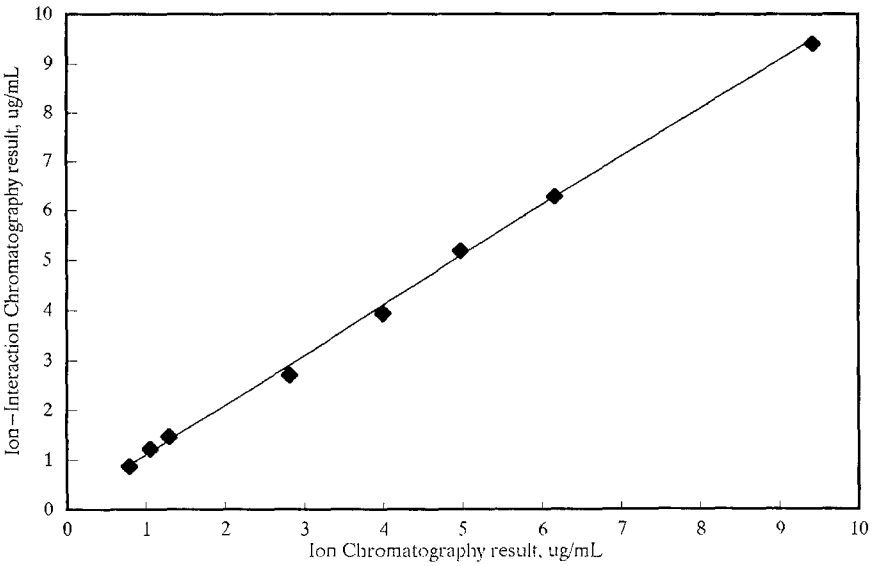

Figure 5. Comparison of results obtained for a series of spiked trypic soy broths by ion-interaction chromatography and ion chromatography. Equation of line is given in text.

respectively. The agreement of the two methods, which separate components by different retention mechanisms, implies that the assay is accurate within the concentration domain studied.

CONCLUSION

Ion-interaction chromatography has been applied to the determination of iron in fermentation broths. The method is sufficiently selective to avoid interference from matrix components and is useful in monitoring the uptake of Fe(III) during the course of fermentation.

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HIGH-SPEED RP-HPLC/FL ANALYSIS OF AMINO ACIDS AFTER AUTOMATED TWO-STEP DERIVATIZATION WITH o-PHTHALDIALDEHYDE/3-MERCAPTOPROPIONIC ACID AND 9-FLUORENYLMETHYL CHLOROFORMATE

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ABSTRACT

This paper describes an automated high-speed RP-HPLC/FL method, including two-step precolumn derivatization with o-phthaldialdehyde/3-mercaptopropionic acid (OPA/3-MPA) for primary amino acids and with 9-fluorenylmethyl chloroformate (FMOC-Cl) for secondary amino acids. The OPA/3-MPA derivatives eluted within 6.5 min, while the retention time of the last eluted amino acid derivative (FMOC-Pro) was 8.0 min. The total analysis time, including precolumn derivatization, separation, column washing and reequilibration cycles, was only 18 min. Therefore, 75-80 samples per day can be analysed during unattended operation. The efficiency of the method was demonstrated by the separation of amino acids extracted from potato tubers.

INTRODUCTION

The qualitative and quantitative determination of amino acids has become increasingly important in the chemical, pharmaceutical and food industries, and also in clinical chemistry and biotechnology. Because of their key role in these areas, increasingly more rapid and sensitive techniques have been developed for their analysis: thin-layer chromatography, gas chromatography, ion-exchange chromatography and high-performance liquid chromatography. Until the early eighties, the most widespread procedure was the cation-exchange chromatography combined with postcolumn derivatization with ninhydrin, as developed by Moore and Stein [1]. Although reliable, this technique is time-consuming and the derivatized compounds can be detected only in the nmol range. The most recent, frequently used methods are based on precolumn derivatization with a number of reagents, such as phenyl isothiocyanate (PITC) [2,3], 5-dimethylamino-naphthalene-1-sulphonyl chloride [4,5], 4-dimethylaminoazobenzene-4'-sulphonyl chloride [6,7], o-phthaldialdehyde (OPA) [8-10], or 9-fluorenylmethyl chloroformate (FMOC-Cl) [11,12]. The reactions of amino acids with PITC, OPA and FMOC-Cl can be automated by using computer-programmable autoinjectors and autosamplers, but phenylthiocarbamyl (PTC) derivative formation requires longer reaction time and these derivatives do not fluoresce. The PTC derivatives can generally be detected with a less sensitive UV detector, but the sensitivity can be improved by electrochemical detection [2]. OPA forms highly fluorescent isoindole derivatives from primary amino acids in the presence of mercaptans (e.g. 2-mercaptoethanol, ethanethiol, 3-mercaptopropionic acid (3-MPA) or 3-mercapto-1-propanol). Isoindole derivative formation needs a short reaction time (1 min) under basic conditions and takes place at room temperature. Unfortunately, the OPA reagent does not react with secondary amino acids such as Pro and HO-Pro and the derivatives have limited stability [13]. To overcome these disadvantages, an automatic two-step derivatization has been developed. In the first step, all primary amino acids react with the OPA reagent containing mercaptan. During the second step, the reaction mixture of isoindole derivatives is treated with FMOC-Cl to derivatize the unreacted secondary amino acids [8,9]. Both reactions are rapid and, since the derivatives are strongly fluorescent, the detection limits are in the low pmol range.

In this article we describe an efficient, high-speed RP-HPLC method for the analysis of amino acids. The method involves automated two-step precolumn derivatization, high-speed separation on a short cartridge-type column and fluorescence detection of the amino acid derivatives.

MATERIALS AND METHODS

Chemicals

For calibration, standard mixtures (20, 50, 100, 200 pmol/μl) of amino acids (Serva, Heidelberg, Germany) were prepared with 0.1 N hydrochloric acid. Each standard mixture contained 100 pmol/μl norvaline (Nval) as internal standard (ISTD). Acetonitrile (MeCN), methanol (MeOH) and tetrahydrofuran (THF) were HPLC grade from Farmitalia Carlo Erba (Milano, Italy). Triethylamine (TEA) was purchased from Sigma (St. Louis, MO, U.S.A.). Borate buffer (0.4 N, pH 10.4) was prepared from boric acid and potassium hydroxide (Reanal, Budapest, Hungary). Sodium acetate (NaOAc) was obtained from Reanal (Budapest, Hungary). For the preparation of buffers (borate and acetate), HPLC grade water was produced by using Nanopure II (Barnstead/Thermolyne Corporation, Dubuque, IA, U.S.A.) cartridge-type water purification equipment with a 0.2 μm final filter.

Preparation of Derivatization Reagents

The primary amino acids were derivatized with OPA/3-MPA reagent. 10 mg of OPA in an amber HPLC autosampler screw-cap vial (Sigma, St. Louis, MO, U.S.A.) was dissolved in 100 μl of HPLC grade MeOH and made-up to 1 ml with borate buffer (0.4 N, pH 10.4). Finally, 20 μl of 3-MPA was added to the vial. Secondary amino acids such as HO-Pro and Pro were derivatized with FMOC-Cl (Sigma, St. Louis, MO, U.S.A.) reagent. The FMOC-Cl (2.5 mg) was dissolved in 1 ml of anhydrous MeCN. Both reagents were stable for at least one week when stored in amber vials in a refrigerator at 4 °C under nitrogen.

Equipment

The analyses were performed on a Hewlett-Packard HP 1090 Series II/M liquid chromatograph supplied with a DR5 binary solvent-delivery system, a variable-volume autoinjector, an autosampler, a temperature-controlled column compartment and a stand-alone HP 1046A programmable fluorescence detector. To minimize noise, second-order or higher reflected light was removed by installing a 289 nm cut-off filter in front of the emission grating. The chromatographic separations were performed through a Hewlett-Packard 100x4 mm ID cartridge-type column filled with 3 μm Hypersil (Shandon, Cheshire, England) ODS particles. The guard column was attached to the analytical column without any capillaries.

Extraction of Free Amino Acids from Potato Tubers

Potato tubers (Solanum tuberosum L. cv. Kondor) were peeled and cut into pieces, and a representative 100 g sample was extracted with 100 ml of boiling water for 2 hours. Thereafter, the extract was centrifuged at 5000 g for 10 min and the supernatant was filtered through a 0.45 μm Millex FH membrane (Millipore, Bedford, MA, U.S.A.). Finally, 100 μl of extract was pipetted into a HPLC autosampler vial and diluted ten-fold with 900 μl of HPLC grade water containing 100 ng of Nval as internal standard.

Automated Precolumn Derivatization

A reliable computer-controlled autoinjector and an autosampler were employed to perform the derivatization of primary and secondary amino acids, with OPA/3-MPA and by FMOC-Cl. The borate buffer and the needle rinsing fluids, together with the reagents, were placed in the first magazine of the autosampler. The derivatization reactions were completed in the injection capillary of the autoinjector, in which the reaction mixture was mixed by moving the plunger of a 25 μl Hamilton syringe (Hamilton-Bonaduz AG, Bonaduz,

Switzerland) back and forth with the aid of a stepping motor. The different steps of the injector program are shown in Table 1.

Chromatographic Conditions

The gradient solvent-delivery system consisted of two solvents. Solvent A was 0.018 M NaOAc supplemented with 0.02% (v/v) TEA and 0.3% (v/v) THF. The THF was added to acetate buffer containing TEA after adjustment of the pH to 7.2 with 1% (v/v) acetic acid (Suprapur, Merck, Darmstadt, Germany). Solvent B was MeCN/MeOH/0.1 M NaOAc pH 7.2 in a ratio of 2/2/1 (v/v). Both solvents were filtered through an 0.45 μm Millex FH membrane (Millipore, Bedford, MA, U.S.A.). Helium degassing was used to avoid bubble formation in the solvent-delivery system and the flow cell of the detector. The column was kept at a constant temperature of 40 °C in the column compartment. The gradient elution started with 100% solvent A and increased in five linear steps to 100% B in 8.5 min with a flow rate of 1.4 ml/min. When the last amino acid derivative (FMOC-Pro) eluted at 8 min, the column was washed with 100% solvent B for 4 min, and reequilibrated to the initial conditions (Table 2). For detection of the OPA/3-MPA derivatives of primary amino acids, the excitation and emission wavelengths of the fluorescence detector were adjusted to 340 and 450 nm, respectively. After the elution of the primary amino acids, at 6.6 min the excitation and emission wavelengths were automatically switched to 264 and 313 nm, to detect the FMOC-Cl-derivatized secondary amino acids. All instrument parameters, including autoinjector, autosampler, solvent-delivery system, column compartment temperature and also fluorescence detector parameters, were accurately controlled by an HP 9000 Series 310 computer supplied with HP 79998A ChemStation software.

RESULTS AND DISCUSSION

The amino acids were derivatized automatically by means of the autoinjector and autosampler according to Schuster [8] with minor alterations. In order to ensure the stability of the FMOC-Cl reagent in the autosampler against its ready

TABLE 1

Injector Program for Two-Step Derivatization of Amino Acids

Line #	Function	Amount (μl)	Details	Substance
1	Draw	5	From vial 2	Borate buffer (0.4 N, pH 10.4)
2	Draw	1	From vial 0	OPA/3-MPA reagent
3	Draw	0	From vial 100	Water for needle wash
4	Draw	1	From sample	Primary and secondary amino acids
5	Draw	0	From vial 100	Water for needle wash
6	Draw	0	From vial 3	Anhydrous MeCN for needle wash
7	Mix	7	Seven cycles	Reactions of primary amino acids
8	Draw	1	From vial 1	FMOC-Cl reagent
9	Draw	0	From vial 4	Anhydrous MeCN for needle wash
10	Mix	8	Four cycles	Reactions of secondary amino acids
11	Inject	8		

TABLE 2

Time-table for Separation and Detection of Amino Acid Derivatives

Time	Gradient parameters (%)		Detection wavelengths (nm)	
(min)	Solvent A	Solvent B	Excitation	Emission
0	100	0	340	450
0.5	94	6		
3.0	80	20		
6.5	50	50		
6.8			264	313
8.0	25	75		
8.5	0	100		
12.5	0	100		
13.5	100	0	340	450

hydrolysis, two washing steps with anhydrous MeCN were applied to remove the water traces remaining on the outer surface of the injection needle. The complete autoinjector program in 11 steps, including drawing, mixing and needle rinsing cycles, required 5 min only. The time necessary to complete the derivatizations did not increase the total analysis time, because the columns were equilibrated with 100% solvent A during the injector program.

The high-speed separation of amino acid derivatives was performed by using a relatively short column (10 cm x 4 mm ID) filled with Hypersil ODS packing. To increase the lifetime of this separation column, use of a guard column is recommended, as otherwise a number of compounds in the crude extract, the reagents and their by-products can quickly destroy the separation column. However, the connection of a guard column to an analytical column usually requires a capillary, which adds an extra delay volume to the system, decreasing the resolution between compounds eluting close to each other (as in this case). Therefore, use of a cartridge-type column with a built-in guard column (no extra delay volume between columns) is the best choice. To separate the derivatized amino acids, a binary gradient in five linear steps was applied through the column. The NaOAc concentration (0.018 M) in solvent A was critical to obtain a good separation for Ala/Arg. When its concentration was raised to 0.02 M or above, the Ala/Arg resolution decreased. On the other hand, when the NaOAc concentration was lower than 0.018 M Gln, His and Gly did not separate well. The underivatized silanol groups in the column packing were blocked by pipetting a small amount of TEA (0.02% v/v) into solvent A. When TEA was omitted or its concentration decreased to 0.01%, His/Gly were not resolved. In solvent B, the mixture of MeCN and MeOH (2 parts each, mixed with 1 part of 0.1 M NaOAc) had a crucial role in separating Trp from Val/Met and Phe/Ile. When MeOH or MeCN was omitted or when they were mixed with NaOAc in other ratios (e.g. 1/3/1or 3/1/1), Trp eluted together with Val/Met or Phe/Ile.

Under the experimental conditions used, all common amino acid standards, including Nval as internal standard, were well separated, with a resolution of 1.64 (Phe/Ile) or better. This resolution resulted in a good separation of the free amino acids in potato tuber extracts as physiological samples (Figure 1). There was no interference with the reagent excess and reaction by-products, since OPA did not fluoresce and FMOC-Cl and its by-products eluted at least 1 min later than FMOC-Pro. The separation of a 50 pmol/μl amino acid standard mixture revealed that the isoindole derivatives eluted from the column in sharp peaks. Their widths

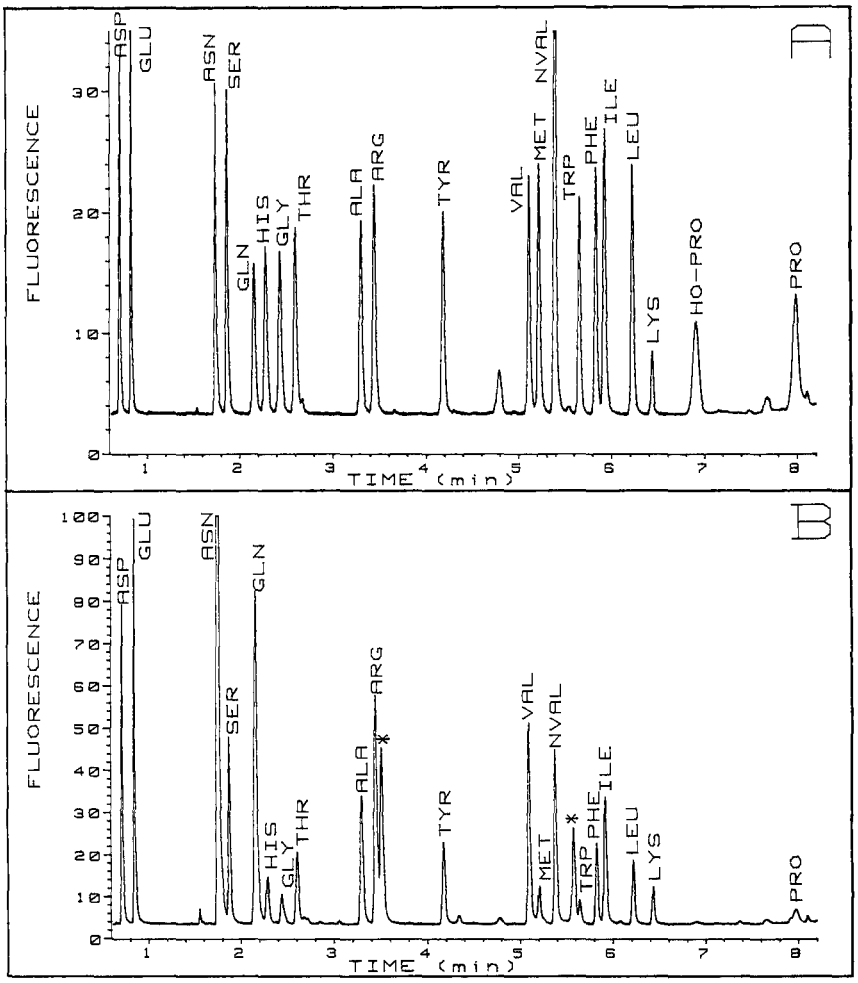

FIGURE 1. High-speed RP-HPLC analysis of derivatized amino acids. A) Separation of an amino acid standard mixture (50 pmol each) including Nval as internal standard (100 pmol). B) Chromatogram of the amino acids extracted from potato tubers including internal standard. The peaks labeled with an asterisk are unknown. Conditions: column, Hewlett-Packard 100x4.0 mm cartridge-type (with built-in guard column) filled with 3 µm Hypersil ODS packing; flow rate, 1.4 ml/min; column compartment temperature, 40 °C. For other conditions (gradient and detection parameters) see Table 2.

at half height and at the baseline ranged between 0.015 and 0.03 min and between 0.03 and 0.072 min, respectively. The excellent peak shape may be due to the limited chromatographic dispersion inside the column (packing with 3 μm particles) and the higher flow rate (1.4 ml/min) through the detector cell. The FMOC derivatives of HO-Pro and Pro eluted in peaks with slightly higher widths at half height (0.065-0.076 min) and at the baseline (0.111-0.125 min). The isoindole derivatives of Cys and Cys-Cys do not fluoresce, and they can therefore be detected with a less sensitive UV detector after formation of a mixed disulfide with 3,3'-dithiodipropionic acid following reaction with OPA/3-MPA to give an isoindole derivative [14,15].

The amino acids containing primary amino group(s) eluted within 6.5 min. The last separated amino acid was the FMOC-Pro, with a retention time of 8 min. The reproducibilities of the retention times and relative retention times were also calculated and are shown in Table 3. With this method, the lowest detectable amounts ranged between 1 and 5 pmol of amino acid at a signal to noise ratio of 5/1. Including the time necessary for derivatization, separation, column washing and reequilibration cycles, samples could be analysed at 18-min intervals, and the sample throughput is therefore up to 80 samples per day during unattended operation.

The calibration curves were linear within the examined concentration range. Due to the peak sharpness, the correlation coefficients were higher when calculated relative to the peak heights rather than to peak areas, except for HO-Pro and Pro derivatized with FMOC-Cl (Table 4).

CONCLUSIONS

This paper describes a rapid, sensitive and reproducible method for the RP-HPLC analysis of amino acids as demonstrated on the example of potato tuber extracts. The method involves an automated precolumn two-step derivatization as published by Schuster [8] and Blankenship et al. [9], a high-speed separation and fluorescence detection. Solvents and an appropriate gradient consisting of several linear steps are applied. Together with the lower cost cartridge-type ODS column this allows shortening of the analysis time considerably, while the resolution remains similar to or is even better than that reported so far. The problem of

TABLE 3

Precision Calculation for Retention Times of Amino Acid Derivatives (n=10)

Amino acid	Retention times (min)		Retention times (min) Relative to Nval ISTD	
	Mean	R.S.D. (%)	Mean	R.S.D. (%)
Aspartic acid	0.709	1.45	0.137	1.13
Glutamic acid	0.831	1.09	0.163	0.86
Asparagine	1.747	0.91	0.330	0.58
Serine	1.871	0.94	0.354	0.61
Glutamine	2.160	1.30	0.412	0.98
Histidine	2.286	1.22	0.435	0.90
Glycine	2.439	1.20	0.465	0.88
Threonine	2.602	0.93	0.494	0.60
Alanine	3.305	0.79	0.622	0.47
Arginine	3.446	0.77	0.649	0.45
Tyrosine	4.185	0.60	0.780	0.27
Valine	5.113	0.35	0.948	0.02
Methionine	5.221	0.39	0.968	0.06
Norvaline	5.394	0.33	1.000	
Tryptophan	5.657	0.36	1.048	0.05
Phenylalanine	5.835	0.32	1.080	0.02
Isoleucine	5.928	0.28	1.096	0.05
Leucine	6.224	0.28	1.150	0.04
Lysine	6.435	0.28	1.186	0.06
HO-Proline	6.903	0.26	1.278	0.06
Proline	7.982	0.17	1.473	0.17

TABLE 4

Comparison of Correlation Coefficients for Peak Areas r(a) and Heights r(h)

Amino acid	r (a)	r (h)	Amino acid	r (a)	r (h)
Aspartic acid	0.992	0.996	Tyrosine	0.996	0.997
Glutamic acid	0.990	0.995	Valine	0.992	0.997
Asparagine	0.993	0.996	Methionine	0.991	0.998
Serine	0.993	0.997	Tryptophan	0.995	0.998
Glutamine	0.990	0.996	Phenylalanine	0.993	0.997
Histidine	0.995	0.995	Isoleucine	0.990	0.996
Glycine	0.986	0.996	Leucine	0.989	0.996
Threonine	0.984	0.997	Lysine	0.990	0.998
Alanine	0.992	0.997	HO-Proline	0.994	0.990
Arginine	0.991	0.997	Proline	0.996	0.991

Correlation coefficients r(a) and r(h) were calculated from 5 analyses of a standard mixture of amino acids ranging from 20 pmol to 200 pmol.

limited stability of the isoindole derivatives can be avoided via the automated derivatization and quite rapid separation time.

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VARIABLES INFLUENCING THE DIRECT DETERMINATION OF HALOACETIC ACIDS IN WATER BY REVERSED-PHASE ION-PAIR CHROMATOGRAPHY WITH INDIRECT UV DETECTION

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ABSTRACT

A reversed-phase ion-pair chromatographic system for the direct determination of haloacetic acids in water has been optimized utilizing indirect photometric detection. Acetic, chloroacetic, bromoacetic, iodoacetic, dichloroacetic and dibromoacetic acids were used to characterize the chromatographic system. The effect of temperature on retention time shows a non linear van't Hoff behavior indicating a change in the mechanism of retention at about 30°C. Above 30°C, retention times decreased proportionally to increases in the temperature of the column. Separations are achieved in the pH range of 3.5 to 6.3 with an optimum at ca. pH 5.4. Increases in the concentrations of KH_2PO_4, 1-hexanesulfonate (competing ion) and acetonitrile result in proportional decreases in capacity factors with some selectivity variations depending on the analyte. Increases in the concentration of benzyltributylammonium ion (ion interaction reagent) resulted in increases in capacity factors with a usable range from 8 to 12 mM. Of the reversed

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phases studied, Spherisorb C-18 yielded the optimal results for the acids studied. Linear calibration curves for these acids were obtained utilizing indirect UV detection with detection limits as low as 2 parts per million.

INTRODUCTION

Studies involving the formation and distribution of haloacetic acids in aquatic environments continue to be important and challenging tasks (1). Currently established methods, including EPA Method 552, involve extraction of the acids into organic solvents followed by derivatization into methyl esters for analysis by gas chromatography (2). Derivatization steps are time consuming and many derivatization reagents including diazomethane, used in EPA method 552, are toxic, carcinogenic and explosive hazards. Therefore, direct methods for the analysis of haloacetic acids without the need for derivatization are desirable. Reversed phase ion pair chromatography (RP-IPC) with indirect detection can be used to simultaneously separate neutral, ionic, and ionizable compounds with or without chromophores. Mobile phases in RP-IPC generally include 5 to 500 mM of an ion interaction reagent (also called a pairing ion or counterion). The most commonly used ion interaction reagent is the tetrabutylammonium ion although many others have been used, including different tetraalkylammonium ions, alkylsulfonic acids and trialkylamines (3-5).

The technique of ultraviolet detection for non ultraviolet absorbing solutes was first described by Schill et. al. (6) and later by Small and Miller (7) who called it "indirect photometry". Indirect fluorescence detection has also been reported (8,9). The choice of the wavelength in indirect photometry depends on the optical density of the ultraviolet absorbing eluent. Values greater than 1.6 may produce excessive noise and lead to a poor signal to noise ratio. Values lower than 0.4 may produce a poor signal. In the range 0.4 to 1.6, electronic compensation of the eluent absorbance to zero is possible without excessive noise (10). It has been suggested that 0.5 is an adequate upper limit for absorbance (11). The ultraviolet-absorbing component in the mobile phase will be in equilibrium with the stationary phase until an injected solutes disrupts that equilibrium either by binding or displacement giving rise to a detector response. This response can be positive or negative and is dependent on the retention and charge of the solute relative to the absorbing pairing ion. It has been suggested that the detection sensitivity for compounds of low retention can be improved if the mobile phase contains two

ultraviolet-absorbing ions with opposite charges and hydrophobicities, but with both having absorptivities in the same wavelength range (12).

The present RP-IPC method has been optimized and the relative effects of variations in the concentration of the ion interaction reagent, the organic modifier, the competing ion, the pH, the ionic strength, and the temperature of the separation have been evaluated.

System peaks

In HPLC, a chromatogram may show more peaks than the number of solutes in the sample, particularly when the mobile phase contains more than one component. These peaks have been called extra peaks, ghost peaks, pseudo peaks (13), vacancy peaks (14) and induced peaks (15), among other terms. The term "system peaks" will be used in this paper to indicate peaks not directly attributed to the actual solutes in the sample. The number of mobile phase components seen as system peaks depends on factors including the response of each particular compound to the detector in use. System peaks contain information about the thermodynamic (i.e., retention) and kinetic (i.e., broadening) processes that occur in the column. Thus, they can lead not only to a better understanding of the chromatographic process, but also to a better evaluation of the nature and amount of the solutes in the sample injected (16).

The origin of the system peaks has been studied in some detail elsewhere (16-18). After the mobile phase has passed through the chromatographic column for a certain period of time, an equilibrium between the stationary phase and the mobile phase constituents is reached and maintained as long as the chromatographic system is not disturbed. If anything in the mobile phase is changed, or if a solute is injected into the column, this equilibrium is disrupted and the chromatographic system immediately responds and adjusts to a new equilibrium state. The resulting injection zone, therefore, contains not only the injected solutes but also some, or all, of the mobile phase components at different concentrations than they are in the bulk mobile phase. System peaks have, in a given chromatographic system, constant capacity factors irrespective of the sample injected. They can appear positive or negative to the detector base line and their areas can depend on the nature of the injected solutes (16).

Mechanisms of retention

Many names have been used to describe ion pair chromatography including soap chromatography, solvent generated ion exchange, dynamic ion exchange, hetaeric chromatography, solvophobic-ion chromatography, ion interaction chromatography and chromatography on sorbed ionic sites. The exact mechanism by which separation occurs in reversed phase ion pair chromatography is still a matter of debate although three main models are popular with none completely satisfactory for all aspects of the phenomenon. Each is considered more an extreme situation than a comprehensive theory (19-21). According to the 'Ion Pair Model' or 'Partition Model' (22,23), ion pair formation occurs in the mobile phase prior to the adsorption or partition of the ion interaction reagent into the stationary phase. This model explains with greater precision the situation when a non-bonded reversed column is used and the stationary phase behaves as a bulk liquid, but is not able to explain ion-pair interactions with chemically bonded reversed phase columns.

The 'Dynamic Ion Exchange Model' (23-25) most closely explains chromatography when bonded reversed columns are in the system. The ion interaction reagent is envisioned to be absorbed onto the stationary phase surface. Once absorbed, the ion interaction reagent behaves as a liquid ion exchanger producing ionic interactions between the ionized solute molecules and the counterions adsorptively bound to the stationary phase. Finally, according to the 'Ion Interaction Model', ion pair formation is not necessary nor is the classical ion exchange interaction required to produce separation and retention. The model explains the process on the basis of a electrical double layer formation at the stationary phase surface as a result of the dynamic adsorption of the ion interaction reagent (19,26). The ion interaction model was used to predict that ultraviolet absorbing lipophilic ions added to a reversed phase eluent would coelute with non ultraviolet absorbing sample molecules to facilitate their detection (27). Figure 1 presents a schematic of some of the possible interactions among the bonded phase (C-18), the ion interaction reagent (benzyltributylammonium (BTA)), the competing ion (1-hexanesulfonate) and one of the compounds studied in this work (dichloroacetic acid).

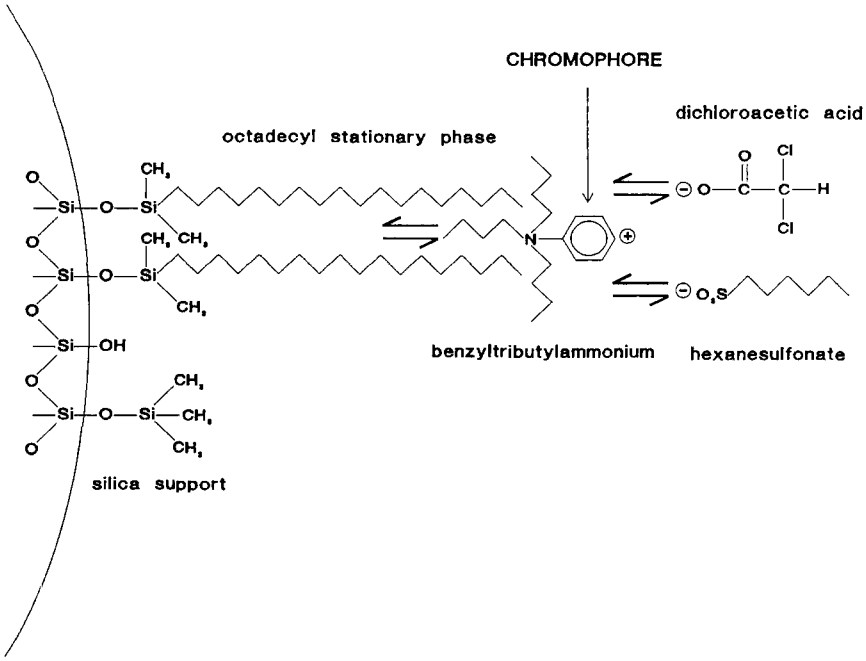

FIGURE 1 Schematic of several possible interactions among the bonded stationary phase (C18), the ion interaction reagent (benzyltributyl-ammonium), the competing ion (1-hexanesulfonate) and one of the haloacetic acids studied (dichloroacetic acid).

MATERIALS

Benzyltributylammonium chloride, 1-hexanesulfonic acid sodium salt, 98% monobasic potassium phosphate, 98% bromoacetic acid, 98% iodoacetic acid, 99% dichloroacetic acid and 90% dibromoacetic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI). HPLC grade water, HPLC grade acetonitrile and certified chloroacetic acid were obtained from Fisher Scientific (Fair Lawn, NJ). Analytical reagent grade glacial acetic acid was obtained from Mallinckrodt (St. Louis, Mo.). All chemicals were used as received, without further purification.

METHODS

Data was collected on a home-built HPLC system designed for portability consisting of a Scientific System Inc. (SSI) Model 300 LC pump (State College, PA), a SSI Model LP-21 LO-PULSE pulse controller, an Omega Engineering (Samford, CT) Type PSW Model 133 8835 gauge, and an E-Lab OMS TECH (Miami, FL) gradient controller and Chromatography System data manager. Sample injection was performed using a Valco (Houston, TX) injection valve with a 20μL sample loop. Temperature control was achieved with an Eppendorf (Madison, WI) TC-50 controller and CH-30 column heater. Detection was performed with an ISCO (Lincoln, NE) V4 UV-Visible absorption detector. The spectrum of the mobile phase was obtained with a Shimadzu (Columbia, MD) UV-2101PC UV-VIS scanning spectrophotometer. The fluorescence of the mobile phase was evaluated with a Perkin Elmer (Norwalk, CT) luminescence spectrometer Model LS50. The solutions were filtered using a filter degasser assembly from Aura Industries Inc. (Staten Island, NY). The columns compared were SGE (Austin, TX) glass-lined stainless steel of 4mmx25cm (IDxL) dimensions containing 5 μm particles with the following chemistry, pore sizes, surface areas and endcapping: Spherisorb C-18 (80Å, 220m^2/g, fully); Phenyl (80Å, 220m^2/g, partly); C-8 (80Å, 220m^2/g, fully); Hypersil C-18 (120Å, 170m^2/g, fully); Nucleosil C-18 (300Å, 190m^2/g, fully); Nucleosil C-4 (300Å, 190m^2/g, fully).

Mobile phases

The mobile phases and solutions were prepared using HPLC grade water. Eluents were filtered through a 0.45 μm Teflon membrane filter and thoroughly degassed with helium prior to use. The components of the mobile phase for this study were similar to that applied to the determination of inorganic anions (28), although using the same concentrations as those reported, resulted in no resolution of the haloacetic acids. Complete optimization procedures are described later. The mobile phases for the pH study were prepared so as to keep the ionic strength of all mobile phases constant by first increasing the mobile phase pH from 4.53 to 8.50 with sodium hydroxide or decreasing the pH to 2.30 with phosphoric acid,

followed by readjustment to the desired pH. The mobile phase showed three UV absorption maxima at 269, 263 and 258 nm. Table 1 summarizes the ultraviolet absorption of the studied acids at these wavelengths. Acetic, chloroacetic, bromoacetic and dichloroacetic acids do not absorb at all, dibromoacetic acid demonstrates minimal absorptivity in the 258-262 region and iodoacetic acid shows a maximum at 365, with a comparable absorptivity to the mobile phase when its concentration is 1318 parts per million (ppm).

Equilibration of system

Equilibration of an IPC system in some cases can take a very long time. Some authors recommend conditioning the system overnight (29); whereas, others suggest it is not necessary (10). Knox and Jurand (30,31) started with a mobile phase at a slightly higher concentration of the ion pair to reach the equilibrium faster. In the present study, equilibration was generally obtained within 1.5 to 2 hours. Once equilibrium has been obtained, to again reach the equilibrium

TABLE 1

Ultraviolet Absorptivities of the Ion Interaction Reagent and the Acids Studied

Compound	Concentration	Absorptivity at 269nm	263nm	258nm
Benzyltributylammonium	8 mM	2.59	2.78	2.66
Acetic acid	800 ppm	0.00	0.00	0.00
Chloroacetic acid	1511 ppm	0.00	0.00	0.00
Bromoacetic acid	858 ppm	0.00	0.00	0.00
Iodoacetic acid	1318 ppm	2.46	2.53	2.51
Dichloroacetic acid	1000 ppm	0.00	0.00	0.00
Dibromoacetic acid	1425 ppm	0.35	0.69	1.10

conditions after changing just one parameter, such as the temperature, generally takes less than 45 minutes. Regular replacement of frits and the pre-column packing also assisted in the rapid establishment of equilibrium and a stable baseline. The following have been observed when regular system maintenance was not performed: strange peak appearance (sometimes presenting a reproducible retention time), long time required for system stability, loss of resolution, erratic base line, unusual system peak patterns, sudden increases in signal (baseline) rising off scale. These problems were avoided by regular replacement of the pre-column packing material and the frits and filters before problems occurred.

Haloacetic acid standards

The toxicity, corrosiveness and hygroscopic properties of the haloacetic acids (see Table 2) make the handling of these compounds and the preparation of solutions at precise and reproducible concentrations difficult. To minimize these difficulties and to assure that the results were comparable, a single stock solution

TABLE 2

Physical and Chemical Properties of the Acids Studied

Acetic Acid	Formula Weight	Melting Point (°C)	Boiling Point (°C)	pKa (25°C)	Toxicity
Acetic	60.05	16	116-118	4.75	C
Chloro-	94.50	62-64	189	2.85	C, HT
Bromo-	138 95	49-51	208	2.69	C, L
Iodo-	185.95	77-79	-	3.18	C, T
Dichloro-	128.94	13.5	194	1.48	C, T
Dibromo-	217.84	48	195	1.39	C, T
Trichloro-	163.39	54-56	196	0.70	C, T

C = corrosive, HT = highly toxic, L = lachrymator, T = toxic

was carefully prepared with the following concentrations: acetic acid (800 ppm), chloroacetic acid (1511 ppm), bromoacetic acid (858 ppm), iodoacetic acid (1318 ppm), dichloroacetic acid (1000 ppm), and dibromoacetic acid (1425 ppm). All samples were then prepared from the same stock solution. The stock solution was regularly tested by looking for any changes in the UV spectra and regularly comparing ion pair chromatograms run under standard conditions to those run when the solution was first prepared.

RESULTS AND DISCUSSION

A mobile phase containing 8.22 mM benzyltributylammonium, 0.13 mM hexanesulfonate, 5.66 mM monobasic potassium phosphate, pH 5.2 and 11 per cent acetonitrile was able to produce the complete separation of the six acids from acetic acid to dibromoacetic acid in less than 20 minutes as shown in Figure 2. The chromatogram shows 6 positive peaks, one for each of the 6 acids injected preceeded by 5 distinct system peaks including a large negative peak which should correspond to the benzyltributylammonium ion. Trichloroacetic acid had a very long retention time (under the isocratic condition used in this study) which resulted in a severely broadened peak and poor detectability, and, therefore, was not included in the present optimization experiments. Our results are in agreement with prevoius workers who report positive UV absorbance (or fluorescence) when the sample and the ion interaction reagent are of opposite charge (as is in this case), and the sample components elute after the system peaks (32).

The capacity factors for all 7 acids listed in Table 2 was measured under isocratic conditions for the pKa comparison shown below, although gradient elution would be necessary to elute trichloroacetic acid with acceptable peak shape for quantitation. The capacity factors for the acids generally correlate with their acid dissociation constants as seen in Figure 3 for acetic acid through trichloroacetic acid. The pKa of the solute has been shown previously to have a significant effect on the retention of ionic solutes in ion-pair chromatography (33). Acids with higher Ka, at a given pH above their pKa, should have more anions available to interact with the ion interaction reagent and therefore should be more strongly retained than other similar acids with lower Ka values.

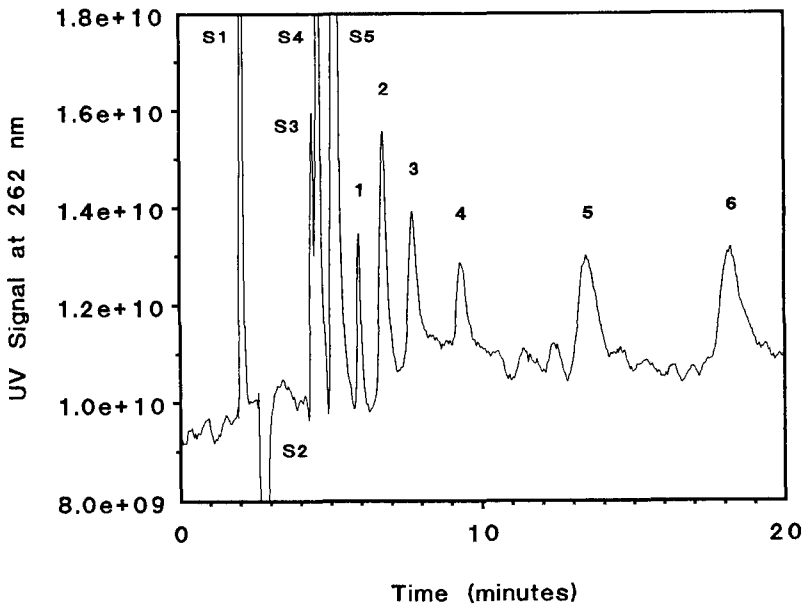

FIGURE 2 Typical isocratic separation of the haloacetic acids employing indirect UV detection. Peak identification: S1-S5 = system peaks; 1 = acetic; 2 = chloro-; 3 = bromo-; 4 = iodo-; 5 = dichloro-; 6 = dibromoacetic acid.

Effect of temperature

The effect of the temperature on the retention time of five haloacetic acids is shown in Figure 4 (complete data is listed in Table 3) using a Spherisorb C18 column. The system pressure fluctuated between 800 pounds per square inch for the higher temperature (58 centigrade degrees) and 1500 pounds per square inch for the lower temperature (22 centigrade degrees). As can be seen, all of the compounds showed non-linear van't Hoff behavior at temperatures below ca. 30°C, and linear behavior above ca. 30°C. Temperatures above ca. 38°C are recommended for good efficiency at an acceptable capacity factor. Linear temperature dependence of retention for C-18 columns using hydro-organic mobile phases has been reported (34-36). Non-linear dependencies have also been

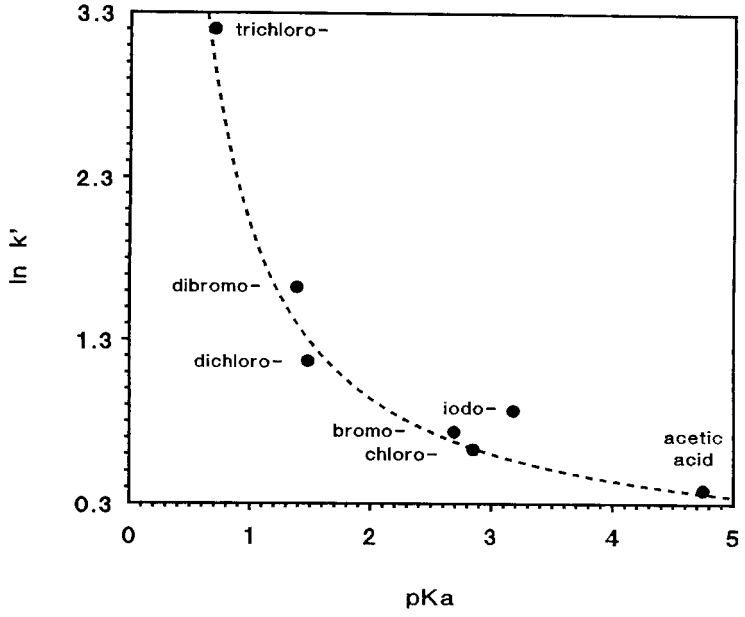

FIGURE 3 Plot of lnk' versus pKa for acetic acid and the following haloacetic acids: trichloro-, dibromo-, dichloro-, iodo-, bromo- and chloro-.

observed for gas chromatography and reversed phase liquid chromatography with silicas chemically bonded to n-octyl, n-octadecyl, aminobutyl and 2,4-dinitroanilinobutyl groups (37-39). A debate has arisen about the nature of phase transitions in these kind of stationary phases. Transitions can occur due to conformational changes in the stationary phase or the solute or due to changes in the ionization of the acid studied. It is unlikely that the solutes studied here would undergo conformational changes under the conditions studied, and the pH of the mobile phase used (5.2) should be high enough to keep the acids (with the exception of acetic acid) in their ionic form. Therefore, it is reasonable to assume that a real phase transition, with a corresponding change in the retention mechanism is occurring.

Our 30°C transition temperature is similar to that reported by other workers. Transitions have been reported for monomeric C-18 stationary phases at

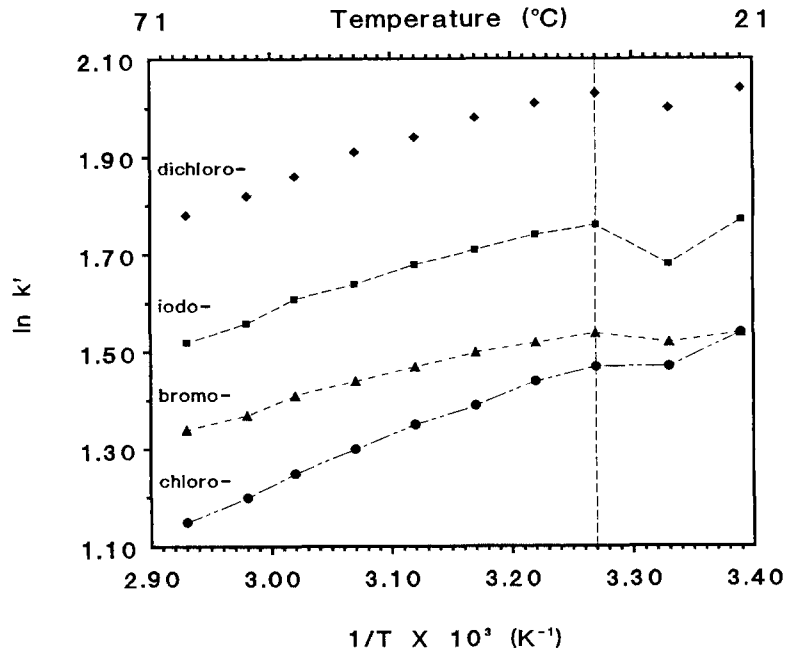

FIGURE 4 Plot of lnk' versus 1/T for chloro-, bromo-, iodo-, and dichloroacetic acid.

ca. 22°C in the absence of solvent using a gas chromatographic technique (40,41) and ca. 30-31°C for monomeric reversed-phase HPLC stationary phases (35,42). The present results indicate that below the phase transition point (30°C) the stationary phase may behave more like a solid (governed by surface adsorption), likely resulting in decreased interaction between the relatively bulky benzyltributylammonium ion and the stationary phase, thereby reducing retention. Above the phase transition point, the stationary phase behaves more like a liquid allowing partitioning and deeper penetration of the ion pair reagent into the stationary phase and therefore increased retention, supported by workers who report a vertical penetration of the solute molecules into the bonded layer (43-45).

TABLE 3

Logarithm of the Capacity Factors Obtained at Different Temperatures. Detector = 260 nm, Flow rate = 0.6 ml/min, [KH_2PO_4] = 5.66 mM, [1-hexanesulfonate] = 0.13 mM, [acetonitrile] = 11%, pH = 5.2, [benzyltributylammonium] = 8.22 mM.

Temp. (°C)	lnk' for -acetic acid				
	Chloro-	Bromo-	Iodo-	Dichloro-	Dibromo-
22.1	1.545	1.545	1.775	2.039	2.686
26.4	1.470	1.515	1.677	2.004	2.642
30.0	1.470	1.541	1.756	2.028	2.664
33.7	1.437	1.524	1.740	2.017	2.626
38.5	1.391	1.500	1.712	1.981	2.590
42.5	1.348	1.475	1.681	1.944	2.542
46.5	1.302	1.442	1.645	1.905	2.480
50.6	1.250	1.409	1.607	1.864	2.425
54.9	1.203	1.374	1.564	1.820	2.365
58.1	1.154	1.338	1.522	1.775	2.303

Effect of phosphate concentration

Figure 5 illustrates the dependence of the lnk' of acetic acid, chloro-, iodo-, and dichloroacetic acid on the monobasic potassium phosphate concentration (complete data in Table 4) employing a Spherisorb C18 column. The capacity factor values of the solutes decrease as the phosphate concentration increases, in agreement with other studies investigating the influence of eluent ionic strength on retention in ion-pair HPLC systems (19,27,28). Using the ion-interaction model, retention begins with the establishment of an equilibrium between the lipophilic reagent ion (ion interaction reagent) and the stationary phase followed by retention of a sample of opposite charge to the reagent ion depending on both electrostatic attraction and a lipophilic adsorptive force. Therefore, increasing ionic strength should decrease retention by influencing the electrostatic attraction (27). We have

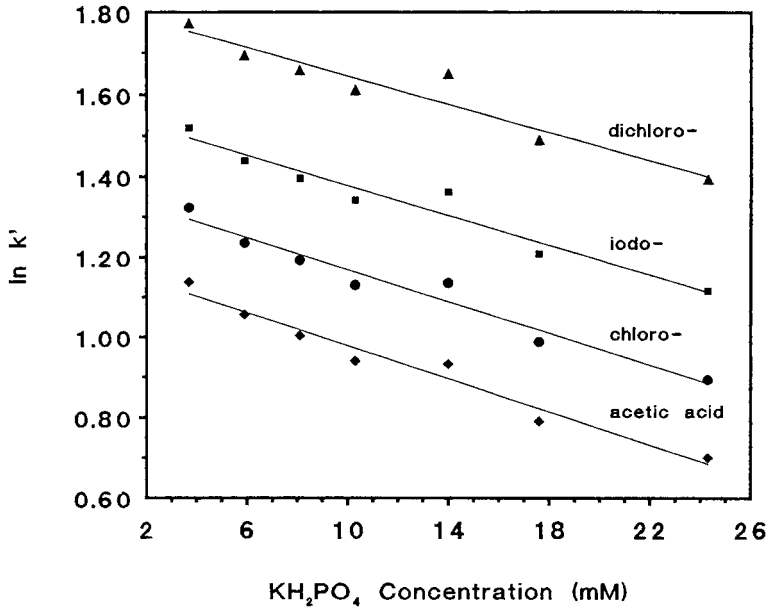

FIGURE 5 lnk' as a function of KH_2PO_4 concentration for acetic acid, chloro-, iodo- and dichloroacetic acid.

also observed that response is increased and peaks shapes are improved at higher concentrations of monobasic potassium phosphate.

Effect of hexane sulfonate concentration

Increasing the concentration of the hexane sulfonate in the mobile phase results in a corresponding decrease in retention for the acids as seen in Table 5 using a Spherisorb C18 column. Figure 6 illustrates the lnk' values of three of the acids as a function of the hexanesulfonate concentration (the effect is similar for all of the acids). Again, this is the expected behavior, given that the hexane sulfonate ions, being of like charge to the solutes, will compete for the ion interaction reagent and decrease the retention of the solutes. There is almost no variation of the selectivity when the concentration of the hexane sulfonate varies. These results

TABLE 4

Logarithm of the Capacity Factors Obtained With Different Concentrations of KH_2PO_4. Flow rate = 0.7 ml/min, temperature = 52.3°C, [1-hexanesulfonate] = 0.13 mM, [acetonitrile] = 11%, pH = 5.2, [benzyltributylammonium] = 8.22 mM.

[KH2PO4]	lnk' for acid					
	Acetic	Cl-	Br-	I-	DCl-	DBr-
3.7	1.138	1.323	1.372	1.520	1.774	2.296
5.9	1.057	1.236	1.295	1.439	1.695	2.227
8.1	1.004	1.193	1.248	1.396	1.659	2.193
10.3	0.941	1.131	1.186	1.342	1.612	2.146
14.0	0.933	1.136	1.193	1.362	1.651	2.200
17.6	0.791	0.989	1.057	1.208	1.490	2.041
24.3	0.700	0.894	0.959	1.115	1.393	1.957

TABLE 5

Logarithm of the Capacity Factors Obtained With Different Concentrations of 1-Hexanesulfonate. Flow rate = 0.8 ml/min, temperature = 51.4°C, [KH_2PO_4] = 5.66, [acetonitrile] = 11%, pH = 5.3, [BTA] = 8.22 mM.

[1-hexane-sulfonate]	lnk' for acid					
	Acetic	Cl-	Br-	I-	DCl-	DBr-
0.05	0.858	1.045	1.078	1.241	1.505	1.982
0.10	0.803	0.983	1.025	1.179	1.438	1.908
0.13	0.852	0.934	0.988	1.128	1.384	1.848
0.16	0.713	0.888	0.932	1.076	1.333	1.793
0.19	0.673	0.841	0.886	1.029	1.280	1.737

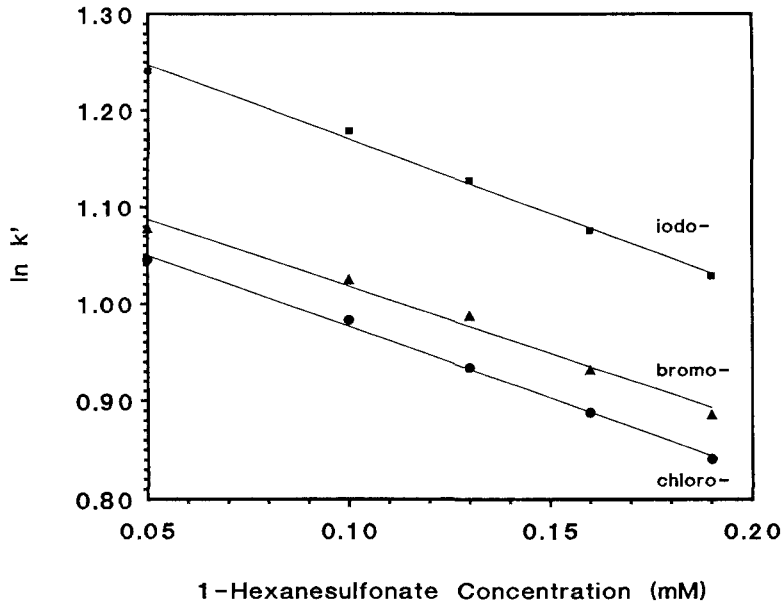

FIGURE 6 lnk' as a function of 1-hexanesulfonate concentration for chloro-, bromo- and iodoacetic acid.

are different than those reported previously for inorganic anions in a similar chromatographic system, where significant variations in selectivity were observed even with small variations in hexane sulfonate concentration (28). In addition to reducing the retention time of the solutes, in the present study, increasing hexane sulfonate concentrations increased efficiency and improved peak symmetry.

Effect of acetonitrile concentration

The effect of acetonitrile concentration on the capacity factors of acetic, chloroacetic, bromoacetic, iodoacetic, dichloroacetic and dibromoacetic acids are shown in Figure 7 (From data in Table 6) employing a Spherisorb C18 column. As expected, the acetonitrile concentration produces a dramatic effect on the retention of all of the solutes. Hydrophobic effects are known to dominate in the

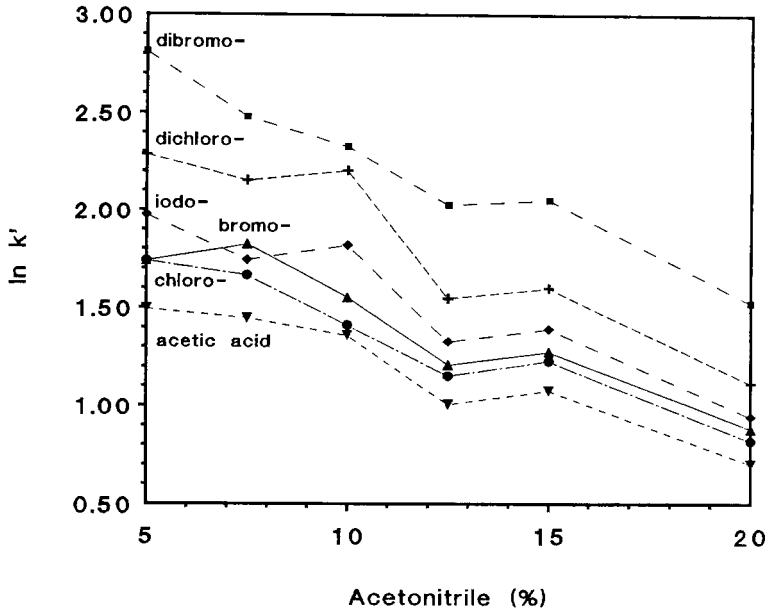

FIGURE 7 lnk' as a function of the percent (v/v) acetonitrile for acetic acid, chloro-, bromo-, iodo-, dichloro- and dibromoacetic acid.

water/organic solvent binary eluent systems commonly employed in RP-IPC (46). Hydrophobic expulsion is generally attenuated by increasing the concentration of organic modifier. Although some selectivity variations are seen at acetonitrile concentrations below ca. 10%, these separations generally yielded broader, less symmetric peaks and poorer reproducibility compared to separations performed at 10% acetonitrile and above. Previous workers have demonstrated linear relationships between the capacity factor of analytes and the ion interaction reagent for various IPC systems (21,33,47).

Effect of the mobile phase pH

Table 7 lists the results obtained when mobile phases are adjusted to different pH values. No separation was observed at pH below 3, and very little

TABLE 6

Logarithm of the Capacity Factors Obtained With Different Concentrations of Acetonitrile. Flow rate = 0.8 ml/min, temperature = 52.2°C, $[KH_2PO_4]$ = 3.68 mM, [1-hexanesulfonate] = 0.13 mM, pH = 5.2, [BTA] = 8.22 mM.

[acetonitrile]	lnk' for acid					
	Acetic	Cl-	Br-	I-	DCl-	DBr-
5.7	1.495	1.739	1.739	1.975	2.287	2.814
7.5	1.448	1.665	1.824	1.743	2.152	2.481
10.0	1.361	1.414	1.554	1.820	2.204	2.329
12.5	1.007	1.153	1.210	1.328	1.549	2.028
15.0	1.078	1.229	1.276	1.393	1.603	2.055
20.0	0.701	0.819	0.881	0.944	1.115	1.526

separation was seen at pH above 7. Reasonable separation was achieved from pH 3.5 to pH 6.3. Figure 8 is a plot of relative capacity factor (k'/k'max) versus pH, (k'max is the maximum capacity factor). The dependence on the pH on retention for the present system is in agreement with results reported by other workers, where, at low pH, the acids are no longer ionized, and, at high pH, hydroxyl ions formed compete with the sample anions leading to a smaller number of sample ion pairs in the stationary phase (48). For the acids in the present study, a pH of ca. 5 was found to be optimal.

Effect of ion interaction reagent concentration

Results for the effect of benzyltributylammonium ion concentration on retention are summarized in Table 8. No separation was obtained with concentrations below 6 mM. At 8 mM, all solutes, with the exception of acetic acid, was achieved. At 14 mM and above, the response of the UV detector used

TABLE 7

Capacity factors obtained with mobile phases adjusted to different pH values. Flow rate = 0.8 ml/min, temperature = 51.5°C, [KH_2PO_4] = 6.0 mM, [1-hexanesulfonate] = 0.13 mM, [acetonitrile] = 12%, [benzyltributylammonium] = 8.22 mM, and stationary phase = C18 Spherisorb.

pH	k' for acid					
	Acetic	Cl-	Br-	I-	DCl-	DBr-
2.41	0.00	0.00	0.00	0.00	0.00	0.00
3.47	2.01	2.41	2.60	3.00	3.76	7.10
4.34	2.09	2.65	2.65	3.27	4.29	7.19
4.58	2.59	3.19	3.19	3.79	4.84	7.82
5 46	2 77	3 33	3.58	4.15	5.50	9.50
6.33	1.65	1.83	2.20	2.36	3.21	6.20
7 26	1.95	1 95	1.95	1.95	2.59	5.09
8.31	1.82	1.82	1.82	1.82	2.28	4.80

(at 262 nm) was off scale. Therefore, for the present study, the useful range was determined to be between the limited range of 10 to 12 mM. Although the data set is too small to generalize any conclusions, the increase in capacity factors with increased ion interaction reagent concentration is in keeping with results reported previously by other workers (20).

Role of the stationary phase

The different analytical columns were compared by injecting the same sample composition under identical instrumental conditions as follows: column temperature = 38°C, flow rate = 0.8 ml/minute, mobile phase containing 12 mM benzyltributylammonium, 0.1 mM hexanesulfonate, 6 mM monobasic potassium phosphate, pH 5.2 and 16 % (v/v) acetonitrile. The Octyl (C-8) and Nucleosil C-4

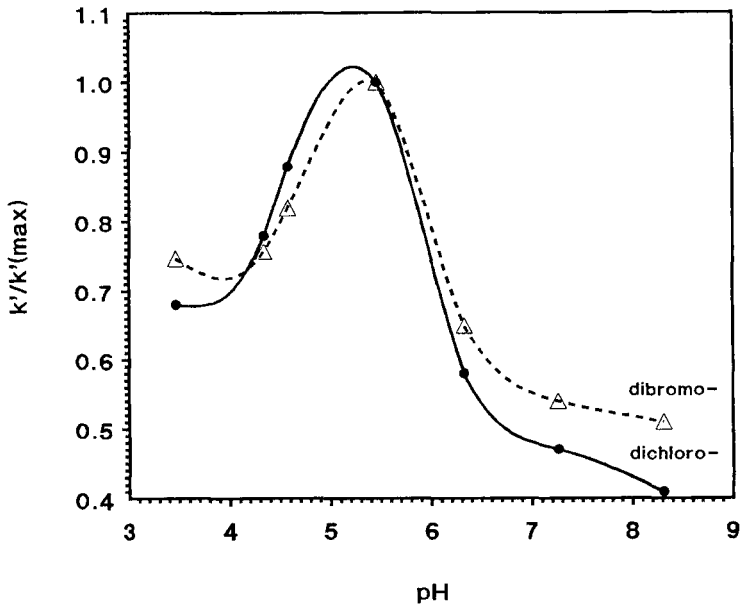

FIGURE 8 Plot of k'/k'(max) as a function of pH of the mobile phase for dibromo- and dichloroacetic acid.

TABLE 8

Capacity Factors Obtained With Mobile Phases Containing Different Concentrations of Benzyltributylammonium Ion. Flow rate = 0.8 ml/min, temperature = 51.5°C, [KH_2PO_4] = 60 mM, [1-hexanesulfonate] = 0.13 mM, [acetonitrile] = 12%, pH = 5.45, and stationary phase = C18 Spherisorb.

[BTA] (mM)	k' for acid					
	Acetic	Cl-	Br-	I-	DCl-	DBr-
6	0.00	0.00	0.00	0.00	0.00	0.00
8	0.00	1.66	1.78	2.06	2.74	4.55
10	1.45	1.75	1.90	2.20	2.92	4.94
12	1.47	1.88	2.09	2.38	3.23	5.08
14	----------MOBILE PHASE OFF SCALE----------					

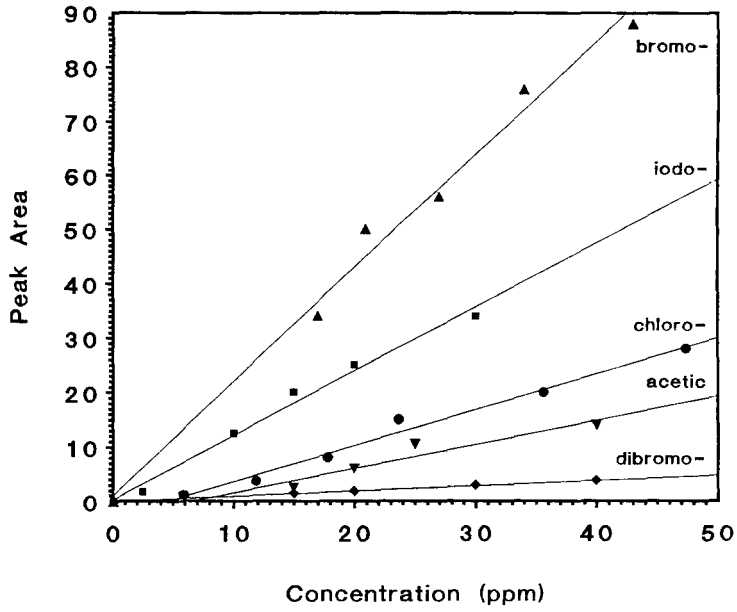

FIGURE 9 Peak areas obtained at 262 nm with different concentrations of acids. Flow rate = 0.6 ml/min, temp. = 38.0°C, [KH2PO4] = 5.66 mM, [1-hexanesulfonate] = 0.13 mM, [acetonitrile] = 11%, pH = 5.2, [BTA] = 8.22 mM, and stationary phase = Spherisorb C18.

yielded the poorest separation, able to separate just a few of the acids. The phenyl column showed very good separation for all compounds (except acetic acid) and very good peak shapes, but capacity factors for the different acids were low, which could be a problem if a more complex sample, containing other compounds with retention times in the same range, were to be analyzed. Hypersil C-18, demonstrated excellent separation, but poor peak shapes for dichloroacetic and dibromoacetic acids. Overall, the best results were obtained for Spherisorb C-18 and Nucleosil C-18, with convenient capacity factors and good peak shapes.

Calibration curves and detection limits

The linearity and the detection limit of the system was evaluated for all of the acids using UV absorption at 262 nm. The mobile phase contained 8.22 mM

benzyltributylammonium, 0.13 mM hexanesulfonate, 5.66 mM monobasic potassium phosphate, pH 5.2 and 11 per cent acetonitrile in water. The flow rate was 0.6 mL/minute at a column temperature of 38°C. All acids were determined in the presence of the other five, in concentrations ranging from 2 to 48 ppm with linear responses seen for all of the acids as seen in Figure 9. The detector response and detection limits were highly dependent on the acid with better sensitivity for the monohaloacetic acids than for the dihaloacetic acids. Detection limits were estimated by determining the lowest concentration which yielded significant and reproducible detector response with a signal-to-noise ratio of ca. 4. Detection limits were in the range 2-15 ppm with correlation coefficients listed below: Acetic acid = 15 ppm (r = 0.949); Chloroacetic acid = 2 ppm (r = 0.993); Bromoacetic acid = 8 ppm (r = 0.994); Iodoacetic acid = 3 ppm (r = 0.994); Dichloroacetic acid = 10 ppm (r = 0.993); Dibromoacetic acid = 15 ppm (r = 0.998).

CONCLUSIONS

Of the commercial columns tested, Spherisorb C-18, yielded the best results for the separation of the haloacetic acids studied. This column exhibited a linear van't Hoff behavior (decrease in lnk' with increasing temperature) in the range of ca. 30°C to 60°C with a phase transition resulting in a loss of resolution below 30°C. The capacity factor of the acids decreases with increases in the phosphate concentration and increases in the concentration of 1-hexanesulfonate in the mobile phase with little variation in the selectivity. Values of pH from 3.5 to 6.3 provided acceptable resolution with optimal conditions seen at ca. pH 5. Retention of the analytes increases with increasing benzyltributylammonium ion with an optimal value between 10 and 12 mM. Increasing the acetonitrile concentration produces one of the most significant effects (in the usable concentration range), resulting in a significant decrease in the retention of the acids studied.

The relative effects of the six variables studied can be compared by processing all of the data for one of the solutes, chloroacetic acid, and calculating the theoretical change which would be required to decrease the k' by one unit (i.e. from 4 to 3), corresponding to a decrease in the lnk' of 0.3 (i.e. from 1.4 to 1.1). This change would require increasing the column temperature by 27°C, increasing the KH_2PO_4 concentration by 15 mM, increasing the 1-hexanesulfonate

concentration by 0.2 mM, decreasing the pH by 2 units (depending on the starting pH, i.e. from 5.5 to 3.5), decreasing the benzyltributylammonium concentration by 10 mM (although this would likely be below the minimum of 8 mM depending on the starting point), or increasing the amount of acetonitrile by 5 % (v/v).

This chromatographic method is able to separate a mixture of acetic, chloroacetic, bromoacetic, iodoacetic, dichloroacetic, and dibromoacetic acids with good resolution in less than 20 minutes under isocratic conditions. Aqueous samples can be injected directly, with no sample preparation and the system has a linear response for all of the acids in the range studied (up to 50 ppm). Detection limits as low as 2 parts per million were possible using a UV-visible detector and lower detection limits may be feasible utilizing indirect fluorescence detection as the UV-absorbing mobile phase employed here is also intensely fluorescent.

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THE DETERMINATION OF METHADONE AND METABOLITES IN HUMAN URINE BY HPLC WITH ULTRAVIOLET, AND PARTICLE BEAM MASS SPECTROMETRIC DETECTION

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ABSTRACT

Liquid chromatography coupled to Mass Spectrometry has been used to identify and quantitate methadone and two of its metabolites following a normal phase separation on a cyano-phase column. Solvent extraction was used to recover the target compounds from urine at the optimal pH of 10, (recovery: 88%, methadone; 75%, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP). Routine analysis may be performed using UV detection at 290 nm. However, for greater selectivity and identification of eluates mass spectrometry is preferred. In the urine samples studied the methadone/EDDP ratio varied from 1:1 to 1:10. Two samples showed evidence for the presence of 1,5-dimethyl-3,3-diphenylpyrrolidone (DDP). Methadone may be

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detected selectively at m/z = 223 but the ion abundance is weak. Detection using the base peak m/z = 72 is preferred because of the increased sensitivity it confers although selectivity of detection is lost.

INTRODUCTION

In 1965 Dole and Nyswander (1) reported the importance and effectiveness of methadone as a treatment for opiate dependence. Since that time, methadone maintenance treatment has become one of the most widely used procedures for opiate dependency treatment. This application generated the need to understand the metabolism of methadone and to monitor the concentration of the drug and its metabolites in vivo. Various chromatographic techniques have been employed including thin layer chromatography (2), gas liquid chromatography (3), gas chromatography-mass spectrometry (4), radioimmunoassay (5) and enzyme immunoassay (6) techniques. More recently HPLC has been used (7) to study methadone metabolism in dogs but metabolite identity was presumed rather than established. An improved method based on HPLC for the determination of methadone and two major metabolites in rat plasma has also appeared (8). Separation is achieved on a C_{18} reversed phase column with an acetonitrile/water mobile phase. Solid phase extraction was used for preparation of the samples and gave excellent recoveries (85-100%). Difenoxin was used as internal standard. Studies of methadone metabolism in humans are less common. However, some results have appeared. For example the influence of liver disease on methadone metabolism has been assessed (9) and found to be negligible. Recent publications have tended to concentrate on the use of methadone to counteract opiate addition with particular emphasis being given to the measurement of the concentration of methadone itself in plasma (10,11). However this method utilises a silica column and UV detection at 215 nm.

Chromatographically silica columns have some disadvantages. Control of activity of the silica surface may be important to the separation and the carry-over of small amounts of water from the sample preparation stage may affect column activity. Pre-saturation of the silica surface with water mitigates this effect. Similarly, presaturation reduces the potential of silica to irreversibly adsorb material at the top of the column. Use of the low detection wavelength (215 nm) is relatively non-specific and may yield a relatively high signal-to-noise ratio. However, this method has been used successfully to study the steady-state pharmacokinetics of methadone in opioid addicts (12).

A major factor influencing methadone metabolism is reported to be the co-administration of other drugs. Methadone kinetics have been reported to be disrupted by carbamazepine (13), barbiturates (14) and amitriptyline (15). Hence methods which identify and quantitate both the parent compound and the major metabolites are needed to allow variation in the metabolism of methadone to be studied We now report some preliminary results on the use of HPLC coupled to particle beam mass spectrometry to identify metabolites of methadone extract from the urine of patients undergoing methadone therapy.

EXPERIMENTAL

Materials

All solvents were of HPLC grade and ammonium acetate was AnalaR grade (BDH Chemicals Ltd). Distilled water was checked for UV absorbence (200 nm - 300 nm) before use. Racemic d, l - methadone (Sigma Chemicals, Poole, Dorset) and EDDP (donated by Leeds Hospital) were used as received.

Equipment

A Philips Analytical PU4015 dual piston pump (Philips Analytical, Cambridge UK), a UV detector (PU4025), (Philips Analytical, Cambridge, UK) fitted with an 8 µl flow cell (10 mm path length) and an integrator (HP3394, Hewlett Packard) were used. The column was Nucleosil 5CN (250 mm x 4.6 mm id) (Phenomenex Cat No OOG-0324-EO) and injection (20 µl or 100 µl) was via a Rheodyne 7010 valve. The mobile phase was 30% 0.1M ammonium acetate adjusted to pH 3.6 with glacial acetic acid: 70% acetonitrile. Batches of mobile phase were degassed with helium (20 minutes) before use. A detector wavelength of 280 nm and a flow rate of 1.0 ml min^{-1} for UV detection and 0.6 ml min^{-1} for mass spectrometric detection was used. The mass spectrometer (TRIO-1) and particle beam interface (LINC type) were supplied by VG MASSLAB Ltd (Crewe Road, Wythenshawe, Manchester M23 9BE, UK).

Extraction Procedures

The pH of 10 ml urine was adjusted to 9.5 - 10.0 with ammonium hydroxide. A 1 ml aliquot was then mixed with 5 ml of 1-chlorobutane (Aldrich Chemical Co Ltd, The

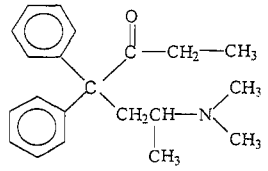
Old Brickyard, New Road, Gillingham, Dorset, SP H4JL) and the sample shaken mechanically for 30 minutes. Centrifugation (2000 rpm, 5 mins) yielded a 1-chlorobutane top layer which was removed to a clean glass vial. 20 µl of dimethylformamide was added to act as a "keeper" solvent to minimise loss of methadone during evaporation of 1-chlorobutane under a stream of dry nitrogen. The residue sample (volume approximately 20 µl) was stored at O °C until reconstituted in the mobile phase (500 µl) immediately prior to injection.

RESULTS AND DISCUSSION

Methadone (I) is extensively metabolised in the body by a variety of pathways. For subjects on a methadone maintenance programme the ratio of the primary metabolite (EDDP) to methadone is normally much higher than in an overdose case. The primary metabolic reaction in man is oxidative N-demethylation to form an intermediate (N-desmethyl -methadone) which spontaneously cyclises to form EDDP (II), which thus contains a pyrrolidine structure. A diphenyl methyl moiety is common to both structures and provides the major source of uv absorbence albeit a relatively weak one due to non-conjugation. Subsequent reactions include hydroxylation of a phenyl ring to yield hydroxy-EDDP (III) which now contains a phenolic function, and demethylation to provide EMDP (IV). Another pathway involves ketone oxidation and oxidative removal of the ethyl group on methadone followed by spontaneous cyclisation to form DDP (V) which contains a pyrrolidone structure.

Chromatography

Polar drugs and their metabolites are usually separated by reversed phase chromatography. The literature indicates that a cyano-bonded phase is most appropriate for methadone and was selected for this work. Selection of the mobile phase was restricted by the need for compatibility with the particle beam interface. Hence, no involatile buffer salts could be used. Likewise, use of ion-pairing agents such as alkane sulphonic acids is excluded because of incompatibility with the use of mass spectrometry as the detection mechanism. The choice of a cyano-bonded phase however, renders their use unnecessary as the cyano group provides the polar functionality necessary for retention. Ammonium acetate was chosen as the buffer salt as it is volatile and hence amenable to use with the particle beam interface and mass spectrometry. It also contributes marginally to improved chromatographic efficiency by moderating peak tailing.



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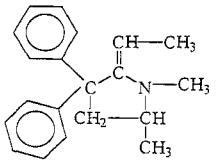

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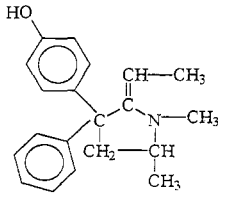

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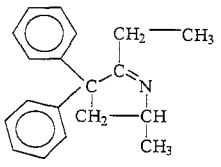

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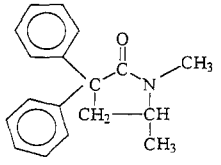

(V)

The most often selected wavelength for detection of methadone is 215 nm. However, given the much greater concentration of methadone present in urine we have investigated the use of higher wavelengths which have provided the added benefit of greater selectivity for our particular compounds of interest. Molar extinction coefficients are generally lower at higher wavelengths but signal-to-noise ratios are better and this fact may be exploited to regain sensitivity of detection. A UV spectrum of methadone in the mobile phase indicated two maxima at 259.8 nm and 293.6 nm of similar intensity [absorbence (259.8 nm) = 0.187, absorbence (213.6 nm) = 0.183, for a 1 mg in 10 ml solution]. The higher wavelength was chosen because of the increase in absorbence at 260 nm caused by the glacial acetic acid. Using the selected chromatographic conditions (1.0 ml min^{-1}, 70% acetonitrile: 30% ammonium acetate, pH = 3.6 with glacial acetic acid, 20 µl injection, 293.6 nm) the retention time for methadone was 5.35 min (k' = 4.94) and for EDDP was 5.70 min (k' = 5.33) for standards injected in the mobile phase. For spiked urine extracts a large amount of co-extracted material elutes at approximately 2.9-3.5 minutes and causes a slight shift of retention to 5.45 minutes (k'=5.05) and 5.90 (k'=5.55) respectively. The nature and complexity of the co-extracted material varies from sample to sample and this is reflected in small changes in retention time for the target analytes. The example shown in Figure 1 is a spiked urine sample which is particularly rich in co-extracted material yet still gives good resolution for methadone (t_R = 5.45, k' = 5.05) and EDDP (t_R = 5.89, k'=5.5).

For mass spectroscopic studies the flow rate was reduced to 0.6 ml min^{-1} to accommodate the capabilities of the particle beam interface. Both the EI and CI modes were evaluated. The mass range used was 65-500 amu. Methadone and EDP standards were used to generate spectra. Urine extracts from known methadone users were screened to attempt to identify other possible metabolites present. Using the EI mode methadone, EDDP and DDP (V) were identified but when CI(methane) was employed hydroxylated EMDP(III) was observed and DDP was not found. Figure 2 shows a summed ion intensity (m/z = 65:500) chromatogram obtained in the EI mode for a urine extract. Comparison of mass spectra obtained indicates that the peaks at t_R = 8.79, t_R 7.04 and t_R = 9.31 are methadone, DDP and EDDP respectively. Examination of the peaks at t_R = 6.16 and ca 8.26 indicate that they do not contain ions representative of other metabolites. Presumably these are co-extracted substances which are not uv responsive at 290 nm (Figure 1).

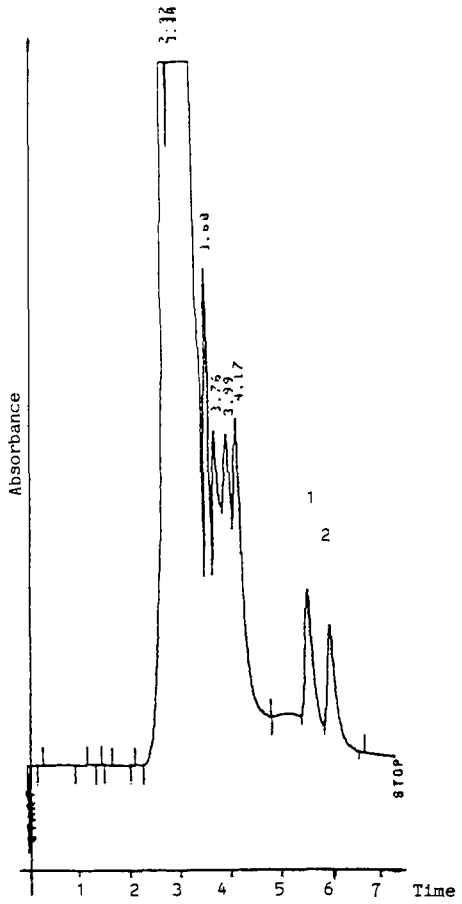

Figure 1 Urine sample spiked with methadone (40 mg l^{-1}, 0.129 mmol) and EDDP (40 ng l^{-1}, 0.144 mmol) chromatographed following extraction with n-butyl chloride, 1 = methadone, 2 = EDDP

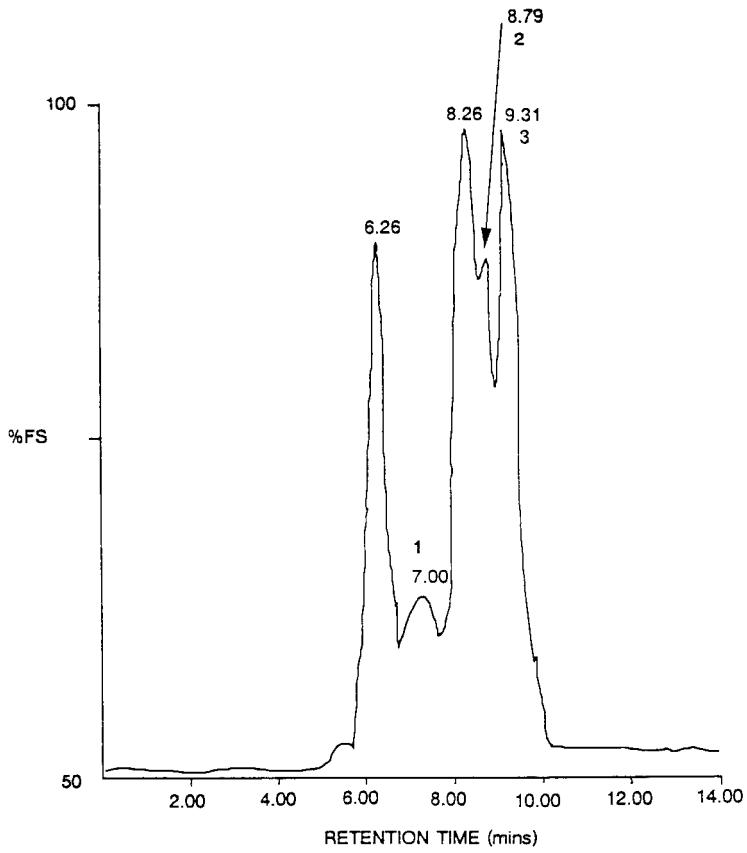

Figure 2 Summed ion intensity chromatogram obtained in EI mode for a urine extract 1 = methadone, 2 = DDP, 3 = EDDP.

Table 1 shows the groups of ions for the various compounds under study. In the EI mode, methadone gives a strong base peak at m/z = 72 corresponding to the $C_4H_{10}N$ fragment from the side chain. Methadone concentrations are frequently found to be too low to use the weak ion at m/z = 223 for monitoring as the EI abundance is only 1.65%. In order to enhance sensitivity the less selective but much more intense base peak (100% relative intensity) at m/z = 72 may be used. No parent ion for methadone (EI mode) at m/z = 310 was observed. Other fragments with masses of

Table 1 Suitable ions for selected ion monitoring for methadone and its major metabolites.

ANALYTE	MW	$[M+1]^+$ (CI)	MONITORING ION	OTHER IONS
METHADONE	310	311	72	223,265,165,179
EDDP	277	278	277	200,234,262,276,106
EMDP	263	264	263	208
HYDROXYLATED EDDP	293	294	-	-
DDP	265	266	265	194,179,130,115

165 and 179 appear to contain the diphenylmethyl moiety. EDDP shows a molecular ion at m/z = 277. The peak at m/z = 208 is reportedly weak (0.8%) and we confirm this observation. Loss of the sidechain is thus not a favoured process. The fragment of mass 106 appears common to both EDDP and DDP. No evidence for the presence of significant concentrations of other metabolites was found in the samples studied.

Calibration

Calibration curves were obtained for methadone and EDDP for use in recovery studies. External calibration was used and uv detection was employed. Calibration curves over the range 0 to 100 mg l^{-1} for both methadone (0-0.323 mmol) and EDDP (0-0.361 mmol) were obtained and found to be linear for both methadone (slope = 1.480 = intercept (y) 3.94 : correlation coefficient = 0.9998 and EDDP, (slope = 1.934: intercept (y) = 3.57, correlation coefficient = 0.9978). The calculated limit of detection with detection in the ultra-violet was ca 20 ng on column, as reported previously by Wolff et al. (11)

Recovery of Methadone and EDDP from Urine

Liquid-liquid extraction methods are most commonly used for the recovery of methadone from urine. Both methadone and its metabolites may be recovered from alkaline urine. A high pH suppresses the protonation of methadone and related compounds rendering them more readily partitioned into an organic solvent. Published extraction methods reveal the use of dichloromethane, hexane, ethylacetate and 1-chlorobutane as partitioning solvents. Attempts to achieve efficient recovery with hexane, dichloromethane and ethylacetate proved unsuccessful. Previous work with 1-chlorobutane gave mixed values for recovery with Wolff et al (11) reporting 98% recovery of methadone (range 90-110%) whereas Buice (16) claims only 63.5%. Sullivan and Blake (17) demonstrated that 1-chlorobutane was an effective extracting solvent. To optimise the extraction procedure, two blank urine samples were spiked with methadone to give concentrations of 1.37 mg l^{-1} (4.4 μmole) and 1.48 mg l^{-1} (4.8 μmole) respectively for recovery at low concentrations and 22, 40, 60 and 80 mg l^{-1} for recovery at high concentrations. Extractions were performed at five different pH values (2.0, 5.0, 8.0, 10.0, 12.0) by adjustment with either glacial acetic acid or ammonium hydroxide solution. 1-Chlorobutane was used as solvent. The extraction time (by shaking) was 30 minutes, followed by centrifugation (2000 rpm, 5 minutes)

and removal of the upper layer . Approximately 20 µl of dimethylformamide (DMF) was added as 'keeper' solvent to minimise loss of analytes during evaporation of the 1-chlorobutane under a gentle stream of dry nitrogen. The oily residue consisting of extracted material with ca 20 µl of DMF was stored at 0 °C until reconstituted with mobile phase immediately prior to analysis. The average recovery for methadone was 88% at pH, 10 with lower values for other pH values.

A similar process was performed for recovery of EDDP from urine spiked at 1.5 mg l^{-1} (5.4 µmole) and at 40 µg l^{-1} (144 µmole) and 60 mg l-1 (216 µmole). Again the pH was varied over the same range as for methadone with the best recovery being obtained at pH = 10 (75%). Other values obtained were 32.5% (pH=12), 42.5% (pH=8) and no detectable amount was recovered at pH2 and pH5.

It should be noted that whilst the extraction of methadone and EDDP is from alkaline urine, the chromatographic separation is carried out at pH 3.6. Furthermore, no methadone or EDDP was recovered into the organic phase at pH 2 or 5. Both methadone and EDDP can be classed as tertiary amines although the nitrogen atom in EDDP is contained in a 5-membered ring. Both compounds are thus likely to display basic characteristics and exist as free bases at the pH of extraction. At acidic pH however the lone pair on the nitrogen will protonate rendering both these structures cationic and thus highly polar. Hence there is a need to use an ion-pairing agent to obtain retention on a C_{18} bonded phase column. When a cyano-bonded phase is used the -C≡N group provides sufficient electron density to retain the protonated species. Hence retention is affected by both solvent composition and pH. In the separation system used it is the acetate ion which acts as the counter ion to the protonated compounds and thus duplicates the role of the ion pairing agent (normally an alkyl sulphonic acid group,) albeit with a smaller effect. This small effect does, however, contribute to an improvement in peak shape. Hence the cyano column operates in a similar retention mode to a silica column (11) but suffers less from irreversible adsorption.

Application to Urine Samples

A total of 16 samples was obtained from a drug therapy clinic. Each sample was extracted using the procedure based on 1-chlorobutane and analysed using both UV and MS detection. Of these 16 samples, 8 revealed no detectable amounts of either methadone or any identifiable metabolite. Of the 8 positive samples all contained

measurable concentrations of both methadone and EDDP. The ratio of the concentrations of methadone to EDDP ranged from <1:1 (3 samples) through an approximate 1:1 ratio (2 samples) to 1:10 (3 samples). These results tend to confirm that methadone metabolism is individually defined. In addition, two samples studied showed evidence (m/z =265) for the presence of DDP. The mass chromatogram (Figure 3) shows the response for DDP at t_R=7.00. The peak at t_R = 6.23 in the ion chromatogram for m/z =72 is not thought to be related to methadone and serves as a reminder of the non-specificity of this ion.

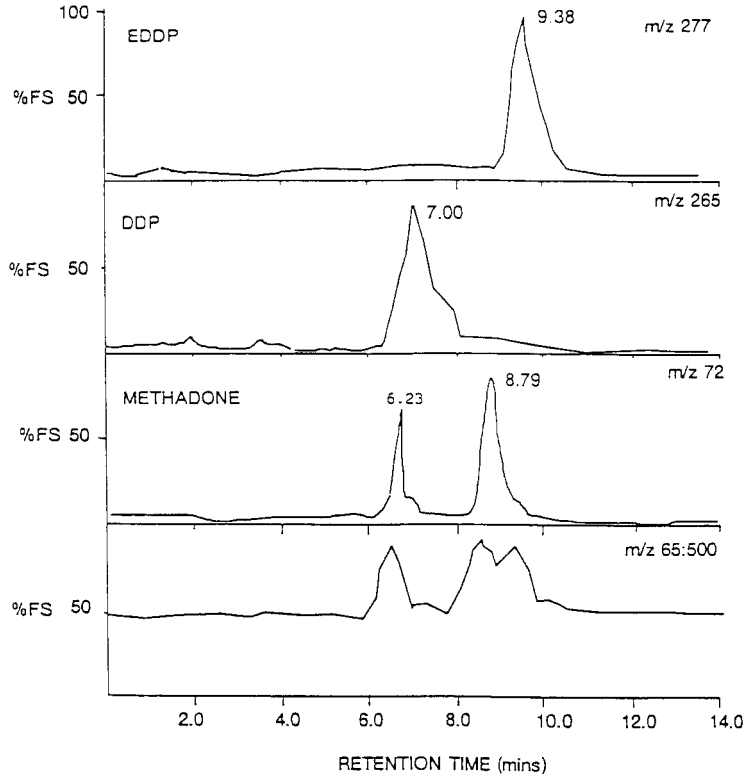

Figure 3 Mass chromatograms obtained in EI mode for a human urine extract indicating the presence of DDP (t_R = 7.00m, m/z = 265) in addition to EDDP (t_R = 9.38m m/z = 377) and methadone (t_R = 8.79m, m/z = 72).

CONCLUSIONS

Methadone and EDDP may be recovered from alkaline urine using 1-chlorobutane as solvent with recoveries of 88% and 75% respectively.

Separation on a cyano-bonded column is achieved with uv detection at 290 nm or by particle beam mass spectrometry. Of 16 samples analysed, 8 were found to contain methadone and EDDP and two of these revealed evidence of an additional metabolite DDP but no standard was available for quantitation. Although analysis may be carried out by using UV detection, mass spectrometry provides useful information about the identity of co-extracted material and provides evidence of identity which can indicate the presence of additional metabolites.

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QUANTITATIVE HPTLC DETERMINATION OF ELEMENTAL SULFUR IN SULFUR TOPICAL MEDICATIONS

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ABSTRACT

A method was developed for determination of elemental sulfur in sulfur topical pharmaceutical preparations involving separation on a preadsorbent HPTLC silica gel plate with fluorescent phosphor, detection by fluorescence quenching, and quantification by densitometric scanning. Sulfur was directly detected and quantified on the plate at levels as low as 800 ng, and no interference was encountered from other ingredients in the medication formulations. The method was applied to the analysis of commercial liquid and cream preparations having respective label values of 5% and 8% sulfur, and recoveries averaged 99.3 and 99.5%. An unknown cream medication was assayed using the method and the accuracy of the result was validated by standard addition. All analyses were carried out with 5 or 6 replicates to evaluate precision, and coefficients of variation ranged from 2-4%.

INTRODUCTION

Elemental sulfur is an active ingredient in a variety of topical ointment, cream, and liquid pharmaceutical preparations for control of acne. The official methods for determination of sulfur in these products involve chemical

oxidation or reduction followed by titration or gravimetric analysis (1). These methods are slow and are not sufficiently selective for many of the complex formulations currently being marketed. A method involving separation of sulfur by TLC on homemade silica gel G plates, recovery by scraping off of the sulfur band and elution with chloroform, and analysis by spectrometry at 265 nm was recently reported (1). In this paper we report a quantitative method that is based on separation of sulfur on commercial high performance preadsorbent silica gel plates containing fluorescent indicator, detection by fluorescence quenching, and in situ densitometric scanning at 277 nm. This direct method is faster, more convenient, and less prone to random loss of analyte during the scraping and elution operations compared to the earlier method.

EXPERIMENTAL

Standard Solution

A standard solution (200 ng/ul) was prepared by adding 10.0 mg of reagent grade elemental sulfur (99.999% purity, Janssen Chimica) to 25 ml of acetone in a 50 ml volumetric flask. The solution was boiled on a hot plate for ca. 30 min, with additions of acetone as needed to maintain the volume, until the sulfur was completely dissolved. The solution was cooled to room temperature and the flask was filled to the line with acetone and mixed thoroughly by repeated inversion for 45 sec.

Thin Layer Chromatography

TLC was carried out on 10 x 20 cm Whatman LHPKDF high performance silica gel plates with 19 lanes, preadsorbent spotting area, and fluorescent phosphor (catalog no. 4806-711). Standard and sample solutions were applied to the preadsorbent using a 10 ul Drummond digital microdispenser. Plates were developed with petroleum ether for a distance of 7 cm beyond the silica gel-preadsorbent junction in a paper-lined, solvent-saturated Camag twin-trough chamber and dried in a fume hood. Separated sulfur zones were detected by inspection under 254 nm UV light in a viewing cabinet and scanned using a Shimadzu CS-930 densitometer in the single beam, reflectance mode at 277 nm.

Analysis of Samples

The samples analyzed to test the new method were an acne medication cream with a label declaration of 8% sulfur, a liquid medication with a label value of 5%, and another cream containing an unspecified amount of sulfur, all purchased in a local pharmacy without prescription. Approximately 250 mg of the 5% liquid was poured into a tared 25 ml volumetric flask, the flask was reweighed to obtain the exact sample weight by difference, the sulfur in the sample was dissolved by boiling with ca. 15 ml of acetone, and the solution was cooled to room temperature and diluted to the line with acetone. Approximately 156 mg of the 8% cream or 250 mg of the unknown were accurately weighed by difference into a tared 50 ml

beaker and quantitatively transferred into a 25 ml volumetric flask with acetone, and the solution was boiled and diluted to the line as described above.

Recovery from the known samples was determined by spotting 4.00, 8.00, and 12.00 ul of standard (containing 0.800, 1.60, and 2.40 ug of sulfur, respectively) and duplicate aliquots of sample solution that represented a theoretical weight equal to the 8.00 ul of standard. Based on the sample weights specified above, 3.20 ul was spotted for both samples (containing 1.60 ug of sulfur for 100% recovery). A calibration curve was constructed from the scan areas and spotted weights of the standards using a Quattro curve-fitting program on an IBM PC, the weight of sulfur in the sample was interpolated from the curve using the average scan area of the duplicate sample aliquots, and percent recovery was calculated by comparing the experimental and theoretical sample weights.

The percent sulfur in the unknown was determined by spotting 4.00, 8.00, and 12.00 ul aliquots of standard and 2.00, 3.20, 5.00, and 8.00 ul of the sample. The scan areas and weights of the standards were used to produce the calibration curve, and the weight of sulfur was interpolated from the curve using the scan area of the 5.00 ul sample zone, which most closely matched the 8.00 ul standard. The percent sulfur was calculated based on the weights of sulfur and sample (250 mg) and the volumes of the total sample (25 ml) and the aliquot spotted (5 ul).

RESULTS AND DISCUSSION

Boiling with acetone completely dissolved the sulfur in the standard and samples, but other insoluble sample ingredients settled to the bottom of the volumetric flasks and did not interfere with spotting. Sulfur produced a tight band with R_f 0.63 across the lane of the high performance preadsorbent silica gel plate when developed with petroleum ether. Despite the presence of many ingredients in the medications analyzed, including resorcinol, fragrances, and methyl and propyl parabens, zones other than sulfur were not detected by fluorescence quenching under 254 nm UV light in any of the chromatograms. Standards and samples were always chromatographed together to correct for the inevitable slight variations in the slope and intercept of the calibration curve on different plates. The calibration curve typically had a linearity correlation coefficient (R value) of 0.99.

The liquid medication with a 5% label value of sulfur was analyzed 5 times, and the percent recovery values obtained were 97.0, 96.9, 98.8, 104, 99.5 (99.3 average, 3.0 standard deviation). Percent recovery values for 6 analyses of the cream with an 8% label value were 98.3, 96.3, 102.3, 102.1, 98.3, 99.5 (99.5 average, 2.3 standard deviation).

The analysis of the unknown cream was replicated 5 times with the following results: 3.20, 3.16, 2.93, 3.28, 3.00%; average 3.11%; standard deviation 0.14%. A standard addition analysis was performed in duplicate using a spiked sample to validate these results for the unknown. A weighed amount of solid sulfur was added to 25.0 ml of preanalyzed sample

solution to exactly double the content of sulfur, and the solution was heated to dissolve the sulfur and diluted to 50.0 ml with acetone. Duplicate 5.00 ul aliquots of the spiked and unspiked sample solutions were chromatographed, and the average scan areas were found to agree within 0.05 and 0.20% (relative error), thereby verifying the original analysis of the unknown.

The quantitative HPTLC method described is simple because layer preparation, sample preparation, or scraping and elution of the separated sulfur zones are not required, and it has high sample throughput because up to 16 samples can be analyzed on a single plate along with the three required standards. It has been shown that the method has the necessary sensitivity, selectivity, accuracy, and precision for routine use in a pharmaceutical analytical laboratory.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF LANATOSIDES IN *DIGITALIS LUTEA* AND *DIGITALIS AMBIGUA* LEAVES

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ABSTRACT

An quantitative method for the determination of lanatoside A and lanatoside B in *Digitalis lutea* and *Digitalis ambigua* leaves by high-performance liquid chromatography (HPLC) is described. The extract of dry leaf powder with chloroform:ethanol (1:2, v/v) was submitted to Sep-Pak cartridges prior to HPLC analysis. HPLC was performed on an ODS column using methanol:water (2:1, v/v) for *Digitalis lutea* and a phenylsilyl bonded silica column with acetonitrile:water (5:8, v/v) for *Digitalis ambigua*. The effluent was monitored by ultraviolet (UV) absorption at 220 nm. The quantitation was carried out by the internal standard method. The present method is sufficiently sensitive and reproducible to assay lanatosides in *Digitalis* leaves.

INTRODUCTION

The cardiac glycosides prepared from *Digitalis* leaves are therapeutically important substances for the treatment of heart disease. For the determination of the glycosides in *Digitalis* leaves, thin-layer chromatography (TLC) has been shown to be a

useful method (1,2). However, HPLC seems to be more efficient for the analysis of the glycosides. The separation of various mixtures of pure cardiac glycosides has been achieved by the use of a normal-phase silica column (3-5) and a reversed-phase column (4,6). In addition, the usefulness of adapting gradient elution to the HPLC separation of the plant extract has been shown (7,8).

The genus *Digitalis* comprises about 25 species, of which *Digitalis lutea* and *Digitalis ambigua* (syn. *Digitalis grandiflora*) are known to contain lanatosides as primary glycosides. The published methods for the analysis of cardiac glycosides in *Digitalis lutea* and *Digitalis ambigua* have utilized paper chromatography (9,10), TLC (11), and HPLC employing gradient elution (12-14). In the previous paper of this serise, we reported the determination of purpurea glycosides in *Digitalis purpurea* leaves (15) and lanatosides in *Digitalis lanata* leaves (16), by means of HPLC. The present paper describes the convenient method for the simultaneous determination of lanatoside A and lanatoside B from the extract of *Digitalis lutea* and *Digitalis ambigua* leaves, which involves clean-up with Sep-Pak cartridges and subsequent separation by reversed-phase HPLC with isocratic elution.

MATERIALS AND METHODS

Chemicals

Lanatoside A and lanatoside B were purchased from E. Merck (Darmstadt, F.R.G.), and their chemical structures are given in Figure 1. 14α,15α-Epoxy-"β"-anhydrodesacetyllanatoside A, used as the internal standard, was synthesized in four steps from desacetyllanatoside A by the method adapted from Sawlewicz *et al.* (17). Desacetyllanatoside A was prepared from lanatoside A according to the procedure of Pekić and Miljković (18). All of these compounds were checked for homogeneity by TLC, and solvents were purified by redistillation prior to use.

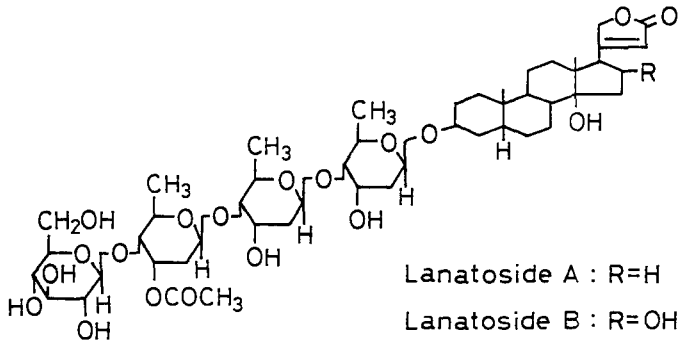

FIGURE 1. Chemical structures of the lanatosides investigated.

Chromatographic Apparatus

The HPLC system consisted of a JASCO 880-PU pump (Japan Spectroscopic, Tokyo, Japan), a Model KHP-UI-130 injector (Kyowa Seimitsu, Tokyo), a Model UV-8010 variable-wavelength detector (Tosoh, Tokyo), and a Chromatopac C-R3A data processor (Shimadzu, Kyoto, Japan). The Cosmosil $5C_{18}$ (5 μm, 150 x 4.6 mm I.D.)(Chemco Scientific, Osaka, Japan) and Cosmosil 5Ph (5 μm, 300 x 4.6 mm I.D.) columns were used at ambient temperature.

Preparation of *Digitalis* Leaf Powder

Leaves of *Digitalis lutea* L. and *Digitalis ambigua* Murr. were collected during the flowering stage in June at the Medicinal Plant Garden (Kanazawa, Japan) of Hokuriku University. These fresh leaves were immediately freeze-dried in a Neocool Model DC-55A apparatus (Yamato Scientific, Tokyo) and then dried using phosphorus pentoxide under reduced pressure at room temperature. The dried leaves were pulverized and sifted through a sieve of mesh width 500 μm. The leaf powder obtained was further dried using phosphorus pentoxide under reduced pressure for five days.

Sample Preparation for HPLC

Leaf powder (*ca.* 50 mg) of *Digitalis lutea* or *Digitalis ambigua* was accurately weighed and extracted with 25 ml of chloroform:ethanol (1:2, v/v) containing 14α,15α-epoxy-"β"-anhydrodesacetyllanatoside A (24.36 μg) as an internal standard. After ultrasonication for 1 hr in an ultrasonic cleaning bath, the extract was filtered and evaporated to dryness using a rotary evaporator. The residue was dissolved in 1 ml of ethyl acetate: ethanol:acetic acid (100:1:0.1, v/v) and subjected to the Sep-Pak silica cartridge (Waters, Milford, MA). Then 19 ml of ethyl acetate:ethanol:acetic acid (100:1:0.1, v/v) and 10 ml of ethyl acetate:ethanol:acetic acid (100:20:0.12, v/v) were successively passed through the cartridge. After evaporation of the latter fraction (10 ml) using a rotary evaporator, the resulting residue was dissolved in 1 ml of methanol:water:acetic acid (20:30:0.05, v/v) and loaded on the Sep-Pak C_{18} cartridge. After washing with 14 ml of methanol:water:acetic acid (20:30:0.05, v/v), lanatosides were eluted with 15 ml of methanol:water:acetic acid (20: 10:0.03, v/v). The eluate was evaporated to dryness *in vacuo*. The material obtained was submitted to HPLC.

HPLC Determination

The HPLC determination of lanatosides in *Digitalis lutea* leaves was achieved by using a Cosmosil $5C_{18}$ column. The mobile phase used for the separation was methanol:water (2:1, v/v) and the flow rate was adjusted to 0.6 ml/min. The HPLC for *Digitalis ambigua* leaves was performed on a Cosmosil 5Ph column with acetonitrile:water (5:8, v/v) at the flow rate of 0.4 ml/min. The effluent was monitored by UV absorption at 220 nm. The extract pretreated above was dissolved in 0.5 ml of the mobile phase and a 10 μl volume of the sample solution was injected into the liquid chromatograph. Lanatoside A and lanatoside B in *Digitalis* plants were determined by the internal standard method. Calibration graphs were constructed by plotting the ratio of the peak area of lanatoside A or lanatoside B to the peak area of the internal

standard against the weight of each compound. The average peak areas from three chromatograms were used for the determination.

TLC Procedure

Normal- and reversed-phase TLC were performed on high-performance silica gel 60 F_{254} plates (5 x 10 cm, E. Merck) and $KC_{18}F$ plates (5 x 10 cm, Whatman, Clifton, NJ), respectively. The plates were developed in glass chamber, checked by UV light around 254 nm, sprayed with concentrated sulfuric acid, and heated at 120 °C for 10 min.

RESULTS AND DISCUSSION

The HPLC determination of lanatosides in the leaves was carried out by the incorporation of an internal standard in order to improve the reproducibility on the clean-up procedure and the chromatographic run. Many compounds were investigated and 14α,15α-epoxy-"β"-anhydrodesacetyllanatoside A, which can be separated satisfactorily from lanatoside A and lanatoside B, was selected. The dried leaf powder was extracted with chloroform: ethanol (1:2, v/v) by ultrasonication. For the purpose of removing the many other plant materials, the extract was submitted to Sep-Pak cartridges packed with silica gel and ODS bonded silica gel prior to HPLC (Figure 2). The purified material was subjected to HPLC on a reversed-phase column. A detection wavelength of 220 nm was used, account being taken of the α,β-unsaturated lactone ring attached at the C-17 position of the steroid nucleus.

For the determination of lanatoside A and lanatoside B in *Digitalis lutea* leaves, the HPLC separation was performed on an ODS bonded silica column using methanol:water (2:1, v/v) as the mobile phase at a flow rate of 0.6 ml/min. Figure 3(a) shows the chromatogram of a standard mixture of lanatoside A, lanatoside B, and the internal standard. The separation is sufficiently good

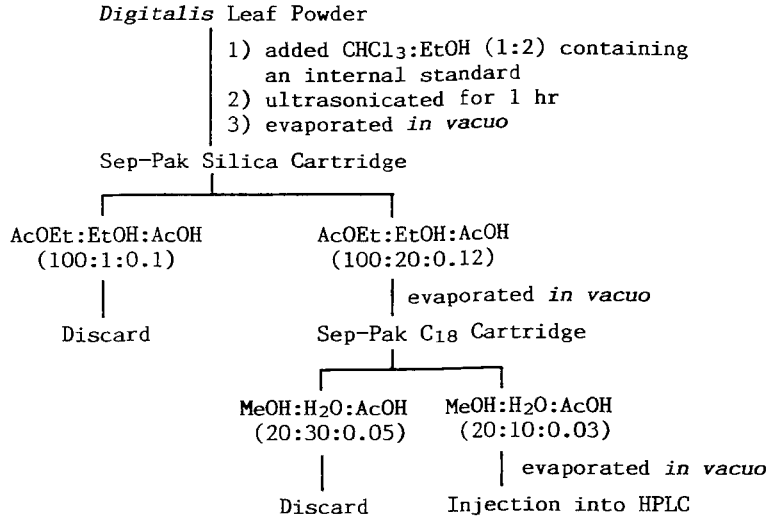

FIGURE 2. Procedure of the sample preparation for the HPLC determination of lanatosides in *Digitalis* leaves.

and reproducible to permit quantitative work. The representative chromatogram of the extract with the internal standard is given in Figure 3(b). From a chromatogram of the extract in the absence of the internal standard, the other substances present in the leaves were ascertained not to interfere with the peak of the internal standard. To make sure that the peaks of lanatoside A and lanatoside B were homogeneous, the eluate corresponding to each peak was collected and analyzed by TLC. Both normal-phase TLC (lanatoside A, R_f 0.48; lanatoside B, R_f 0.42) using chloroform:methanol:water (80:20:2.5, v/v) as developing solvent and reversed-phase TLC (lanatoside A, R_f 0.45; lanatoside B, R_f 0.55) using acetonitrile:0.5 *M* sodium chloride (10:13, v/v) indicated single components. For the HPLC quantitation, linear calibration graphs were prepared by plotting seven data points in the ranges of 10∿80 μg for lanatoside A and 5∿40 μg for lanatoside B. The regression equations and correlation coefficients (r) were deter-

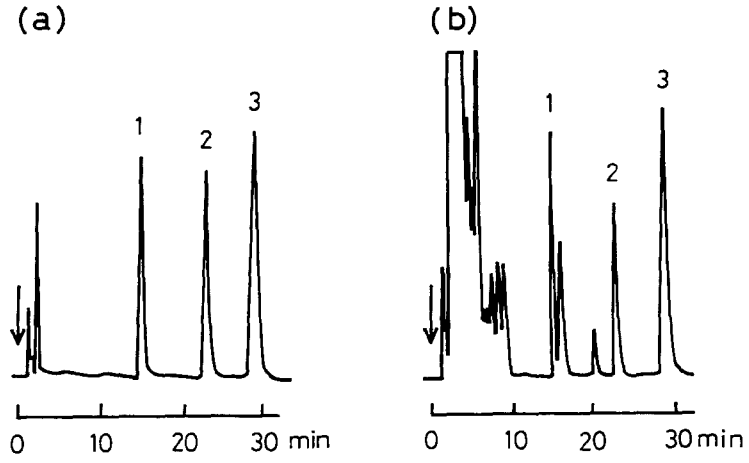

FIGURE 3. HPLC separations of lanatoside A, lanatoside B, and an internal standard for *Digitalis lutea* leaves. Peak identification: 1=lanatoside B; 2=14α,15α-epoxy-"β"-anhydrodesacetyllanatoside A; 3=lanatoside A. Chromatographic conditions: Cosmosil $5C_{18}$ column (150 x 4.6 mm I.D.); mobile phase, methanol:water (2:1, v/v); flow rate, 0.6 ml/min; UV detection at 220 nm; sample volume, 10 µl. Chromatogram(a): mixture of pure compounds. Chromatogram(b): extract of *Digitalis lutea* leaves with an internal standard.

mined as $y=0.0505x + 0.0231$ ($r=0.997$) for lanatoside A and $y=0.0472x - 0.0115$ ($r=0.998$) for lanatoside B, where y represents the peak area ratio of lanatosides to the internal standard and x the amount (µg) of lanatosides. The assay results obtained from ten dry leaf powder samples of *Digitalis lutea* are compiled in Table 1. The data indicate that the average contents of lanatoside A and lanatoside B per 100 mg of the leaf powder were 77.7 and 43.6 µg, respectively, with good reproducibility. The amount of lanatoside A in *Digitalis lutea* was 1.8 times higher than that of lanatoside B.

The determination of lanatosides in *Digitalis ambigua* leaves was also undertaken. When an ODS bonded silica column was used, the presence of co-extracted constituents of the leaves inter-

TABLE 1

Contents of Lanatoside A and Lanatoside B in *Digitalis lutea* Leaves Determined by the Present Method

Glycoside	Found* (μg)		Mean ± S.D. (μg)	C.V. (%)
Lanatoside A	81.3	78.5		
	77.1	73.2		
	74.8	77.6		
	77.9	75.6		
	77.7	83.3	77.7 ± 3.0	3.9
Lanatoside B	44.1	44.2		
	43.8	43.1		
	43.1	43.7		
	43.0	43.8		
	43.8	43.3	43.6 ± 0.4	0.9

*Values are the amount of lanatoside A or lanatoside B per 100 mg of a dry leaf powder sample.

fered with the peaks of lanatosides. For the complete HPLC separation of lanatosides in the leaves, a phenylsilyl bonded silica was employed as the stationary phase. Figure 4(a) depicts the chromatogram of a mixture of lanatoside A, lanatoside B, and the internal standard. These compounds were separated by using a solvent system consisting of acetonitrile:water (5:8, v/v) at a flow rate of 0.4 ml/min. The typical chromatogram of the extract after incorporation of the internal standard is illustrated in Figure 4(b). The purity of the peaks of lanatoside A and lanatoside B was also checked by both normal-phase TLC and reversed-phase TLC in the same manner as described above. The calibration graphs were obtained by plotting the peak area ratios (y) of lanatosides to the internal standard against the amount (x μg) of lanatosides. The regression equations were $y=0.0501x + 0.0080$ ($r=0.998$) for lanatoside A and $y=0.0462x - 0.0073$ ($r=0.998$) for lanatoside B. The quantitative analysis indicated that *Digitalis ambigua* leaves contained 62.6 μg of lanatoside A and 27.8 μg of lanatoside B per 100 mg of the dry leaf powder (Table 2). The content of lanatoside A in *Digitalis ambigua* leaves was about 2.3 times that of lanatoside B.

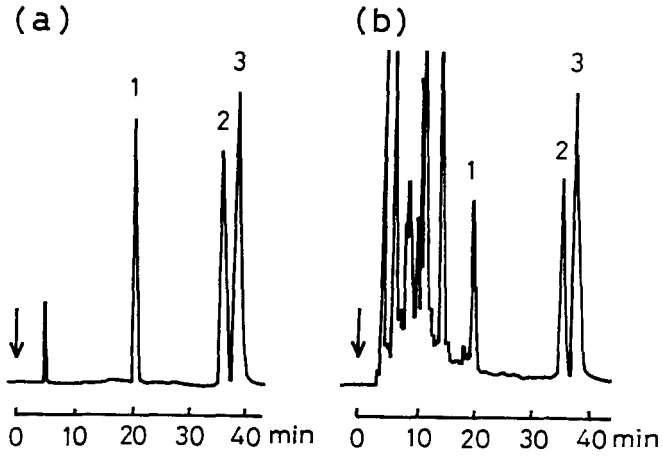

FIGURE 4. HPLC separations of lanatoside A, lanatoside B, and an internal standard for *Digitalis ambigua* leaves. Peak identification: 1=lanatoside B; 2=14α,15α-epoxy-"β"-anhydrodesacetyllanatoside A; 3=lanatoside A. Chromatographic conditions: Cosmosil 5Ph column (300 x 4.6 mm I.D.); mobile phase, acetonitrile:water (5:8, v/v); flow rate, 0.4 ml/min; UV detection at 220 nm; sample volume, 10 µl. Chromatogram(a): mixture of pure compounds. Chromatogram(b): extract of *Digitalis ambigua* leaves with an internal standard.

TABLE 2

Contents of Lanatoside A and Lanatoside B in *Digitalis ambigua* Leaves Determined by the Present Method

Glycoside	Found* (µg)		Mean ± S.D. (µg)	C.V. (%)
Lanatoside A	62.9	61.2		
	61.2	62.8		
	64.1	63.9		
	62.4	62.5		
	62.2	62.4	62.6 ± 1.0	1.6
Lanatoside B	30.3	26.5		
	28.5	25.4		
	27.0	29.2		
	28.6	27.7		
	29.3	25.6	27.8 ± 1.7	6.1

*Values are the amount of lanatoside A or lanatoside B per 100 mg of a dry leaf powder sample.

In the present study, the HPLC determination of lanatoside A and lanatoside B in *Digitalis lutea* and *Digitalis ambigua* leaves was achieved under an isocratic elution by the internal standard method. The values of lanatosides in *Digitalis lutea* were higher than those in *Digitalis ambigua*. The pretreatment procedure using Sep-Pak cartridges before the HPLC analysis was of great importance in the elimination of interfering peaks of the chromatogram. In conclusion, the proposed method is precise and selective for the determination of lanatosides in the leaves of *Digitalis lutea* and *Digitalis ambigua*. This technique can be useful for estimation of the quality of the leaves.

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COMPARATIVE HPLC ANALYSIS OF POLYPHENOLIC COMPOUNDS IN FOUR SPECIES OF GALIUM L.

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ABSTRACT

Four species of Galium L. (Rubiaceae) were comparatively studied by analytical HPLC for the chlorogenic acid and the flavonoid pattern. The comparison of the HPLC profiles proved a striking correlation of the flavonoid pattern with the classification of the sectios and groups giving a promising feature in the chemotaxonomic study.

INTRODUCTION

Galium is a large and taxonomically difficult genus of the Rubiaceae family, comprising many species with known therapeutic properties [1,2]. In the present work HPLC is used to record a profile of the polyphenolic content of four species of Galium L.. These species are Galium aparine L., G. tricornutum Dandy, G. heldreichii Hal. and G. melanantherum Boiss.. The first two are annuals belonging to the sectio Kolgyda Dumort. (sectio Aparine (D.C.) Griseb). The other two are perennials belonging to the series Erecta Pobed. of the sectio Leiogalium Lebed.. G. aparine is a very variable cosmopolitan

species. G. tricornutum is a european and asiatic species. G. heldreichii and G. melanantherum belong to two different groups of closely related taxa. The first of them belongs to the group of G. mollugo and the second to the G. incurvum group. Both have a relatively small area of distribution: the first is an Aegean element and the second is an endemic of S.E. Greece [3,4].

In the recent years HPLC has been used for the investigation of polyphenolics as an accurate and sensitive technique which gives results rapidly compared to the more classical procedures [5,6]. In the HPLC chromatograms we can notice the difference in the polyphenolic patterns of the species belonging to different sectios and groups.

EXPERIMENTAL

Plant Material

The aerial parts of the above species were collected in June from Attiki. The plant material was dried in a cool dark place and powdered. Voucher specimens are deposited in the laboratory of Pharmacognosy of the University of Athens. 1 g of each specimen was extracted with methanol under reflux for 3 hrs. The extracts were concentrated to 2 ml and adjusted to a volume of 25 ml with methanol.

Chromatography

The above mentioned methanolic solutions were filtered through acrodisc CR 0.45 μm (Gelman) cartridges and 5 μl of the filtrates were injected into HPLC column. The HPLC isocratic analysis was carried out with a Waters Liquid Chromatograph Model 590 equipped with U6K injector, Waters Lambda-Max model 481 variable wavelength detector and Lichrosorb RP-18 column (25 cm x 4.6 mm), 10 μ. The mobile phase was methanol-acetic acid 5% (40-60% by volume). The mobile phase components were degassed in an ultrasonic bath and filtered through a Millipore HA (0.45 μm) membrane filter. The flow rate was 2 ml/min, the UV detector was monitored at 340 nm (0.01 aufs), the chart speed was 1 cm/min and the chromatography run time 10 min.

The interpretation of the peaks was performed by comparison of the retention time with those obtained from reference substances chromatographed under the same conditions. The reference substances were previously isolated and identified with standard procedures from G. melanantherum [7] and G. heldreichii [8].

The HPLC chromatograms of the four species are illustrated in figures 1 and 2.

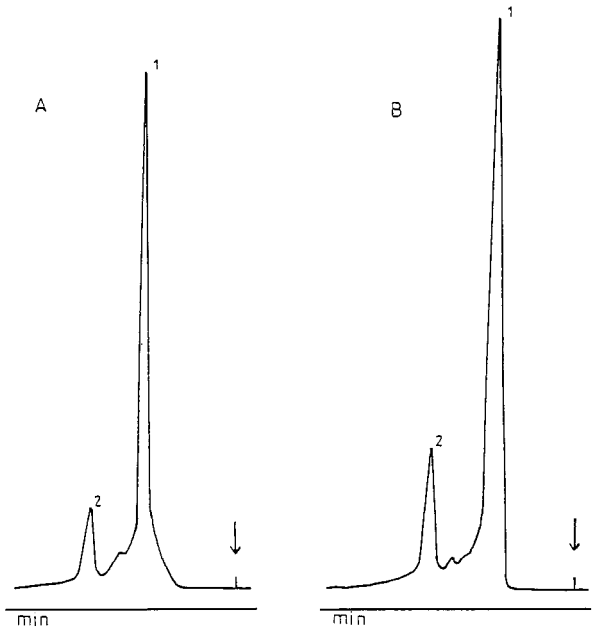

FIGURE 1. HPLC profiles of G. aparine (A) and G. tricornutum (B). 1: chlorogenic acid (t_R 2.08 min), 2: rutin (t_R 3.45 min).

RESULTS AND DISCUSSION

By examining the HPLC profiles we observe that chlorogenic acid is predominant in all species and the differences are noticed in the flavonoid pattern between the sectios. The two species (G. aparine and G. tricornutum) belonging to the sectio Kolgyda Dumort are very poor in flavonoids and they are characterized by the presence of rutin (quercetin-3-rutinoside). In the contrary the two species (G. melanantherum and G. heldreichii) belonging to the sectio Leiogalium Lebed. are richer in flavonoids and they are characterized by the presence of flavone and flavonol mono- and di-glycosides: luteolin-7-glucoside, luteolin-7-diglucoside, rutin, isoquercitrin (quercetin-3-glucoside), kaempferol-3-rutinoside. The presence of C-glucosides (orientin: luteolin-8-C-glucoside and vitexin: apigenin-8-C-glucoside) in G. melanantherum contrary to their absence in G. heldreichii is an additional differentiation feature between these species belonging to different groups.

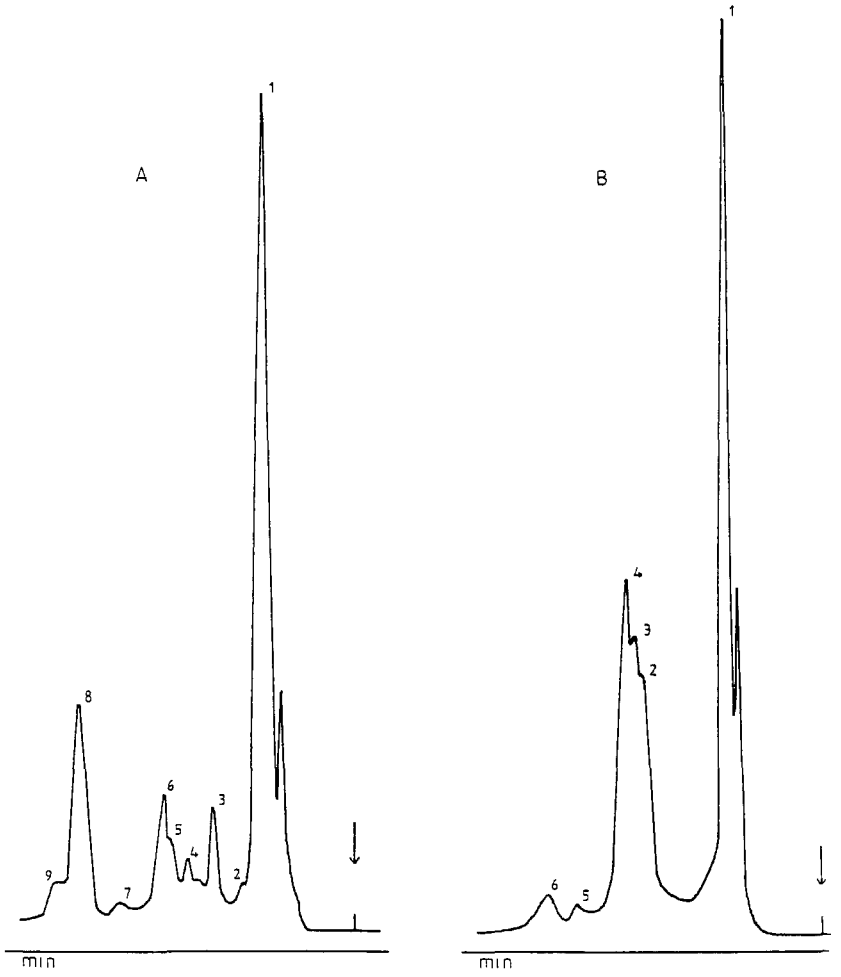

FIGURE 2. HPLC profiles of G. melanantherum (A) and G. heldreichii (B). Peaks of (A) = 1: chlorogenic acid (t_R 2.19 min), 2: orientin (t_R 2.70 min), 3: vitexin (t_R 3.35 min), 4: luteolin-7-diglucoside (t_R 3.99 min), 5: luteolin-7-glucoside (t_R 4.38 min), 6: rutin (t_R 4.50 min), 7: isoquercitrin (t_R 5.65 min), 8: unidentified, 9: kaempferol-3-rutinoside (t_R 7.05 min). Peaks of (B) = 1: chlorogenic acid (t_R 2.18 min), 2: luteolin-7-diglucoside (t_R 3.95 min), 3: luteolin-7-glucoside (t_R 4.18 min), 4: rutin (t_R 4.45 min), 5: isoquercitrin or/and hyperoside (t_R 5.65 min), 6: unidentified.

Isocratic HPLC analysis was chosen because of its reproducible results. The aglyka were not considered because under these conditions of chromatography they possess longer retention times and hence very broad peaks (tailing).

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DETERMINATION OF 2-KETO ACIDS AND AMINO ACIDS IN PLANT EXTRACTS

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ABSTRACT

2-keto acids and amino acids were extracted using liquid nitrogen and 0.25 N HCl. The keto acids and amino acids were seperated by cation exchange chromatography on AG50W-X8 resin. The cation exchange chromatography is vital for the determination of keto acids. The keto acids were derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB), a specific derivatizing agent for 2-keto acids. The derivatized keto acids were quantified by reversed phase high performance liquid chromatography (HPLC). This assay is highly sensitive and can measure as low as 10 fmole of the keto acids per 10 µl injection. The amino acids were analyzed by an automatic amino acid analyzer. These methods were used to show that 2-ketobutyrate (2-KB) and 2-aminobutyrate (2-AB) accumulate in plants treated with an acetohydroxyacid synthase inhibiting herbicide.

INTRODUCTION

Imidazolinones, sulfonylureas and triazolopyrimidines kill plants by inhibiting acetohydroxyacid synthase, a key enzyme leading to the biosynthesis of valine, leucine and isoleucine (1). This enzyme condenses two moles of pyruvate to produce acetolactate or a mole of pyruvate and a mole of 2-KB to produce acetohydroxybutyrate. It has been suggested that inhibition of AHAS by sulfonylureas leads to accumulation of 2KB which is toxic to microbes (2-

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5). Based on this finding, it has been speculated that AHAS inhibitors kill plants due to the accumulation of 2KB, the reaction product of threonine dehydratase. Due to this reason we have been interested in quantitation of 2-KB in plants.

There are several published methods for the determination of 2-KB and other 2-keto acids in plasma and urine (6-10). However, there is no sensitive assay for determination of 2-KB in plant extracts. Derivatizing agents such as dihydrophenylhydrazine, o-phenylenediamine and 1,2-diamino-4,5-methylenedioxybenzene have been used for spectrophotometric or fluorometric determinations of 2-keto acids. Several published methods used for quantitation of keto acid in plasma or urine (6-10) were attempted, however, none of them were successful because the concentration of keto acids in plant extracts is very small and the spiked keto acids were lost when mixed with the plant extracts. Here, we report a method of extraction and derivatization that has been successfully used for the determination of 2-keto acids in various plant extracts. Furthermore, the same extraction method allowed preparation of samples for amino acid analysis.

MATERIALS AND METHODS

Plant Material

Plants were grown in a growth chamber at 30/20°C day/night temperature and 16 h daylength. Maize seeds were germinated on a wet paper toweling and then five-day old seedlings were transferred to 50 ml plastic tubes covered with aluminum foil to eliminate light and containing 35 ml of a complete nutrient solution (11) which was changed daily. Plants were treated with 10 μM imazaquin when the fourth leaf began to emerge from the whorl. The leaf sheath/shoot meristem region was extracted to measure the effects of treatments on keto acids and amino acids. At least two replications with 2-5 plants per replication were used for various measurements. The experiments were conducted several times, however, the data for one example of each representative experiment is presented.

Extraction of keto acids and amino acids

The plant material was pulverized in liquid nitrogen and then further ground in 0.25N HCl containing 0.1 mg/ml 2-oxopentanoate (internal standard for keto acid analysis) and 500 nmol/ml L-α-amino-β-guanadinopropionic acid (internal standard for amino acid analysis). Two ml of extraction solution was used for each g of tissue fresh weight. The extract was centrifuged at 25,000 g for 15 min. An aliquot of the supernatant (0.25 ml) was loaded on a cation exchange column (AG 50W-X8 from Bio Rad, Richmond, CA; resin bed volume=4ml) pre-equilibrated with 0.01 N HCl. The column was washed with 1.5 ml of 0.01 N HCl and then the keto acids were eluted in 2 ml of 0.01 N HCl. Amino acids bind with this resin and were eluted with 4 x 4 ml aliquots of 9N ammonium hydroxide.

Derivatization of keto acids

The method of derivatizing the keto acids to form quinoxalones was adapted from Nakamura et al. (9) and Wang et al. (10). A 5 mM solution of DMB was prepared in a fresh solution of 1.5 N HCl containing 20 mM sodium dithionite and 1 mM β-mercaptoethanol. A 250 µl aliquot of the solution containing the keto acids was mixed with an equal aliquot of DMB solution. The mixture was vortexed and then heated in a boiling water bath for 45 min. The derivatized keto acids solution was diluted in the HPLC running buffer for analysis.

HPLC of keto acids

The HPLC conditions are identical to those described previously (9). The HPLC system consisted of a Beckman 112 solvent delivery module (Beckman, Fullerton, CA), DYNAMAX Model FL-1 fluorescence detector (Rainin, Woburn, MA), a WISP 710B automatic sampler and a Waters 840 data integration system (Waters Assoc., Milford, MA). A Radial-PAK cartridge C_{18} reversed-phase column (5-µm particle size; 100 x 8 mm i.d.) was used which was connected with a stainless steel guard column packed with C_{18} resin. The mobile phase (acetonitrile-methanol-40 mM phosphate, pH 7, 12/13/25, v/v/v) was run at a flow rate of 1.5 ml/min. For the fluorometric analysis, the excitation and emission wavelengths were 367 and 446 nm, respectively.

Amino acid analysis

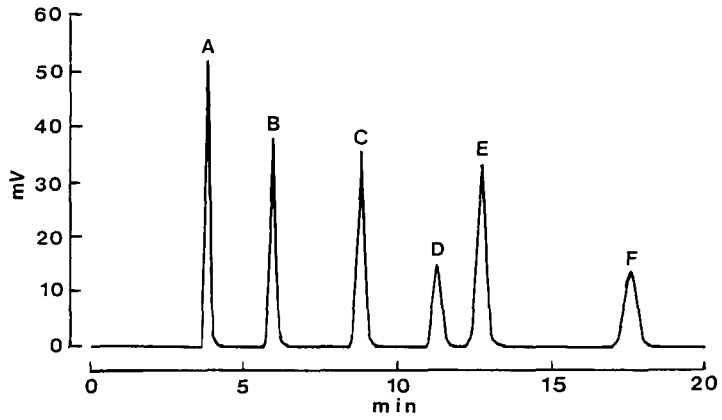
Amino acids eluted from the cation exchange column were freeze dried and then dissolved in Na-S buffer (Beckman, Fullerton, CA). The solution was filtered to remove the particulate matter and the amino acid composition was determined on a Beckman 7300 amino acid analyzer.

RESULTS AND DISCUSSION

o-Phenylenediamine was used for determination of keto acids in preliminary experiments, however, almost all of the keto acids spiked in the plant extracts were lost during the derivatization process. Once the extraction and derivatization conditions were optimized, the sensitivity of detection using *o*-phenylenediamine was not high enough to detect 2-KB in plant extracts. It has been reported that DMB is a specific fluorogenic reagent for 2-keto acids (9). DMB was shown to be the best precolumn derivatization reagent, in terms of sensitivity and reactivity, in the HPLC evaluation of eight 1,2-diaminobenzene derivatives. Due to this reason, DMB was chosen and used in our subsequent experiments.

Several different extraction conditions were evaluated. Pulverizing plant material using liquid nitrogen allowed rapid cell breakage, preparation of the sample in powder form, and reduced enzymatic attack on the compounds of interest. This step is especially important for plant material containing high polyphenol oxidase activity which makes the sample dark brown in a very short time and thereby interferes with analyses. Further grinding the sample in strong acidic conditions (0.25N HCl) denatures proteins and thereby reduces the risk of enzymatic attack on keto acids and amino acids.

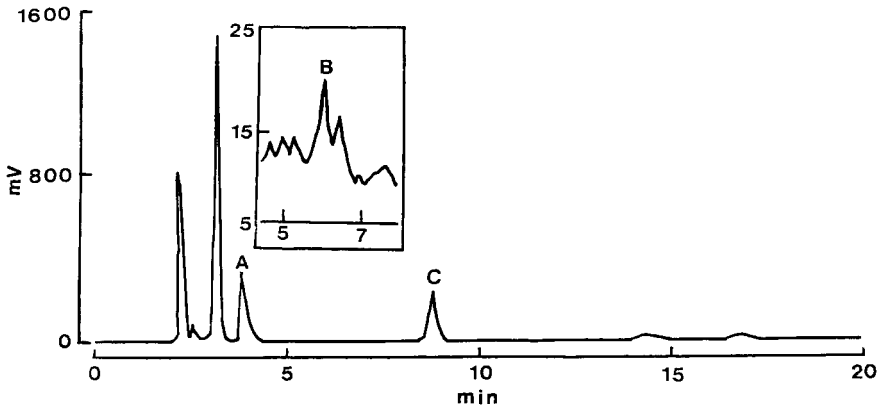
There was poor recovery of keto acids spiked in the crude extracts of plants prepared as described above. Apparently, there was some interference from the metabolites present in the plant extracts. It was found that cation exchange chromatography on AG 50W-X8 (Bio Rad, Richmond, CA) removed the interfering compound(s). The keto acids do not bind with the resin and are collected in the passthrough. Amino acids present in the extract bind with the resin which were eluted with 9N ammonium hydroxide. Routinely, greater than 90% of the spiked keto acid and amino acid were recovered using this extraction procedure.



1. HPLC of authentic pyruvate (peak A), 2-KB (peak B), 2-ketopentanoate (peak C), 2-ketoisovalerate (peak D), 2-ketoisocaproate (peak E) and 2-ketomethylvalerate (peak F) following derivatization with DMB.

The conditions for derivatization with DMB and HPLC are almost identical wth those described previously (9, 10). Figure 1 shows a typical chromatogram obtained with a standard mixture of 2- keto acids. Each of these keto acids gave single peaks with identical retention times when they were subjected to the same procedures individually. 2-ketopentanoate was not seen in the plant extracts in the preliminary experiments, therefore, this keto acid was used as an internal standard in all subsequent experiments. Using this protocol, as low as 10 fmol of a keto acid per 10 µl injection was detected. The extraction and derivatization procedure described here worked for all samples that we examined. These included samples from tissue culture (Black Mexican Sweet corn cells), dicots (cocklebur, lima bean, and sunflower) and monocot (corn). In this paper we have presented results obtained only from corn.

Several peaks of different keto acids were detected in the extracts of corn shoots (Fig. 2). In addition to the retention times of the standards, spiking the samples with different keto acids led to the identification of various keto acids. Of all keto acids quantified, pyruvate was present



2. HPLC of DMB-derivatized keto acids in extracts of corn seedlings. The inset shows the enlarged view of the chromatogram between 5 and 7 min where 2-KB (Peak B) elutes. Pyruvate (Peak A) and 2-ketopentanoate (internal standard; Peak C) are shown in the main chromatogram.

in the highest concentration (Table 1). Only trace amounts of other keto acids of the branched chain amino acid biosynthetic pathway were present in corn shoot extracts. Some other keto acids were present in high concentration (Fig. 2), however, We made no attempts to identify these keto acids.

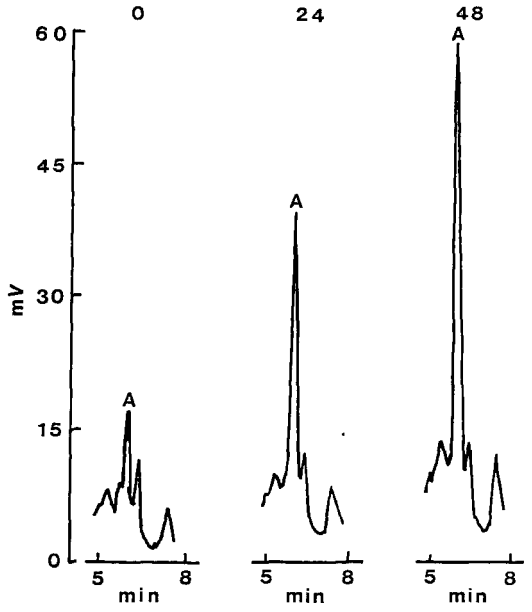
Imazaquin at 10 μM caused accumulation of 2-KB in corn shoots (Fig. 3, Table 2). This result is consistent with the previously reported accumulation of 2-KB in *Salmonella* grown in the presence of an AHAS inhibiting herbicide (2-5). A progressive increase in accumulation of 2-KB over time indicates that imazaquin prevents utilization of 2-KB by AHAS. Since the pathway before AHAS is unaffected, carbon continues to flow in the aspartate pathway leading to the accumulation of 2-KB.

Amino acids eluted from AG 50W-X8 resin were freeze dried and then dissolved in Na-S buffer and analyzed on Beckman 7300 amino acid analyzer. Excellent recovery of amino acids

Table 1

Levels of different keto acids of the branched chain amino acid biosynthetic pathway in corn seedling.

Keto acid	nmoles / g fresh weight
Pyruvate	85.0
2-ketobutyrate	0.2
2-ketoisovalerate	0.2
2-ketoisocaproate	< 0.05
2-ketomethylvalerate	< 0.05



3. Accumulation of 2-KB (Peak A) in corn seedlings treated with 10 μM imazaquin at 0, 24 and 48 h.

Table 2

Accumulation of 2-KB and 2-AB in corn seedlings treated with 10 μM imazaquin.

Time (h)	2-KB	2-AB
	(nmoles / g fresh weight)	
0	0.2	1
24	4.8	384
48	10.3	1269

was obtained in plant samples prespared in this way (chromatogram and data not shown). Corn shoots contain very low levels of 2-AB (Table 2). However, imazaquin treatment caused accumulation of 2-AB which increased progressively with time. Interestingly, the levels of 2-AB were about 100-fold higher than the levels of 2-KB. Since 2-AB is a transamination product of 2-KB, our observation suggests that the two compounds are in equilibrium in vivo and the equilibrium is in favor of 2-AB.

In summary, a method of simultaneous extraction of 2-keto acids and amino acids has been developed. The keto acids and amino acids are separated by cation exchange chromatography. This chromatography is also vital for the determination of keto acids. The keto acids are derivatized with DMB and then analyzed by reversed phase HPLC. The amino acids are quantitated by an amino acid analyzer. These methods were used to demonstrate that 2-KB and 2-AB accumulate in imazaquin treated corn shoots.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TRIPTOLIDE AND TRIPDIOLIDE IN AN ETHYL ACETATE EXTRACT OF *TRIPTERYGIUM WILFORDII HOOK F.*

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ABSTRACT

A new analytical method for the determination of triptolide and tripdiolide in ethyl acetate extracts of *Triterygium wilfordii Hook F.* is described. The procedure consists of preliminary enrichment by Sep-Pak alumina B cartridge chromatography followed by HPLC analysis. HPLC is performed with a stainless steel column packed with Nova-Pak C18, using acetonitrile-water (19 : 81) as a mobile phase for triptolide and acetonitrile-water (11 : 89) for tripdiolide. The effluent is monitored by ultraviolet detection at 214 nm. Quantitative analysis of triptolide is then carried out by comparison to an internal standard, and of tripdiolide by the external standard method. The amounts of triptolide and tripdiolide per 100 mg of the ethyl acetate extract were determined to be 19.88 ug and 9.58 ug respectively. The method is sufficiently sensitive and specific to assay the diterpenes found in *Tripterygium wilfordii Hook F.* accurately.

INTRODUCTION

Tripterygium wilfordii Hook F. (TWHF) is a medicinal plant that has been intensively studied[1]. The ethyl acetate extract of TWHF has been reported to be effective in the treatment of many autoimmune diseases, including rheumatoid arthritis[2,3]. Although the active ingredients of

TWHF have not been completely delineated, triptolide and tripdiolide are thought to be two of the more potent compounds, accounting for much of the efficacy and toxicity of this plant [4-9].

The techniques that have been employed to analyze the active components of TWHF involve thin-layer chromatograghy scanning densitometry and have quantitated triptolide only[10-12]. The TLC-Scanner procedure requires considerable expertise for its accurate application[17] and therefore, has not been widely applied. The total diterpene content of TWHF has been determined by a spectrophotometric method[13-15]. However, this method and TLC require visualizing the components with the Kedde reagent, that is usually not very stable[16]. Capillary gas chromatography (GC) has been used recently to the assess triptolide content of TWHF[18]. In contrast to high performance liquid chromatography, GC requires high temperature to evaporate the diterpene sample that may alter the components. In addition, it is difficult to use GC to prepare samples for biologic use. Since the extracts of TWHF are being more widely studied in clinical trials in both China and western countries, an accurate, sensitive and reliable method to separate and analyze its components is required. In this report, a new efficient and convenient method for the quantitation of the major active components of TWHF, triptolide and tripdiolide, using reverse phase high performance liquid chromatography (HPLC) is described.

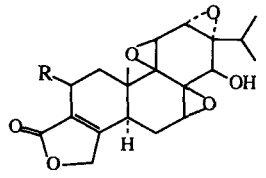
EXPERIMENTAL

Instruments

The Waters (Milford, MA) liquid chromatograph employed was configured with two Model 510 pumps, a Model U6K injector and a Model 441 UV detector set up at 214 nm. The data was processed with Millennium software, Version 1.10 (Waters Assoc.). The stainless steel column (150 mm x 3.9 mm I.D.) was packed with Nova-Pak C18, particle size 4 um (Waters Assoc.). An HPLC pre-column, with an insert packed with Nova-Pak C18, (Waters Assoc.) was used to extend the column life. The model ULTRAsonik 2QT / H ultrasonic water bath used in the solvent degassing and sample preparation was purchased from NEY Barkmeyer Division (Yucaipa, CA.).

Chemicals and reagents

Triptolide and tripdiolide were prepared from the ethyl acetate extract of TWHF by silica gel column chromatography successively with chloroform, chloroform-ether and chloroform-ethyl acetate as the eluents. The fractions containing triptolide and tripdiolide were purified on preparative HPLC with a Nova-Pak C18 column, 25 x 100 mm, using acetonitrile-water as the mobile phase. The compounds were recrystalized from n-hexane-dichloromethane. Triptolide was identified by UV, IR, proton NMR and mass spectrums. Tripdiolide was identified by HPLC, TLC and proton NMR and comparison with the known laboratory product provided by Boehringer Ingelheim Pharmaceuticals Inc. (Ridgefield, Connecticut). Acetonitrile was HPLC grade purchased from Aldrich Chemical Co. (Milwaukee, WI), water was Millipore pure, and other



Triptolide: R=H
Tripdiolide: R= β-OH

Fig. 1. Structures of triptolide and tripdiolide.

solvents were GR grade. The mobile phases were degassed by vacuum in conjunction with sonication just before use. The Sep-Pak Plus alumina B cartridge was purchased from Waters Assoc.(Milford, MA); Acetophenone, selected as an internal standard for the triptolide assessment, was purchased from Sigma Chemical Co. (St. Louis, MI). The chemical structures of triptolide and tripdiolide are shown in Fig. 1.

Preparation of the ethyl acetate extract of TWHF

The roots of TWHF were collected from Fujian province, China. The skin was removed from the roots and the woody portion of the roots was ground to coarse powder. 1000 g of the coarse powder was extracted with ethanol three times. The ethanol solutions were combined and evaporated under reduced pressure. The residue was then extracted with ethyl acetate. Concentration of the solution under reduced pressure yielded 22 g of the ethyl acetate extract.

Enrichment procedure

About 50 mg of the ethyl acetate extract was weighed accurately and dissolved in 10 ml of chloroform in an ultrasonic bath for 25 minutes. The extract solution was filtered and the residue was washed with 10 ml of chloroform-ethyl acetate (9 : 1). The washings combined with the original chloroform solution were applied to the Sep-Pak cartridge. 25 ml of chloroform-ethyl acetate (9 : 1) and 15 ml of ethyl acetate-methanol (9 : 1) were successively passed through the cartridge. The chloroform-ethyl acetate fraction, used for the determination of triptolide, was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved with 1.00 ml of acetophenone solution, that was prepared by dissolving acetophenone in methanol to obtain a solution having a concentration of 12.5 ug per ml. The dissolved residue was diluted with acetonitrile-water (19 : 81) to 2.00 ml. The ethyl acetate-methanol fraction was evaporated. The residue was dissolved in 1.00 ml of acetonitrile-water (11 : 89) solution and used to analyze for tripdiolide content.

Determination of diterpenes

A 10 ul volume of each purified sample solution was injected into the liquid chromatograph. The mobile phase for each separation is listed with the individual chromatogram.

Triptolide was determined by comparison to an internal standard. The reference solutions containing 1.83, 3.66, 7.32, 16.08 and 36.18 ng ul^{-1} of triptolide and 6.25 ng ul^{-1} of acetophenone for each solution were prepared in acetonitrile-water (19 : 81). The reference solutions of tripdiolide were prepared in acetonitrile-water (11 : 89) at the concentrations of 1.28, 2.55, 5.10, 10.20, 20.40, 30.60, and 40.80 ng ul^{-1}. Two replicates of each were injected into the HPLC system. The resulting chromatograms yielded data for the standard curves. The contents of triptolide and tripdiolide were calculated and expressed per 100 mg of the dried extract (drying at 80^0 C to a constant weight [19])

RESULTS AND DISCUSSION

This study focused on the development of a reliable method to analyze the two major components of TWHF. The enrichment procedure and chromatographic separation as well as the selection of an internal standard are three major problems in HPLC analysis of crude plant extracts. Many enrichment procedures were investigated during the preliminary phases of this study. These included different absorbents, such as silica gel, alumina N, florisil, diol, aminopropyl NH_2, cyanopropyl CN, activated carbon and polyamide. In addition, different solvent systems were tested. Sep-Pak Plus alumina B cartridge was found to be an efficient and convenient purifying method that involved the minimum number of steps. HPLC was performed with a Nova-Pak C18 column using acetonitrile-water as a mobile phase system. This resulted in a better separation of triptolide, tripdiolide and acetophenone from other components of the plant than did the use of methanol-water as a mobile phase. A detective wavelength of 214 nm was employed because of the α,β-unsaturated lactone ring in the diterpene structures. Acetophenone was found to be the most suitable internal standard for the determination of triptolide. Because of interference from other components, attempts to use an internal standard in the determination of tripdiolide were unsuccessful. Fig.2 illustrates the chromatogram of triptolide and acetophenone. The retention times of the two compounds were 11.35 min. and 8.15 min. respectively. Fig. 3 shows the chromatogram of tripdiolide. The retention time was 10.3 min.

The separation of triptolide, acetophenone and tripdiolide from the extracts of TWHF by HPLC was achieved using the method described above. This approach provided a good quantitative and reproducible recovery. Fig. 4 depicts a typical chromatogram of the extract for the determination of triptolide after addition of acetophenone. It is apparent that the other components present in the extract did not alter the internal standard peak. Fig. 5 shows a typical chromatogram of the extract for the determination of tripdiolide.

The peak purity was tested by collecting the fractions corresponding to both of the compounds and analyzing them by HPLC on the same column using methanol-water (30 : 70) as a mobile phase and adjusting the flow rate to 1.0 mL per min. The results indicated that a single component with the retention times corresponding to triptolide (5.1 min.) or tripdiolide (16.7 min.) had been isolated.

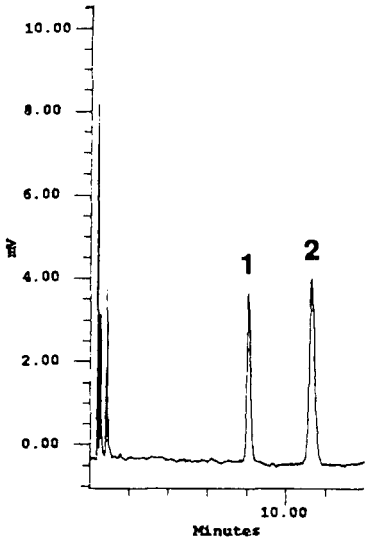


Fig. 2. Chromatogram of triptolide and acetophenone. Peaks: 1 = acetophenone; 2 = triptolide. Conditions: Nova-Pak C18 stainless steel column (150 mm x 3.9 mm I.D.); mobile phase, acetonitrile-water (19 : 81); flow-rate, 1.5 ml/ min.; UV monitor at 214 nm; sample volume 10 ul.

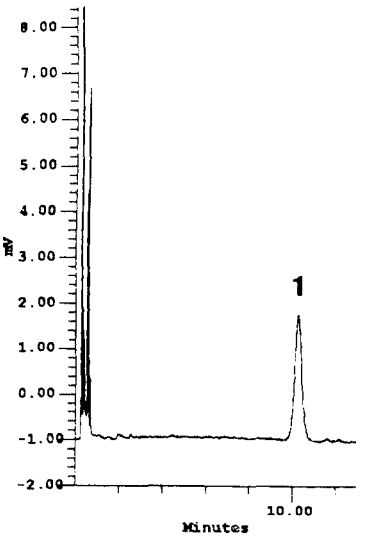


Fig. 3. Chromatogram of tripdiolide. Peak: 1 = tripdiolide. Mobile phase acetonitrile-water (11 : 89); flow-rate, 2.0 mL/ min. Other conditions as in Fig. 2.

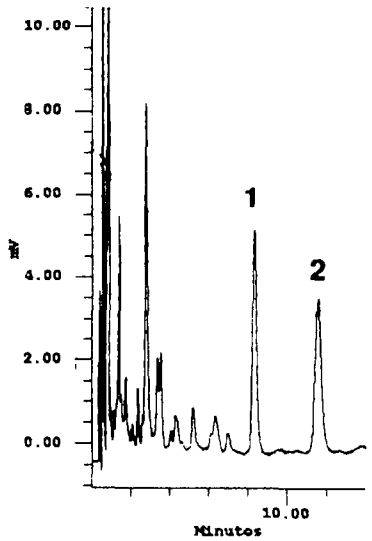


Fig.4. Chromatogram of the extract of Tripterygium wilfordii Hook F. with the internal standard for the determination of triptolide. Peak: 1 = acetophenone; 2 = triptolide. Conditions as in Fig. 2.

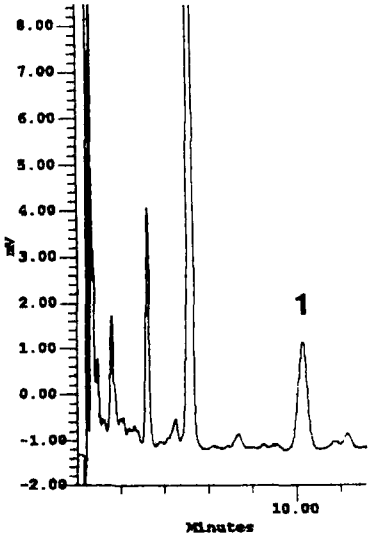


Fig. 5. Chromatogram of the extract of Tripterygium wilfordii Hook F. for the determination of tripdiolide. Peak 1 = tripdiolide. Conditions as in Fig. 3.

A linear calibration graph for triptolide was obtained by plotting the ratio of the peak area of triptolide to the internal standard (y) versus the amount of triptolide (x, ng). The regression equation and correlation coefficient (r) were $y = 0.025x - 0.049$, $r = 0.9999$, $n = 5$. The linear calibration graph of tripdiolide was obtained by plotting the peak area response of tripdiolide (y) versus the amount of tripdiolide (x, ng). The regression equation and correlation coefficient were $y = 744.2x - 2123$, $r = 0.9998$, $n = 7$. The range of the calibration curve was from 18.3 ng to 361.8 ng for triptolide and from 12.8 ng to 408.0 ng for tripdiolide.

The detection limit[20,21] was determined at very small concentrations using the described method. The minimum detectable amounts of triptolide and tripdiolide were 4.77 ± 0.66 ng ($n = 4$) and 9.05 ± 0.66 ng ($n = 3$) respectively.

The recovery test was carried out by adding pure triptolide and tripdiolide to the extract and assaying with the same procedure described above. The recoveries (mean % $\pm$ SD) of triptolide was 98.34 ± 1.54 ($n = 4$) and tripdiolide was 95.85 ± 1.49 ($n = 4$).

The assay results are displayed in table 1. Each term is the mean of two injections. The contents of triptolide and tripdiolide in 100 mg of the extract of TWHF were 19.88 and 9.58 ug respectively.

TABLE 1

CONTENTS OF TRIPTOLIDE AND TRIPDIOLIDE IN THE EXTRACT OF *TRYPTERYGIUM WILFORDII HOOK F.* DETERMINED BY HPLC.

Diterpenes	Amount in individual Determinations (ug per 100 mg of extract)	Mean $\pm$ SD	Relative Standard Deviation (%)
Triptolide	18.45 20.90 21.05 18.95 19.82 20.12	19.88 $\pm$ 1.04 n = 6	0.052
Tripdiolide	10.30 10.03 10.17 9.64 9.72 10.14 10.14 9.51 9.12 8.75 8.98 9.01 8.98 9.11 10.06	9.58 $\pm$ 0.54 n = 15	0.056

The values from individual experiments are the amounts per 100 mg of dry ethyl acetate extract.

In conclusion, this report describes a new accurate, sensitive and reliable method for the determination of triptolide and tripdiolide in an extract of TWHF. The pretreatment of samples with the Sep-Pak alumina B cartridge before HPLC represents a fast, simple and effective enrichment procedure with a very satisfactory recovery of the compounds. The successful employment of the internal standard greatly improved the accuracy and reproducibility for the triptolide assay. Triptolide and tripdiolide have been thought to be two of the major diterpene compounds contained in TWHF. This study provides the first quantitative data about the tripdiolide content in TWHF. Combined with the capacity to analyze triptolide, the approach makes it possible to evaluate the efficacy and toxicity of the TWHF extract and control the quality and safety of the preparation of this material for clinical trials and animal experiments.

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SIMULTANEOUS HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AMPROLIUM, ETHOPABATE, SULFAQUINOXALINE AND N4-ACETYLSULFAQUINOXALINE IN CHICKEN TISSUES

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method is described for the quantitative simultaneous residue determination of amprolium with fluorometric detection using post-column reaction, and ethopabate, sulfaquinoxaline and its major metabolite, N4-acetylsulfaquinoxaline with UV detection, in chicken muscle, liver, kidney, skin and plasma. Average recoveries from chicken tissues fortified with 0.1 μg/g of the four compounds tested were ranged from 81.0 to 103.8 % for individual compounds from individual tissues. Coefficients of variation were ranged from 1.1 to 8.6 %. Detection limits were 0.002-0.004 μg/g for each compound. The applicability of this method was demonstrated by determining concentrations of the four compounds in tissues from chickens administered with the three parent compounds.

INTRODUCTION

Amprolium (AMP), Ethopabate (EB) and Sulfaquinoxaline (SQ) are widely used to prevent coccidiosis and leukocytozoonosis in chickens. Since they are usually used as a combination of AMP + EB, AMP + SQ or AMP + EB + SQ, it is very useful and important to establish a simultaneous determination method with them in chicken tissues. Determination of N^4-acetyl SQ (ASQ) residue in chicken tissues should be also important, because ASQ, a major metabolite of SQ, can be detected in most of edible tissues from chickens administered SQ (1) and will be reconverted to SQ after their being uptaken in human body (2).

Several analytical methods involving gas chromatography (GC;3,4) and high-performance liquid chromatography (HPLC) with ultraviolet (UV; 5,6,7,8), fluorescence (9,10) detection have been reported for detecting AMP, EB and SQ individually, or the combination of EB and SQ in chicken muscle and liver. Nose et al.(11) have been reported to detect AMP, EB, SQ and other seven compounds simultaneously in chicken muscle with GC, though the GC conditions were separated in each compound and detection limits were

not enough for residue analysis. However, any methods to determine AMP, EB, SQ and ASQ simultaneously in most of edible chicken tissues including skin using HPLC with a low sensitivity limit have not yet been reported.

The purpose of the present study was to develop a simultaneous quantitative determination method with HPLC for AMP, EB, SQ and ASQ from chicken muscle, liver, kidney, plasma and skin. Further, applicability of this method was ascertained to determine the four tested compounds in tissues from chickens administered a commercial preparation containing AMP, EB and SQ.

MATERIAL AND METHODS

Reagents

(a) Solvents - Acetonitrile (MeCN), methanol (MeOH), n-hexane and 2-propanol (Wako Pure Chemical Industry Ltd., Osaka, Japan).

(b) Anhydrous sodium sulfate, disodium hydrogenphosphate 12-water, potassium dihydrogenphosphate, sodium 1- hexanesulfonate, sodium hydroxide and potassium ferricyanide (Wako Pure Chemical Industry Ltd.).

(c) Alumina - Alumina B Akt. I (ICN Biomedicals, Eschwege, FRG).

(d) Coccidiostats - AMP-HCl and SQ (Sigma Chemical Co., St. Louis, MO) and EB (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan).

(e) Metabolite - ASQ was synthesized by the method reported previously (12).

(f) Internal standard - Chloramphenicol (CP, Sigma Chemical).

(g) Standard solutions - Stock solutions in concentration of 25 - 100 μg/ml were prepared in MeCN for EB,SQ and ASQ and in 2% water-MeCN for AMP, and stored in dark at 4°C. A working solutions of lower concentrations were prepared from this solution by dilution with MeCN.

(h) Quartz wool - Fine (Nippon Chromato Works, Ltd., Tokyo, Japan).

(i) Reaction solution - Dissolve 50 g sodium hydroxide and in water, add 0.8 g potassium ferricyanide, and dilute to 1 liter with water.

Apparatus

(a) Homogenizer - Bio-mixer BM-2 (Niti-on, Tokyo, Japan).

(b) Evaporator - Rotary evaporator MINI model RE-21 (Yamato Scientific Co., Tokyo, Japan).

(c) Centrifuge - Model 8800 (Kubota Co., Tokyo, Japan).

(d) Cleanup column - A small quartz wool plug was placed at the bottom of a 30 cm x 15 mm id column, 6 g alumina was packed into the column with MeCN - MeOH (6:4, V/V), and the column was washed with 30 ml of the same solution before use.

(e) HPLC system and conditions - The HPLC system comprised a Model PU-980 pump (Japan Spectroscopic Co., Tokyo, Japan), a Model LC-9A pump (Shimadzu Co., Kyoto, Japan), a Model SIL-6A autoinjector (Shimadzu Co.), a Model 860-CO column oven (Japan Spectroscopic Co.), a Model 875-UV detector (Japan Spectroscopic Co.) placed between the column and the reactor coil, a Model RF-535 spectrofluorometer (Shimadzu Co.) and Model C-R5A integrators (Shimadzu Co.). The column was a 25 cm x 4.6 mm id stainless steel ODS column (L-column ODS, Chemicals Inspection and Testing Institute, Tokyo, Japan). The reactor coil placed in the column oven was 10 m x 0.25 mm id stainless steel tube. The mobile phase-1 and the mobile phase-2 were consisted of 0.2 M potassium dihydrogenphosphate - MeCN (85:15, V/V) containing 5 mM sodium 1-hexanesulfonate and 10 mM phosphate buffer (pH 5.0) - MeCN (79:21, V/V), respectively. The injection volume was 20 μl. The flow-rates of the mobile

phase-1 and reaction solution were both 0.7 ml/min, and the flow-rate of the mobile phase-2 was 1.0 ml/min. The fluorescence of AMP derivative converted by oxidation with ferricyanide in alkaline solution were detected at 367 nm excitation and 470 nm emission in spectrofluorometer by using mobile phase-1, and EB, SQ and ASQ were detected at 270 nm with spectrophotometer by using mobile phase-2. The column and the reactor coil temperature was 40°C. The chromatograms were recorded with a chart speed of 5 mm/min.

(f) Photodiode-array system - The detector was a Model SPD-M6A (Shimadzu Co.) interfaced with a PC-9801 VX personal computer (NEC Corporation, Tokyo, Japan). The recorder was a Model UP-2000 (Shimadzu Co.).

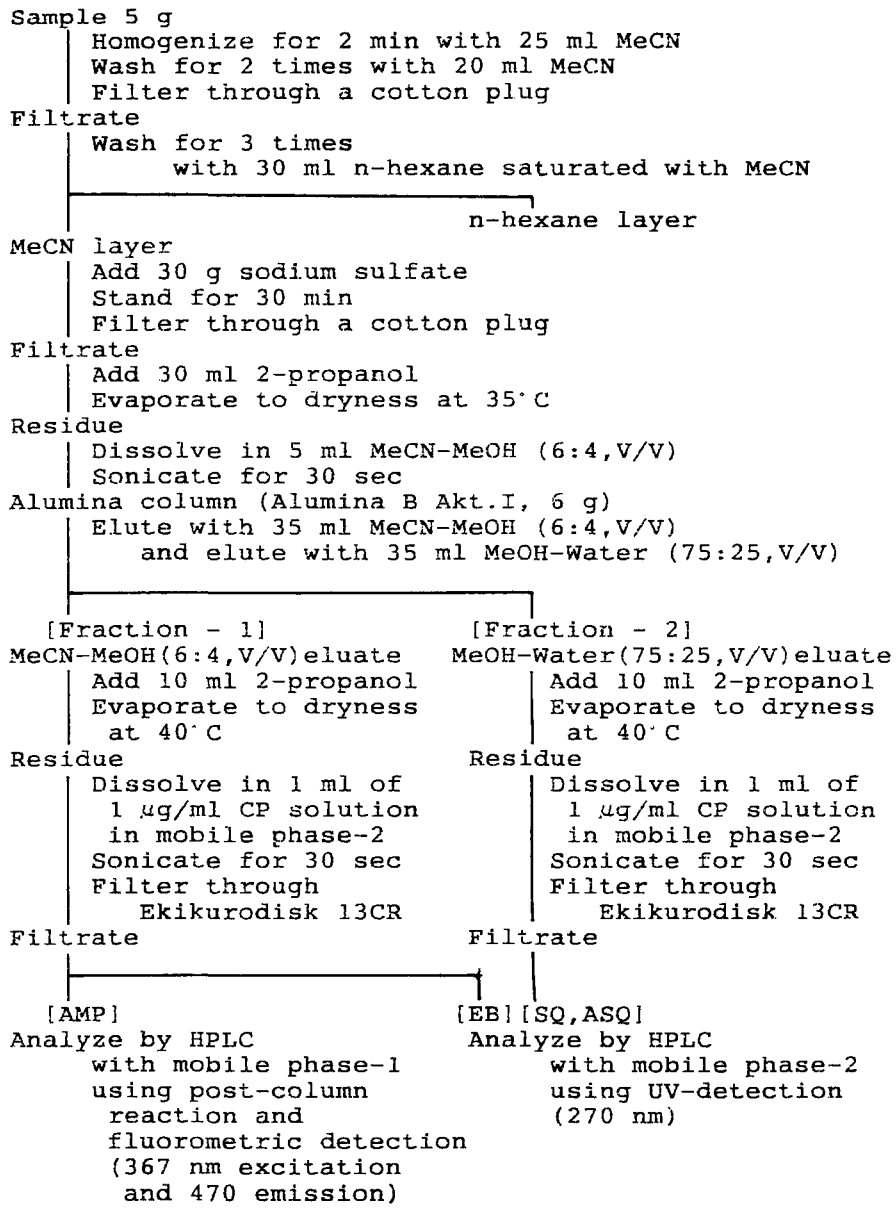
Control tissue samples

Ten non-medicated White Leghorn chickens (Nisseiken Co., Ltd., Tokyo, Japan) were sacrificed after bleeding, and the muscle, liver, kidney and skin were removed. The plasma after centrifuged at 3,000 rpm for 5 min and tissue samples were stored frozen at - 80°C until analysis.

Sample preparation

Sample preparation procedure was shown in Scheme 1.

5 g of chopped muscle, liver, kidney, skin or plasma was homogenized for 2 min with 25 ml MeCN. The homogenizer and glassware were washed twice with 20 ml MeCN. The mixture was filtered through a cotton plug, washed with 30 ml n-hexane saturated with MeCN for three times, and 30 g anhydrous sodium sulfate was added to the filtrate. The mixture was allowed to stand for 30 min at room temperature, filtered through a cotton plug, and 30 ml 2-propanol was added to the filtrate. The filtrate was evaporated to dryness at 35°C, and the residue was dissolved in 5 ml MeCN-MeOH (6:4, V/V), sonicated, and applied to an alumina column. AMP and EB were eluted with 35 ml MeCN-MeOH (6:4, V/V) which was named as fraction-1, then SQ and ASQ were eluted with MeOH-water (75:25, V/V) which was named as fraction-2. The both fractions were added 10 ml 2-propanol and evaporated to dryness at 40°C. These residues were dissolved in the mobile phase-1 containing with 1 μg/ml CP. The solutions were filtered through Ekikurodisk 13 CR (Gelman Sciences Japan, Tokyo, Japan) and subsequently injected into HPLC system. The resulting solution of fraction-1



SCHEMA 1. Analytical Procedure.

was analyzed by using the mobile phase-1, and AMP was detected by using spectrofluorometer at 367 nm excitation and 470 nm emission after mixing with the reaction solution in reactor coil to convert of AMP to a fluorescent derivative by oxidation with ferricyanide in alkaline solution. The resulting solution of fraction-1 (EB) and fraction-2 (SQ and ASQ) were analyzed by using the mobile phase-2 and detected by using spectrophotometer with detection wavelength of 270 nm.

Recovery

Recovery values of AMP were evaluated by comparing peak-areas of AMP extracted from fortified tissue samples with peak-areas of the standard solutions. Recovery values of EB, SQ and ASQ were evaluated by comparing peak-area ratios of each compound extracted from fortified tissue samples with peak-area ratios of the standard solutions.

Application

Two White Leghorn chickens (Nisseiken Co.) of 7 weeks old were used. They were kept in cages individually and provided non-medicated feeds and water ad libitum. They were administered 0.4 g/kg PANCOXIN (Dainippon Pharmaceutical Co.) containing

AMP (200 mg/g), EB (10 mg/g) and SQ (120 mg/g) orally with catheter. 6 and 24 hours after the administration they were sacrificed after bleeding, and the muscle, liver, kidney and skin of trunk were removed. Plasma and tissue samples were stored frozen at -80°C until analysis.

RESULTS AND DISCUSSION

Sample preparation

In the present study the sample preparation method developed was based on our previous reports concerning residue analytical methods of some coccidiostats (1,13). It is very useful and convenient to develop a universal method that would be applicable to determine all residual coccidiostats in animal tissues. In our previous report samples applied to an alumina column were firstly washed with 15 ml MeCN-MeOH (6:4, V/V) to remove lipo-soluble tissue components from samples. Since AMP and EB were eluted with this solution, we could not use the solution for this purpose. We selected the procedure of washing with n-hexane saturated with MeCN was repeated three times before applying to alumina column for this purpose.

Profiles of compounds eluted from the alumina column, which the sample solution from a control kidney added 1 μg of each compounds (1 μg/ml working standard solutions) was loaded on to, were shown in Fig.1.

EB was eluted by MeCN-MeOH (6:4, V/V) from the first eluate fraction (5 ml) and AMP was eluted in succession. 30 ml MeCN-MeOH (6:4, V/V) was necessary to elute AMP completely. In the case of plasma, only AMP was delayed one fraction (5 ml) to be eluted, but 35 ml MeCN-MeOH (6:4, V/V) was enough to be eluted. Recoveries of EB and AMP were 99.5 and 96.1 % , respectively. Then, 35 ml MeCN-MeOH (6:4,V/V) was selected as the first elution solution. After eluted EB and AMP, three different MeOH-water solutions, 85:15, 75:25 and 50:50 (V/V), were applied to elute SQ and ASQ from the alumina column. Increasing water content of elution solution, the total volume of solution was decreased, but recoveries of SQ and ASQ were 95 - 96, 98 - 100 and 76 - 79 % in 85:15, 75:25 and 50:50 (V/V) MeOH-water, respectively. Then, 35 ml MeOH-water (75:25, V/V) was selected as the second elution solution.

HPLC conditions

HPLC condition with UV detection was used for

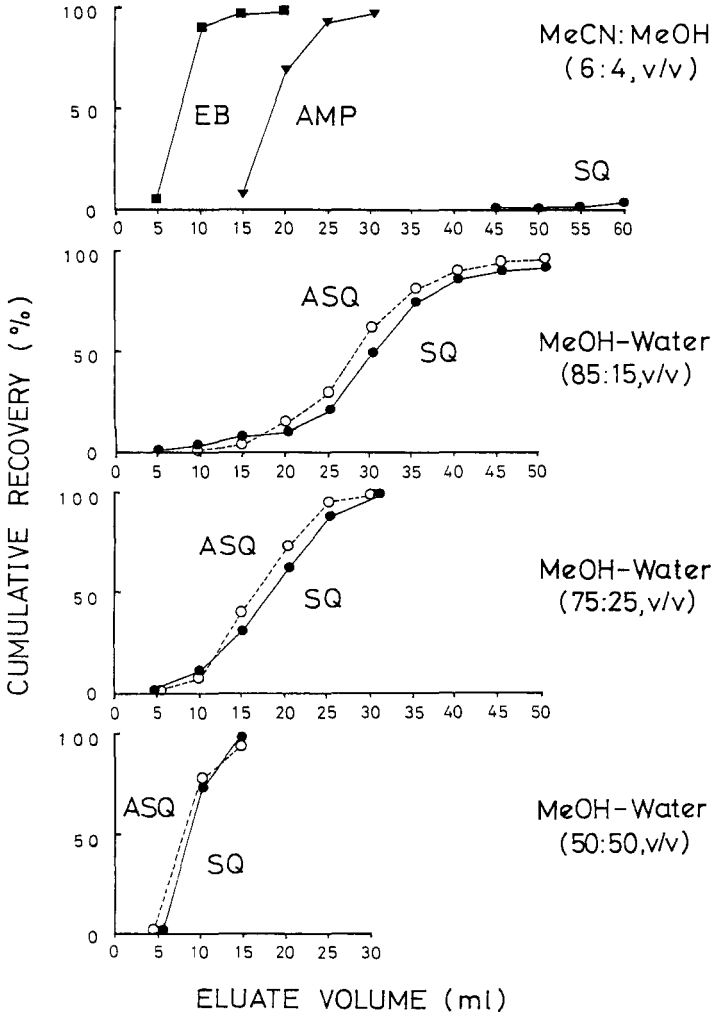


FIGURE 1. Elution Profiles of AMP(—▼—), EB(—■—), SQ(—●—) and ASQ(---○---) from an Alumina Column with the First Elution of MeCN-MeOH (6:4, V/V) and the Second Elution Using Three Kinds of Solvents, MeOH-Water (85:15, 75:25, 50:50, V/V).

determination of sulfonamides on the basis of our previous studies (1). But, we could not use a single HPLC condition to determine four compounds tested, because AMP was eluted at a very short retention time in the condition, and could not be separated from tissue components (tR=3.1 min in mobile phase-2). Then, fluorometric detection using post-column reaction was selected for AMP detection on the basis of previous study (10).

HPLC condition of AMP was changed a little from the previous study (10). For the purpose of ensuring conversion of AMP to a fluorescent derivative more, volume of reactor coil was increased about two fold, column temperature was raised from 30 to 40°C, and the content of MeCN in mobile phase-1 was decreased.

Optimal HPLC condition with UV detection was determined using tissue sample solutions described in sample preparation. Column was selected after some trials using 5 kinds of ODS columns, Capcell Pak C18 (Shiseido Company Ltd.,Tokyo, Japan), TSKgel-80Ts (Tosoh Co. Ltd., Tokyo, Japan), Chemco Pak-Nucleosil 5C18 (CHEMCO Scientific Co. Ltd., Osaka, Japan), Senshu Pak-ODS-1251-SS (Senshu Scientific Co. Ltd., Tokyo, Japan) and L-column ODS. Optimal mobile phase for each tissue was selected after trials of varying

pH from 5.0 to 5.8, mixture rate of MeCN in phosphate buffer from 15 to 25 %.

The retention time of tested compounds and other sulfonamides, their N^4-acetyl metabolites, diaminopyrimidines and other drugs which are used for poultry diseases by using mobile phase-2 with UV-detection are shown in table 1. All tested compounds except sulfadimethoxine were not interfered for determination by the compounds tested in the present study.

Chromatograms

Fig.2-(a), Fig.3-(a) and Fig.4-(a) show typical chromatograms of standard solutions of AMP, EB, SQ, ASQ and internal standard, CP. Fig.2-(b-f) shows typical chromatograms of fraction-1 of five tissue extracts from a control chicken using fluorometric detection. Fig.3-(b-f) and Fig.4-(b-f) show typical chromatograms of fraction-1 and fraction-2 of five tissue extracts from a control chicken using UV detection, respectively. Several peaks derived from tissue components appeared in the chromatograms, but all compounds tested were not interfered by them. AMP and EB in fraction-1 were not interfered each other, because AMP was detected at very short

TABLE 1

Retention Time of Compounds in UV-detection Method.

Compounds	Retention Time (min)
Sulfonamides	
SQ	20.7
sulfadiazine	4.8
sulfamethazine	6.2
sulfachloropyridazine	3.3
sulfamonomethoxine	8.5
sulfamethoxazole	11.2
sulfadimethoxine	20.8
sulfadoxine	11.1
N4-acetyl sulfonamides	
ASQ	16.7
N4-acetyl sulfamonomethoxine	7.2
N4-acetyl sulfadiazine	4.8
N4-acetyl sulfamethazine	6.2
N4-acetyl sulfamethoxazole	10.0
N4-acetyl sulfadimethoxine	18.1
Diaminopyrimidines	
trimethoprim	5.1
ormethoprim	5.8
pyrimethamine	21.4
diaveridine	4.5
Others	
EB	20.0
CP	18.8
AMP	3.1
oxolinic acid	16.1
nalidixic acid	33.8
nitrofurazone	6.1
furazolidone	8.7
thiamphenicol	6.1

retention time with UV detection, and EB was not detected with fluorometric detection.

Calibration curves and detection limits

The calibration curves of four compounds were

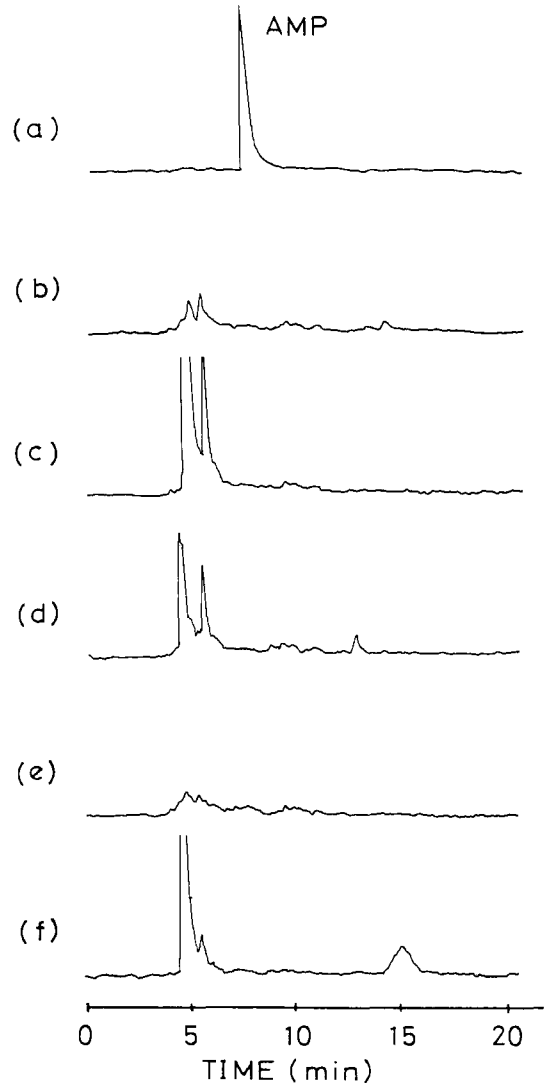


FIGURE 2. Typical Chromatograms of Standards containing 0.1 g/ml AMP (a) and Fraction - 1 of Control Tissue Extracts, muscle (b), Liver (c), Kidney (d), Skin (e) and Plasma (f).

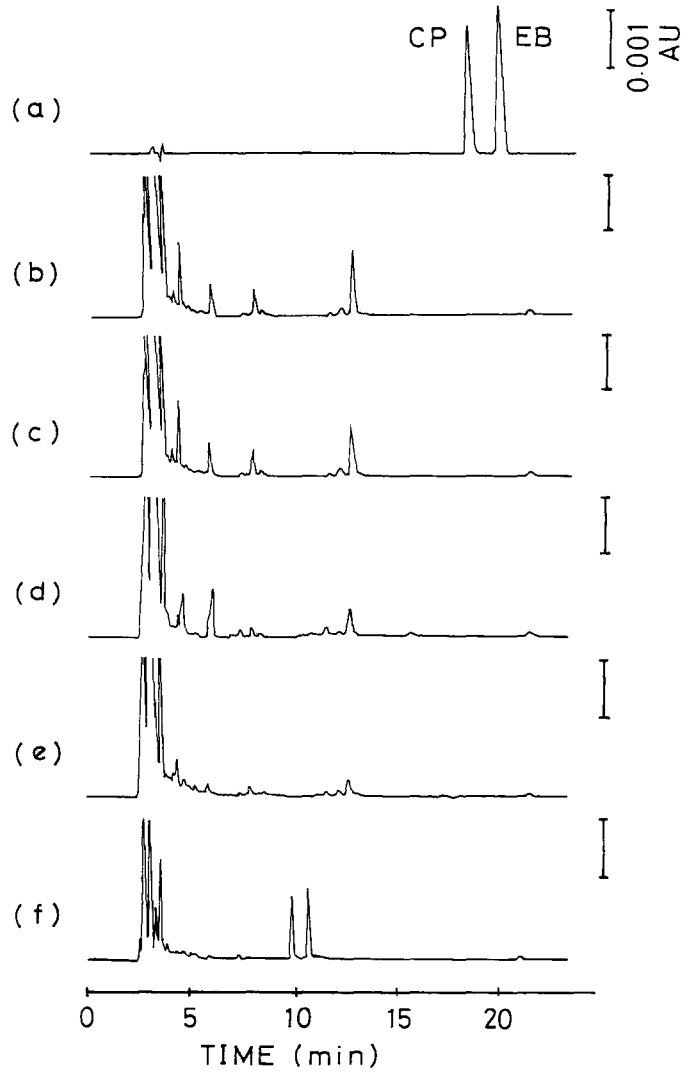


FIGURE 3. Typical Chromatograms of Standards containing 1.0 g/ml EB (a) and Fraction - 1 of Control Tissue Extracts, muscle (b), Liver (c), Kidney (d), Skin (e) and Plasma (f).

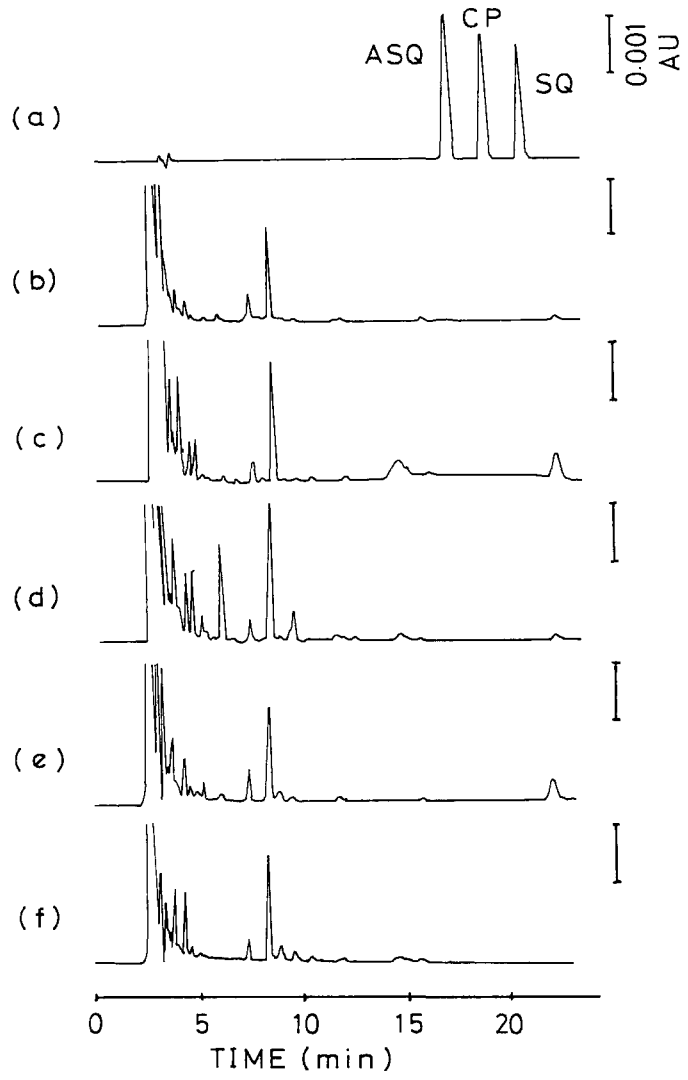


FIGURE 4. Typical Chromatograms of Standards containing 1.0 g/ml SQ and ASQ (a) and Fraction - 2 of Control Tissue Extracts, muscle (b), Liver (c), Kidney (d), Skin (e) and Plasma (f).

linear and reproducible through the investigated concentration range of 0.05 - 50 μg/ml, which is equivalent to 0.01 - 10 μg/g in tissue (R=0.999, n=5).

The detection limits shown in Table 2 (signal-to-noise ratio of 3) were satisfactory for residue analysis. These detection limits of SQ and ASQ were more sensitive than those in our previous report (1). This improvement might be caused by using L-column ODS for HPLC analysis and sample pretreatment of washing with n-hexane for three times.

Recovery

Recovery studies were conducted by adding 0.1 μg/g of AMP, EB, SQ and ASQ to each 5 g of control tissue sample. The extract from each sample was analyzed by the present method. Table 3 shows recovery data of the four compounds from tissues. Recoveries ranged from 83.8 to 103.8 % for individual compounds from individual tissues. Recoveries of SQ and ASQ from plasma were a little lower than those of other tissues, and this tendency was similar to that of our previous report (1). This tendency might be caused by delaying of SQ and ASQ elution from the alumina column. Coefficient of variation (C.V.)

TABLE 2

Detection Limits of AMP, EB, ASQ and SQ in Chicken Tissues.

Tissue	Detection Limit (μg/g)			
	AMP	EB	ASQ	SQ
Muscle	0.003	0.002	0.003	0.003
Liver	0.002	0.003	0.003	0.003
Kidney	0.004	0.003	0.003	0.003
Skin	0.002	0.003	0.003	0.003
Plasma	0.003	0.003	0.003	0.003

TABLE 3

Recoveries from Chicken Tissues Fortified with 0.1 μg/g of AMP, EB, ASQ and SQ.

Tissue	Recovery (%) (Coefficient of variation (%))			
	AMP	EB	ASQ	SQ
Muscle	90.2 (5.1)	99.7 (1.7)	99.7 (1.3)	103.8 (3.4)
Liver	85.8 (5.5)	97.3 (1.8)	89.4 (4.8)	97.4 (5.3)
Kidney	93.5 (4.0)	99.3 (1.1)	92.8 (3.1)	99.1 (3.3)
Skin	94.6 (8.6)	95.6 (3.8)	95.0 (4.6)	100.1 (4.2)
Plasma	98.1 (5.3)	90.8 (6.8)	83.8 (2.6)	81.0 (6.5)

n=5

ranged from 1.1 to 8.6 %. The recoveries were satisfactory for residue analysis.

Application

The application study was made to confirm whether the present method is applicable to quantitative assay of AMP, EB, SQ and ASQ in tissues from chickens administered AMP, EB and SQ.

Typical chromatograms of a liver extract are shown in Fig.5. The four compounds from five tissues were well separated not only from each other but also from tissue components. Further, the purity of SQ and ASQ peaks from individual tissues were determined by using the photodiode-array detector. Purity indices were 0.9999 for SQ and ranged from 0.9991 to 0.9999 for ASQ.

Table 4 shows concentration of four compounds in tissues. Though EB could not been detected in all samples because of a small dosage amount in the commercial preparation, AMP, SQ and ASQ were detected in all samples. AMP concentration was low in spite of high dosing, but SQ concentration was very high, especially in plasma which was three-fold greater than in muscle. ASQ concentration was low, and ratios of ASQ to SQ ranged from 0.9 to 4.3 % and was highest in liver.

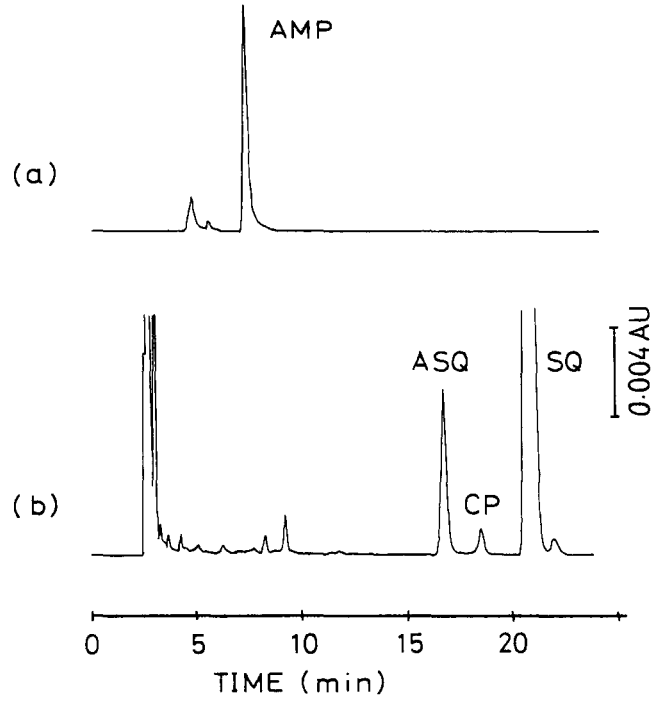


FIGURE 5. Typical Chromatograms of Ffraction - 1 with Fluorometric Ddetection (a) and Fraction - 2 with UV-detection for SQ and ASQ (b) of a Liver Extract from a Chicken 24 Hours after Orally Administration of AMP, EB and SQ.

CONCLUSION

A simultaneous HPLC residue analytical method with fluorometric detection using post-column reaction of AMP and with UV-detection of EB, SQ and ASQ in chicken muscle, liver, kidney, skin and plasma has been developed. This method was shown to be

TABLE 4

Concentration of AMP, EB, ASQ and SQ in Tissues from Chicken Administered with Commercial Drug Containing AMP 200 mg/g, EB 10 mg/g and SQ 120 mg/g.

Tissue	chicken[1)]	Concentration in Tissues (μg/g)			
		AMP	EB	ASQ	SQ
Muscle	1	0.45	-[2)]	1.51	53.58
	2	0.37	-	1.67	52.68
Liver	1	3.76	-	4.33	101.61
	2	1.41	-	3.98	111.70
Kidney	1	3.29	-	2.36	167.02
	2	0.59	-	2.06	163.66
Skin	1	0.59	-	2.08	80.29
	2	0.28	-	1.77	72.96
Plasma	1	0.31	-	1.54	171.49
	2	0.21	-	1.63	172.20

1) Chicken 1 and 2 were sacrificed 6 and 24 hours after single oral administration of 0.4 g/kg commercial drug, respectively.
2) Not detected.

applicable to tissue samples from a drug administered chicken. The detection limits and recoveries were satisfactory to residue analysis.

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DETERMINATION OF FAT-SOLUBLE VITAMINS BY LIQUID CHROMATOGRAPHY IN PEDIATRIC PARENTERAL NUTRITIONS

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ABSTRACT

A reversed phase high performance liquid chromatographic method using narrow-bore columns packed with 3 µm particles is described for the simultaneous determination of trans-retinol, ergocalciferol, DL-α-tocopherol and phytomenadione. The fat-soluble vitamins were separated in a C_{18} bonded phase column and eluted with methanol as the eluent pumped at a flow rate of 0.2 mL/min. A spectral detector was used and the wavelenghts were set at 325, 265, 284 and 250 nm for vitamin A, D_2, E and K_1 respectively. All vitamins were separated in less than 13 minutes. This method was applied to the determination of fat-soluble vitamins in the pediatric parenteral nutritions. The effect of the nutrition composition, daylight and the plastic infusion tubing on the stability of the vitamins was studied. Recovery studies showed good results for all solutes (91.0% - 110.5%) and the coefficients of variation were always less than 3%.

INTRODUCTION

The aim of parenteral nutrition is to improve or prevent malnutrition in those patients whose physio-pathological circumstances do not allow them to carry out certain necessities. Therefore, nutrient admixtures (aminoacids, glucose, vitamins, electrolites, oligoelements, water and in certain cases fats) are elaborated for intravenous administration, in accordance with different protocols.

During the neonatal period, the particular need to supply those nutritive elements which are necessary to achieve an adequate maturing of organs and systems is evident. This, together with metabolic and digestive immaturity, makes this subject group particularly sensitive to the administration of inadequate quantities of nutrients, especially the new-born who, due to incomplete fetal development or because of certain illnesses, can only receive an intravenous intake via parenteral nutrition during their first few days, weeks, or even months of life.

Clinical studies exist which relate fat-soluble vitamin deficiency states in newly-borns with certain pathologies (broncopulmonar dysplasia associated with a deficiency in retinol, haemolitic anaemia due to a lack of vitamin E or haemorraghing illness related to low plasma concentrations of clotting factors which depend of vitamin K), but, at the same time, clinical cases have been described which were triggered by high doses of fat-soluble vitamins (late growth owing to an excess in vitamin D or sepsis associated with a high vitamin E intake (1,2).

This all indicates that, for a correct supply of vitamins, the stability of said vitamins in pediatric parenteral nutritions must be known.

The mixing of the nutrients in a single container gives rise to problems of physico-chemical stability in some vitamins, yet in studies of said stability in parenteral nutrition, the data obtained to date with respect to degradation is disperse (3-5), due, in our opinion, not only to the distinct factors which affect the integrity of the vitamins (composition of the admixtures, the base products, the container and administration materials, exposure to the light, etc.) which depend on the protocols of elaboration and administration employed by each Hospital Nutrition Team, but also the methods and techniques of analysis employed.

The application of high performance liquid chromatography (HPLC) to the quantification of fat-soluble vitamins has been gaining acceptance as a sensitive and rapid analytical tool. In recent years many HPLC assays have been published, the principles of wich have been summarised (6,7). With respect to the evolution of the vitamin content in parenteral nutritions, most of the studies concentrate on the analysis of vitamin A, as it is one of the more labile vitamins and, what is more, recent papers (8,9), in which several vitamins are studied, excude vitamin D, probably since the vitamin is present in a very low concentration and the analytical methodologies used are pre-established, the latter are not sensitive enough,

especially when the concentration of vitamin D in the nutrition is expected to decrease with time.

This paper describes a practical and sensitive method for the simultaneous determination of fat-soluble vitamins in infant parenteral feeds by HPLC using narrow-bore columns (2.1 mm i.d.) packed with octadecylsilane. The proposed method was applied to the study of the evolution of the content in vitamins in infant parenteral feeds as a function of the influence of the feed composition, the infusion equipment employed and daylight.

EXPERIMENTAL

Reagents and Samples

Methanol was HPLC grade and was employed as supplied by the manufacturers. Ultrapure water was obtained though a Millipore Milli-Q system (Mildford, M.A., U.S.A.). Analytical grade trans-retinol, ergocalciferol, DL-α-tocopherol acetate and phytomenadione standard supplies by Merck (Darmstadt, Germany) were used. Butylated hydroxytoluene (BHT) was purchased from Sigma (St Louis, M.O., U.S.A.). Absolute ethanol and hexane were purchased from Romil (Loughborough, Leics, U.K.).

Individual stock solutions of each vitamin were prepared in ethanol with 0.025% of BHT to provide a concentration of 5 mg/mL for trans-retinol and phytomenadione and 2.5 mg/mL for DL-α-tocopherol acetate

and ergocalciferol. These solutions were degassed with helium and stored in dark glass flasks under -18°C refrigeration.

Individual or mixed standard solutions were prepared by appropiate dilution of the stock solution and filtered through a 0.45 µm membrane (Millex-HV 13, Millipore) before being injected into the chromatographic system.

The parenteral nutritions formulas investigated are shown in the Tables I and II. All components were mixed in a glass container and transferred to polyvinyl chloride (PVC) (Viaflex 500 mL, Baxter) plastic bags. Both the composition of the parenteral feed as the well as the plastic bags employed were similar to those used in the neonatal intensive care unit at Central Hospital of Asturias (Spain).

Apparatus and Conditions

The experiments were carried out using an HPLC system equipped with two Kontron 422 pumps; a 8125 Rheodyne injector with a 5 µL injection loop; a Kontron model 430 UV-VIS detector with a 3 µL flow cell and a data station with Data System 450 software (Kontron Instruments, Milan, Italy). The wavelenghts of the detector were switched at 325, 265, 284 and 250 nm for vitamins A, D_2, E and K_1 repectively.

The column employed was a Spherisorb ODS-2 (100 x 2.1 mm i.d., 3 µm). An ODS guard column was used to protect the analytical column. The mobile phase was pumped at a flow rate of 0.2 mL/min and used in

TABLE 1
Parenteral Nutritions Content

	PN-1 mL	PN-2 mL	PN-3 mL	PN-4 mL	PN-5 ml
Trophamine (Aminoacids Solution, 6%)	12	12	16	24	32
L-Cisteine Hydrochloride 8.8%	0.6	0.6	0.8	1.2	1.6
Glucose 50%	10	15	17	20	20
Sodium Chloride 0.9%	20	16	16	13	13
Potassium Chloride 18.5%	0.2	0.2	--	--	--
Calcium Gluconate 9.2%	2.9	2.9	3.5	4.0	4.1
Potassium Monohydrogenphosphate 1M	0.5	0.5	0.6	0.7	0.8
Magnesium Sulphate 15%	0.2	0.2	0.4	0.4	0.4
Oligoelements Pediatric Solution	0.7	0.7	0.7	0.7	0.7
Sodium Heparine 1%	0.2	0.2	0.2	0.2	0.2
Pediatric Multivitamins	5	5	5	5	5
Nonpyrogenic Sterile Water	47.7	46.7	39.8	30.8	22.2

isocratic mode (100% methanol). Before use, the mobile phase was vacuum-filtered 0.45 µm nylon filter and degassed with helium.

The chromatographic experiments were carried out at room temperature (20 ± 2°C).

TABLE 2.Pediatric Multivitamin Composition

FAT-SOLUBLE VITAMINS	
Trans-Retinol	0.7 mg
Ergocalciferol	10 μg
DL-α-Tocopherol Acetate	7 mg
Phytomenadione	0.2 mg
WATER-SOLUBLE VITAMINS	
Riboflavine	1.4 mg
Pyridoxine Hydrochloride	1.0 mg
Niacinamide	17.0 mg
Pantothenic Acid	5.0 mg
Thiamine	1.2 mg
Biotin	20 μg
Folic Acid	0.14 mg
Cyanocobalamin	1 μg
Ascorbic Acid	80 mg
EXCIPIENTS	
Mannitol	375 mg
Polysorbate 80	50 mg
Polysorbate 20	0.8 mg
Butylated Hydroxytoluene	58 μg
Butylated Hydroxyanisole	14 μg

RESULTS AND DISCUSSION

Using previous studies as a basis, we chose high performance reversed-phase liquid chromatography for the separation and determination of the fat-soluble vitamins in pediatric parenteral nutritions (PPN). We studied the effect of the elution conditions, particularly mobile phase composition and flow, on the resolution of fat-soluble vitamins. This was achieved with narrow-bore columns because, in comparising with ordinary columns, it is clear that less mobile phase solvent is consumed and the analytical cost is therefore less. Furthermore, the detection limits obtained with the narrov-bore columns are lower than those provided by the ordinary column. This is quite significant for the analysis of samples such as the PPN in wich the vitamin concentration, because of its marked lability, decrease with the time.

Taking into account the important structural differences among the vitamins to be separated, as well as the high retention capacity of the octadecylsilane packing chosen as the stationary phase, mobile phases with a high elution capacity, such as methanol or mixtures of methanol with extremely low water contents (99:1), were employed. With respect to the mobile phase flow employed, this was modified over a range of values between 0.15 and 0.25 mL/min, the resolution obtained in all cases always being higher than 1.0. The optimum working conditions were found to be the following: mobile phase 100% methanol, flow rate 0.2 mL/min, isocratic mode, room temperature.

A chromatogram of a mixture of standards demonstrating the separation of vitamins A, D_2, E and K_1 is presented in Figure 1. As can be seen, the analysis time is less 13 minutes under isocratic conditions, thus avoiding re-equilibration delay between runs. Retention times of analysis performed on the same day were reproducible within ±1% (Table III).

Figure 2 shows the chromatogram obtained, under optimum experimental conditions, for an ethanolic solution of the vitamin complex wich is added to the nutrient units. As can be seen, a considerable front of scarcely retained or unretained compounds appears which interferes wich the chromatogram peaks corresponding to

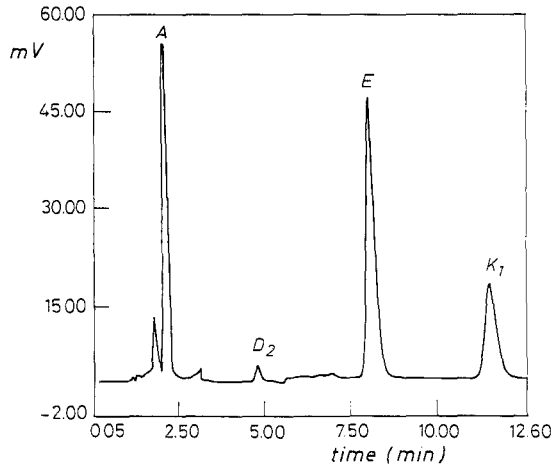

FIGURE 1. Chromatogram obtained from standard solutions of fat-soluble vitamins by using a Spherisorb ODS-2 (100x2.1 mm I.D. 3 µm). Mobile phase, 100% methanol. Flow-rate, 0.2mL/min.

TABLE 3

Retention Times And Their Repeatability In The Investigated Vitamins

Vitamin	Retention Time (min)	C.V. (%)
A	2.18	0.69
D_2	5.03	0.82
E	8.77	0.83
K_1	12.45	0.93

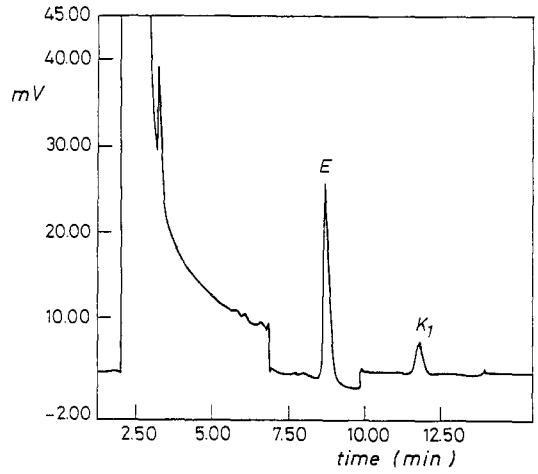

FIGURE 2. Typical chromatogram of fat-soluble vitamins contained in an ethanolic solution of the multi-vitamin added to the nutritional formulas. Column and chromatographic conditions as in Figure 1.

vitamins A and D_2. Since the number of species present in the nutritions is even higher, a clean-up of the sample was carried out using hexane as extractant according to the following procedure: PPN (1mL) was transferred into a 10 mL glass centrifuge tube and 3 mL of hexane were added and the admixture was vortexed for 5 min. The solution was then centrifuged at 2000 g for 10 min. The organic layer was transferred and the extraction process was repeated with 3mL of hexane. The organic layers were jointed and washed with 2 mL of methanol-water (9:1). The organic upper layer was separated and passed through a 0.45 µm filter. After this, it was evaporated under nitrogen until dry and reconstituted in 1 mL ethanol with the help of an ultrasonic bath. A 5 µL aliquot of this solution was injected into the HPLC system.

Since vitamins oxidise easily, as well as being photo-sensitive and thermo-labile, the preparation of the samples has to be carried out under diffuse light, in the presence of an anti-oxidant, and it must then be preserved at low temperatures. In fact, the nutrition samples tested contain butylated hydroxytoluene (BHT) as anti-oxidant. Although this compound is extractable in hexane, considering that the solubility of oxygen in hexane is greater than in ethanol, it was decided to add an equivalent quantity of BHT (0.58ppm) to the hexane employed in the extraction so as to preserve the vitamins extracted whilst these remained in hexane.

Figure 3 shows the chromatograms obtained on extracting two similar aliquots of the nutrition in hexane, with and without adding BHT in the organic

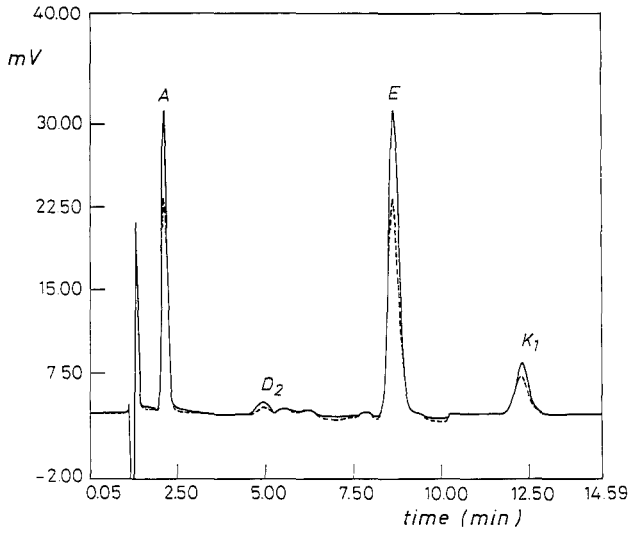

FIGURE 3. Chromatograms of hexane extract of a pediatric parenteral nutrition admixture:
— with BHT as antioxidant in the organic layer.
-- without BHT in the organic layer.
Chromatographic conditions as in Figure 1.

layer, where it can be seen that their behaviour is practically the same, although an important increase in the peaks corresponding to vitamins A and E is appreciated with BHT, for which reason said anti-oxidant was systematically incorporated in the preparation procedure of the sample.

Table IV lists the minimum quantities detectable for the four vitamins based on a signal-to-noise ratio of 3:1. In order to check the accuracy of the proposed method, we determined recoveries by analysing PPN as such and spiked with known amounts of the vitamins. All analyses were carried out in triplicate at three

TABLE 4

Detection Limits Of Fat-Soluble Vitamins Determined By Using Narrow-Bore Column

Vitamin	D.L. (ng)
A	0.75
D_2	0.85
E	4.85
K_1	0.95

concentration levels. For determination of vitamin D_2 in the samples, a preconcentration step was neccesary. The results obtained are given in Table V. The average recoveries obtained, wich ranged between 91.0±0.6 and 110.5±0.9 testify to the accuracy of the proposed method.

The precision of the method was checked using five different PPN and by analysing each sample in triplicate. The coefficients of variation were always less than 3%.

Under the above described working conditions the vitamins in the PPN were quantified by the external standard method. Standards were injected and the resulting integrator response factors were computed and then processed by the integrator to deliver the unknown concentrations. The injected volume used was always 5 µL and the amount of each vitamin present was directly obtained from the data module. Regular recalibrations were carried out.

TABLE 5

Recovery Studies Of Fat-Soluble Vitamins Added To The Nutritional Formulation.

Vitamin	Amount in sample µg/mL	Amount added µg/mL	Amount found µg/mL	% Recovery
A	6.63	1.04	7.60	99.1±4.2
		2.08	7.93	91.0±0.6
		3.12	10.02	102.8±2.6
D_2	0.08	0.03	0.103	94.0±1.8
		0.06	0.154	110.1±0.3
		0.09	0.188	110.5±0.9
E	67.7	5.00	73.35	100.9±5.9
		10.0	79.64	102.5±2.7
		20.0	78.40	94.8±0.6
K_1	1.85	0.3	2.35	109.4±1.0
		0.6	2.44	99.4±2.5
		0.9	2.79	101.5±2.4

To check the influence of nutritional parenteral composition on vitamin stability, five formulas were prepared (see Table I) the vitamin content being determined over time (recently prepared, at 10 hours and after 24 hours). The bags were maintained at room temperature and unprotected from the light. From the results obtained, it can be seen that the composition of the nutritions has not notable influence on the evolution of the vitamin content, or at least does not

have the same degree of influence as the other external factors. As an example, Figure 4 represents the decrease that the concentration of vitamin K_1 suffers with respect to the composition of the sample over time.

The photo-sensitivity of the different vitamins in the bags of nutrition during administration was studied in one of the samples (PN-2). In order to do this, two bags were prepared, one of which was protected from the light. Its vitamin content being determined when recently prepared and after 24 hours. As can be seen in Figure 5, the degradation of the vitamins in the bag exposed to the light is greater than in the protected bag, vitamin E being the least affected by this factor. The small variation in the concentration of vitamin E may be attributed to other factors such as oxidation, due, amongst other causes, to the permeation of atmospheric oxygen through the bag.

Vitamin A is affected to an important degree by the light, in fact, protecting it enables around 30% more to be recuperated after 24 hours. Vitamin D_2, as opposed to the other three, seems to degrade fundamentally because of this factor. The concentrations of vitamins A, E, D_2 and K_1 decrease over time, independently of whether they are exposed to the light or not, as a consequence of the other causes mentionet, vitamin A being the most affected.

Another possible cause of loss of vitamin content in the parenteral nutritions may be attributed to proccesses of adsorption onto the plastic material with

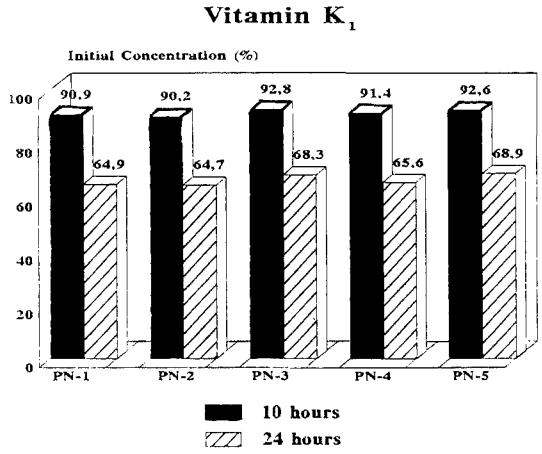

FIGURE 4. Evolution of vitamin K_1 concentration according to the pediatric parenteral nutrition admixture composition over time.

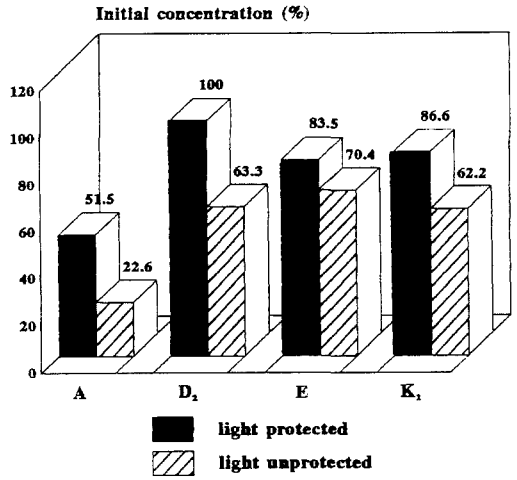

FIGURE 5. Stability of fat-soluble vitamins in a pediatric parenteral nutrition admixture, in a polyvinyl chloride plastic bag, hanging with and without to protection from daylight.

which the intravenous perfusion equipment is elaborated. In order to study this possibility, the same nutrient mix (PN-2) was passed through the infusion equipment (Abbot Venisystems, Lifecare 5000 IV). After purging the system, a flow of 3 mL/hour was established and aliquots were taken from the bag and from the distal end of the equipment (connection point with the patient's intravenous administration tube) which was subject to room tempetarure and protected from the light. The chromatographic analysis carried out shows that vitamin A is highly absorbed, there existing an average difference in concentration between the distal end of the tube and the bag of 41.9%, while the rest of the vitamins practically do not experiment interaction with the perfusion equipment -which may be relevant with respect to therapy- the slight differences obtained were attributed to error in the method.

CONCLUSIONS

Reversed-phase chromatography using narrow-bore columns packed with 3 µm particles provides a rapid and simple alternative to the determination of fat-soluble vitamins. The method described in this report is suitable for determining the vitamins A, D_2, E and K_1 in pediatric parenteral nutritions (PPN).

The results of the experiments in this study demonstrate that under the conditions of preparation and administration employed, vitamins losses, particularly vitamin A, appear in PPN infused to neonatal patients. Given the clinical importance of intravenous delivery of vitamin A for premature neonates the use of more inert

bags and perfusion equipment protected from the light is considered highly convenient.

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^{18}O AND U^{13}C LABELING OF PHOTOSYNTHETIC AND RELATED QUINONES AND THEIR PURIFICATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Different photosynthetic quinones have been labeled on the carbonyl groups by oxygen exchange with $H_2{}^{18}O$ and purified by high performance liquid chromatography on normal phase column with non aqueous solvent mixtures. U^{13}C quinones have been extracted from Spirulina maxima, Synechocystis 6803 and E. coli, grown on ^{13}C labeled medium and purified on reversed phase columns. Vitamin K_1, plastoquinone PQA_{45}, ubiquinone Q_8 have been obtained in mg amounts, while different other quinones (menaquinone MK_8, ubiquinones Q_2, Q_7, Q_9, Q_{11}, plastoquinones A) were present only in trace amounts. Several of these quinones have been used in the reconstitution of the photosynthetic reaction centers, in order to assign the quinone bands in the light-induced Fourier transformed infrared difference spectra of the photoreduction of the primary quinone acceptor.

INTRODUCTION

The identification of the different bands observed in the light-induced FTIR difference spectra of the photoreduction of the primary quinone acceptor Q_A and their assignment to the protein or the quinone moiety are greatly facilitated by the use of bacterial reaction centers reconstituted with ^{18}O and ^{13}C quinones (1). In this work, the ^{18}O labeling has been obtained by isotopic exchange with $H_2{}^{18}O$ on commercially available quinones and the uniformly ^{13}C labeled quinones have been extracted from bacteria or algae grown on ^{13}C labeled culture medium. In the two cases the quinones have been purified in one or several steps by high performance liquid chromatography.

MATERIAL AND METHODS

1) Labeling

a/ ^{18}O labeling

5 to 50 mg of quinone (vitamin K_1, Q_{10}, dimethylnaphtoquinone, 2-3 dimethoxy 5 methyl 1-4 benzoquinone, 2-3 dimethoxy 5-6 dimethyl 1-4 benzoquinone, tetramethyl 1-4 benzoquinone) (Sigma, Aldrich, Apin Chemicals) were incubated, in 1 ml vials stoppered with teflon faced liners, at 37°C during 1 to 15 days, under nitrogen, with 90 µl $H_2{}^{18}O$ (Service des Molécules Marquées CEA) and 60 µl trifluoroacetic acid (TFA, Merck), dissolved in 350 µl tetrahydrofuran (THF, Merck).

b/ $U^{13}C$ labeling

E. coli strain BL 21 (2) was grown on mineral medium M9 added with 0.66 g/l of ammonium sulfate and 1.5 g/l of ^{13}C D glucose as sole carbon source. The medium was supplemented by 5 mg/l thiamine, minerals (0.5 mM $FeCl_3$, 0.1 mM $CaCl_2$) and contained 25 mg/l chloramphenicol and 150 mg/l ampicillin. The bioreactor was LSL Biolaffite 30 Al. The medium was oxygenated with air (12 l/min) at 37°C and the pH was continuously adjusted at 7.2 with 1 M NaOH. The final volume was 10.2 l.

Synechocystis PCC 6803 and Spirulina maxima were both cultivated in Zarrouk medium (3) containing 1.05 g/l of ^{13}C sodium carbonate as sole carbon source. Culture medium was maintained at 29°C pH 9.3 under 600 µE/sm^2 continuous incandescent illumination per absorbance unit at 366 nm. The final volume was 28.5 l.

For all samples, 50 µg of dried biomass were isotopically diluted with 1 mg casein and combusted in an oxygen/helium atmosphere. The gaseous product was analysed using a mass spectrometer. The isotopic enrichment of the preparation calculated from the $^{13}CO_2/^{12}CO_2$ ratio, was 98 % in the case of E. coli, 99 % in the case of Synechocystis and 96 % for Spirulina.

2) Purification

a) ^{18}O labeled quinones

THF, TFA and the mixture of $H_2{}^{16}O$ and $H_2{}^{18}O$ were evaporated under nitrogen stream and the residue of labeled quinone and impurities was dissolved in 500 µl chloroform (Lichrosolv, stabilised with 2 methyl 2 butene, Merck). High performance liquid chromatography was applied with a Waters Ass. liquid chromatograph composed of a M 510 pump, U_6K injector and 490 E detector. Preparative runs were performed on Partisil 10 M9 Whatman columns (9 mm x 50 cm),

with chloroform-ethanol mixtures (0.2 to 0.4 %) as eluent, or chloroform-hexane mixture (4/1 V/V), in the case of tetramethylbenzoquinone. Optical absorption was followed at the corresponding uv maximum.

b) $U^{13}C$ labeled quinones

- Quinones from E. coli

The culture medium was centrifuged for 20 min at 7,500 RPM. 25 g of wet bacteria were collected and extracted three times by agitation during 10 min with 100 ml of pure ethanol (eventually followed by acetone, then hexane). The suspension was filtered on glass filter, the different extractions were pooled and dried under vacuum. The residue was then extracted with hexane (2 ml), the solution was washed with water, separated and evaporated under vacuum. The new residue was dissolved in ethanol and injected on a Partisil 10 ODS_2 M20 Whatman column (20 mm x 50 cm). The different components were eluted with pure ethanol (14-18 ml/min). The quinone fractions were collected, evaporated and further purified on Ultrasphere ODS 5 µm (Beckman) (4,6 mm x 25 cm), with pure ethanol as eluent (1 ml/min), and finally on Partisil 10 M9 as above, with chloroform containing 0.2 % ethanol (6 ml/min).

- Quinones from Spirulina maxima

175 g of wet centrifuged Spirulina were successively extracted with 250 ml of the following solvents : methanol (four times), ethanol (twice), acetone, heptane, chloroform-acetone-methanol mixture (1/2/1) (twice). The different filtrates (obtained by passage on glass sintered filters) were dried under vacuum, the residues were dissolved in minimal ethanol or isopropanol volume, filtered and injected on Partisil 10 ODS_2 M20, with pure or 2 % H_2O-containing ethanol as eluent (18 ml/m). Before chromatography, the methanol fractions, containing mainly chlorophyll a, were prepurified by absorption on a small column (diameter 2 cm, height 5 cm) of Bakerbond octadecyl (JJ Baker, BV), washing, and elution with the minimum of ethanol.

The optical density of the eluate was followed at two wavelengths, generally 260 nm and 450 or 530 nm. The fractions corresponding to a UV/visible absorption ratio greater than unity were repurified by one or two passages on µ Bondapak C18 Waters column (3,9 mm x 30 cm), with a mixture ethanol-water as eluent (gradient from 90 % to 100 % ethanol in 10 min, at the rate of 1 ml/min.

- Quinones from Synechocystis PCC 6803

175g of centrifuged Synechocystis (wet weight) were extracted four times successively with ethanol (total volume 2.4 l), then with a mixture chloroform-acetone-methanol (1/2/1) (total volume 800 ml). The different filtrates were pooled, dried under vacuum and the residue was

dissolved with ethanol, then isopropanol. These solutions were injected on a Partisil 10 ODS_2 M20 as above, with pure ethanol or 10 % isopropanol containing ethanol as eluent, at the rate of 18 ml/min.

In this case, the Millenium 2010 Chromatography Manager (Millipore) with the 996 Photodiode Array Detector were used to detect the minute quantities of vitamin K_1 and other quinones, which otherwise would not be visible among predominant pigments. The quinone containing fractions were pooled, dried under vacuum and rechromatographed on the same column as above, with 1 % H_2O-containing ethanol or pure ethanol as eluent, and finally on a μ Bondapak C18 column with a mixture ethanol-water (same gradient as for the quinones of Spirulina).

Analytical supercritical fluid chromatography was performed on a Jasco apparatus (880 PU pumps, 875 UV detector, 880-81 back pressure regulator), on a μ Bondapak C18 column, at 50°C, and 100 bars of pressure, with methanol as modifier (20 %).

UV-visible spectra were performed on a Shimadzu UV 160 A and infrared spectra were obtained on films using a Nicolet 60 SX FTIR spectrometer.

RESULTS

1) ^{18}O labeled quinones

After unsuccessful efforts to label the CO groups of the quinones through the formation of acetals or oximes, we have tried the oxygen exchange route with $H_2{}^{18}O$. A previous report (4) related a 95 % exchange of oxygen by continuous stirring of Q_{10} with $H_2{}^{18}O$ in a dichloromethane - methanol mixture containing trifluoroacetic acid, during four months. In order to accelerate the exchange, we have studied the respective influences of the solvents (dichloromethane, methanol, dioxane, tetrahydrofuran) and of acids (trifluoroacetic, acetic or hydrochloric acids) or base (triethylamine). The incubation was performed at 37°C, to increase the velocities of exchange and to avoid separation of the mixture of solvents into two phases. In the conditions described under Material and Methods, the equilibrium was reached between 5 and 15 days. The maximum attainable percentage of labeling was theoretically around 82 %, mainly limited by the concentration of the TFA (bringing exchangeable ^{16}O) which was necessary for the exchange to occur. It was approximately that value (70 - 75 %) which was calculated from the infrared spectra (Fig. 1) and this labeling has been shown to be sufficient for the assignment of the bands in the light induced FTIR difference spectra (1). The purification was performed on a normal phase column, in non aqueous medium (chloroform containing a low concentration of ethanol). In these conditions, there is no oxygen exchange during the chromatography, contrary to the case where this operation would be done in aqueous medium, on reversed phase column.

The chemical yields decreased with time of incubation, due to slow degradation. With dimethylnaphtoquinone for instance, it was 85 % after 5 days and 65 % after 11 days.

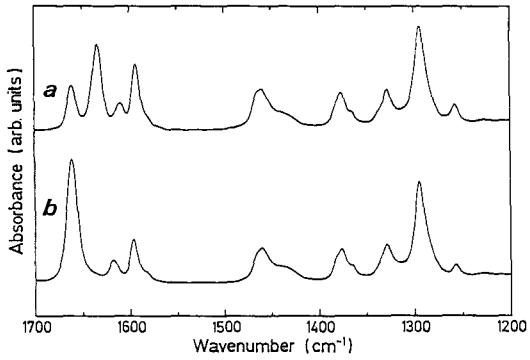

Fig. 1 Infrared spectra of Vitamin K_1 : a) ^{18}O, 70 % labeled, b) ^{16}O.

2) U^{13} labeled quinones of E. coli

Several quinones have been shown to be produced by E. coli : menaquinone (MK8) and demethyl menaquinone (5), ubiquinones from Q_1 to Q_7 (6) which are synthetized in low amounts, and Q_8 which is the major quinone found (6-7). We have purified the main quinone by high performance liquid chromatography on reversed (Fig. 2) and normal phases columns, as described in Materials and Methods. It was identified as pure by uv-visible and infrared spectra and the length of the isoprenoid chain has been determined by comparison of the retention time on reversed phase column with those of different ubiquinones of known chain length. (Fig 3). The quantity of Q_8 purified from ^{12}C or ^{13}C E. coli was 0.063 ± 0,018 mg/g of wet weight of bacteria (7 preparations). This value is similar to that given by Bishop et al (7).

Q_7 and Q_9 were also separated and identified by their spectra and retention time on reversed phase column. Menaquinone was identified by its spectrum and retention time as MK8, in accord with (5). Their concentrations were respectively 2.66, 3.18 and 3.99 µg/g wet weight of bacteria. Contrary to Dave et al (6), we have found in E. coli an ubiquinone with a side chain with more than 8 isoprene units. The menaquinone concentration was much lower than that reported by Bishop et al (7).

3) $U^{13}C$ quinones from Spirulina

After chromatography on Partisil ODS_2 M20 preparative column of the different extracts of Spirulina, and one or two passages on µ Bondapak C_{18} column of the fractions showing a uv/visible absorption ratio greater than unity, several quinones were separated.

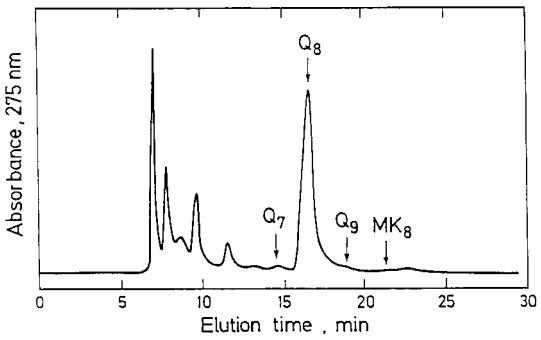

Fig. 2 Chromatogram of E. coli extract.
Conditions : Magnum 20 Partisil ODS_2 column (20 mm x 50 cm), elution with pure ethanol, 14 ml/min.

A major quinone identified by its spectrum as plastoquinone, already proved to be PQA_{45} (8) the concentration of which was found to be 0.016 mg/g wet weight of algae.

Another plastoquinone, with a greater retention time on reversed phase column, in a ten times smaller amount.

Three ubiquinones, identified by their spectra and by their retention time : Q_2, Q_{11} and (probably) Q_{15}, by extrapolation of the curve of Fig 3. Their concentrations were respectively 0.048, 0.38 and 0.37 µg/g wet weight of algae.

Only a small quantity of vitamin K_1 could be separated and purified from Spirulina (0.03 µg/g wet weight). It is possible that some loss has occured during the chromatography, when vitamin K_1 absorbance was masked by other dominant pigments. The photodiode array detector was not yet available for these experiments. However, supercritical fluid chromatography of Spirulina extracts showed that the vitamin K_1 content was very low in this algae: 0.7 % of that of chlorophyll a, 4 % of that of PQA_{45}. Compared to Spirulina, Synechocystis 6803 was found to be much richer in vitamin K_1 (Fig. 4) : from 2.7 to 6 times, on the basis of the chlorophyll a or PQA contents. It is the reason why we have used this cyanobacteria for the purification of labeled vitamin K_1.

4) $U^{13}C$ quinones from Synechocystis 6803

The same protocol as above was used for the purification of the quinones of Synechocystis 6803, except that the detection system used was the Millenium 2010 Chromatography Manager (Millipore) equiped with the 996 Photodiode Array Detector (Waters). This system allows to visualize the spectrum of the eluate as it gets out of the column, and thus

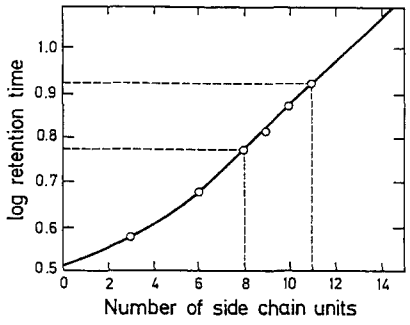

Fig. 3 Relation between retention time and length of the isoprenoid chain.
Conditions : μ Bondapak C18 column (3,9 mm x 30 cm), elution with ethanol containing 2 % H_2O, 1 ml/min.

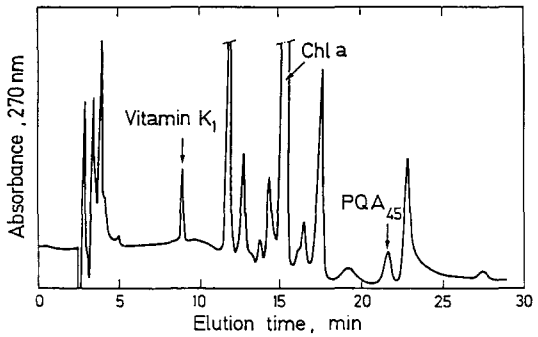

Fig. 4 Supercritical fluid chromatography of Synechocystis 6803 extract.
Conditions : μ Bondapak C18 column (3,9 mm x 30 cm), CO_2 pressure : 100 bars, temperature : 50 °C, Methanol concentration : 20 % during 5 minutes, then linear gradient to 25 % during 10 minutes, 1 ml/mn.

to collect more efficiently the fractions containing substances present only transiently, in low concentrations, and mixed with other strongly absorbing materials. Vitamin K_1 was purified from the ^{12}C and ^{13}C labeled algae (respectively 0.027 and 0.041 mg/g wet weight), as PQA45 (0.052 mg/g) for the normal culture, but surprisingly, 0.004 mg/g for the ^{13}C labeled sample). In addition, two other plastoquinones (255 nm absorbing materials) were eluted after PQA45, in very small amounts (respectively 0,072 and 0,12 μg/g). The reduced form of vitamin K_1, is also

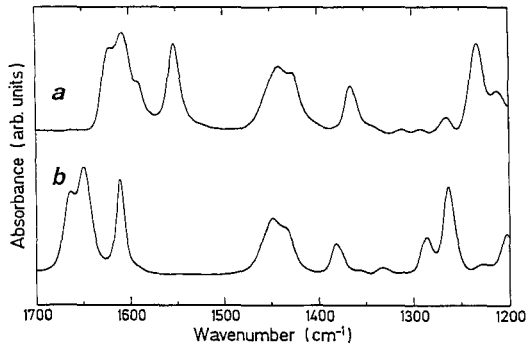

Fig. 5 Infrared spectra of Ubiquinone Q_8 : a) ^{13}C, b) ^{12}C.

present in traces (0,27 µg/g)). It has been identified by its spectrum (maxima at 240 and 300 nm) and by formation of vitamin K_1as the major reaction product after oxidation with potassium ferricyanide (spectrum and retention time).

CONCLUSION

It is possible to label carbonyl groups of photosynthetic and related quinones by oxygen exchange with $H_2{}^{18}O$ and to purify them by HPLC on normal phase column, with non-aqueous solvents. $U^{13}C$ quinones as vitamin K_1, plastoquinone PQA45 and ubiquinone Q_8 have been extracted from Synechocystis Spirulina and E. coli respectively, grown on labeled medium and purified in mg amounts by HPLC on reversed phase column. These materials have been used then for the reconstitution of the photosynthetic reaction centers and for the assignment of the quinone bands in the light-induced FTIR difference spectra of the photoreduction of the primary quinone acceptor Q_A

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DETERMINATION OF LINOPIRDINE AND ITS MONO-N-OXIDE METABOLITE IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Sensitive and selective high-performance liquid chromatographic methods for the determination of linopirdine, a novel cognitive enhancer, and a major metabolite, linopirdine mono-N-oxide, in human plasma and urine are described. For plasma, alkalyzed samples were extracted with ethyl acetate. For urine, neutral samples were extracted with ethyl acetate and further treated by solid-phase extraction. The plasma residues were chromatographed on a Beckman CN HPLC column and the urine residues on a Jones Apex II CN HPLC column (both 4.6 x 25 cm). The mobile phase consisted of acetonitrile-ammonium acetate mixed with glacial acetic acid, 1-octane sulfonic acid and triethlyamine. The flow rate was 1.5 ml/min and the compounds were detected by UV at 254 nm. The lower limits of quantification for linopirdine and linopirdine mono-N-oxide were 2.5 ng/ml in plasma and 10.0 ng/ml and 100 ng/ml, respectively, in urine. The precision and accuracy, expressed as the percent coefficient of variation and percent difference, respectively, were <20 percent for the assays. The methods were used to study the pharmacokinetics of linopirdine and linopirdine mono-N-oxide in human subjects.

INTRODUCTION

Linopirdine, 3-3-bis(4-pyridinylmethyl)-1-phenylindolin-2-one, a phenylindolinone derivative, is being clinically evaluated as a potential cognitive enhancer for the treatment of Alzheimer's disease (1,2). Linopirdine is extensively metabolized in animals and man and linopirdine mono-N-oxide has been identified as a major metabolite (3). The mono-N-oxide metabolite is biologically inactive in an anti-amnestic screen in rats (data on file). However, quantification of linopirdine mono-N-oxide was included in the event that the metabolite might produce other pharmacological effects or assist in eludicating linopirdine disposition. To quantify both linopirdine and linopirdine mono-N-oxide in human plasma or urine, separate selective and sensitive high-performance liquid chromatographic methods were developed and validated. These procedures have been used to assay plasma or urine samples in a number of clinical trials.

MATERIALS

Linopirdine, linopirdine mono-N-oxide and the internal standard, E-4953, (3,3-bis(2-pyridinylmethyl)-1-phenyl-indolin-2-one hydrochloride) (Figure 1) were all obtained from The DuPont Merck Pharmaceutical Company (Wilmington, DE). HPLC grade acetonitrile, ethyl acetate, methanol, and reagent grade sodium hydroxide and hydrochloric acid were obtained from J. T. Baker (Phillipsburg, NJ). HPLC grade ammonium acetate, triethylamine, glacial acetic acid, certified reagent grade pH 9 buffer (0.1 M) and ACS certified reagent grade tris(hydoxymethyl)amino-methane (tris) were obtained from Fisher

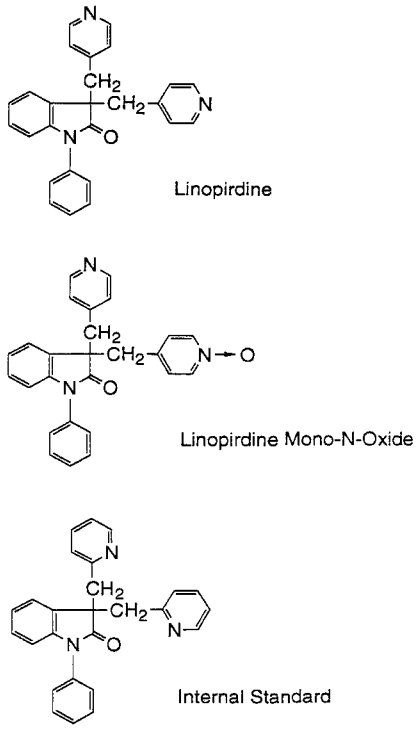

FIGURE 1. Chemical structures of linopirdine, linopirdine mono-N-oxide and internal standard.

Scientific (Fair Lawn, NJ). HPLC grade 1-octane sulfonic acid was obtained from Eastman Kodak (Rochester, NY) and certified reagent grade pH 7 phosphate buffer was obtained from VWR Scientific (Bridgeport, NJ). Drug-free control human plasma was obtained from Biological Specialty Corp. (Lansdale, PA) and control human urine was obtained from in-house donors.

METHODS

Instrumentation

The chromatography systems consisted of a Spectra-Physics SP8810 isocratic pump (Palo Alto, CA), a Beckman 160 fixed wavelength UV detector (Fullerton, CA) and a Waters 712 autosampler (Milford, MA). The mobile phase consisted of a mixture of the following: acetonitrile: 0.05 M ammonium acetate:glacial acetic acid:0.1 M 1-octane sulfonic acid:triethylamine, 24:74.6:0.8:0.5:0.1 and 30:68.6:0.8:0.5:0.1 by volume for plasma and urine, respectively. With a flow rate of 1.5 ml/min, the retention times of linopirdine, linopirdine mono-N-oxide and internal standard in plasma and urine were 14.8, 7.3, and 17.7 minutes and 9.5, 5.0, and 11.0 minutes, respectively. Data acquisition and integration was achieved using a PE Nelson Turbochrom data acquisition system (PE Nelson, Cupertino, CA, USA) for plasma assays and a Chromperfect data acquisition system (Justice Innovations, Mountain View, CA) for urine assays.

Standard Preparation

Calibration standards were prepared from working stock solutions of linopirdine/linopirdine mono-N-oxide in methanol. A 200-µl aliquot of each stock solution was added to a glass culture tube. The working stock solution was evaporated to dryness under nitrogen. One ml of control plasma or urine was added and the tube was then vortexed to mix. Linopirdine/linopirdine mono-N-oxide standards were prepared at concentrations of 2.5 to 1500 ng/ml in plasma, 10 to 505 ng/ml for linopirdine in urine, and 100 to 10000 ng/ml for linopirdine mono-N-oxide in urine.

Plasma Extraction Procedure

Plasma samples (1.0 ml) were spiked with internal standard (0.2 ml of 1.0 μg/ml) and pH 9 buffer (1.0 ml). Ethyl acetate (5.0 ml) was added to the mixture and mixed. Following centrifugation, the ethyl acetate fraction was transferred to a clean tube. Extraction of the aqueous phase was repeated (an additional 5.0 ml of ethyl acetate) and the organic phases were combined. One ml of 0.1 N hydrochloric acid was added to the organic phase. It was then mixed and centrifuged. After discarding the organic phase, 0.1 N sodium hydroxide (1.25 ml) was added to the remaining aqueous portion and this mixture was extracted with ethyl acetate (5.0 ml). The ethyl acetate fraction was transferred to a clean tube, evaporated to dryness under nitrogen, and the residue was reconstituted in mobile phase (0.2 ml).

Urine Extraction Procedure

Urine samples (1.0 ml) were processed as described above for plasma, however, pH 7 phosphate buffer was substituted for pH 9 buffer. In addition, a solid phase extraction was employed to further isolate the compounds of interest. The final organic residue was reconstituted in methanol (0.2 ml) and 0.1 M pH 10 tris buffer (1.0 ml). To a conditioned Baker CN (500 mg) solid phase extraction column was added the sample mixture containing methanol and tris buffer layered between two aliquots of tris buffer (1.0 ml each). The buffer and sample mixture was passed through the column using vacuum. Following three water rinses (1.0 ml each), the column was eluted with three aliquots of methanol (0.5 ml each) and the eluent was evaporated to dryness under nitrogen. The residue was then reconstituted in mobile phase (0.2 ml).

Validation of the Assays

For calibration, plasma or urine standards were spiked with known amounts of internal standard and with linopirdine and linopirdine mono-N-oxide at concentrations ranging between 2.5 to 1500 ng/ml and 10.0 to 10,000 ng/ml for plasma and urine, respectively. Calibration curves were obtained by power curve fit of the peak height ratio of linopirdine or linopirdine mono-N-oxide to the internal standard against the corresponding known concentration. The intra- and interday precision of the assays for linopirdine and linopirdine mono-N-oxide were estimated by measuring plasma/urine quality control samples (standards were used instead for linopirdine in urine) at day 1 (n=5 or 6 per concentration) and on two subsequent assay days (n=3 per concentration). The accuracy of the assays was evaluated by assaying quality control samples containing known amounts of each compound. The extraction efficiency was determined by comparing the peak heights of extracted standards to the corresponding unextracted standards. Selectivity and specificity was determined by injection of standard solutions of 42 various drugs which were selected based on concomitant medication information from linopirdine clinical trials. Any peak with a relative retention time ± 0.5 minutes of linopirdine, linopirdine mono-N-oxide or the internal standard was considered a potential interference. If a potential interference was observed, that drug was spiked into drug-free control human plasma, extracted, and chromatographed. The stability of linopirdine and linopirdine mono-N-oxide in human plasma and urine stored at -20°C was determined. Plasma samples were spiked at concentrations of 166 or 1660 ng/ml of linopirdine and 50 or 1000 ng/ml of or linopirdine mono-N-oxide. Urine samples were spiked

at concentrations of 40 or 400 ng/ml of linopirdine and 320 or 840 ng/ml of linopirdine mono-N-oxide. Samples were assayed immediately and at various times after storage.

Application to Clinical Trials

The assays were used in a clinical study to assess the safety and pharmacokinetics of linopirdine after a single 40 mg oral dose in healthy elderly volunteers. Urine and venous blood samples were collected at various time points. Aliquoted plasma and urine samples remained frozen (-20°C) until assayed.

RESULTS AND DISCUSSION

The methods described in this paper were developed to quantitatively assay linopirdine and a major metabolite, linopirdine mono-N-oxide in human plasma and urine. The procedures consisted of a series of extractions and, for urine only, an additional isolation step employing a solid phase extraction column. Separation of the analytes and internal standard was achieved using a cyano HPLC column followed by ultraviolet detection at 254 nm. Typical chromatograms of blank and 'spiked' human plasma and urine are shown in Figure 2. Also shown in Figure 2 are chromatograms of a plasma sample extract from an elderly patient taken 3 hours after receiving a 30-mg oral dose of linopirdine (c) and a urine sample extract from an elderly volunteer taken at a 0-6 hour collection interval after receiving a 40-mg oral dose of linopirdine (f). The lower limits of quantification (i.e., the lowest concentration that could be determined with precision and accuracy estimates of less than or equal to 20%) for linopirdine and linopirdine

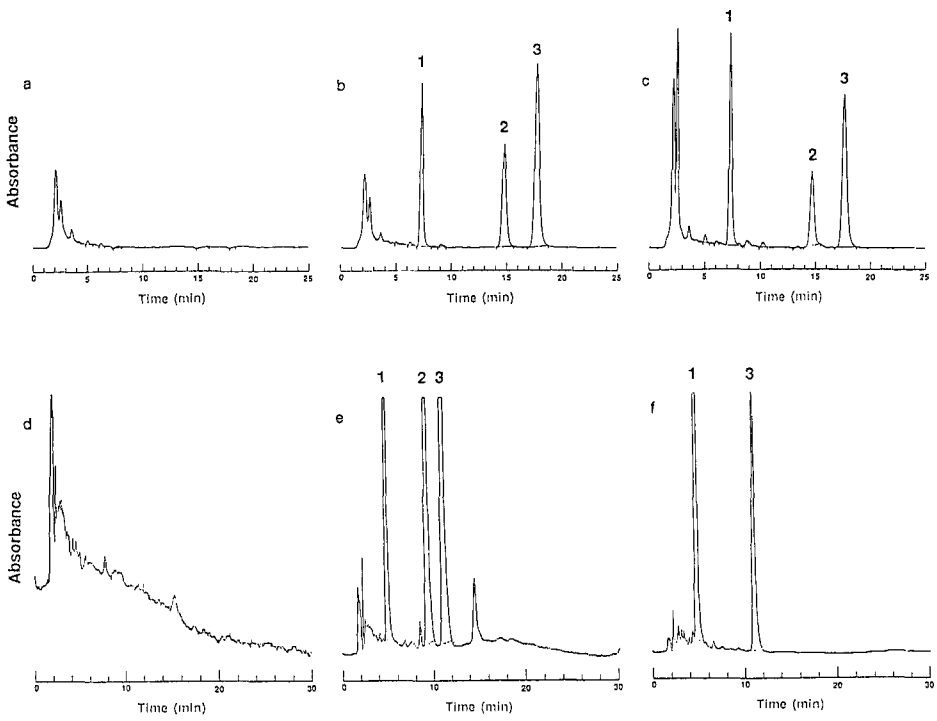

FIGURE 2. Chromatograms of (a) an extract of drug-free human plasma, (b) a spiked human plasma sample containing 100 ng/ml each of linopirdine and linopirdine mono-N-oxide, (c) a patient's plasma sample containing 85 ng/ml linopirdine and 160 ng/ml linopirdine mono-N-oxide, (d) an extract of drug-free human urine, (e) a spiked human urine sample containing 1500 ng/ml each of linopirdine and linopirdine mono-N-oxide, and (f) a subject's urine sample containing 4200 ng/ml linopirdine mono-N-oxide. Linopirdine mono-N-oxide, linopirdine, and the internal standard (E4953) are denoted 1, 2, and 3, respectively.

mono-N-oxide were 2.5 ng/ml in plasma and 10.0 ng/ml and 100 ng/ml, respectively, for urine. The intraday precision and accuracy of the assays for the determination of linopirdine and linopirdine mono-N-oxide in human plasma and urine are shown in Tables 1 and 2, respectively. The %CV ranged from 1.2% to 12.5% and 3.0% to 10.6% for linopirdine in

TABLE 1

Intraday Precision and Accuracy of Linopirdine and Linopirdine Mono-N-oxide in Human Plasma

Concentration Added (ng/ml)	Difference (%)		Mean Concentration Found ± SD (ng/ml)	Mean Difference (%)	CV (%)
Linopirdine					
2.5	-8.0	-12.0	2.4 ± 0.3	-4.0	12.5
	-16.0	16.0			
	4.0				
10.0	5.0	-1.0	10.2 ± 0.3	2.0	2.9
	6.0	1.0			
	1.0				
100.3	1.2	0.9	100.1 ± 1.2	-0.2	1.2
	-1.3	-1.3			
	-0.7				
1003.0	1.5	0.3	1021.2 ± 13.0	1.8	1.3
	1.3	2.2			
	3.8				
1504.5	3.6	-0.9	1542.2 ± 56.9	2.5	3.7
	-1.8	4.3			
	7.3				
Linopirdine Mono-N-oxide					
2.5	4.0	-4.0	2.7 ± 0.4	8.0	14.8
	0.0	32.0			
	8.0				
10.0	4.0	-3.0	10.1 ± 0.5	1.0	5.0
	8.0	-1.0			
	-1.0				
100.4	1.0	-3.3	95.4 ± 3.8	-5.0	4.0
	-8.1	-7.3			
	-7.2				
1004.5	-2.1	-3.9	985.7 ± 14.8	-1.9	1.5
	-2.0	0.3			
	-1.7				
1506.8	-1.1	-7.1	1463.2 ± 51.7	-2.9	3.5
	-5.6	-2.0			
	1.3				

TABLE 2

Intraday Precision and Accuracy of Linopirdine and Linopirdine Mono-N-oxide in Human Urine

Concentration Added (ng/ml)	Difference (%)		Mean Concentration Found ± SD (ng/ml)	Mean Difference (%)	CV (%)
Linopirdine					
10.1	-6.9	5.9	10.4 ± 1.1	3.0	10.6
	1.0	18.8			
	-10.9	9.9			
25.2	7.1	5.2	26.0 ± 1.6	3.1	6.2
	4.4	-9.5			
	7.9	3.6			
101	-4.7	-6.3	98.7 ± 3.0	-2.3	3.0
	0.0	-2.5			
	2.0	-2.2			
505	10.9	2.6	518 ± 23.2	2.6	4.5
	0.2	4.0			
	-2.2	0.0			
Linopirdine Mono-N-oxide					
100	-9.0	-6.0	91.5 ± 2.9	-8.5	3.2
	-8.0	-5.0			
	-13.0	-10.0			
300	-0.7	-0.3	304 ± 5.6	1.3	1.8
	2.7	0.0			
	3.0	3.3			
750	-2.3	1.7	747 ± 11.9	-0.4	1.6
	0.8	-0.9			
	0.4	-1.9			
3000	2.9	0.5	3104 ± 54.1	3.5	1.7
	4.1	3.9			
	3.2	6.0			
10000	-1.6	-0.7	9825 ± 498	-1.8	5.1
	-2.7	-5.2			
	7.1	-7.4			

plasma and urine, respectively. The corresponding values for linopirdine mono-N-oxide were 1.5% to 14.8% and 1.6% to 5.1%. The mean percent difference for linopirdine ranged from -4.0% to 2.5% and -2.3% to

3.1% in plasma and urine, respectively. The corresponding values for linopirdine mono-N-oxide were -5.0% to 8.0% and -8.5% to 3.5%. The interday precision of the assays is shown in Tables 3 and 4. The %CV ranged from 0.1% to 8.5% and 2.0% to 8.0% for linopirdine in plasma and urine, respectively. The corresponding values for linopirdine mono-N-oxide were 0.0% to 6.2% and 0.5% to 3.9% The overall

TABLE 3

Interday Precision of Linopirdine and Linopirdine Mono-N-oxide in Human Plasma

Concentration Added (ng/ml)	Mean Concentration Found[a] (ng/ml)	CV (%)
Linopirdine		
2.5	2.3	4.3
5.0	4.7	8.5
10.0	10.0	4.0
15.0	14.8	5.4
25.1	24.8	2.0
50.2	50.4	0.2
100.3	100.0	1.9
501.5	504.0	2.2
1003.0	1017.7	0.1
1504.5	1549.3	1.3
Linopirdine Mono-N-oxide		
2.5	2.6	0.0
5.0	4.9	2.0
10.0	9.7	6.2
15.1	14.5	2.8
25.1	24.0	2.5
50.2	49.6	4.6
100.4	97.9	4.9
502.2	489.5	3.2
1004.5	1016.2	3.1
1506.8	1521.0	1.7

[a] n=3.

TABLE 4

Interday Precision of Linopirdine and Linopirdine Mono-N-oxide in Human Urine

Concentration Added (ng/ml)	Mean Concentration Found[a] (ng/ml)	CV (%)
Linopirdine		
10.1	10.1	8.0
25.2	24.8	3.9
101	102	3.1
505	507	2.0
Linopirdine Mono-N-oxide		
100	95.1	3.9
200	205	1.0
400	390	1.0
1500	1489	0.7
5000	5012	0.8
7000	6973	1.3
10000	10101	0.5

[a] n=3.

extraction recovery averaged 60.7% and 74.3% for linopirdine and 43.3% and 50.9% for linopirdine mono-N-oxide in plasma and urine, respectively. Of the 42 drugs tested for potential interference, only bupropion and nifedipine yielded a relative retention time within ± 0.5 minutes of linopirdine, linopirdine mono-N-oxide, or internal standard. However, no chromatographic interferences were present following extraction of bupropion and nifedipine in control human plasma. A list of the drugs tested is shown in Table 5. The stability of linopirdine and linopirdine mono-N-oxide in frozen plasma and urine was studied. In plasma, no degradation of linopirdine or linopirdine mono-N-oxide was observed following storage at -20°C for at least 13

TABLE 5

Relative Retention Times for Potentially Interferring Drugs in Plasma Assay[a,b,c]

Drug	Retention Time Relative to Linopirdine Mono-N-oxide (min)	Retention Time Relative to Linopirdine (min)	Drug	Retention Time Relative to Linopirdine Mono-N-oxide (min)	Retention Time Relative to Linopirdine (min)
Acebutolol	-3.4	-11.1	Levothyroxin	17.2	9.3
Acetominophen	-4.3	-11.5	Lidocaine	-3.1	-10.9
Allopurinol	-5.3	-13.0	Lorazepam	2.8	-4.9
Alprazolam	5.6	-2.1	Metoprolol	-3.2	-11.0
Atenolol	-4.6	-11.9	Metronidazole	-4.3	-11.8
Brompheniramine	2.8	-4.9	Nadolol	-4.3	-11.8
Bupropion	-0.5	-8.2	Nifedipine	11.0	3.6
Chlorpheniramine	1.8	-5.7	Nortriptyline	17.7	9.8
Colchicine	-2.6	-10.0	Promethazine	12.5	4.6
Dextromethorphan	2.6	-5.3	Ranitidine	-2.7	-10.4
Diltiazem	8.7	0.8	Salbutamol	-4.6	-12.1
Dipyridamole	2.3	-5.1	Sulfacetamide	-4.2	-12.1
Doxylamine	-1.5	-9.0	Tetracycline	4.0	-3.3
Ephedrine	-4.4	-12.2	Theophylline	-4.7	-11.9
Famotidine	-4.7	-12.2	Timolol	-3.0	-10.3
Haloperidol	19.2	11.7	Triamterene	-3.1	-10.3
Hydralazine	-4.5	-11.7	Trimethoprim	-3.7	-11.2
Labetalol	2.0	-5.5	Verapamil	26.4	18.7

[a] Relative retention times were calculated as the difference between the retention time of test drug to the retention time of either linopirdine or linopirdine mono-N-oxide.

[b] Test solutions contained 50 µg/ml except lorazepam (1000 µg/ml), alprazolam (250 µg/ml), and bupropion (25 µg/ml).

[c] Meclizine, terfenadine, pilocarpine, thioridazine, phenylpropranolamine, and doxycycline were also tested and no chromatographic peak was observed within 60 minutes following injection.

months and 5 months, respectively. In urine, the corresponding stability values were 4 months and 18 months.

The assays were used to quantitate plasma and urine concentrations in volunteers after oral administration of linopirdine. Figure 3 shows a semi-logarithmic plot of mean plasma concentrations of linopirdine and linopirdine mono-N-oxide in elderly volunteers (aged 67-79 years) following a single oral dose of 40-mg linopirdine. In six subjects, the mean Cmax linopirdine plasma concentration was 775 ng/ml. The mean Cmax linopirdine mono-N-oxide plasma concentration was similar,

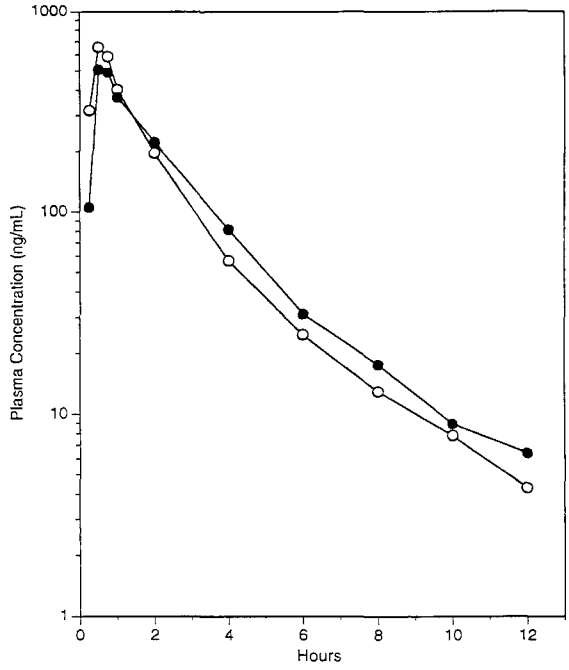

FIGURE 3. Mean plasma concentration versus time plot in healthy elderly subjects (n=6) following a single oral dose of 40-mg linopirdine. (O, linopirdine; ●, linopirdine mono-N-oxide.)

653 ng/ml. The urinary excretion of linopirdine was negligible as can be seen in Figure 2(f). The urinary excretion of linopirdine mono-N-oxide over 24 hours ranged from 2.2% to 8.6% of the linopirdine dose.

In summary, separate sensitive and selective high-performance liquid chromatographic methods for the determination of linopirdine and a major metabolite, linopirdine mono-N-oxide, in human plasma and urine were developed. They were successfully used to study the pharmacokinetics of linopirdine in humans.

ACKNOWLEDGMENTS

The authors would like to thank Hoa Nguyen, Cynthia Robinson, and Suzanne Maebert Young for their technical assistance and Barbara Campbell for her assistance in the preparation of this manuscript.

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2. B. Saletu, A. Darragh, H. P. Breuel, W. Herrmann, P. Salmon, R. Coen, P. Anderer, Human Psychopharmacology 6:267-275 (1991).

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JOURNAL OF LIQUID CHROMATOGRAPHY, 17(20), 4557-4558 (1994)

THE BOOK CORNER

THIN LAYER CHROMATOGRAPHIC Rf VALUES OF TOXICOLOGICALLY RELEVANT SUBSTANCES ON STANDARDIZED SYSTEM, 2nd revised and enlarged edition, VCH Publishers, Weinheim, 1992, 308 pages, DM 138.00; ISBN 3-527-27397-2

This book is prepared under the auspices of the Committee for Systematic Toxicological Analysis of the International Association of Forensic Toxicologists (TIAFT). It represents a sound, practical guide for substance identification by thin layer chromatography (TLC) which is considered to be a popular, state-of-the-art analytical technique for toxicologic analysis because of its speed, reliability and low cost.

The book outlines the principles for choosing proper TLC systems for screening purposes and the methods for using those systems. It also compiles a large collection of over 1600 substances including pharmaceutical drugs, illicit drugs and pesticides for the purpose of identification under standardized systems which have been proven to be reliable and reproducible.

The volume consists, mainly, of 16 tables listing those drug substances, in alphabetical order, versus Rf data and chemical abstract registry numbers (CAS No.), and

- Rf data for ten TLC systems in ascending numerical order versus substance names.
- CAS registry numbers in numerical order versus substance names.

This compilation is a valuable resource for forensic and toxicological laboratories, hospitals and academic institutions.

Reviewed by
Hassan Y. Aboul-Enein, PhD, FRSC
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THIN-LAYER CHROMATOGRAPHY: REAGENTS AND DETECTION METHODS. VOLUME 1B, H. Jork, W. Fisher, W. Funk and H. Wimmer, VCH Weinheim, Germany,1994; xvi + 496 pp., DM 198.00. £81.00: ISBN 3-527-28205-X

This volume is the second book of a practice-oriented series of two books published by VCH pubtishers and authored by well-recognized scientists in the field. It offers a valuable practical guide to all practitioners of TLC.

The book consists of two parts. Part I discusses the specific detection methods which include

(a) Activation reactions e.g. photochemical activation, thermochemical activation and electrochemical activation and

(b) Reagents applied for the recognition of functional groups.

Part II deals with description of the preparation of about 65 reagents, arranged in alphabetical order. Each reagent discussed gives a brief note on its application, detailed methodology for its preparation, including helpful hints and precautions, chemical reactions involved, if known, and finally, an example for the procedure tested for the reagent, supplemented by literature references.

The book is well illustrated, as it contains 116 figures, 31 of which are in color and also 19 tables. A copy of this book should be available in every analytical chemistry and quality control laboratory dealing with TLC. It is highly recommended as a valuable reference for analytical chemists, organic chemists, biochemists, among other professionals in both pharmaceutical and chemical industries and academic institutions.

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LIQUID CHROMATOGRAPHY CALENDAR

1994

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

1995

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

1996

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

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

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

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

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

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

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

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

1997

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

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

1998

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

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

1999

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

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

2000

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

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

2001

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

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

2002

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

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

The Journal of Liquid Chromatography will publish, **at no charge**, announcements of interest to liquid chromatographers in every issue of the Journal. To be listed in Meetings & Symposia, we will need to know: Name of the meeting or symposium, sponsoring organization, when and where it will be held, and whom to contact for additional details. Incomplete information will not be published. You are invited to send announcements to **Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P.O. Box 2180, Cherry Hill, NJ 08034-0162, USA.**

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In the first quarter of 1995 MDPC will be offering *Quality Auditing for the Industrial Laboratory* led by Donald C. Singer and Ronald P. Upton. This conference will be held on February 6, 1995 in San Francisco, CA; February 8, 1995 in Chicago, IL; and on February 10, 1995 in Saddle Brook, NJ. All those attending will receive Marcel Dekker, Inc. publications that reinforce and extend the subject being addressed.

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In the first quarter of 1995 MDPC will be offering *Biotechnology Law for the Non-Lawyer*, led by Gale R. Peterson and Robert A. Armitage. This conference will be held on March 13, 1995 in San Francisco, CA; March 15, 1995 in Research Triangle Park, NC; and on March 17, 1995 in Boston/Cambridge, MA. All those attending will receive Marcel Dekker, Inc. publications that reinforce and extend the subject being addressed.

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Effective immediately, manuscripts will be accepted on computer diskettes. A printed manuscript must accompany the diskette. For approximately one year, the diskettes will be used, on an experimental basis, to produce typeset-quality papers for publication in the Journal of Liquid Chromatography. Diskettes must be in an IBM-compatible format with MS-DOS Version 3.0 or greater. The following word processing formats can be accommodated:

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* The **preferred** word processor is **WordPerfect 5.1.**

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Journal of Liquid Chromatography
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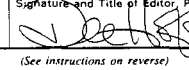

PS Form 3526, January 1991 *(See instructions on reverse)*

INSTRUCTIONS TO AUTHORS

Journal of Liquid Chromatography is published in the English language for the rapid communication of research in liquid chromatography and its related sciences and technologies.

Directions for Submission

One typewritten manuscript, suitable for direct reproduction, and two (2) clear copies with figures must be submitted. Since the Journal is produced by direct photography of the manuscripts, typing and format instructions must be strictly followed. Non-compliance will result in return of the manuscript to the author and will delay its publication. To avoid creasing, manuscripts should be placed between heavy cardboards before mailing.

Manuscripts may also be submitted on **computer diskettes**. A printed manuscript must also be submitted with diskettes because, at the present time, we are experimenting with manuscripts on diskettes. Diskettes must be readable with an IBM-compatible computer (Macintosh or other type not acceptable) and must be formatted with MS-DOS 3.1 or greater. Be sure to indicate the word processing software that was used to prepare the manuscript diskette.

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1. The general format of the manuscript should be:
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   Author(s)' names and full addresses
   Abstract
   Text Discussion
   References

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

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

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

4. **Text Discussion:** Whenever possible, the text discussion should be divided into major sections such as

INTRODUCTION
MATERIALS
METHODS
RESULTS
DISCUSSION
ACKNOWLEDGEMENTS
REFERENCES

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

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

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

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

Following are acceptable reference formats:

Journal:

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

Book:

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

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

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

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

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

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

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

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