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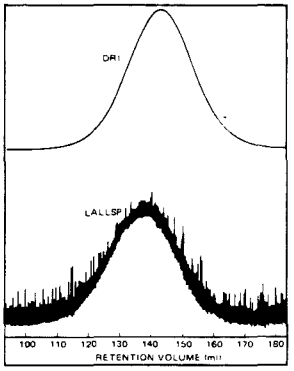

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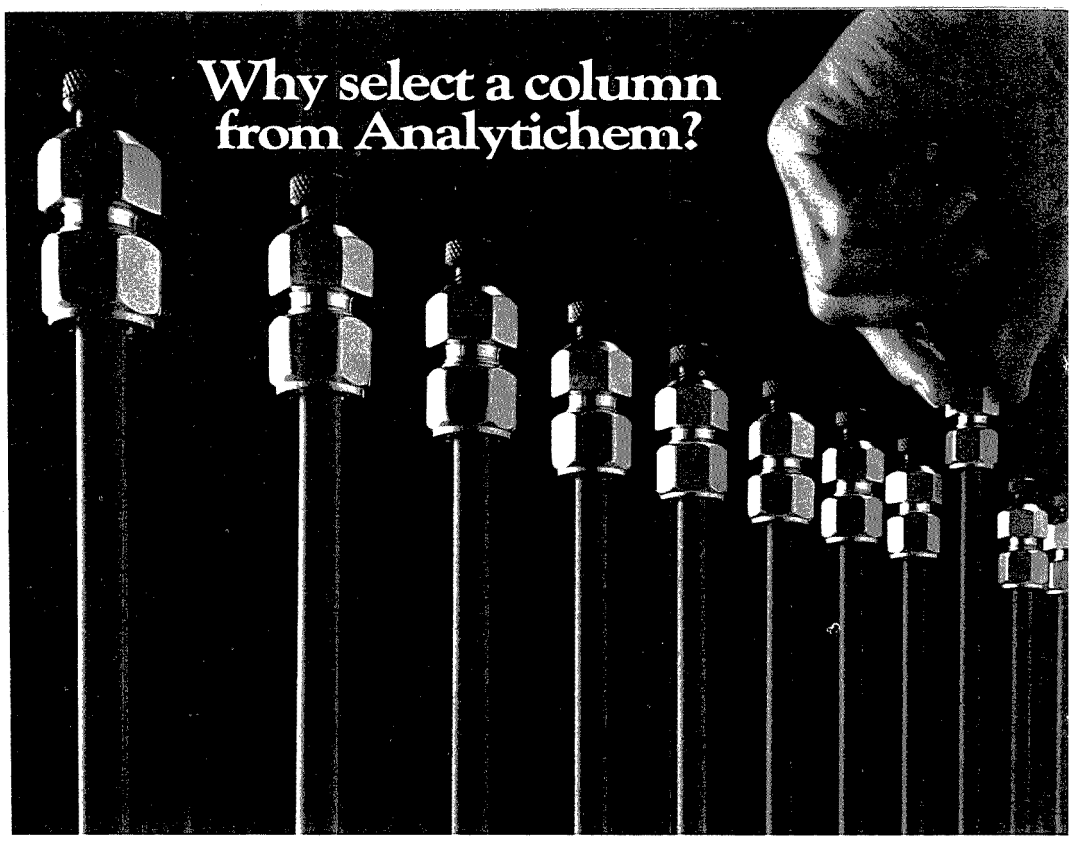

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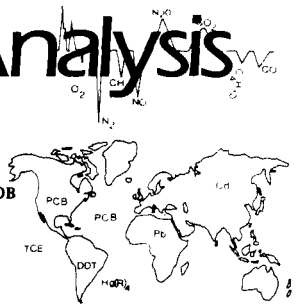

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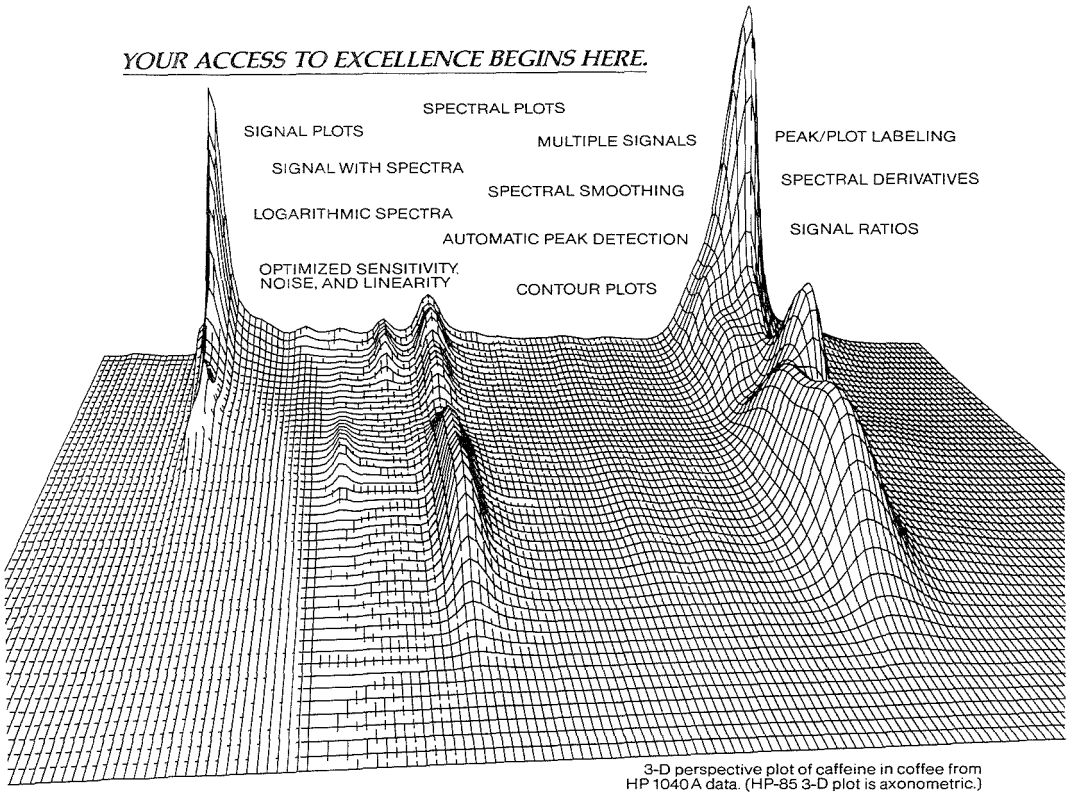

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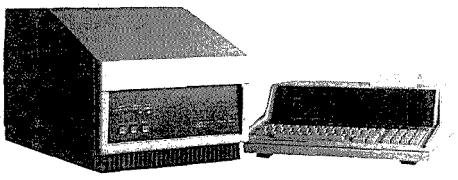

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(6), 1061-1078 (1984)

A MODEL ACCOUNTING FOR CONCENTRATION EFFECTS IN EXCLUSION CHROMATOGRAPHY

J.E. Figueruelo, A. Campos, V. Soria and R. Tejero

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ABSTRACT

A model has been developed that gives a quantitative description for the dependence of the elution volume, V_e, on the concentration of injected solute, c, in exclusion chromatography (SEC). The concentration-dependent shrinkage of coils has been evaluated from the intrinsic viscosity displayed by a polymer in a binary dilute solution formed by itself (at c concentration) and the eluent. In the derived equation, concentration effects are mainly governed by the Huggins coefficient, k_H, which includes hydrodynamic as well as thermodynamic interactions. Comparisons of predicted and experimental elution volumes for diverse literature polymer/eluent/gel systems show that the model quantitatively correctly describes the dependences of concentration effects on polymer molecular weight and on thermodynamic quality of eluent.

INTRODUCTION

In exclusion chromatography (SEC) the elution volume of a macromolecular solute varies with the concentration of injected solution as it has experimentally been found both for synthetic (1-3) and for biological macromolecules (4-6). The higher the solute molecular weight and the narrower its molecular weight distribution and the better the solvent the more pronounced is the variation (7-11), a linear correlation between concentration and the elution volume at the maximum of the chromatogram, V_e, is usually found. Coil shrinkage (12-14) and viscous fingering (15,16)

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occuring with increasing concentration are claimed to be the causing agents of V_e retardation, the joint contribution of both effects being conventionally known as "concentration effects" in SEC and not included in this concept are other secondary effects as column overloading or solute sorption onto the gel (17), which also retard V_e.

SEC is conventionally used as a tool for the evaluation of molecular weight averages of polymers, through a calibration equation, usually linear, which relates the logarithms of the hydrodynamic volumes of samples with their V_e's at infinite dilution.In practice, the universal calibration curve (18) is determined by injecting solutions of standard samples at finite concentrations and although a progressive dilution of injected solutions takes place along the column, obtained V_e values are at finite concentrations and they must be corrected at infinite dilution. Many empirical correction methods (2,11,19-21) have been suggested and different theoretical models have also been proposed intending to quantitatively predict concentration effects. In Jancas's model (16,22,23) the causing agents of concentration effects are assumed to be the viscosity gradient in the zone moving along the column and the coil shrinkage, whereas in the semiempirical Rudin's model (12,24) and in Bleha's one (25) only the last effect is considered.

The concentration-dependent shrinkage of coils is usually believed to be the result of thermodynamic interactions through complicated intra- and inter-molecular excluded-volume effects (26-29). In the model proposed in this paper, it is assumed that coil shrinkage, and therefore concentration effects, are due to the joint contribution of thermodynamic and hydrodynamic interactions. Both are introduced in the model through the Huggins coefficient, k_H, on which predicted elution volumes depend.

EXPERIMENTAL

Specific viscosities at six or seven concentrations were measured with a conventional Ubbelhode viscometer. From intercepts

and slopes of Huggins plots, (η_{sp}/c vs. c), Huggins coefficients, k_H were evaluated according to

$$\frac{\eta_{sp}}{c} = [\eta] + [\eta]^2 k_H c$$

The polymers used for k_H evaluations were polystyrene (PS) monodisperse samples (standard Waters Assoc.) and poly(methyl methacrylate), PMM, narrow distribution fractions (I < 1.2).

THEORY

A universal calibration is usually applied in SEC in order to evaluate molecular weight averages of polymers. The universal calibration concept (18) assumes a linear functionality between the log of the solute hydrodynamic volume at infinite dilution, $V_h(0)$, and solute elution volume

$$\log V_h(0) = \log M[\eta_A] = Q - PV_e(0) \qquad (1)$$

were M and $[\eta_A]$ respectively stand for molecular mass and intrinsic viscosity of a solute A and Q and P are calibration constants. $V_e(0)$ is the elution volume at infinite dilution and it cannot directly be evaluated from the chromatogram because this is run at finite concentration, c_A; of course $V_e(0) = V_{e_{c_A \to 0}}$. At a c_A concentration, coil shrinkage takes place, the hydrodynamic volume of solute A being $V_h(c_A)$ and strongly depending on c_A. Assuming that the dilution of the injected solution taking place along the column is negligible, an injected solution at c_A concentration will yield an elution volume, $V_e(c_A)$, related with $V_h(c_A)$ through eq. (1):

$$\log V_h(c_A) = Q - PV_e(c_A) \qquad (2)$$

The polymer A in a bynary mixture formed by solvent and by itself at a concentration c_A will display an intrinsic viscosity $[\eta_A]_{c_A}$ different to that in the pure solvent. Assuming that $V_h(c_A) = M[\eta_A]_{c_A}$, eq. (2) may be written as

$$\log M[\eta_A]_{c_A} = Q - PV_e(c_A) \qquad (3)$$

Subtraction of eqs. (1) and (3) yields:

$$- P(V_e(c_A) - V_e(0)) = \log ([\eta_A]_{c_A} / [\eta_A]) \qquad (4)$$

On the other hand, the intrinsic viscosity ($[\eta_A]_{c_B}$) of a polymer A in a binary dilute solution formed by a polymer B (at concentration c_B) in a solvent is given by (30-32)

$$[\eta_A]_{c_B} = \frac{[\eta_A] + c_B b_{AB}}{1 + c_B[\eta_B] + c_B^2 b_B} \qquad (5)$$

where $[\eta_A]$ and $[\eta_B]$ are the respective intrinsic viscosities of both polymers in the pure solvent and the coefficients b_A, b_B, b_{AB} characterize the interactions of like (AA, BB) and unlike (AB) molecules, respectively.

In the present study, we are not interested on the viscometric behaviour of a polymer in a solution of a different polymer but on the viscosity of polymer A in a solution of itself at c_A concentration. In this case, eq. (5) is transformed on:

$$[\eta_A]_{c_A} = \frac{[\eta_A] + 2 c_A b_A}{1 + c_A[\eta_A] + c_A^2 b_A} \qquad (6)$$

Eq. (6) holds for dilute solutions, that is, for low c_A values. Neglecting higher terms than c_A^2 in the expansion McLaurin's serie of the above eq., dividing both members by $[\eta_A]$, rearranging terms in c_A powers, and applying log, this is obtained:

$$\log \frac{[\eta_A]_{c_A}}{[\eta_A]} = \log \left\{ 1 + \frac{2b_A - [\eta_A]^2}{[\eta_A]} c_A + \frac{[\eta_A]^3 - 3b_A[\eta_A]}{[\eta_A]} c_A^2 \right\} \qquad (7)$$

Changing log in the second member to ln and recalling that $\lim_{x \to 0} \ln (1 + x) \simeq x$ and that the interaction coefficient b_A is related to the Huggins coefficient (k_H) through $b_A = k_H[\eta_A]^2$ (31,32), eq. (7) is transformed on:

$$\log \frac{[\eta_A]_{c_A}}{[\eta_A]} = 0.4343 \ln \left\{ 1 + (2k_H - 1)[\eta_A] c_A + (1 - 3k_H)[\eta_A]^2 c_A^2 \right\} =$$

$$= 0.4343 \left\{ (2k_H-1)[\eta_A]c_A + (1-3k_H)[\eta_A]^2 c_A^2 \right\} \quad (8)$$

Finally, substitution of eq. (8) in eq. (4) yields, removing subscripts

$$V_e(c) = V_e(0) + \frac{0.4343}{P} \left\{ (1-2k_H)[\eta]c + (3k_H-1)[\eta]^2 c^2 \right\} \quad (9)$$

Eq. (9) shows that concentration effects in SEC for a solute depends on its intrinsic viscosity, $[\eta]$, its Huggins coefficient, k_H, and its concentration, c. Eq. (9) indirectly depends on the slope of the linear part of the universal calibration, P, defining the selectivity of the separation in a column.

RESULTS AND DISCUSSION

In good solvents and at the low injected concentrations usually used in SEC (< 5 mg/ml), the experimental evidence so far accumulated is that a linear dependence of V_e on c usually holds, steeper the slope, the higher the molecular weight of polymeric solute (7,8,10). In eq. (9) two terms account for concentration effects, one linear and the other quadratic in c. In good solvents, $k_H = 0.25 - 0.4$, therefore $|(1-2k_H)| > |(3k_H-1)|$. Moreover, in the usual measurement conditions, $[\eta]c < 1$, therefore, $[\eta]c > [\eta]^2c^2$. Both combined inequalities give the following result $(1-2k_H)[\eta]c >> (3k_H-1)[\eta]^2c^2$, the quadratic term being negligible and a practically linear variation of V_e with c being predicted by the present model. On the other hand, when M increases, k_H decreases and both the coefficient $(1-2k_H)$ and $[\eta]$ increase; as a result, a steeper dependence on c of V_e is also predicted. In same polymer-solvent systems, at concentrations higher than $c \simeq 0.5$ mg/ml, the inicial straight lines V_e vs. c become curved either upwards or downwards depending on the polymer eluent system and both curvatures becoming steeper with increasing molecular weight. Eq. (9) also accounts for both experimental behaviours. So, when $k_H > 1/3$ rising curvatures must be expected, whereas downward ones are predicted in better solvents, $k_H < 1/3$. Of course, when $k_H=1/3$, linearity holds even at moderate concentrations. Qualitatively,

then, the present model is able to account for the different concentration effects experimentally observed in good eluents.

Difficulties arise, however, when intending to compare quantitative predictions of this model with literature experimental results. On one hand, because of the scarcity of literature references, in which both elution volumes and their corresponding viscosity coefficients for the polymeric solutes are given. On the other hand, when looking for elution volumes and k_H data separately, not very accurate values may be found because:

i) Although plentiful concentration effects data may be found in the literature, most of these are shown in a graphic form, and in a single figure data for several polymer samples with very different molecular weights are usually represented. The differences in elution volumes between different polymer samples are usually several magnitude orders higher than the differences caused by concentration effects, therefore, the uncertainty in the V_e numerical values extracted from literature figures may be about the same magnitude order than the expected concentration effects.

ii) Besides that, the chances of finding appropriate k_H values for the above eluent-polymer sample-temperature systems are remote. What makes such undertaking particularly difficult is that k_H depends not only on polymer-solvent system, but also on molecular weight distribution of polymer, branching degree of the chain and velocity gradient (33). In the present context, the last two effects may be neglected. No agreement exists on the dependence of k_H on molecular weight; it seems that with low molecular weight k_H decreases as M increases to a certain M limit, beyond which k_H is practically insensitive to M (33,34). Fortunately, it seems that molecular weight distribution does not significantly influence k_H (33).

iii) Added to the above problems is the rather considerable limit of error of experimental k_H values, which can amount to 10-20 % (33).

Taking into account the above considerations, literature references on concentration effects may be grouped in three categories:

1. Those in which both numerical values for V_e (usually tables) and k_H values for the polymeric solutes are given.

2. Literature reports in which V_e's are also given in tabulated form,but k_H values for the measured polymers are not reported. In these cases, k_H values must be searched for in other literature sources. Three possibilities, in a decreasing order of preference, have been followed in this paper:

a) When existing k_H - M data, the corresponding interpolated values have been used.

b) If possibility a) does not exist, used k_H values are those found in the literature for the closest temperature and/or molecular weight polymer.

c) If even this possibility fails, k_H experimental evaluations were undertaken. The polymers used for those evaluations were monodisperse samples or narrow distribution fractions with the closest possible molecular weight to the chromatographic samples.

3. Most of the literature references on concentration effects fall into this group. V_e's are given here in figures. When existing k_H values in the same reference, those have been used; when not, the considerations above made have also been followed.

As far as we know, only one system, namely polystyrene, PS, (M_w = 867000)/tetrahydrofuran, THF, falls into group 1. It was measured by Janca to test the realibility of its own concentration effects model (16). In table 1, concentration effects predicted by Janca (ΔV_J) and by the present model (ΔV_F) are shown at different concentrations of injected solution. Concentration effects (ΔV_e) are defined as the difference between the elution volumes at

TABLE 1

Concentration Effects in the System PS3(M_w = 867000)/THF. Comparison between Experimental (16), ΔV_e, and predicted Values from Janca's Model (16), ΔV_J, and from the present Model, ΔV_F.

c g/100 ml	V_e counts	ΔV_e counts	ΔV_J counts	ΔV_F^* counts
0.4	22.8	1.3	0.2	0.9
0.2	22.3	0.8	0.02	0.4
0.1	21.8	0.3	0.00	0.2
0.05	21.7	0.2	-0.05	0.1
0.025	21.6	0.1	-0.07	0.04
0	21.50			

* Data needed to calculate ΔV_F are from ref. (16) and are in Table 3.

concentration c, $V_e(c)$, and at infinite dilution, $V_e(0)$, in eq. (9).

In Table 2 elution volumes at several concentrations as predicted by the present model are given for systems falling into group 2. The first two systems were, respectively, used by Janca (16) and by Rudin (12) as test systems of their own models. The three different behaviours theoretically predicted by our model in good solvents, that is, a practically linear, a curved upwards and a curved downwards dependence of V_e are illustrated in Figure 1, for several systems falling into group 3. Data for eq.(9) application in good solvent systems are enclosed in Table 3. Expected elution volumes from Bleha's and Rudin's models are also depicted in Figures 1b) and 1c), respectively. No significative differences, from a quantitative point of view, are found in good solvents between our predicted elution volumes and those derived from other models, as Figures 1b) and 1c) show. Of course, if the quadratic term in eq. (9) is neglected, as it does not play an important role in the systems so far represented in Figures 1b)

TABLE 2

Concentration Effects in Different Polymer/Eluent/Gel Systems. Comparison between Experimental, V_e, and Calculated with eq. (9), $V_e(c)$, Elution Volumes.

a) PS/THF/rigid porous glass at 25°C (16)

Sample	c,%	V_e, counts	$V_e(c)$,* counts	Sample	c,%	V_e, counts	$V_e(c)$,* counts
	0.8	25.8	25.3(24.5)		0.4	29.2	29.1(28.9)
	0.4	25.2	24.7(24.4)		0.2	28.9	29.0(28.8)
PS 4	0.2	24.7	24.4(24.3)	PS 6	0.1	28.9	28.9(28.8)
498000	0.1	24.4	24.2(24.2)	200000	0.05	28.9	28.9(28.8)
	0.05	24.2	24.2(24.2)		0.025	28.9	28.9(28.8)
	0.025	24.1	24.1(24.2)		0	28.95	
	0	24.1					

b) PMMA/THF/styragel at 25°C (35)

Sample	$c.10^3$ g/ml	V_e, ml	$V_e(c)$, ml	Sample	$c.10^3$ g/ml	V_e, ml	$V_e(c)$, ml
	0	102.8			0	114.9	
	1.13	103.5	103.5		1.64	115.5	115.3
Fract. A1	3.13	104.5	104.6	Fract. B2	3.28	116.0	115.7
$M_w=246.10^4$	6.46	106.0	106.5	$M_w=596.10^3$	6.58	117.0	116.5
	9.74	108.0	108.2		9.55	118.0	117.3
	12.7	111.0	109.8		13.15	119.5	118.4
	0	117.1			0	123.0	
	1.59	117.5	117.4		1.61	123.0	123.2
Fract. B1	3.15	118.0	117.7	Fract. C2	3.21	123.5	123.4
$M_w=440.10^3$	6.66	119.0	118.4	$M_w=240.10^3$	6.35	124.0	123.8
	9.77	120.0	119.1		9.66	124.5	124.2
	13.2	121.0	119.9		13.15	125.0	124.7

c) PS/THF/μstyragel at 25°C (36)

Sample	$c.10^3$ g/ml	V_e, counts	$V_e(c)$, counts	Sample	$c.10^3$ g/ml	V_e, counts	$V_e(c)$, counts
	0	26.18			0	27.04	
PS	1.4	26.27	26.29	PS	1.1	27.10	27.11
620.10^3	2.2	26.34	26.36	412.10^3	1.3	27.12	27.13
	3.4	26.43	26.48		2.1	27.15	27.17
					2.9	27.20	27.23

* Values in parenthesis are $V_e(c)$ predicted by Janca (16).

TABLE 3

Parameters used for the Evaluation of Concentration Effects in Good Solvents

System	Sample	P	$[\eta]$ ml.g^{-1}	k_H
PS/THF (16), Table 1	PS 3	0.145 counts^{-1}	212	0.365
PS/THF (16), Table 2a	PS 4	0.145 counts^{-1}	142	0.366 a
	PS 6		74	0.367 b
PMMA/THF (35), Table 2b	Fraction A1	0.079 ml^{-1}	329	0.33
	Fraction B2		124	0.35
	Fraction B1		100 (12)	0.36 c
	Fraction C2		66	0.36
PS/THF (36), Table 2c	PS 620000	0.30 counts^{-1}	185	0.366
	PS 412000		150	0.367 a
PS/THF (37), Figure 1a	1.8×10^6 PS	0.068 ml^{-1}	422	0.325 c
	860000 PS	0.068 ml^{-1}	240	0.365 (16)
	200000 PS	0.075 ml^{-1}	78	0.367 b
PS/Toluene (25), Figure 1b	$M=4.98\times10^5$	0.068 ml^{-1}	141	0.33 (33)
	$M=3.20\times10^5$		88	0.35 (33)
PS/Butanone (38), Figure 1c	$M=4.98\times10^5$	0.256 counts^{-1}	81	0.55
	$M=3.35\times10^6$		271	0.48 c

a. Interpolated values from k_H vs. M data in ref. (16).
b. Extrapolated values from k_H vs. M data in ref. (16).
c. Measured in this lab.

and 1c), the new eq. describing concentration effects (eq. (10)) obeys a similar functionality to those derived from Bleha's model (eq. (11)) and Rudin's model (eq. (12)) :

$$V_e(c) = V_e(0) + \frac{0.4343}{P}(1-2k_H)[\eta]c \qquad (10)$$

$$V_e(c) = V_e(0) + \frac{1.303}{P} A_2 M F c \qquad (11)$$

$$V_e(c) = V_e(0) + \frac{0.352}{P} (1 - [\eta]_\theta/[\eta])[\eta]c \qquad (12)$$

Eq. (12) proceeds from a rearrangement of the original Rudin's eq. (see eq. (24) in ref. (24)).

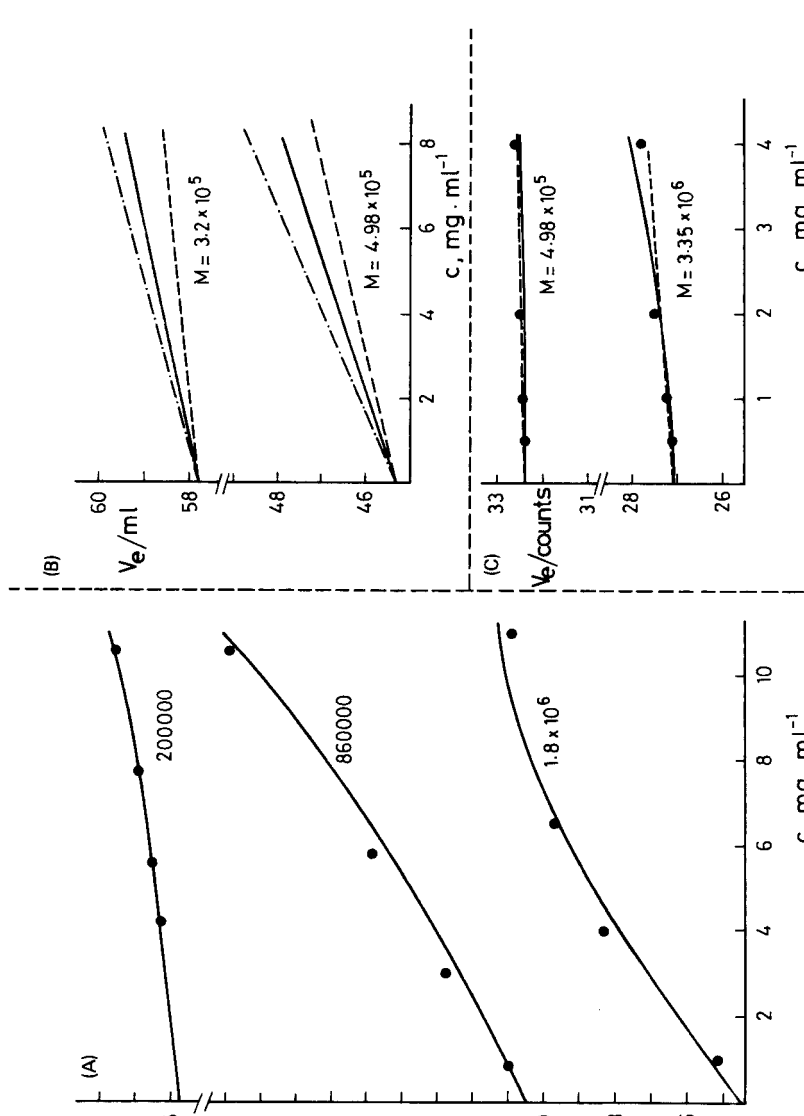

FIGURE 1. Calculated and experimental concentration effects for the systems: a) PS/THF; (●) experimental values (37); (———) predicted with eq.(9). b) PS/toluene; (—.—.—)experimental values (25); (———) predicted with eq.(9); (– – – –) predicted with Bleha's model (25). c) PS/butanone; (●) experimental values (38); (———) predicted with eq.(9); (– – – –) predicted with Rudin's model.

The small differences, among the three models, then, must be looked for in the coefficient of the variable c/P and those differences may be caused mainly by the different experimental magnitudes on which the coefficients depend on. Bleha's model(25) is based upon Yamakawa's theory (29), whereas the late Rudin's model (24), even though it was firstly derived in a semiempirical way (12), also agrees with Yamakawa theory relating concentration and effective hydrodynamic volume of solvated polymers in moderately concentrated solutions. Therefore, both models are two different ways of presenting Yamakawa theory, in Bleha's model elution volumes are given in terms of equilibrium properties ($A_2 \equiv$ second virial coefficient) wherein Rudin's model they are a function of transport properties ($[\eta] \equiv$ intrinsic viscosity). However and in spite of the almost quantitative agreement found between our model and those based on Yamakawa theory, a qualitative disagreement, intrinsic in nature, exists.So, wherein the models based in Yamakawa theory the concentration-dependent shrinkage of coils is a result of thermodynamic interactions including inter- and intramolecular excluded-volume effects, our model depends on k_H, which certainly includes thermodynamic interactions, but also includes hydrodynamic and other types of interactions, being the most important both thermodynamic and hydrodynamic interactions (31-33). This conceptual difference is not largely reflected on the predicted elution volumes in good solvents, as previously shown, but it is however at θ conditions. In fact, at θ conditions thermodynamic interactions vanish, the ratio $[\eta]/[\eta]_\theta$ and the expansion factor being equal to unity and the function F being zero. As a result, see eqs. (11) and (12), a universal behaviour is predicted by both Bleha's and Rudin's model: elution volumes do not depend on concentration at θ conditions, which is not the behaviour predicted by our model. Elution volumes in our model depend on k_H and since not a single, therefore universal, k_H value is found at θ conditions, either theoretical or experimental, not an universal concentration dependence of V_e must be expected. Among other theoretical

predictions, Yamakawa arrives at $(k_H)_\theta = 0.50$, taking only into account the effect of the concentration dependence of the molecular dimensions (39). Peterson and Fixman predict that $(k_H)_\theta = 0.833$ facing the hydrodynamic problem (40) and a value $(k_H)_\theta = 0.52$ is claimed by Sakai (41). On the other hand, experiments show that $(k_H)_\theta$ lies between 0.5 and 0.7 (31,42,43) and it is molecular weight dependent. In this context, it is obvious that not a universal behaviour of elution volumes as concentration independent must be expected in θ eluents.

Before presenting quantitative results in θ eluents, a qualitative analysis of the results predicted by our model may be worthwhile . At an hypothetical $(k_H)_\theta = 0.50$, in accordance with the Yamakawa's theoretical $(k_H)_\theta$ value, the linear term in eq.(9) vanishes as it also happens with thermodynamic interactions according to Yamakawa (39), but the quadratic term still remains. In this context, the quadratic term looks like accounting for hydrodynamic and other kinds of interactions, which are responsible of the residual concentration dependence of elution volumes at those θ conditions. Of course, $(3k_H-1)$ coefficient is larger here than in good solvents, whereas $[\eta]_\theta$ is smaller than $[\eta]$. The increase in the former is cancelled by the decrease in the latter and as a result the value of the quadratic term is small and about in the same order as in good solvents. A small positive V_e vs. c trend must, then, be expected in $(k_H)_\theta = 0.50$ systems. For those systems, with $(k_H)_\theta$ values close to the limiting 0.50, the linear coefficient $(1-2k_H)$ becomes negative and, of course, much smaller than the quadratic coefficient, $|(1-2k_H)| << |(3k_H-1)|$. However, here again, as in good solvents, $[\eta]_\theta c > [\eta]_\theta^2 c^2$, and the linear term provokes a decrease in the small predicted dependence of V_e with c followed at $(k_H)_\theta$ 0.50. As long as $(k_H)_\theta$ departs farther from 0.50, the differences between linear and quadratic terms become smaller, the elution volumes depending less on c and that dependence vanishing at a limit $(k_H)_\theta$ value and even becoming negative at low concentrations and

for $(k_H)_\theta$ values above the limit. On the other hand, below a given molecular weight, $(k_H)_\theta$ strongly increases with decreasing molecular weight (42,34), high $(k_H)_\theta$ values being reached for low molecular weight polymers and a negative dependence of elution volumes with c must, then, be expected. However, in these low molecular weight polymer systems, $[\eta]_\theta$ is small and the expected negative dependence, if any, will be negligible. In any case, and as a summary, at θ conditions and for high molecular weight polymers a small positive, if any, V_e dependence on c must be expected, which will gradually diminish as long as the polymer molecular weight decreases and for very low molecular weight polymers even can become negative.

The difficulties appearing when intending to compare our quantitative predictions for θ systems with literature experimental results are enhanced with respect to those found and discussed before for good solvent systems. Here, tabulated V_e vs. c were not found and we must retort upon data of group 3. Besides that, and because of the small differences in elution volumes usually found, close to the accuracy in the V_e experimental measurements, horizontal straight lines in the plots V_e vs. c are usually depicted, masking the experimental points and therefore spreading the generalized opinion of the V_e independence with c at θ conditions. The uncertainty in numerical values extracted from literature figures here may be higher than expected concentration effects.

The three different behaviours theoretically predicted by our model in θ conditions, that is, a positive, a null and a negative concentration dependence of V_e, as long as $(k_H)_\theta$ increases, are illustrated in Figure 2, where predicted and experimental V_e vs. c plots for polystyrene (PS) in different θ mixtures are compared. For $(k_H)_\theta$ close to 0.50, as it occurs in the benzene/methanol (78/22, v/v) θ mixture, a small positive concentration dependence of V_e is experimentally observed (7), as explicitly confirmed by authors, and in accordance with

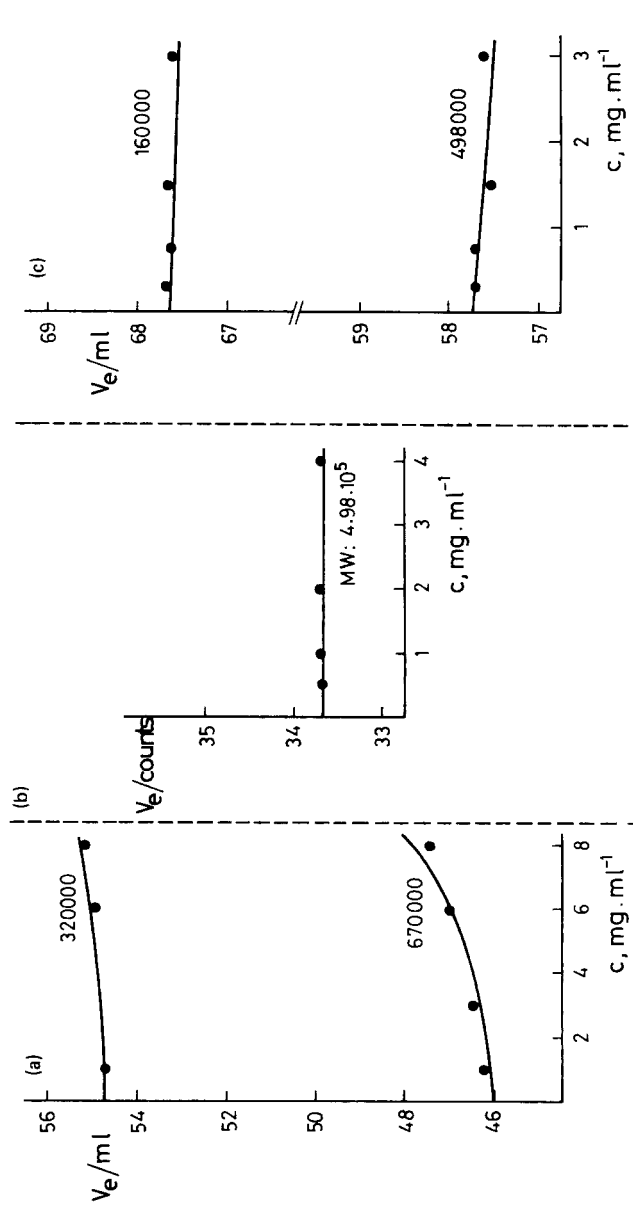

FIGURE 2. Calculated and experimental concentration effects for PS in θ eluents; (●) experimental values; (——) predicted with eq.(9). a) benzene-methanol(78/22) (7). b) butanone-methanol (88.7/11.3) (19). c) butanone-n,heptane(50/50) (44).

TABLE 4

Parameters used for the Evaluation of Concentration Effects in θ Eluents.

System	Sample	P	$[\eta]$ $ml.g^{-1}$	k_H
PS/benzene-methanol (78/22),v/v) (7), Figure 2a.	670000 320000	0.069 ml^{-1}	93 60	0.50 a 0.51 a
PS/butanone-methanol (88.7/11.3,v/v) (19) Figure 2b.	4.98×10^5	0.261 $counts^{-1}$	51 a	0.63 a
PS/butanone-n,heptane (50/50,v/v) (43), Figure 2c.	498000 160000	$0.074ml^{-1}$	59.5 a 33.7 a	0.73 a 0.70 a

a. Measured in this lab.

theoretical predictions. For intermediate $(k_H)_\theta$ values a null dependence must be expected, as it is experimentally confirmed in the θ mixture butanone/methanol (88.7/11.3, v/v) (19) and for high $(k_H)_\theta$ values a very small negative dependence must be obeyed, as also happens in the mixture butanone/n-heptane (50/50, v/v) (43). Data for eq. (9) application in θ eluents are enclosed in Table 4.

Finally and as a conclusion, the applicability of the present model deserves some comments. An appropriate calibration in SEC demands $[\eta]$ values, but the evaluation of $[\eta]$'s yields at once the corresponding k_H values. No additional data, then, is needed to calculate concentration effects, since these only depend on $[\eta]$ and k_H.

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SOLVENT COMPOSITION - CAPACITY FACTOR DEPENDENCIES OF IODOAMINO ACIDS.*

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ABSTRACT

The retention behaviour of several iodoamino acids on μBondapak C_{18} columns has been investigated as the volume fraction, ψ_s, of the organic solvent modifier was varied over the range $0.08 < \psi_s < 0.8$ under low pH, low ionic strength conditions. Bimodal plots of the logarithmic capacity factor, log k', versus ψ_s were observed with selectivity reversals from a reversed phase to a polar phase elution mode occurring at ψ_s *ca* 0.6 for acetonitrile-based eluents. The calculated slope parameters (s-values) of the iodoamino acids were similar to those found with other low molecular weight solutes but were considerably smaller than s-values observed with polypeptides or proteins of comparable capacity factor characteristics.

INTRODUCTION

Over the past several years, the relationship between mobile phase composition and retention behaviour for peptides and proteins separated on alkylsilicas has attracted increasing

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* High Performance Liquid Chromatography of Amino Acids, Peptides and Proteins Part LII. For Part LI see ref. 12.

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attention. In particular, studies[1-4] on the influence of the organic solvent modifier on the chromatographic properties of these ionogenic substances have been undertaken not only to validate predictions based on solvophobic theory[5] but also to permit practical interconversions between isocratic and gradient elution data (and vice versa). These investigations, which have allowed improved criteria to be established for resolution optimisation, have also demonstrated that in general peptides and proteins do not show linear dependencies of their logarithmic capacity factors, log k', on the volume fraction, ψ_s, of the organic solvent modifier. Instead, curvilinear and even bimodal concave plots are commonly observed. It is also clear that the slope of the tangent (s-value) of the plots obtained from these measurements depends on the molecular characteristics of the solute as well as the choice of chromatographic conditions. With well defined elution conditions and a specified hydrocarbonaceous stationary phase, the slopes of the plots of log k' versus ψ_s for a series of polypeptides[3,4], as well as neutral solutes such as polystyrenes[6], tend to increase with molecular size. However, molecular size per se is known[1,2] not to be the dominant parameter in controlling the retention of polypeptides and related ionogenic solutes to alkylsilicas but rather the interfacial hydrophobic contact area established between the solute and the hydrocarbonaceous ligand.

Many low molecular weight amino acid derivatives, such as the non-polar dansyl amino acids and phenylthiohydantoin amino acid derivatives, exhibit comparable retention with alkylsilicas in terms of their capacity factors (and thus comparable hydrophobic contact areas) to that shown by larger peptides. However, the retention behaviour of amino acid derivatives is generally more predictable and the overall chromatographic performance in terms of resolution and peak shape is usually superior to that observed with larger molecules. Mobile phase composition is known to play

a important role in the expression of these differences which are believed to arise from more complex secondary equilibrium and kinetic processes. The present study examines one aspect of these differences, namely the solvent composition-capacity factor dependencies using as model compounds several hydrophobic iodoamino acid derivatives. The study reveals that in common with other low molecular weight neutral or acidic organic compounds, the s-values of iodoamino acids are significantly smaller than those observed for polypeptides. Further, elution order reversals typified by polar phase selectivity are observed at high organic solvent content.

MATERIALS AND METHODS

Reagents: Acetonitrile was purchased from Waters Assoc. (Aus.) Pty. Ltd. Sodium sulphate, orthophosphoric acid, sulphuric acid and triethylamine were all AnalaR grade reagents from BDH (Poole, Great Britain) or May & Baker (Dagenham, Great Britain). Water was quartz distilled and deionised using a Milli-Q system (Millipore, Bedford, MA.). The iodoamino acid derivatives were obtained from Henning (Berlin, G.F.R.). Stock solutions of the iodoaminoacid derivatives were prepared by dissolving the compounds in 1% methanolic NH_4OH at a concentration of ca 10 mg/ml.

Apparatus: All chromatographic data were collected using a Waters Assoc. (Milford, M.A.) HPLC system which consisted of a M6000A solvent delivery unit, a U6K universal liquid chromatographic injector, and a M440 UV absorbance detector coupled to a M720 data module. Sample injections were made with SGE model 50A syringes (SGE, Melbourne, Aus.). The pH measurements were performed with a Radiometer PH M-64 meter equipped with a combination glass electrode. The μBondapak C_{18} columns were purchased from Waters Assoc. (Aus.).

Chromatographic Procedures

A flow rate of 1.0 ml/min was used for this investigation. The solvent reservoirs, precolumn delivery systems and columns were maintained at 20°. Bulk solvents and appropriate isocratic mobile phases were prepared and degassed by sonication as reported previously[3]. The μBondapak C_{18} columns were equilibrated to new mobile phase conditions for at least 30 mins. Sample sizes varied between 1 μg and 5 μg of iodoamino acid and were within the linear range of the adsorption isotherms. The capacity factors for isocratic retention experiments were calculated by established methods with all data points representing an average of triplicate measurements. The precision of the measurements was generally 2%. The s-values for the various solutes were calculated from the curve tangent or by linear regression analysis of the isocratic retention data using a Hewlett-Packard-97 calculator. The ionic strength and pH of the eluents were selected on the basis of previous experience[3,4] with peptides and other ionogenic solutes to minimised changes in retention as a consequence of changes in ionisation state and/or extent of solvation.

RESULTS AND DISCUSSION

Under chromatographic conditions where the extent of ionisation, solvation and buffer ion interactions for a group of polar, ionogenic solutes remains essentially constant, the dependency between the logarithmic capacity factor, log k', and the volume fraction, ψ_s, of the organic solvent modifier for solutes separated by reversal phase-HPLC may by approximated[3,4] by a linear relationship of the form

$$\log k'_i = \log k'_{i,w} - s_i\psi \qquad (1)$$

where $k'_{i,w}$ is the capacity factor of the solute in a neat water eluent, i.e. at $\psi = 0$, and s_i is the slope parameter for the

solute. The magnitude of the s-value depends on the molecular characteristics of the organic modifier, the stationary phase surface, and the solute itself. Generally, over the range of k'-values of interest in reversed phase HPLC, i.e. $0 < \log k' < 1.3$, reasonable linearity in the plots of logk' versus ψ_S is observed[7,8] for neutral and anionic solutes. However, the dependency of logk' on ψ_S over the ψ_S range corresponding to acceptable solute solubilities, i.e. usually over the range $0<\psi_S<0.8$, in the reversed phase HPLC of polypeptides and related ionised solutes is believed in general to be a polynomial which can be approximated[8,9] over a more limited range of solvent compositions to a quadratic relationship of the form

$$\log k' = A + B\psi_S + C\psi_S^2 \qquad (2)$$

As is evident from Figs. 1a,b the iodoamino acids (compounds 1-6, Table) also exhibit non-linear dependencies of their logk's on ψ_S over the range $0.08<\psi_S<0.80$. In these studies, two primary eluents were chosen to permit the evaluation of the dependency of logk' on ψ_S in the absence and presence of a fixed concentration of a cationic modifier (in this case a trialkylammonium salt) with both the ionic strength and pH held essentially constant. Data obtained with the primary eluent composed of aqueous 4mM sodium sulphate - 15mM orthophosphoric acid - 8% acetonitrile (pH 2.2) (elution system 1) as the volume fraction of acetonitrile is varied up to $\psi_S = 0.8$ are summarised in Fig. 1a. The corresponding experiments with the primary eluent composed of aqueous 4mM sulphuric acid - 15mM orthophosphoric acid - 15mM triethylamine - 8% acetonitrile (pH 2.2) (elution system 2) are summarised in Fig. 1b.

Several salient features are evident from these Figures. Firstly, with all the iodoamino acids examined the plots of logk' versus ψ_S pass through minima. Secondly, at high acetonitrile content in the mobile phase selectivity reversals are observed for

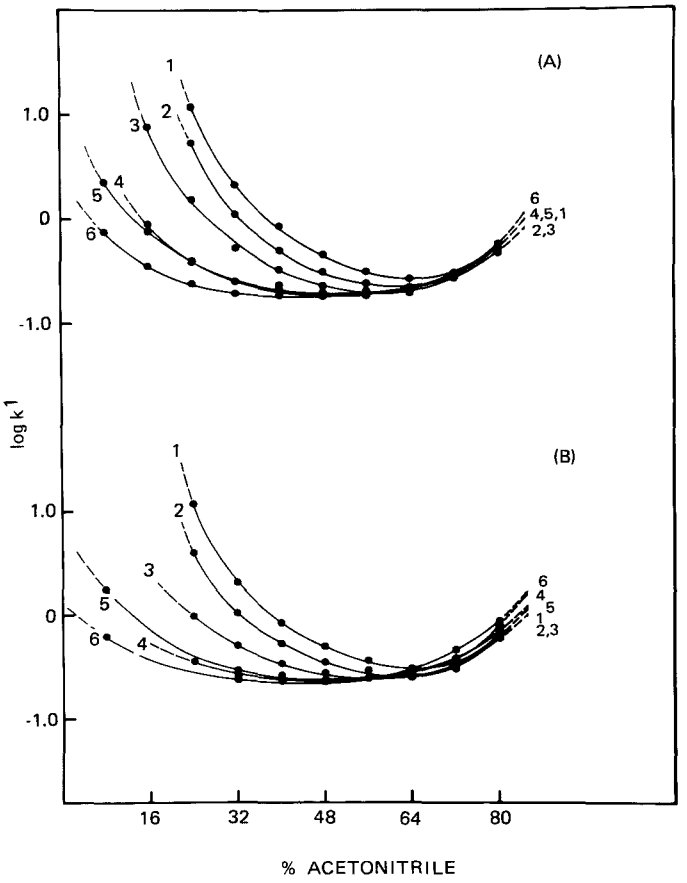

FIGURE: Plots of the logarithmic capacity factor, logk', for several iodoamino acids against the volume fraction, ψ_s, of the organic solvent in water-acetonitrile isocratic mobile phases. Conditions: column, μBondapak C_{18}; flow rate, 1.0 ml/min.; primary mobile phases (a) water - 4mM sodium sulphate - 15 mM orthophosphoric acid, pH 2.2, and (b) water - 4 mM sulphuric acid - 15 mM orthophosphoric acid - 15 mM triethylamine with the acetonitrile content adjusted over the ψ_s range 0.08 - 0.80. The compound key is listed in the Table.

all the iodoamino acids with both elution systems. Triethylamine and related alkylamines have been previously employed with low pH mobile phases in both the reversed phase and size exclusion HPLC of polypeptides. The efficacy of alkylamines to mask coloumbic solute-silanol interactions as well as to participate in pairing ion phenomena with polar solutes has been well documented[1,2]. In this context it is interesting to note the similarity of the plots for the iodoamino acids in the absence and presence of 15mM triethylamine under the various elution conditions studied. The most noticable differences in elution behaviour in the presence and absence of triethylamine were not associated with abolition of the enhanced retention at higher solvent content (a finding which suggests that the polar phase selectivity seen at high ψ_S values is not due to direct electrostatic interaction between ionised silanol and solute groups) but rather were associated with more subtle changes in selectivity such as those associated with reversal of the elution order of thyronine (T_O) and diiodotyrosine (DIT) or the percentage organic modifier at which overall selectivities become changed.

The data obtained with these solutes provide further support for the proposition that the mechanism of retention of ionised solutes, such as amino acid derivatives or peptides, on alkylsilicas involves composite reversed phase and polar phase adsorption components. The importance of each class of adsorption phenomenon will reflect the relative accessibility of appropriate 'solvophobic' or 'silanophilic' binding sites on the stationary phase surface as the water content is varied. As demonstrated elsewhere[1,4] the interplay of such composite retention processes can prove advantageous as far as the control over selectivity. For example, with hydrocarbonaceous stationary phases, such as the μBondapak C_{18} supports where enhanced retention is observed with polar solutes at high organic solvent content, then retrogradients can be employed from high to intermediate solvent content with very non-polar amino acid or peptide derivatives[2]. Such elution

TABLE

Structures and Calculated Retention Parameters of Iodoamino Acids Used in Present Study

No.	Structure	Abbreviation	Elution System 1		Elution System 2	
			s_i	$\log k'_w$	s_i	$\log k'_w$
1.	3,3',5,5'-Tetraiodothyronine	T_4	7.07	2.77	6.95	2.80
2.	3,3',5-Triiodothyronine	T_3	6.42	2.27	5.28	1.90
3.	3,5-Diiodothyronine	T_2	5.72	1.81	2.75	0.70
4.	Thyronine	T_0	3.77	0.90	1.50	0.0
5.	3,5-Diiodotyrosine	DIT	3.07	0.60	2.63	0.45
6.	3-Iodotyrosine	MIT	1.88	0.02	1.25	-0.10
7.	Tyrosine	Y	0.48	-0.58	0.18	-0.59

procedures are usually nor required with the iodoamino acids since adequate selectivity can be maintained under regular reversed phase conditions to resolve all the known iodothyronines and thyroacetic acids[10] with a single gradient of increasing organic solvent composition. However, retrogradients may be usefully employed to permit resolution of biological conjugates such as the N-acetyl derivatives or alternatively the phenolic O-glucuronide or O-sulphate derivatives since polar phase selectivity is maintained in this elution mode.

As is evident from the Table, the s-values for the iodoaminoacids ranged between 1.2 and 7.1. Introduction of iodo-groups into the thyronine nucleus results in increased relative hydrophobicity of the solutes and is also associated with progressively larger s-values (compare data for compounds 1-4). The incremental effect of the iodo-group on the retention of the iodoamino acids to alkylsilicas has been recognised in several previous studies. For example, plots of logk' versus molecular hydrophobic surface area[11], and logk' versus iodine atom per aromatic nucleus[12] have been found to follow linear relationships for these compounds. The above retention data confirm that with homologous solutes the overall hydrophobicity of a solute is a key parameter in determining the magnitude of the solute's s-value. Furthermore, the calculated s-values of compounds 1-7 eluted with either elution system from octadecylsilica are similar to those observed for small polar organic molecules[6] but considerably smaller than s-values of polypeptides and proteins where s-values in the range of 20-30 have been described[3,4,7,8]. The practical consequences of low s-values (and also low k'_w-values) with these iodoamino acids are regular reversed phase chromatographic selectivity with good peak shape and symmetry over a wide range of eluent compositions. In addition, low s-values for a solute imply linear elution development over a considerable range of eluent compositions and a relative insensitivity of the capacity factor to small changes in organic solvent content with water-rich mobile

phases. Such solvent composition-capacity factor relationships are important requirements where reproducible analytical precision is essential, e.g. in the assay of thyronine derivatives in biological samples.

Acknowledgements

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ASSESSMENT OF THE PLASMA-INDUCED CHANGES UNDER ACIDIC CONDITIONS IN THE APPARENT MOLECULAR VOLUME OF β-ENDORPHIN, β-LIPOTROPIN AND γ-LIPOTROPIN BY GEL PERMEATION AND REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.

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SUMMARY

Addition of radiolabeled β-endorphin, β-lipotropin and γ-lipotropin to plasma at acid pH results in an apparent reduction in size of these molecules as evidenced by gel permeation chromatography. These acid plasma-treated molecules, however, are indistinguishable from the untreated radiolabeled polypeptides when subjected to high performance liquid chromatographic separations suggesting no differences in molecular composition. As these apparent changes in polypeptide molecular volume are prevented by addition of Trasylol or sodium azide to the plasma, a likely explanation would appear to be an enzyme-dependent production of anionic lipids in plasma which at acid pH bind to the lipotropins and endorphins reducing their molar volume.

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INTRODUCTION

Recent investigations have established that β-lipotropin (βLPH), γ-lipotropin (γLPH) and β-endorphin (βEP) are present in the peripheral circulation in man (1,2,3) and that they are secreted into the circulation from the pituitary gland. βLPH, a 91 amino acid residue polypeptide contains within its structure the sequences of γLPH (βLPH-(1-58)), β-melanotropin (βMSH) (βLPH-(41-58)), βEP (βLPH-(61-91)), γ-endorphin (γEP) (βLPH-(61-77)) and α-endorphin (αEP) (βLPH-(61-76)). Consequently, it has been suggested that βLPH may serve as a prohormone for some or all of these peptides (4). In the course of experiments designed to separate βLPH and βEP in plasma by chromatographic techniques, we noted that acidification of the plasma caused these peptides to elute at positions corresponding with smaller apparent molecular size than when the same peptides were treated and chromatographed under neutral conditions. The present study was undertaken to investigate these observations and establish the possible role of fragmentation or deamidation processess.

MATERIALS AND METHODS

Synthetic human βEP, αEP and γEP were purchased from UCB Bioproducts (Brussels, Belgium). γLPH and βLPH were kindly supplied by Dr. P. Lowry (St. Bartholomew's Hospital, London, U.K.) and by Dr. C.H. Li (Hormone Research Laboratory,

San Francisco, Ca., U.S.A.) respectively. βMSH was donated by CIBA-Geigy through the U.S.A. National Pituitary Agency (NIAMDD).

Preparation of Radiolabeled Peptides

All peptides were labeled with Na ^{125}I using chloramine T according to the method of Hunter & Greenwood (5). Purification was performed using columns packed with Sephadex G75 for βLPH, G50 for γLPH, βEP and βMSH and Sphadex G25 for αEP and γEP.

Gel Filtration

Sephadex G50 Fine columns (1.3 x 70cm) were pre-equilibrated with either 0.05M phosphate buffer (pH 7.4) or 0.1N acetic acid (pH 2.8). Both eluants contained 0.1% bovine serum albumin (fraction V, Sigma Chemical Co., St. Louis, Mo., U.S.A.). Purified radiolabeled peptides (500,000 cpm) and Na ^{125}I (100,000 cpm) were added to 1ml aliquots of either human plasma or eluant, incubated at 20^{o} for 5 min before loading onto the column, and eluted from the columns at a flow rate of 10ml h^{-1} at 4^{o}. Fractions (1ml) were collected and peaks of radioactivity pooled and lyophilised prior to analysis using high performance liquid chromatographic techniques (HPLC). In those experiments investigating the effects of enzyme inhibitors, Trasylol (Aprotinin, Bayer Pharmaceutical Co. N.S.W., Australia; 1000 KIU ml^{-1}), or sodium azide (0.018%) were added to the plasma and the

eluant prior to addition of the radiolabeled polypeptides.

High Pressure Liquid Chromatography

The HPLC techniques used in this study were based on procedures previously described by Hearn (6) and Hearn and Grego (7). In brief, radiolabeled peptide standards and plasma-treated peptides were chromatographed on a μBondapak C_{18} column (30 x 0.4cm I.D., 10um) using a 60 min linear gradient generated from aqueous 15mM orthophosphoric acid and acetonitrile (0 → 50% v/v) at a flow rate of 2ml min^{-1}. Fractions were collected every 30 sec.

RESULTS

Figure 1(a) shows the elution profiles for radiolabeled βLPH, γLPH, and βEP chromatographed on Sephadex G50 under varying conditions. Treatment of these peptides with plasma at neutral pH resulted in no significant change in the elution profiles when compared with those chromatograms obtained running the peptides in the absence of plasma at both pH 7.4 and 2.8. In contrast, when plasma-treated radiolabeled βLPH, γLPH and βEP were chromatographed under acid conditions, there was an increase in elution volume of the radioactive peak indicating that the labeled moiety was now contained in molecules of apparently smaller size. These apparently "smaller" peptides eluted in a similar position to that

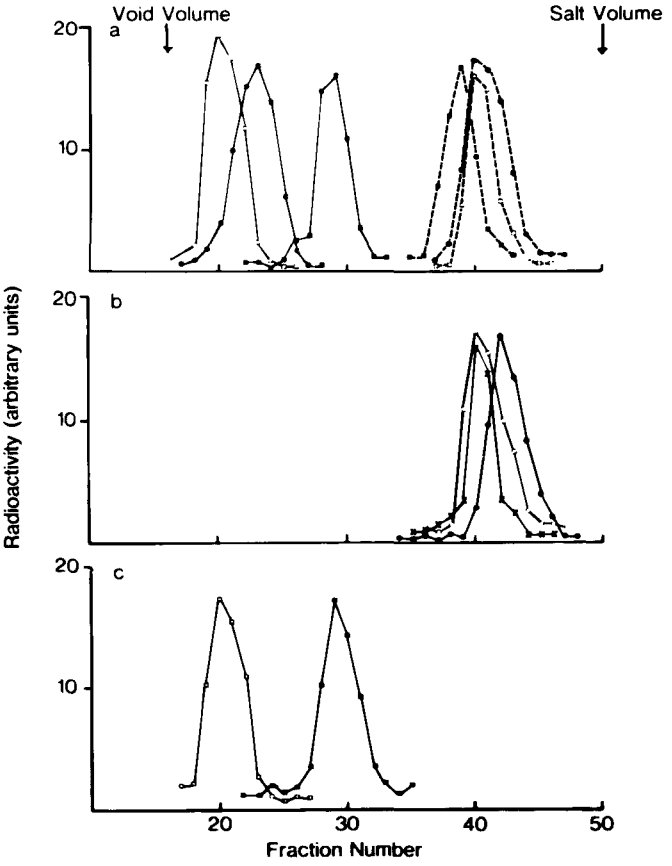

Figure 1. Gel filtration of radiolabeled polypeptides on Sephadex G50. See text for experimental details.

a. Radiolabeled polypeptides eluted + plasma, pH 7.4 or 2.8.

_____ Elution position of polypeptides prior to acid plasma treatment.

----- Elution position of polypeptides after acid plasma treatment.

b. Radiolabeled polypeptides eluted ± plasma, pH 7.4 or 2.8.

c. Radiolabeled polypeptides eluted + plasma, pH 2.8 with Trasylol or sodium azide present.

X βMSH; ● αEP; ○ γEP; ■ βEP; □ βLPH; ⊕ γLPH

found for the peptides βMSH, αEP and γEP (Fig. 1,b). However, The elution volume of these latter peptides (as well as radiolabeled insulin) was unaffected when the samples were treated with acid plasma.

Addition of the enzyme inhibitors Trasylol or sodium azide to the plasma and eluant prevented the apparent change in elution volume for βLPH and βEP when the peptides were run in acid plasma (Fig. 1,c). Furthermore, collection of the eluted βEP peak after an acid plasma run and subsequent rechromatography under neutral conditions resulted in the peak of radioactivity eluting in the same position as observed in the absence of plasma with eluants at either pH 7.4 or 2.8. This suggests that the observed change is freely reversible.

Following gel chromatography, recovered peaks of radioactivity were analysed by HPLC and the resulting chromatograms shown in Fig. 2. It should be noted that radiolabeled βLPH chromatographed as two peaks, βLPH and βLPH', an unexplained observation previously noted (M.T.W.Hearn, unpublished results). A similar result was obtained using a different batch of βLPH supplied by Dr. P. Lowry. As shown in the chromatogram, acid plasma-treated βLPH and βEP eluted in positions coincident with those of untreated βLPH and βEP respectively indicating no change in composition of these polypeptides.

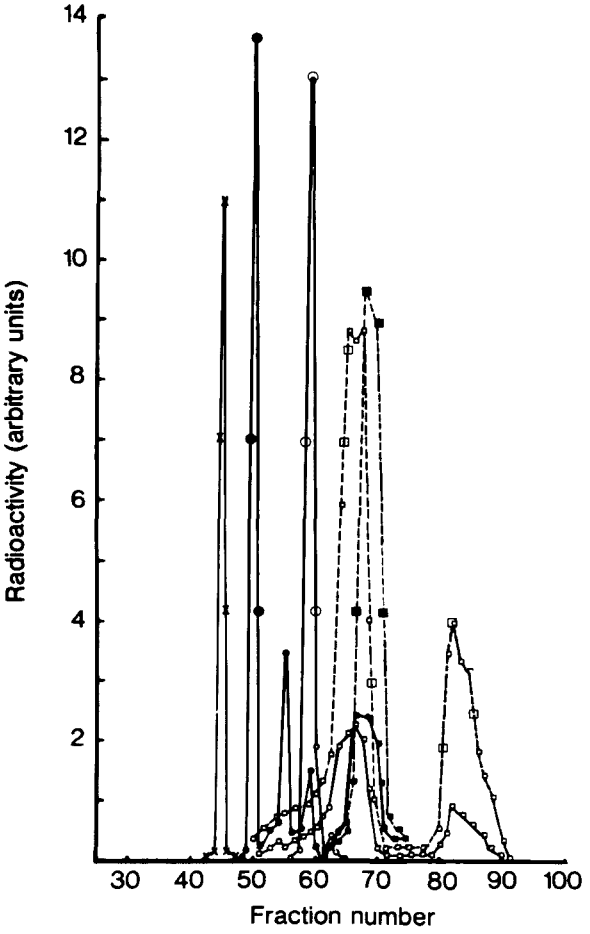

Figure 2. HPLC separation of labeled peptides using a reversed phase uBondapak C_{18} column. See text for experimental details.

_____ Elution position of polypeptides prior to acid plasma treatment.

----- Elution position of polypeptides after acid plasma treatment.

X βMSH; ● αEP; ○ γEP; ■ βEP; □ βLPH

DISCUSSION

Treatment of radiolabeled βEP, βLPH and γLPH with plasma at acid pH resulted in a considerable change in their apparent molecular volumes as assessed by changes in their gel permeation elution volumes. As all chromatograms were carried out on columns of identical dimensions with the same flow rate, gel bed and fraction size, it is unlikely that the enhanced permeation shown by the acid plasma-treated samples could be attributed to physical changes in elution volume arising from eluant viscosity or bed permeability changes. Rather, these changes are indicative of a more compact molecular form for these polypeptides, with smaller apparent Stokes radii.

From the data obtained from HPLC investigations, it can be concluded that there is no change in composition of the peptides following acid plasma treatment and therefore, the changes in elution volumes cannot be attributed to a proteolytic degradation process or fragmentation. A likely explanation is that at acid pH and in the presence of plasma, conformational changes occur in the molecules resulting in a decrease in molecular volume. There is now considerable evidence to suggest that in the presence of acidic lipids, an appreciable helical structure is formed by βLPH and βEP (8,9,10,11), resulting in these peptides occupying a smaller molecular volume. Hence, in water at neutral or acid pHs both polypeptides appear to have little, if any secondary structure whereas upon interaction with various anionic lipids these

polypeptides undergo a conformational change which includes a significant amount of α-helix. Mattice & Robinson (10) predict that it is the carboxyl terminal half of βEP and βLPH which shows high helix potential in the presence of acidic lipids and that αEP, which is the first sixteen amino acid residues of βEP, has a very low helix profile in both water and in anionic lipids. This explanation is also in accord with our observation that αEP and γEP do not undergo an apparent reduction in molecular volume when chromatographed with acid plasma. Assuming that this explanation is correct, the finding that γLPH but not βMSH exhibits changes in molecular size in acid plasma suggests that helical content is also contained between residues 1 to 41 at the amino terminal of βLPH. Furthermore, the observation that these changes in apparent molecular volume are abolished by Trasylol and sodium azide suggest either that an enzyme(s) is present in plasma that promotes rapid conformational changes of these molecules or that binding of β-LPH and β-EP to acidic lipids in plasma is enzyme and pH dependent. The latter possibility is favoured.

It has been suggested that it is the helical segment of the βLPH-related polypeptides which participates in effective receptor binding, the receptor membrane containing anionic lipids (9). Hence, these peptides must assume some ordered conformation to elicit their biological activity and explains why βEP shows significantly higher opiate activity than does αEP (10).

In summary, the present study indicates that an interaction

between β-endorphin and β- and γ-lipotropins with acidic lipids may occur in plasma at low pH. The conformational changes resulting from this interaction give rise to polypeptides with smaller apparent molecular volumes and thus greater permeation on Sephadex gel columns.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF LOW MOLECULAR WEIGHT PROTEINS ON A NON-IONIC MACRORETICULAR POLYSTYRENE RESIN

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ABSTRACT

A high performance liquid chromatography system is presented for analytical and preparative separation of proteins. The method utilizes a macroreticular polystyrene resin having no specific functional groups, and proteins are eluted by the use of linear gradient of acetonitrile (20% - 75%, v/v) in 0.1% (v/v) trifluoroacetic acid. In this standard elution system, twenty proteins having a molecular weight of 4,200 - 58,000 and an isoelectric point of 3.9 - 11.0 have been chromatographed successfully within 80 min. The method allows a rapid, sensitive, and high resolution separation of relatively low molecular weight proteins, where the isolated proteins can be used for subsequent biochemical determinations.

INTRODUCTION

Purification of proteins is often an essential step in molecular studies of biological events, and this has been tried by various physical and chemical means. Among these methods, the chromatography technique is one of the most common and convenient approach with respect to simple operation, considerable

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resolution, and easy recovery of separated proteins. However, classical chromatography procedures utilizing conventional dextran or agarose based supports are time consuming, and require relatively large quantity of starting material essentially because the efficiency of such a column is usually low. For more impact approach to the biology field, it seems necessary to devise more rapid, sensitive, and high resolution methods for the purification and analysis of proteins.

Recent advances of high performance liquid chromatography (HPLC), such as development of reverse phase HPLC using silica-based supports, have significantly improved this underlying problem. It has been shown that the reverse phase HPLC is powerful for the separation of polypeptides such as opioid peptides (1,2) and peptides derived from limited proteolysis (3-5). This method has been also applied for the purification of a number of proteins such as interferons (6), insulin-like growth factors (7), histons (8), and trypsin and chymotrypsin (9). However, the reverse phase separation of proteins seems to be not yet widely accepted as a substitute for the "conventional" chromatography procedures, presumably because the reverse phase supports, available so far, often bind proteins nonspecifically and tightly. On the other hand, HPLC of the gel permeation type has been widely used for the separation of proteins (10), but only for group separation of proteins or separation of rather simple protein mixtures.

In the previous papers (11-13) we have demonstrated that the macroreticular ion-exchange polystyrene resin have many advantageous characteristics in the chromatography of peptides. It is comparable to the silica-based reverse phase supports in high resolution, with added advantages in chemical stability and wide sample capacity range. In these studies we have noticed that relatively large peptides such as those produced by CNBr cleavage or partially degradated proteins are eluted from the resin column in a considerable recovery. This observation has

led us an attempt to apply a macroreticular resin for the separation of proteins.

This paper describes an application to the separation of proteins of the macroreticular polystyrene resin, having no specific functional groups other than the styrene group. The method showed remarkable peak resolution for twenty tested proteins having a molecular weight of 4,200 - 58,000 daltons, where the separation occured in less than 80 min with manogram to milligram quantities of samples.

MATERIALS AND METHODS

Proteins: Calmodulin, S100a protein, micro glutamic acid-rich protein, neuron specific enolase, and DEK protein were purified from bovine brain by ammonium sulfate fractionation of brain soluble extracts and following column chromatography procedures. The purification of calmodulin, S100a protein and micro glutamic acid-rich protein has been described (14-16), and that of other proteins is described elsewhere. Bence-Johns protein, NIG-64, was purified from urine of a patient of primary amyloidosis (17). Streptomyces subtilisin inhibitor was kindly donated by Dr. T. Kainosho of Tokyo Metropolitan University, ion-type superoxide dismutase of *Pseudomonus ovalis* by Dr. F. Yamakura of Juntendo University (Narashino, Japan), phospholipase C (α-toxin) of *Clostridium perfringens* by Dr. Y. Yamakawa of National Institute of Health, Japan, cancer cachexia specific protein by Dr. O. Oda of the Bio-dynamic Research Institute of the Sinseikai Foundation (Nagoya, Japan). Synthetic parathyloid hormone (residues 1 - 34) and C-reactive protein were the products of Toyo Jozo Co. Ltd. Other proteins were obtained from sources indicated in parentheses; L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Millipore Corp.), α-chymotrypsin (Worthington Biochemicals Corp.), thermolysin (Sigma Chemicals), lysozyme (egg white, Seikagaku Kogyo Ltd.), cytochrome c (horse heart, Seikagaku Kogyo Ltd.), and insulin (Calbiochem-Behring Corp.).

Chemicals for chromatography; A macroreticular polystyrene resin, Hitachi-Gel 3013, with a particle size distribution of 5 - 7 μm and a cross-linkage of 35%, was obtained from Hitachi Ltd. (Tokyo, Japan). Acetonitrile (chromatography grade) and trifluoroacetic acid (sequanal grade) were purchased from Wako Pure Chemical Industries (Tokyo). Water was distilled, passed through a mixed-bed ion-exchange resin, and redistilled before use.

Apparatus; A Shimadzu Corporation (Kyoto, Japan) Model LC-3A liquid chromatograph, equipped with a dual wavelength u. v. detector (8 mm light path), was employed. The Hitachi-Gel 3013 resin was made in a slurry in 50% acetonitrile in water and packed into a stainless-steel column (250 mm x 4.6 mm ID) at a pressure of 30 kg/cm^2. The solvent used was 50% acetonitrile, which was then replaced by 20% acetonitrile for most compact packing.

Chromatographic procedure; Sample proteins were introduced onto a column of Hitachi-Gel 3013 and eluted at 40 °C by the use of linear gradient from 20% (v/v) to 75% acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.5 ml/min. The gradient was formed by placing 20 ml of the initial solvent in a gradient mixer, into which the final solvent was added at a constant flow rate of 0.25 ml/min. Where necessary, column effluent was collected either manually or automatically and aliquots were removed for subsequent measurements. The removed effluent was lyophilized after dilution with water (for measurements of enzymatic activities), or dried up under N_2 stream (for amino acid compositional analyses). After completion of one analytical run, the column was reequilibrated for 30 - 60 min with the initial solvent for the next analysis.

Chemical modification and amino acid analysis of proteins; Oxidation with performic acid and S-aminoethylation with ethylene imine were performed essentially as described (17-19). Amino acid analysis was performed with a modified Hitachi Model KLA-5 analyzer as described (16). Proteins were hydrolized with 250 μl of 6 M HCl for 24 h at 110 °C in evacuated, sealed tubes.

RESULTS AND DISCUSSION

Application of the method

In order to evaluate the present chromatography system, we have applied twenty test proteins having different physicochemical properties in terms of molecular weight (4,200 - 58,000) or isoelectric point (pI 3.9 - 11.0)(Table 1). Upon mobile phase gradient of acetonitrile in 0.1% trifluoroacetic acid, all these proteins were eluted from the resin column within 80 min. The chromatograms are shown in Fig. 1, a to t, and the retention time for each protein is incorporated in Table 1. Some comments on the chromatograms are described below.

[1] micro glutamic acid-rich protein (microGluP); MicroGluP is a brain protein having a novel amino acid composition; there are abundant glutamic acid (51% of the total composition) and no hydrophobic amino acids such as Val, Met, Ile, Leu, Tyr, Phe, and Trp (16). This protein exhibits no absorption at 280 nm due to the lack of aromatic residues, and has the smallest retention time among the proteins tested (Fig. 1, c).

[2] streptomyces subtilisin inhibitor (SSI); The preparation of SSI was separated into at least two major peaks in our chromatography system (Fig. 1, e). This corresponds to an argument that the SSI molecule may be heterogeneous in the amino-terminal region (20)

[3] calmodulin; Calmodulin, a calcium binding protein having a repetitive amino acid sequence of an "EF-hand" (21,22), gave rise to a major peak at 52.5 min with a slight shoulder at the leading edge (Fig. 1, g). This would be due to des(Ala-Lys)calmodulin which was present in the calmodulin preparation in almost 10% quantity (12). The oxidation of nine methionyl residues in the protein reduced significantly the retention time of calmodulin (Fig. 1, h), suggesting that the oxidation caused considerable structural changes on the surface of molecule.

[4] S100a protein; S100a protein is a dimer of non-covelently associated subunits, α and β, which have highly homologous amino acid sequence (18,19). Under the solvent conditions employed,

Table 1. A list of proteins subjected to HPLC

Protein	Source	Molecular Weight	Isoelectric point	Retention time (min)
parathyroid hormone (1-34)	chemical synthesis	4,200	9.7*	31.5
insulin	bovine pancreas	5,700	5.3*	36.0
micro glutamic acid-rich protein	bovine brain	10,000	3.9	18.5
cytochrome c	horse heart	11,000	6.5	33.5 34.0
streptomyces subtilisin inhibitor	Streptomyces subtilis	11,500	4.5*	40.5 42.0
lysozyme	chicken egg	14,300	10.5-11.0	37.5
calmodulin	bovine brain	16,700	4.0	52.5
oxidized calmodulin	(modification)	17,000	> 4.0	45.0
S100a protein	bovine brain	10,400(α) 10,500(β)	4.2 4.5	60.0 64.0
C-reactive protein	human urine	20,900	5.8	47.0
superoxide dismutase	Pseudomonus ovalis	23,000(x2)	4.5	51.5
S-aminoethyl superoxide dismutase	(modification)	23,000	< 4.5	52.0
S-aminoethyl Bence-Jones protein NIG-64	human urine (modification)	23,400	7.7*	34.5
trypsin	bovine pancreas	23,900	10.1	37.5 38.0
cancer cachexia specific protein	human serum	25,000	6.5	51.5
α-chymotrypsin	bovine pancreas	25,100	8.1-8.6	44.5
thermolysin	Bacillus thermo-proteolyticus	34,400	4.6*	52.0
phospholipase C	Clostridium perfringens	43,000	5.5	40.5
neuron-specific enolase	bovine brain	50,000(x2)	4.5	49.5
DEK-protein	bovine brain	58,000	4.3	42.5

*Values are estimated from the amino acid compositions by the micro computer method of Manabe (32).

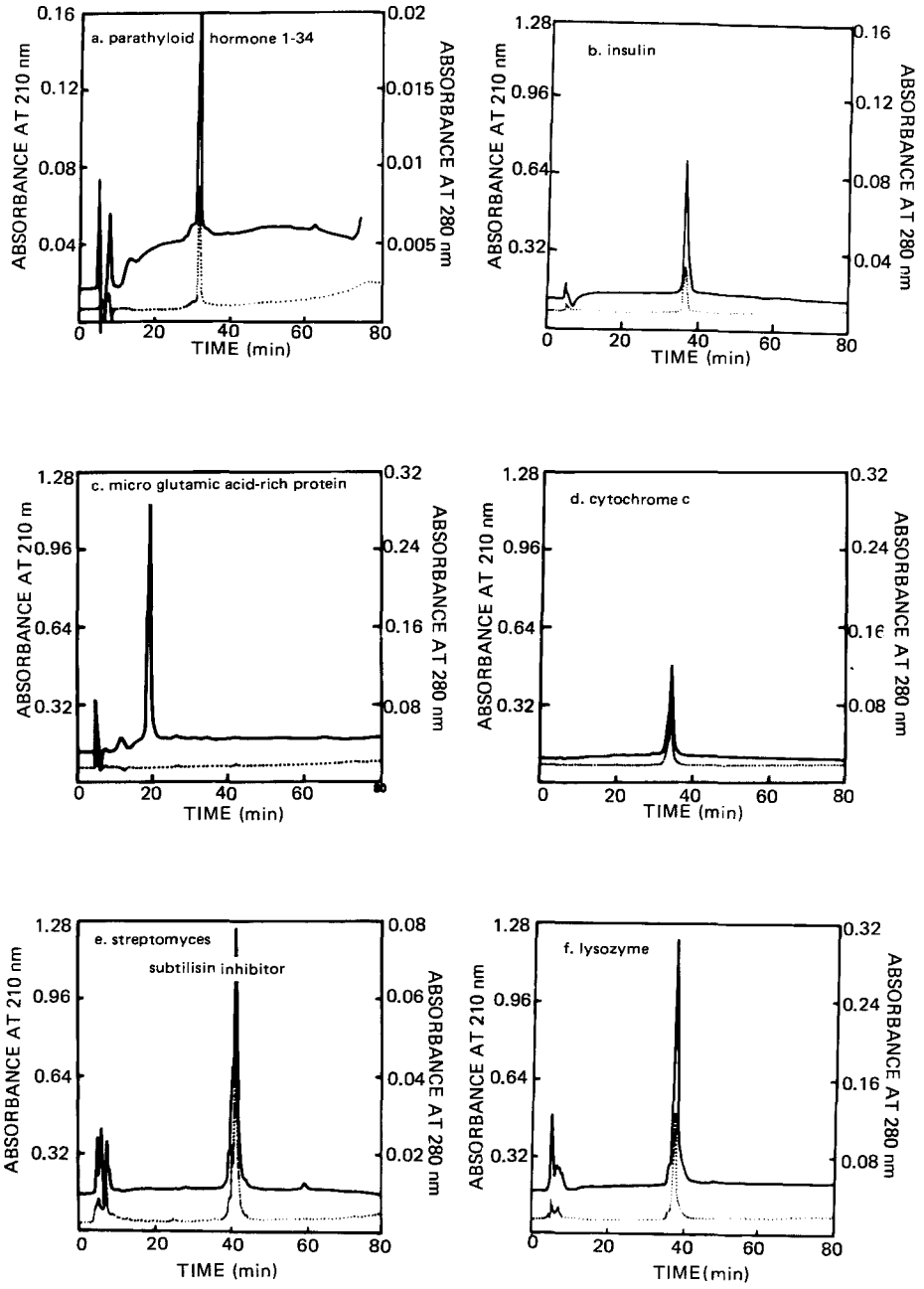
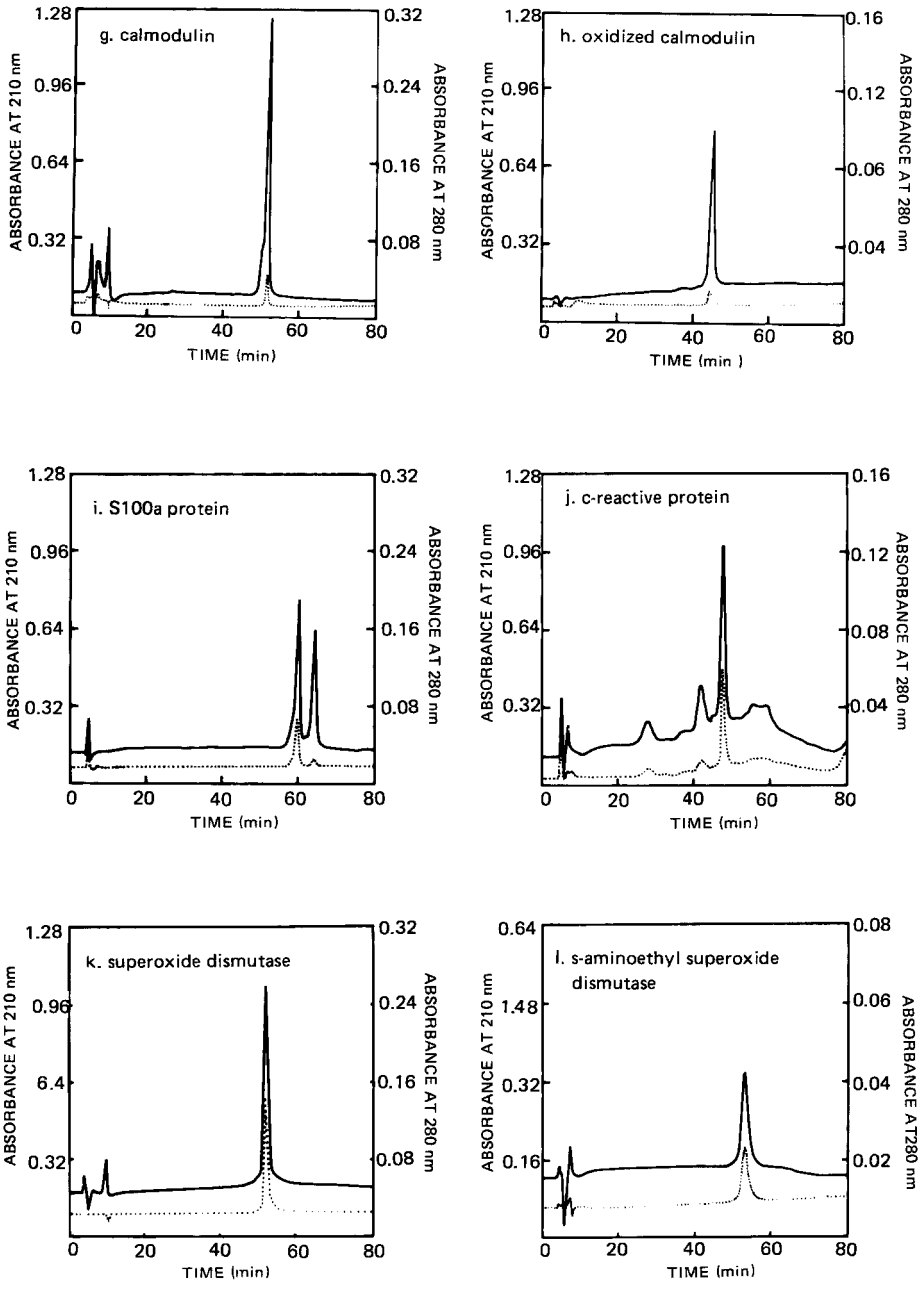


FIGURE 1

High performance liquid chromatography of proteins on a non-ionic macroreticular polystyrene resin. The proteins (1 - 50 µg) were applied to a column (25 x 0.46 cm i.d.) of Hitachi-Gel 3013, and eluted by the use of linear gradient of acetonitrile (20% - 75%) in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. Other conditions are given under Methods.



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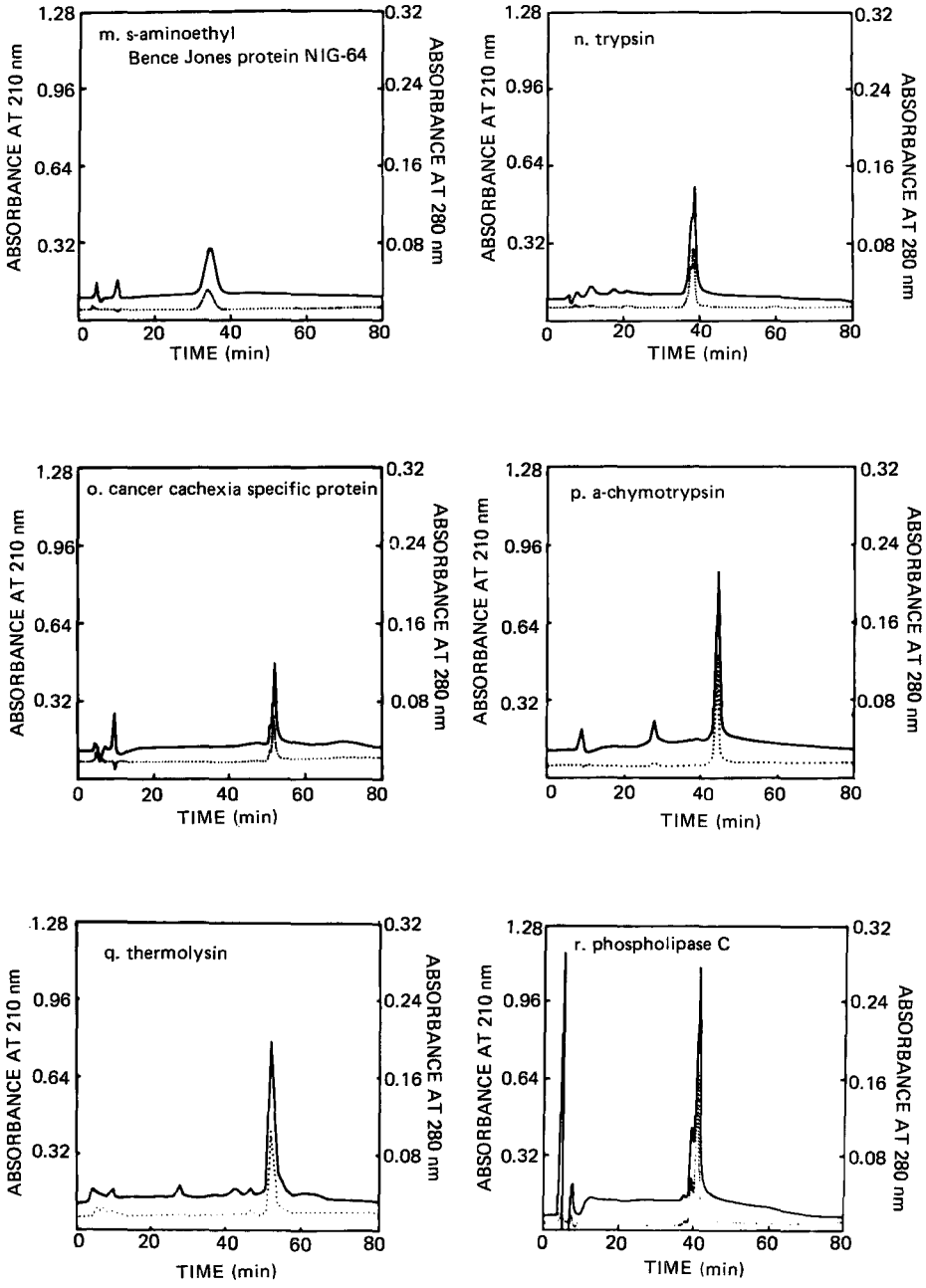

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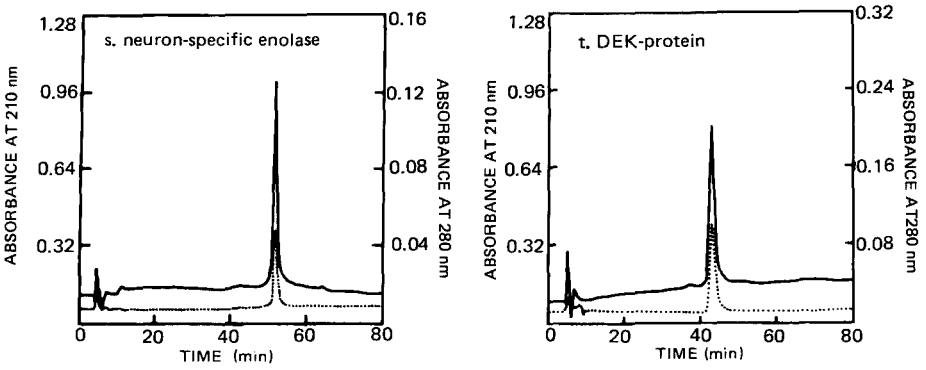

FIGURE 1 (continued)

these subunits of S100a protein were dissociated, and separated from each other as shown in Fig. 1, i. This chromatography procedure could be therefore applied successfully to the estimation of subunit compositions of a series of S100 protein species purified from bovine brain, as well as from other vertebrate sources (23).

[5] superoxide dismutase; Ion-type superoxide dismutase from Pseudomonus ovalis (24) gave rise to a single peak at a retention time of 51.5 min (Fig. 1, k). Aminoethylation of four cystein residues in the protein under reduced, denaturing condition (in 8 M urea) resulted in broadning of the protein peak without significant alteration in the retention time (Fig. 1, l).

[6] trypsin; Commercial trypsin gave rise to two partially-separated peaks (Fig. 1, n). These peaks should be due to α and β forms of trypsin respectively, where α-trypsin was derived from the β form by an internal cleavage at Lys_{131}-Ser_{132} (25). The present chromatography system enables to separate trypsin from α-chymotrypsin (Fig. 1, p) which is often present in trypsin preparations, although we are not able to detect this contaminated chymotrypsin at this scale of experiment.

[7] neuron-specific enolase; Neuron-specific enolase is a dimer

of two homogeneous, non-covalently linked subunits with a molecular weight of about 50,000 (26). The chromatography of this protein showed a single symmetrical peak at 49.5 min (Fig. 1, s).

[8] DEK protein; The brain DEK protein has a monomer molecular weight of 58,000, and is the largest molecule among the proteins tested. This protein was eluted in a single peak at a retention time of 42.5 min (Fig. 1, t).

Separation principle

The present method utilizes a macroreticular resin of the styrene-divinylbenzene type, where proteins are eluted by a mobile phase gradient of acetonitrile in 0.1% trifluoroacetic acid at pH 2. Since this resin has no functional group other than the styrene matrix, the separation should have occured on the basis of interaction between proteins and the styrene matrix. This type of interaction is called "matrix effect" by Regnier et al (27), and is frequently observed in the "ion-exchange" chromatography using polystyrene resins with ionic groups. Thus, the present method is analogous, in separation principle, to the reverse phase HPLC using silica-based supports.

Such a separation principle is particularly clear when the separation of peptides is concerned; where the retention time correlates to the hydrophobicity of peptides estimated from the hydrophobic fragmental constant provided by Recker (28), or modified by Sasagawa et al (29)(Kurosu et al, manuscript in preparation). For the protein molecules, however, this correlation was ambiguous by relatively large deviation. This indicates that proteins are in partially folded conformation even under rather denaturing conditions employed, so that only a portion of the residues is available to interaction with the polystyrene resin. It should be noted that the "intact" proteins appeared in sharp, and narrow peaks as compared to the proteins modified after denaturation (Fig. 1, h, l, m), suggesting that a conformational factor, in fact, contributed to the separation.

It is also noted that the S100a protein has been dissociated into subunits during the chromatography (Fig. 1, i). This could

be attributed to the solvent conditions employed, high organic solvent concentration and acidic pH, which would desrupt both electrostatic and hydrophobic interaction between the subunits. We assume that the subunit separation has also occured for other dimeric proteins such as superoxide dismutase and neuron-specific enolase, although we can not evaluate this assumption as the subunits are identical in both these cases.

Recovery of proteins

We have estimated the recoveries of some proteins tested (c.f. calmodulin, S100a protein, superoxide dismutase, thermolysin, and neuron specific enolase) by means of amino acid analysis of proteins before and after the chromatography, and obtained the values of 85%, 90%, 55%, 60%, and 56% for these proteins, respectively. These results suggest that the recoveries are considerable high for small proteins, and are within an acceptable extent even for relatively large proteins.

Recovery of activities of proteins

Titani et al (9) purified commercial trypsin and chymotrypsin by mean of reverse phase HPLC with almost complete recovery (70 - 104%) of their proteolytic activity. Since the separation conditions employed in this study and that of Titani et al are analogous, a similar extent of activity recovery could be expected for these enzymes.

We found that the trypsin, recovered after this chromatography step, was sufficiently active for peptide mapping studies of proteins. Phospholipase C was also active towards p-nitrophenyl-phosphorylcholine (Y. Yamakawa, personal communication), and two calcium binding proteins, calmodulin and S100a protein, retained their calcium binding abilities. We anticipate therefore that the procedure could be useful for purification of any acid-stable proteins and enzymes.

Maintenance of the column

As noted in the previous reports (11,13,30,31), the macroreticular resin is definitely stable both chemically and mechanically. This means that practically any solvents can be used for

elution. For instance, 2 M NaOH containing acetone or isopropanol was effective in regeneration of the column, which might be necessary after repeated application of crude protein mixtures. However, introduction of large volume of aqueous solution, without organic solvent, should be avoided because this causes rapid increase in back pressure. Note that the polystyrene resin without ionic group has very low affinity against water. We are including 20% acetonitrile in the initial solvent for routine maintenance of the resin column. In our experience, all proteins are adsorbed to the column under this solvent condition.

In conclusion, the chromatography using a macroreticular polystyrene resin appears to be a useful tool for rapid, sensitive, and high resolution separation of proteins. The method is applicable either for analytical or preparative separation of proteins because the resin column has large sample capacity due to its large surface area and porocity (11,13). However, application of this method is limited so far for relatively low molecular weight proteins, apparently because large protein molecules have generally high hydrophobicity and little solubility in the elution solvent. Therefore, the problem remains to devise an equivalent technique that is effective in separation of larger protein molecules.

ACKNOWLEDGEMENT

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SEPARATION OF ORTHOPHTHALALDEHYDE/ETHANETHIOL DERIVATIVES OF TAURINE AND CLOSELY ELUTING AMINO ACIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

In studies of the reverse phase, HPLC analysis of amino acids employing precolumn derivatization with o-phthalaldehyde and ethanethiol, it was shown that α-amino-n-butyric acid, β-amino-isobutyric acid and taurine coeluted in the acetonitrile/aqueous phosphate solvent system. By using a ternary solvent system of acetonitrile/tetrahydrofuran/aqueous phosphate buffer and efficient 5- and 10-μm octadecylsilane packings, the co-elution problem has been resolved. This modified chromatographic system is now being used to quantitatively determine taurine and other closely eluting amino acids in a variety of physiological fluids in order to clarify the role of taurine in human development.

INTRODUCTION

There have been many recent reports describing the reverse phase, HPLC separation followed by fluorescent detection of derivatized amino acids and amino sulfonic acids (1-9). The method involves performing a precolumn derivatization of the amino acids (I) with o-phthalaldehyde (OPA)(II) in the presence of a thiol (III) to form highly fluorescent substituted isoindoles (IV) as

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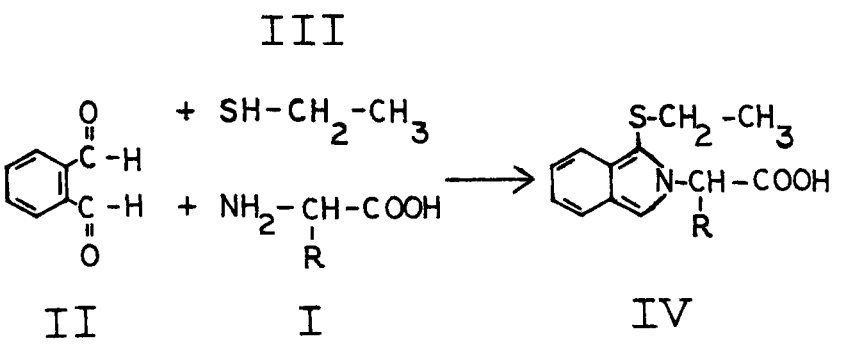
shown below;



The OPA/ethanethiol (ETSH)(III) derivatization reaction, first described by Simons and Johnson (10), was used in earlier reports (1,2,4). It was found that the fluorescent products of this reaction are more stable in comparison to those formed in the OPA/2-mercaptoethanol derivatization reaction. The fluorescent isoindoles formed by the reaction of OPA/ETSH with primary amino acids are formed within seconds at room temperature and in most cases are quite stable in aqueous solution for periods of minutes to hours.

In a recent paper, Hill, Burnworth, Skea and Pfeifer (4) have presented information to characterize the optimal experimental parameters necessary for using the precolumn OPA/ETSH derivatization reaction. They reported that there is a linear increase in peak area versus amino acid concentration over a range from 5 to 500 nmol/mL for each of the twenty amino acid standards studied. Linear regression correlation coefficients of 0.999 or better were obtained. Reasonable agreements for fifteen of the amino acids

between the values obtained by the conventional ion-exchange method, using a Beckman 119C Amino Acid Analyser and the precolumn OPA/ETSH HPLC fluorescent method was reported. Of importance to this report is the fact that Hill, et al., (4) reported that in their acetonitrile/aqueous phosphate system that α-amino-n-butyric acid (α-ABA) and β-aminoisobutyric acid (β-ABA) co-eluted with taurine.

Thalmann et al. (7), reported the use of the precolumn OPA/ETSH method to determine the levels of nineteen of the free amino acids found in the inner ear and cerebrospinal fluids of guinea pigs. They choose to omit reporting the levels of taurine because, in communication with Hill, they reported the coelution of α-ABA and β-ABA with taurine under their specific assay conditions. Turnell and Cooper (8) had reported a clinical study employing the precolumn OPA/2-mercaptoethanol derivatization reaction followed by the reverse phase HPLC separation and fluorescent detection of an estimated thirty-one amino acids in human serum and urine. Acceptable accurate and precise values of all of the common amino acids were obtained except for taurine whose value was reported to be altered by co-elution with β-aminoisobutyric acid (β-ABA). In their chromatographic system, α-ABA was found to elute several minutes after taurine from the 5-μm Ultrasphere ODS (Altex Scientific Co.) column. A mixed gradient solvent system of water/stock sodium proprionate solution/acetonitrile (72/20/8 by vol.) to water/acetonitrile/methanol/dimethylsulfoxide (42/30/25/3 by vol.) was employed. An interesting table in their

publication detailed the stability, in terms of half-life, for the fluorescent products of amino acids/OPA/2-mercaptoethanol (8).

Wheler and Russell (9) reported the use of the precolumn OPA/ETSH HPLC-fluorescent method to separate taurine, from its precursor hypotaurine. The method was also able to separate cysteine sulfinic acid and α-glycerophosphoryl ethanolamine known to be present in mammalian brain fluids. They reported that their method provided upto a 500-fold improvement in sensitivity over the ion-exchange, post-column ninhydrin absorption method (9). However, it appears that these authors were not aware of the co-elution problem of α-ABA and β-ABA with taurine in their specific assay conditions.

Structurally, taurine (TAU), 2-aminoethanesulfonic acid,

$$H_2N - CH_2 - CH_2 - SO_3H \qquad \text{(Structure I)}$$

is a β-amino sulfonic acid. It is readily separated and detected along with the many amino acids in physiological fluids. Taurine is unusual because it occurs in high concentrations in mammalian tissue; it is chemically and metabolically stable, yet it is not a constituent of proteins. Three books (11-13), a marked increase of references to taurine in the literature, and an upcoming review chapter on the liquid chromatographic determination of taurine (14) attest to the growing recognition of the interest in determining the biological functions of taurine.

Normal plasma concentrations of taurine in adult humans have been reported as 59 nmol/mL, with a standard deviation of ± 12 nmol/mL and a range of 41 to 78 nmol/mL (15,16). In the same

study, α-amino-n-butyric acid had a mean value of 20 nmol/mL with a standard deviation of 6 nmol/mL and a range of 9-35 nmol/mL. No values were reported for β-aminoisobutyric; its level is expected to be at least 10-fold lower than for taurine.

High levels of taurine are found in the plasma of human newborns. Within a few days, the taurine concentration in the plasma decreases about three-fold, with a concurrent increase excretion in the urine. The importance of TAU in the developmental changes of humans continues to be an active area of research making the use of high performance liquid chromatography with fluorescence detection a very attractive analytical method to use.

This report details how, by using a ternary solvent system of tetrahydrofuran/acetonitrile/aqueous phosphate system, as has been suggested earlier for the separation of threonine from glycine (4), and by the use of efficient 5-μm octadecylsilane bonded packings, resolution of α-ABA, β-ABA and taurine as their OPA/ETSH derivatives has been achieved.

MATERIALS

A Waters Associates Liquid Chromatographic System equipped with a Model 6000A pump, and a Model U6K injector was used. The fluorescent detector was a Schoeffel/Kratos FS-970 Spectrofluoro Monitor with a standard 5-μL cell. This detector was adjusted to the following parameters: excitation wavelength - 229 nm; emission cut-off filter - 480 nm; time constant - 0.5s; a fine sensitivity setting of 5.00 on the 1.0 μA full-scale range setting.

The chromatograms were recorded using a two pen, 10-mv, electronic integrating recorder, Houston Instr. OmniScribe Model 5213-15, connected in parallel to a Hewlett-Packard Reporting Integrator, Model 3390A, on which the peak retention times, peak areas, ratio of peak area/height and relative peak areas were obtained.

Three different reverse-phase, octadecysilane columns were used and compared. A μBondapak C_{18} column (Waters Assoc. Part No. 27324, Serial No. 13909) was packed with 10 μm, irregularly shaped particles and had column dimensions of 30 cm x 3.9 mm i.d. The second column was a Partisil PXS 10 ODS-3 column, (Whatman Inc. Part No. 4228-111, Serial No. 1P3513) which was packed with 10 μm irregularly shaped silica and had column dimensions of 25 cm x 4.6 mm i.d. The third column was a Partisil PXS 5 ODS-3 column (Whatman Inc. Part No. 4238-111, Serial No. IS2004) which was packed with 5 μm irregularly shaped and also had column dimensions of 25 cm x 4.6 mm. i.d. In front of each one of the analytical columns, a guard column, hand-packed with 37-75 μm C_{18}-Corasil packing having dimensions of 5 cm x 4.6 mm i.d., was used. The temperature of the analytical column was controlled by a constant temperature, 27.5 ± .5°C, circulating water bath (Model T3, P.M. Tamson, Holland). The temperature was monitored by a Model 49TA digital thermometer from YSI Scientific. The column jacket was a precisely grooved aluminum piece made by the University of Connecticut, Chemistry Department's machine shop. The column conditioning recently described by Hill, et al., (4) was carefully followed.

Chemicals

Individual crystalline samples of taurine (TAU), DL-β-aminoisobutyric (β-ABA), and ethanethiol (ETSH) were obtained from Aldrich Chemical Co. DL-α-amino-n-butyric (α-ABA) was obtained from Sigma Chemical Co. The o-phthalaldehyde (OPA) (Fluoropa) and solid amino acid standard H (Part No. 20088) were obtained from Pierce Chemical Co. The acetonitrile (UV grade), methanol and tetrahydrofuran (UV grade) were of HPLC quality and obtained from either Burdick and Jackson Laboratories, Inc. or the MCB Manufacturing Chemists, Inc. The water used in preparing the reverse phase chromatographic solutions was first condensed from steam, then passed thru two mixed-ion exchange resins, one organic removal cartridge followed by a 0.20 μm submicron filter (NANOPURE, 4 module with pump, Sybron/Barnstead). The resulting water would qualify as reagent grade, type I-ASTM standard water having a specific conductivity greater than 10 Mohms/cm. All solutions and samples involved in the liquid chromatographic separation were filtered through 0.22 μm Millipore filters prior to use.

METHODS

Preparation of Solutions

Stock solutions, 1.00×10^{-4} M of each individual amino acid were carefully prepared in a 100-mL volumetric flask. The amino acids were dissolved and diluted with 0.1N aqueous hydrochloric acid solution. The o-phthalaldehyde solution was prepared by dissolving 1.00 g of OPA into 50 mL of methanol. This stock

OPA solution was protected from the light and kept refrigerated at +1°C when not in use. A dilute ethanethiol solution was prepared by adding 500 µL of ethanethiol to 25 ml of methanol, and the resulting solution was passed thru a Sep-Pak® C_{18} cartridge, Waters Assoc., in order to remove impurities. Because of the smell and the volatility of ETSH, all handling of these solutions should be done in an efficient hood. To reduce volatilization, it was found that the diluted ETSH/methanol solutions could be kept for periods of 3 to 5 days at -18°C. The derivatization of the amino acid standards was performed as follows: 20-100 nmol of the amino acid solutions in volumes of 200-1000 µL were placed in a 10-mL amber volumetric flask having a molded teflon stopper, exactly 1.00 mL of a borate buffer (4), 1.00 mL of the OPA solution and 1.00 mL of the ETSH solution were added. The resulting solution was diluted to the 10 mL mark with methanol, shaken and allowed to remain at room temperature for at least 10 min for completion of the reaction, and then analyzed by injecting 10 µL onto the HPLC system.

The tetrahydrofuran/acetonitrile/aqueous phosphate (10/10/80 by volume) solvent mixture was prepared as follows: to prevent the precipitation of the phosphate salt in tetrahydrofuran, it was found necessary to first mix the tetrahydrofuran with the acetonitrile. The stock aqueous phosphate buffer was prepared by mixing 14.10 g of $NaH_2PO_4 \cdot H_2O$ and 36.76 g of Na_2HPO_4 in reagent grade water and diluting to 1.0L. The resulting aqueous buffer solution had a pH of 7.15. Upon dilution, the solvent system consisted of

10 percent by volume of tetrahydrofuran, 10 percent acetonitrile and 80 percent aqueous phosphate buffer. This diluted solvent system was calculated to be 31.0 mM in sodium ion and 18.1 mM in phosphate ion. Previous reports have shown that it is important to control the phosphate concentration of the solvent system in order to optimize the separation of closely eluting peaks (1,4).

RESULTS AND DISCUSSION

Figure 1 shows the isocratic, acetonitrile/aqueous phosphate buffer (20/80 by vol) elution of the OPA/ETSH derivatized products of arginine (ARG), taurine (TAU), α-amino-n-butyric acid (α-ABA) and β-aminoisobutyric acid (β-ABA). The flow rate was 1.0 mL/min and the column was a μBondapak C_{18}. It should be noted at this optimized flow rate and without the use of a gradient, β-ABA co-eluted with TAU.

Various combinations of a ternary solvent system, acetonitrile/tetrahydrofuran/aqueous phosphate buffer were used. It was found that while maintaining a final organic volume ratio of 20 percent by volume, an interesting selectivity for the separation of α-ABA, β-ABA and Taurine could be achieved. By keeping the acetonitrile volume between 10 to 13 percent and by adjusting the tetrahydrofuran volume between 10 to 7 percent, β-ABA can be made to elute after α-ABA and taurine. This selectivity is important when studying the composition of physiological fluids because the concentrations of β-ABA, are often very low (15), and hence it is important to have β-ABA well separated from other

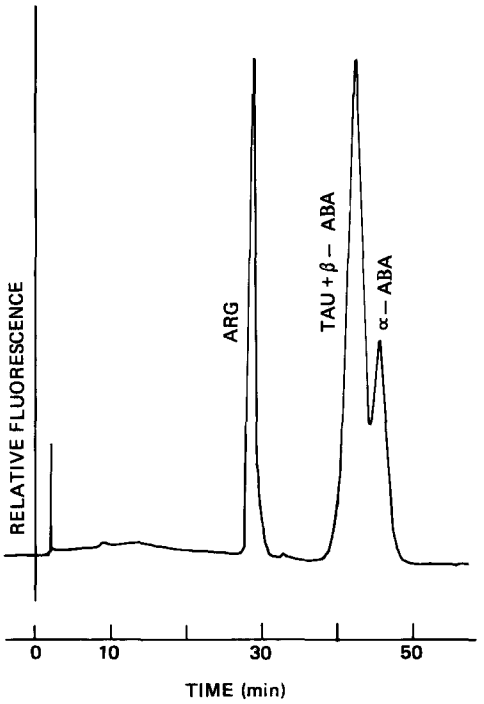


FIGURE 1. Isocratic Elution Profile of the OPA/ETSH derivatized arginine, taurine, α-amino-n-butyric and β-aminoisobutyric acids. Conditions: Column, μBondapak C_{18}, Solvent 20 percent acetonitrile/80 percent aqueous phosphate buffer, flow rate 1.0 mL/min.

closely eluting, derivatized amino acids. In each case, the individual derivatized standards was chromatographed separately to provide peak identification.

Figure 2 shows the improved resolution that is achieved upon using the efficient, 5- or 10-μm ODS columns. The chromatographic system used to compare the three reverse phase columns was identical in all other respects, involving isocratic elution at a flow rate of 1.0 mL/min. Table 1 summarizes the capacity factors, in

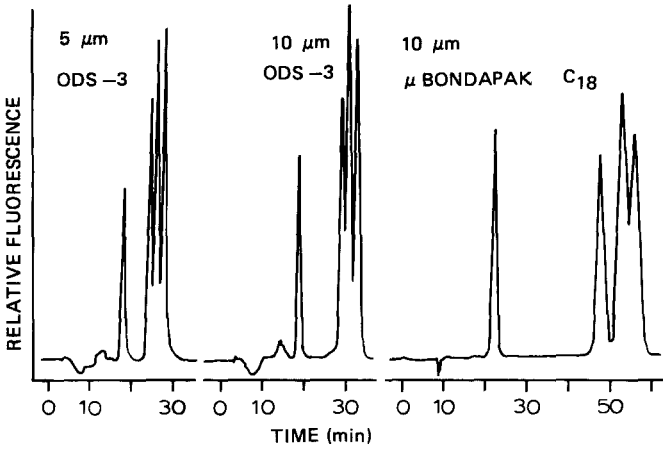


FIGURE 2. Comparison of the Elution Profiles from three different octadecylsilane, reverse phase columns, the 5- and 10-μm ODS-3 and the μBondapak C_{18}. Elution order of the OPA/ETSH derivatized compounds are in all cases arginine, α-Amino-n-butyric, taurine then β-aminoisobutyric acid. Solvent 10 percent acetonitrile/10 percent tetrahydrofuran/80 percent aqueous phosphate buffer, flow rate 1.0 mL/min for each column.

terms of k' for the three closely eluting, OPA/ETSH derivatives of α-ABA, β-ABA and taurine. Also included in Table 1 is the capacity factor for derivatized arginine which was used as a reference point. It has been shown that the retention time of the arginine does not vary with the change in the aqueous buffer concentration because the ARG/OPA/ETSH derivative is a zwitterion at the pH of 7.15 in the aqueous phosphate buffer system (3,4).

Figure 3 depicts the isocratic elution order of a series of standard OPA/ETSH derivatized amino acids marketed by the Pierce Chemical Co. as amino acid standard H, co-injected with derivatized α-ABA, β-ABA and TAU. It can be seen that with a ternary solvent system of acetonitrile/tetrahydrofuran/aqueous phosphate

TABLE 1

Capacity Factors For Taurine and Closely Eluting Amino Acids on Different C_{18} Columns[1]

Amino Acid	5 μm ODS-3	10 μm ODS-3	10μm μBondapak
Arginine	3.9	4.5	7.0
α-amino-n-butyric acid	5.7	7.6	15.7
taurine	6.1	8.1	16.9
β-aminoisobutyric acid	6.7	8.7	17.5

1. Isochratic solvent conditions, acetonitrile/tetrahydrofuran/aqueous phosphate buffer, pH=7.15, 31.0 mM Na and 18.1 mM PO_4, 10/10/80 by vol, flow rate 1.0 mL/min.

buffer and with the use of the efficient 5- or 10-μm reverse phase packings, adequate resolution of α-ABA, β-ABA and TAU is possible. This modification of Hill's et al., (4) solvent system and the use of 5-μm octadecylsilane packings is presently being used to resolve α-amino-n-butyric acid, β-aminoisobutyric acid and taurine in various psysiological fluids.

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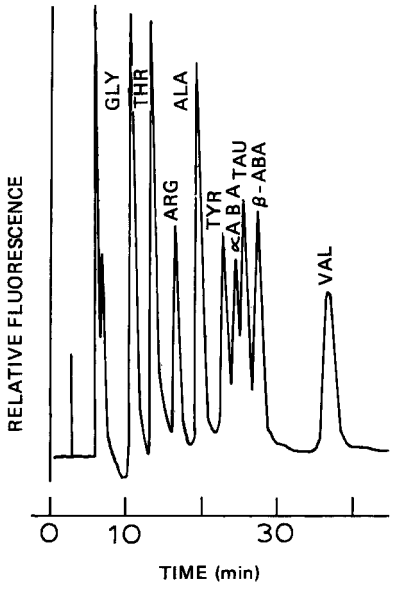

FIGURE 3. Isocratic Elution Profile of Certain of the OPA/ETSH derivatized amino acids: Column 5-um ODS-3, Solvent 10 percent acetonitrile, 10 percent tetrahydrofuran, 80 percent aqueous phosphate buffer, flow rate 1.0 mL/min.

the nature of the ODS-3 columns. Portions of this paper were presented at the 34th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Atlantic City, N.J., March 1983, Paper no. 829. Financial support for purchase of the instrumentation used in this research was provided by the National Institutes of Health Research Grant No. ES-01056, the Department of the Interior, Office of Water Resources and Technology Grant No. A-089-CONN and the University of Connecticut Research Foundation Grant 5171-35-453, for which publication costs for this paper were also received.

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HPLC RETENTION INDEX SCALE FOR NITROGEN-BRIDGED COMPOUNDS

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ABSTRACT

Retention indices of some nitrogen-bridged compounds having pharmacological activity have been determined. A retention index scale based on the relative retention of a homologous series of C_3-C_{23} 2-keto alkanes has been worked out. Linear relationships were found between RI and logP, allowing a prediction of retention indices.

The relationships between the structures and the retention indices of these compounds have been interpreted.

INTRODUCTION

Unlike gas chromatography, retention index RI for characterization of HPLC behaviour has been rarely used and only a few papers have been published [1,2]. In this work, we tried to determine HPLC retention indices to prove that RI can be used also in HPLC, i.e. may be useful in the field of SAR-research. In addition, are tried to predict the retention indices of these compounds using the linear correlation between logP and RI which was obtained. A series of nitrogen-bridged compounds, synthesized at our laboratory [3,4], seem to be good examples

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for our purpose (see Tables 1,2,3). The correlation if it exists between logP and RI may be useful to predict logP values i.e. biological activity for other compounds of the same structural type.

EXPERIMENTAL

Materials

All the model substances have been synthesized at our laboratory [3,4]. The identification and quality control of these compounds was made by melting point determination and chromatography.

All chemicals were analytical (Merck), and were used without further purification.

All solvents were HPLC grade (Merck), and were used without further purification.

Apparatus

The HPLC apparatus was from LABOR MIM LIQUOCHROM, MODEL 2010, Budapest, Hungary.

The reversed phase C_{18} column was 250 mm X 4.6 mm prepacked with 5 um particle size materials (Beckman).

10 ul of sample solutions (0.1 mg/ml in methanol) were injected.

Procedure

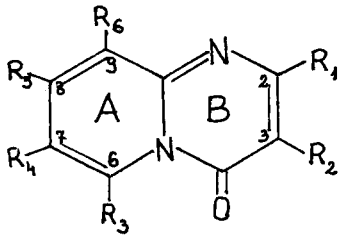
Three concentrations of methanol have been used: 70 %, 60 %, 50 %; each contained a 0.025 M NaH_2PO_4 buffer that had been adjusted to pH 7.0 before adding the methanol.

The flow rate was 0.7 ml/min.

Table 1

Structure of model substances

pyridopyrimidines with unsaturated "A"-ring

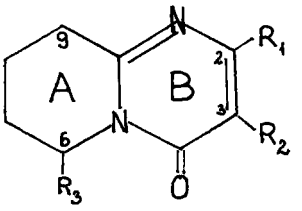


Nr	Substituents on C_2	C_3	C_6	C_7	C_8	C_9
1	H	H	H	H	H	H
2	CH_3	H	H	H	H	H
3	H	CH_3	H	H	H	H
4	H	H	CH_3	H	H	H
5	H	H	H	CH_3	H	H
6	H	H	H	H	CH_3	H
7	H	H	H	H	H	CH_3
8	CH_3	CH_3	H	H	H	H
9	CH_3	H	CH_3	H	H	H
10	CH_3	H	H	H	H	CH_3
11	H	CH_3	CH_3	H	H	H
12	CH_3	H	C_2H_5	H	H	H
13	H	C_2H_5	CH_3	H	H	H
14	CH_3	C_2H_5	CH_3	H	H	H
15	CH_3	C_2H_5	CH_3	H	CH_3	H
16	C_2H_5	CH_3	CH_3	H	H	H
17	C_3H_7	C_2H_5	CH_3	H	H	H

Table 2

Structure of model substances

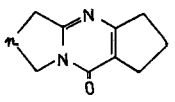
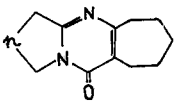
pyridopyrimidines with saturated "A"-ring



Nr	Substituents on			
	C_2	C_3	C_6	C_9
18	H	H	H	H
19	CH_3	H	H	H
20	H	H	CH_3	H
21	CH_3	CH_3	H	H
22	CH_3	H	CH_3	H
23	H	CH_3	CH_3	H
24	CH_3	C_2H_5	CH_3	H
25	CH_3	H	H	CH_3

Table 3. Structure of model substances

Three ring systems with different ring size

Number of compounds			Number of compounds		
26.	n = 1		38.	n = 1	
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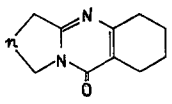
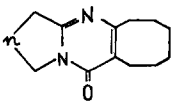
28.	n = 3		40.	n = 3	
29.	n = 4		41.	n = 4	
30.	n = 1		42.	n = 1	
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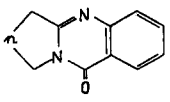
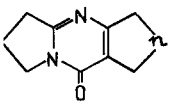
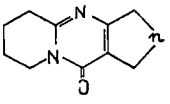
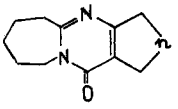
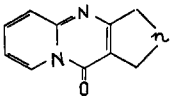
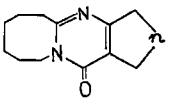
32.	n = 3		44.	n = 3	
33.	n = 4		45.	n = 4	
34.	n = 1		46.=26.	n = 1	
35.	n = 2		47.	n = 2	
36.	n = 3		48.	n = 3	
37.	n = 4		49.	n = 4	
34.a	C_8 Me		47.a	C_8 Me	
34.b	C_3 Me				
36.a	C_3 Me				
50.	n = 1		58.	n = 1	
51.=31.	n = 2		59.	n = 2	
52.	n = 3		60.	n = 3	
53.	n = 4		61.	n = 4	
54.	n = 1		62.	n = 1	
55.	n = 2		63.	n = 2	
56.	n = 3		64.	n = 3	
57.	n = 4		65.	n = 4	

All the experiments were run at room temperature 25 °C.

Measurement of retention indices

The retention indices have been calculated using the following equation:

$$I = 100 \frac{\log K_D - \log K_N}{\log K_{N+1} - \log K_N} + 100\ N \qquad \text{equ.n. 1.}$$

where

K_D = the capacity factor of the drug

K_N + the capacity factor for 2-keto alkane eluting just before the test compound.

K_{N+1} = the capacity factor for 2-keto alkane eluting just after the test compound.

Results and discussion

Retention indices of about 40 compounds have been calculated using equation 1. LogP values for the same compounds have been published earlier [5,6]. The correlations between logP and RI values are shown in Fig. 1, 2 and in Table 5.

The best linear relationship was found between logP and measured RI for the 2-ring and 3-ring nitrogen-bridged compounds at a solvent composition of 70 % methanol and 30 % buffer (pH 7) using a C_{18} column (see Fig. 1 and Fig. 2). From this correlation, either retention index or the logP of the same compounds may be obtained. Table

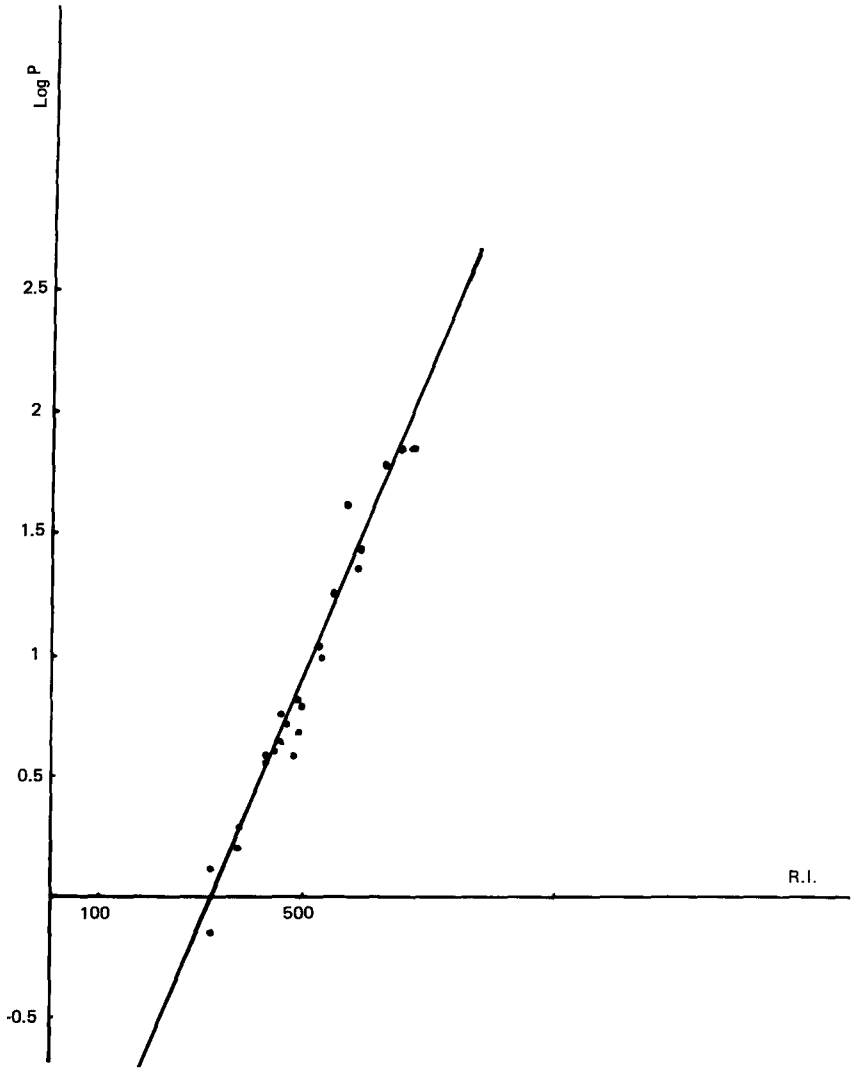


Fig. 1 Relationship between logP and RI of 2-ring compounds (saturated, unsaturated)

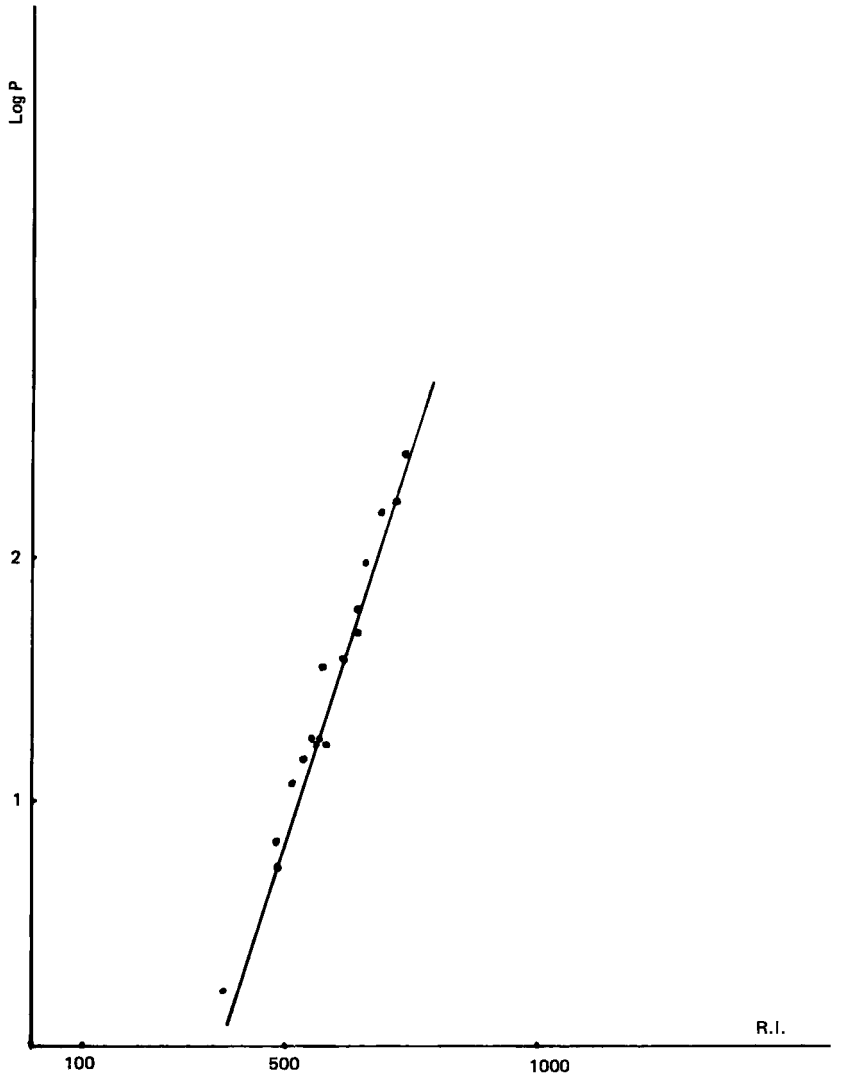


Fig. 2 Relationship between logP and RI of 3-ring compounds (saturated, unsaturated)

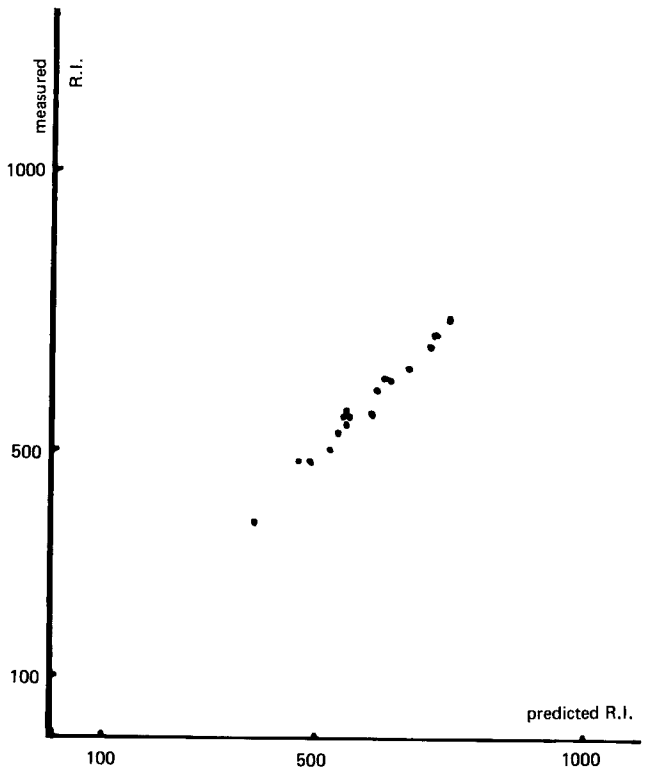
4, (a,b,c,d) and Fig. 3 also show that the measured and the predicted retention indices are very close.

As may be expected, a change in mobile phase concentration has almost no effect on the retention index values for the 2-ring and 3-ring compounds (Fig. 4).

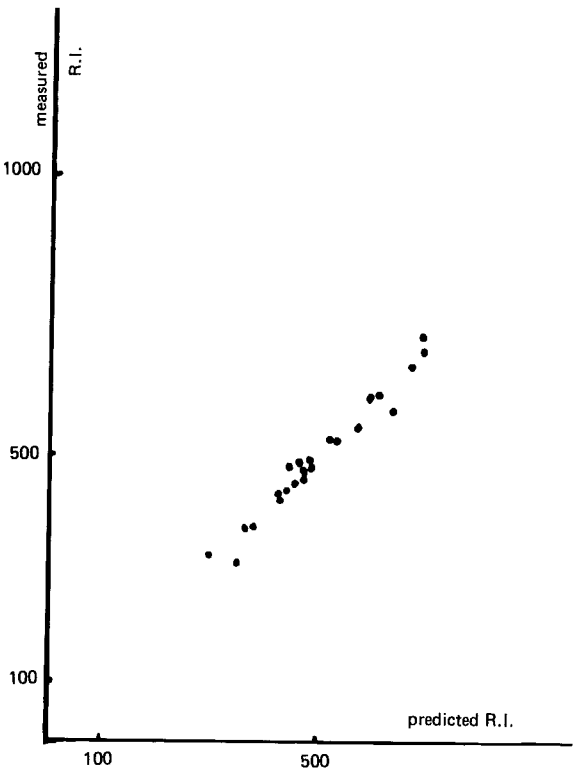
As an alternative, the RI values of the model compounds can be determined by graphical interpolation of a plat of the capacity factor vs. retention index of a standard series of 2-ketoalkanes. Now, the measured logK of the test compound, as well as the assigned values of the ketoalkanes e.g. acetone 300, 2-butanone 400, etc. will be used see (Fig. 5).

In general we can see that the RI values of unsaturated compounds are higher than those of the saturated compounds; this due to that the saturated compounds have been found more polar than the unsaturated. The difference in RI is lower for the unsubstituted pairs of compounds unsaturated-saturated Nr. 1, 18 than in case of methylsubstituted pairs (2, 19; 4, 20; etc.). This may be due to the increasing of the hyperconjugation and the electronic activity of the methyl group for the unsaturated compounds. This conclusion was found for the 2-ring compounds as well as the 3-ring compounds (see the RI values of compounds No. 2-8-9-10, and No. 19-21-22-25 resply Table 4/a, 4/b and Fig. 6).

As expected, RI values increase systematically on increasethe number of carbon atoms; the increment is nearly constant regardless of the position of the rings.



B



A

Fig. 3 Comparison of predicted and measured HPLC retention indexes for 2-ring compound A and 3-ring compound B

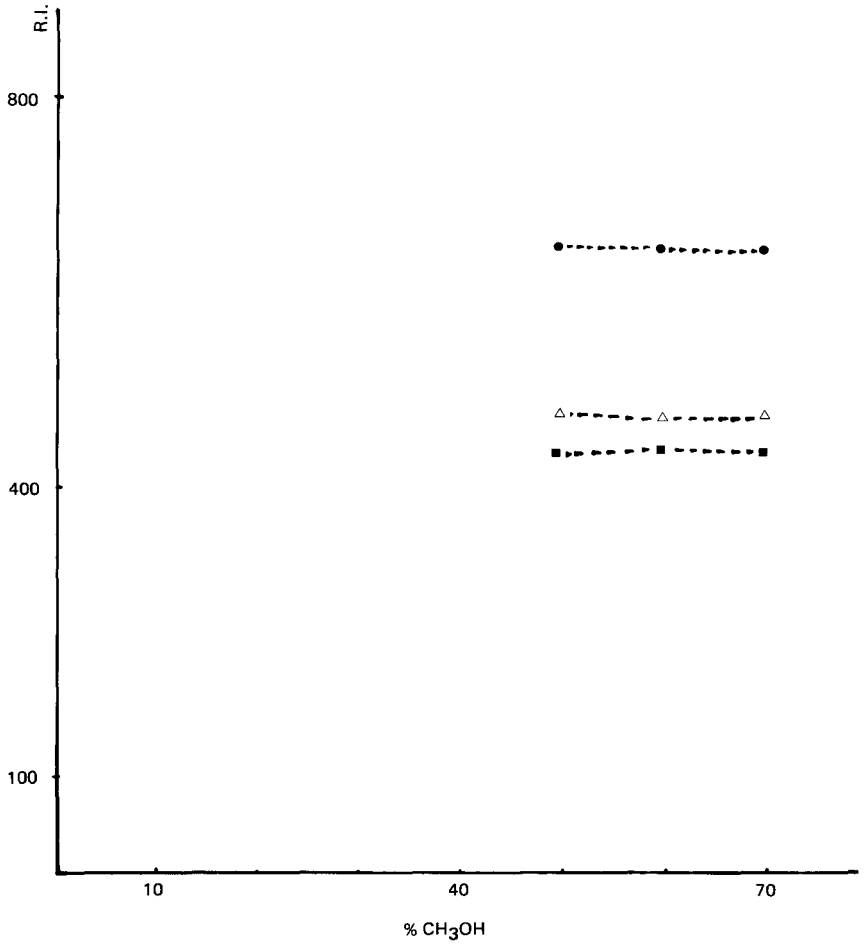


Fig. 4 Effect of methanol concentration of RI of some selected drugs: ● compound n. 32; △ compound n. 4; □ compound n. 2

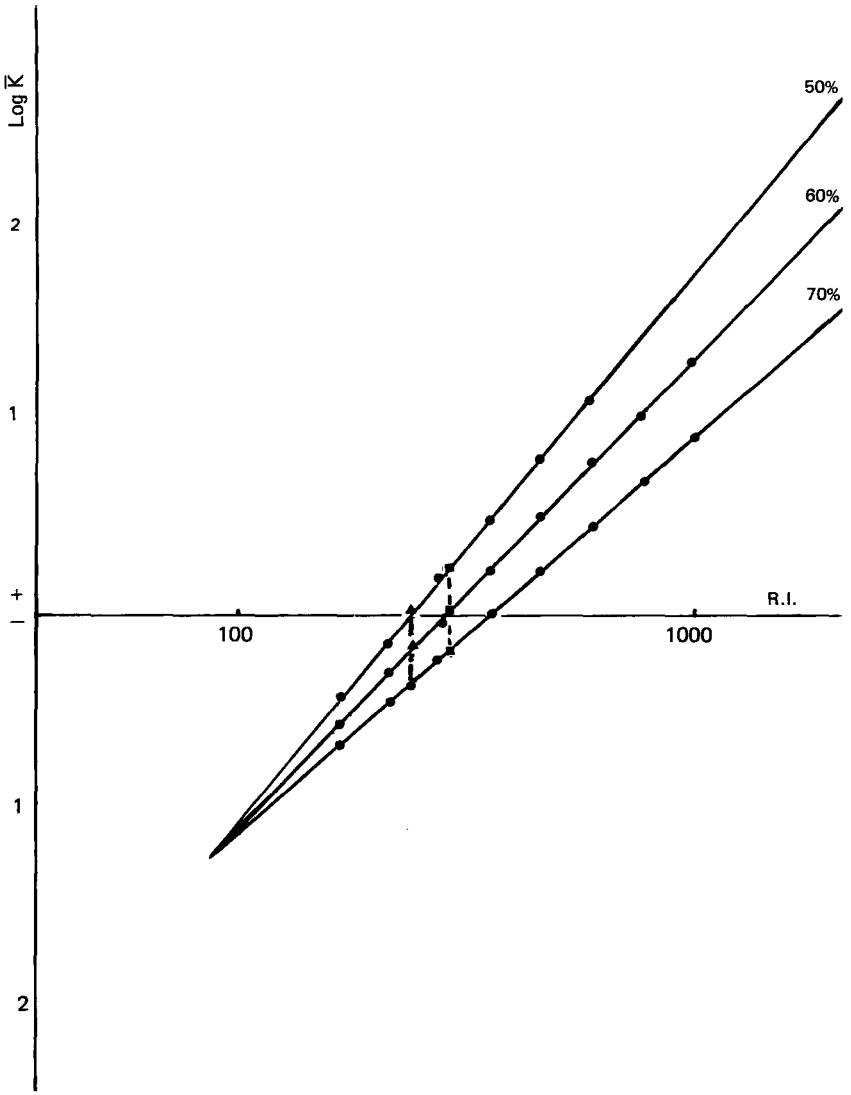


Fig. 5 Determination of RI of some selected drugs (△compound n. 5 and □compound n.34) using series of 2-keto-alkanes at three different concentrations of methanol

Table 4. /a/

Number of compounds	RI		logP	
	measured	calculated	measured	calculated
1	377	366	0,2043	0,2562
2	427	439	0,5724	0,5082
3	458	479	0,7712	0,6643
4	473	472	0,7360	0,7399
5	443	446	0,6086	0,5888
6	443	437	0,5624	0,5888
7	499	483	0,7940	0,8709
9	535	535	1,0530	1,0523
11	558	577	1,2646	1,1683
8	535	524	1,0007	1,0523
10	608	596	1,3641	1,4202
12	585	648	1,6254	1,3043
13	661	681	1,7926	1,6873
15	696	694	1,8561	1,8636
16	719	696	1,8670	1,9795

Table 4. /b/

Number of compounds	RI		logP	
	measured	calculated	measured	calculated
18	331	297	-0,1436	0,0244
19	314	350	0,1212	-0,0612
20	377	385	0,2989	0,2562
21	487	444	0,5969	0,8105
22	452	458	0,668	0,6341
23	487	489	0,8220	0,8105
25	499	462	0,6894	0,8709
24	619	612	1,4416	1,4756

Table 4. /c/

Number of compounds	RI		logP	
	measured	calculated	measured	calculated
30	487	484	0,831	0,8465
31	578	549	1,231	1,4869
32	644	641	1,799	1,8164
33	696	705	2,196	2,1376
26	377	385	0,221	0,1670
27	487	465	0,716	0,8465
28	551	554	1,262	1,2419
40	716	712	2,238	2,2611
47/a	565	547	1,222	1,3283
48	565	552	1,249	1,3283
55	639	625	1,703	1,7855
54	535	543	1,194	1,1430

Table 4. /d/

Number of compounds	RI		logP	
	measured	calculated	measured	calculated
34	509	523	1,074	0,9824
35	-	595	1,515	-
36	649	672	1,995	1,8472
34/a	565	606	1,586	1,3283
34/b	617	607	1,591	1,6496
34/b	743	744	2,439	2,4279

Table 5

Relationship between RI and logP for 2- and 3-ring compounds

A: 2-ring compounds:	n = 23
m	= 0.005038
b	= -1.64308
r^2	= 0.95536
r	= 0.97742
$S_{RI\ measured} / S_{RI\ calculated}$	= 97.80 %
$S_{logP\ measured} / S_{logP\ calculated}$	= 97.73 %
B: 3-ring compounds	n = 17
m	= 0.0061774
b	= -2.161824
r^2	= 0.96657
r	= 0.983143
$S_{RI\ measured} / S_{RI\ calculated}$	= 98.75 %
$S_{logP\ measured} / S_{logP\ calculated}$	= 98.71 %

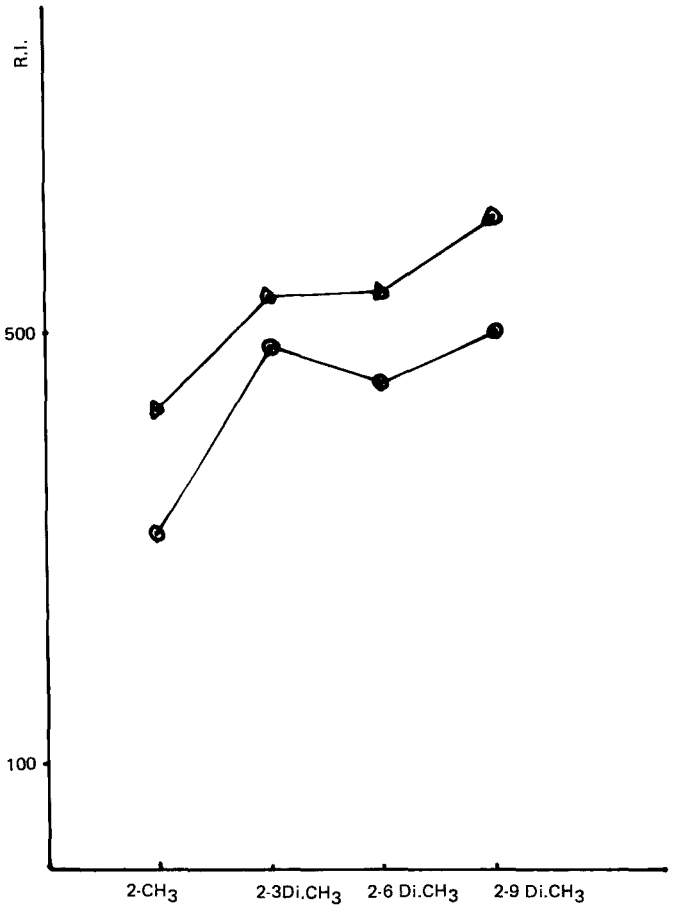


Fig. 6 Relationship between RI and the structure of some tested compounds (△unsaturated 2-ring compounds and ○saturated 2-ring compounds)

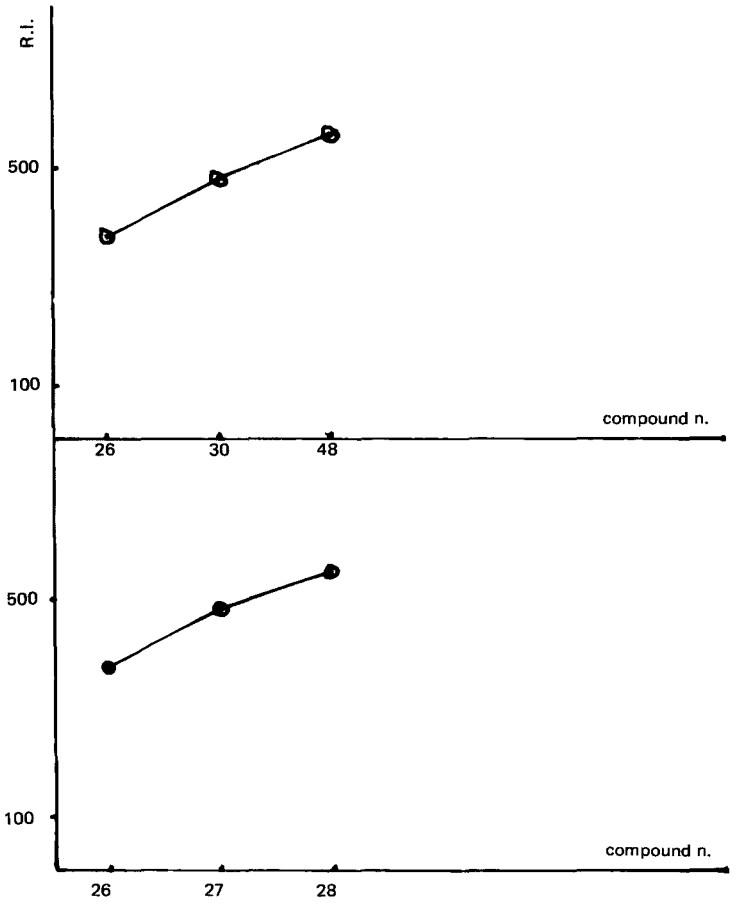


Fig. 7 Relationship between RI and some tested 3-ring compounds

This is illustrated in Fig. 7 compounds No. 26, 30, 48 where the increment in RI values are 110, 78 and also in compounds number 26, 27, 28 where the increments in RI are 110, 74 respectively . We can see, in the same figure, the similarity in RI between compounds number 27 and 30, and compounds No. 48 and 28 which differ from each other only in the positions of the rings. A similar observation was mode for the unsaturated compounds see Table 3, compound No. 34 and 54 .

ACKNOWLEDGEMENT

One of the Authors A.S. would like to thank Dr. H.J. Issaq and Dr. H. Bauer for their encourgement and sincere support.

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HPLC OF NITROGEN-BRIDGED COMPOUNDS

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*Physiological Chemistry Institute, Tübingen University, West-Germany

ABSTRACT

In the area of structure-activity relationships of nitrogen bridged compounds, certain structure-coherent physical properties with ion exhange HPLC behaviour has been studied. This paper illustrates the results in finding the best conditions to separate the various structural types of model compounds. For this purpose, ion exhange HPLC technique has proved as highly advantageous.

INTRODUCTION

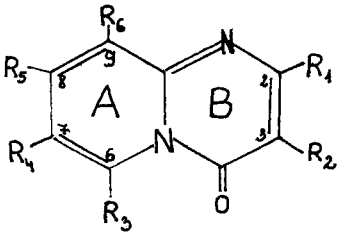
In our previous work[1], we reported the results we achieved by the same chromatographic technique C_{18} reversed phase HPLC for the same model compounds[2], pyridopyrimidines with unsaturated and saturated A-ring Table 1, Table 2 and three ring systems with different ring size Table 3 .

 0148-3919/84/0706-1151$3.50/0

Table 1.

Structure of model substances

pyridopyrimidines with unsaturated "A"-ring

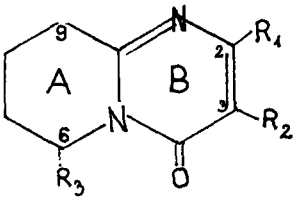


Nr	Substituents on C_2	C_3	C_6	C_7	C_8	C_9
1	H	H	H	H	H	H
2	CH_3	H	H	H	H	H
3	H	CH_3	H	H	H	H
4	H	H	CH_3	H	H	H
5	H	H	H	CH_3	H	H
6	H	H	H	H	CH_3	H
7	H	H	H	H	H	CH_3
8	CH_3	CH_3	H	H	H	H
9	CH_3	H	CH_3	H	H	H
10	CH_3	H	H	H	H	CH_3
11	H	CH_3	CH_3	H	H	H
12	CH_3	H	C_2H_5	H	H	H
13	H	C_2H_5	CH_3	H	H	H
14	CH_3	C_2H_5	CH_3	H	H	H
15	CH_3	C_2H_5	CH_3	H	CH_3	H
16	C_2H_5	CH_3	CH_3	H	H	H
17	C_3H_7	C_2H_5	CH_3	H	H	H

Table 2

Structure of model substances

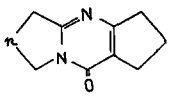
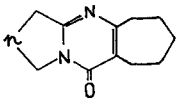
pyridopyrimidines with saturated "A"-ring



Nr	Substituents on			
	C_2	C_3	C_6	C_9
18	H	H	H	H
19	CH_3	H	H	H
20	H	H	CH_3	H
21	CH_3	CH_3	H	H
22	CH_3	H	CH_3	H
23	H	CH_3	CH_3	H
24	CH_3	C_2H_5	CH_3	H
25	CH_3	H	H	CH_3

Table 3. Structure of model substances

Three ring systems with different ring size

Number of compounds			Number of compounds		
26.	n = 1		38.	n = 1	
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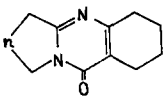
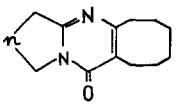
30.	n = 1		42.	n = 1	
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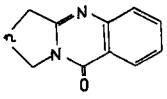
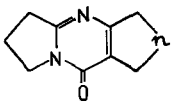
34.	n = 1		46.=26.	n = 1	
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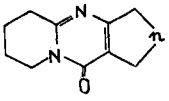
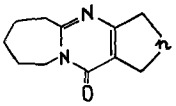
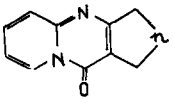
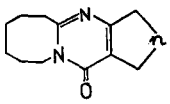
34.b	C_3 Me				
36.a	C_3 Me				
50.	n = 1		58	n = 1	
51.=31.	n = 2		59.	n = 2	
52.	n = 3		60.	n = 3	
53.	n = 4		61.	n = 4	
54.	n = 1		62.	n = 1	
55.	n = 2		63.	n = 2	
56.	n = 3		64.	n = 3	
57.	n = 4		65.	n = 4	

Table 4

The capacity factor and resolution factor of the HPLC system for some pairs

The pair of compounds	K						R_s					
	40%			6,7 pH			40 %			6,7 pH		
	4.7	5.5	6.7	30 %	40 %	50 %	4,7	5,5	6,7	30 %	40 %	50 %
1 18	1.421 1.578	1.421 0.895	1.105 1.105	1.631 1.526	1.105 1.105	0.421 0.421	2,5	2,4	0.0	0.19	0.0	0.0
2 4	2.473 3.947	1.210 1.684	1.473 1.842	2.263 2.842	1.473 1.842	0.578 0.526	1.8	1.8	1.8	0.69	1.8	0.27
4 9	3.947 6.736	1.684 2.368	1.842 2.474	2.842 3.947	1.842 2.474	0.526 0.578	2.7	2.2	2.2	1.08	2.2	0.25
9 12	6.736 8.368	2.368 2.895	2.474 3.368	3.947 5.821	2.474 3.368	0.578 0.684	1.59	1.4	2.5	1.03	2.5	0.5
18 19	1.578 1.842	0.895 1.033	1.105 1.210	1.526 1.105	1.105 1.210	0.421 0.421	0.46	0.7	0.44	0.95	0.44	0.0
20 22	1.421 2.263	0.789 1.053	1.00 1.368	1.589 2.263	1.00 1.368	0.368 0.368	1.7	1.1	1.5	1.5	1.5	0.0
26 27	1.736 3.315	1.210 1.474	1.631 2.474	2.526 4.157	1.631 2.474	0.578 0.593	2.02	0.9	2.6	1.9	2.6	0.5
30 31	3.105 8.578	1.947 3.263	2.526 3.789	4.316 6.578	2.526 3.789	0.736 0.842	5.2	3.3	2.6	2.3	2.5	0.5
34 35	2.789 6.00	1.263 2.842	2.47 3.94	4.894 7.315	2.474 3.947	0.631 0.842	2.93	0.6	1.25	1.15	1.25	0.2
38 28	4.157 3.315	2.105 2.105	2.842 2.789	5.00 4.736	2.842 2.789	0.632 0.632	0.9	0.0	0.12	0.25	0.12	0.0

Stationary phase: Nucleosil 5 SA /NH/
Mobile phase: Methanol-water /KH_2PO_4 / solvent mixture
Methanol content : 30 %, 40 %, 50 %; pH of the mixture 4.7-5.5-6.7 respectively

Table 4.a

	$\bar{k}$ at different pH using 40 % CH_3OH			$\bar{k}$ at different concentration of $\bar{e}$ CH_3OH $\bar{e}$ constant pH 6.7		
Compounds number	4.7	5.5	6.7	30 %	40 %	50 %
1	1.421	1.421	1.105	1.631	1.105	0.421
2	2.473	1.210	1.473	2.263	1.473	0.578
3	2.578	1.316	1.736	2.631	1.736	0.526
4	3.947	1.684	1.842	2.842	1.842	0.526
5	2.473	1.526	1.684	2.684	1.684	0.526
6	2.894	1.447	1.736	2.578	1.736	0.526

7	1.789	1.105	2.157	2.210	1.789	0.421
8	5.736	2.105	2.421	3.947	2.421	0.631
9	6.736	2.368	2.474	3.947	2.474	0.578
10	2.631	1.474	1.947	3.210	1.947	0.473
11	7.421	2.632	2.263	4.737	2.263	0.684
12	8.368	2.895	3.368	5.821	3.368	0.684
13	35.105	3.842	4.210	10.052	4.210	0.789
14	19.315	6.789	5.263	10.210	5.263	0.921
15		18.895	12.631		12.631	1.368
16	33.42	5.895	5.109	18.046	5.105	0.947
17		16.211	10.579		10.579	1.210

Table 4.b

Compounds number	$\bar{k}$ at different pH using 40 % CH_3OH			$\bar{k}$ at different concentration of CH_3OH & constant pH 6.7		
	4.7	5.5	6.7	30 %	40 %	50 %
18	1.578	0.895	1.105	1.526	1.105	0.421
19	1.842	1.033	1.210	1.105	1.210	0.421
20	1.421	0.789	1.000	1.589	1.000	0.368
21	4.684	1.694	1.815	3.158	1.815	0.578
22	2.263	1.053	1.368	2.263	1.368	0.368
23	2.894	1.316	1.632	2.684	1.632	0.447
24	2.157	0.895	1.211	2.00	1.211	0.368
25	6.578	2.605	2.789	5.158	2.789	0.526

Table 4.c

Compound's number	$\bar{k}$ at different pH using 40 % CH_3OH			$\bar{k}$ at different concentration CH OH e constant pH 6.7		
	4.7	5.5	6.7	30 %	40 %	50 %
26	1.736	1.210	1.631	2.526	1.631	0.578
28	3.315	2.105	2.789	4.736	2.789	0.632
30	3.105	1.947	2.526	4.316	2.526	0.736
31	8.578	3.263	3.789	6.578	3.789	0.842
38	4.157	2.105	2.842	5.00	2.842	0.632
47.d	16.578	2.211	2.894	5.210	2.894	0.684
48	4.157	2.105	2.842	5.00	2.842	0.632
49	4.157	2.421	3.421	6.263	3.421	0.684
50	3.315	1.474	2.474	4.157	2.474	0.684
51	8.578	3.263	3.789	6.578	3.789	0.842
59	8.157	3.211	3.263	8.263	3.263	0.789
60	9.421	3.579	4.842	9.526	4.842	0.842
63	10.369	3.947	5.263	10.684	5.263	0.842
65		6.263	8.894	21.631	8.894	1.105

Table 4.d

Compounds number	$\bar{k}$ at different pH using 40 % CH_3OH			$\bar{k}$ at different concentration of CH_3OH $\bar{e}$ constant pH 6.7		
	4.7	5.5	6.7	30 %	40 %	50 %
34	2.789	1.263	2.474	4.894	2.474	0.631
35	6.00	2.842	3.94	7.315	3.947	0.842
36	6.052	2.578	4.631	9.00	4.631	0.789
34.a	3.526	2.158	3.00	5.684	3.000	0.631
34.b	4.789	2.316	4.263	8.210	4.263	0.789
36.a	11.473	6.211	8.526	20.894	8.526	1.053

To find the optimum conditions for the separation of the previous compounds, the capacity factor has been calculated in various pH's and concentrations of methyl alcohol (see Table 4, a,b,c,d); also, the resolution factor for some pairs of these compounds have been calculated.

EXPERIMENTAL

1. Materials

All model substances have been synthesed at our laboratory; the identification and control of these compounds was made by melting point determination and chromatography.

All other chemicals were analytical grade Merck, West Germany and used without further purification.

The HPLC grade of the solvents (Merck, West Germany) was used without further purification.

2. Apparatus

Biotronic UV Detector BT 3030

Biotronic HPLC pump BT 3020

3. Chromatographic procedure

A 250 mm x 4,6 mm Nucleosil column 5 SA/mn was used.

10 ul sample solutions 0.1 ug/ml in methanol were injected.

Experiments were run at room temperature using a mobile phase flow rate 1 ml/min.

Results and discussion

Table 4 shows the retention parameters and resolution factors for some pairs of homologues and structural isomers.

The pK values of these weak bases fall within a relatively narrow range (3.2-4); therefore at, pH 4.7, the compounds may be taken as partially protonated, while at pH 6.7 only the base form is assumed to exist. Consequently the chromatographic process is governed by the following equilibria

I $R - SO_3H + BH^+X^-$ $R - SO_3BH + HX$
At pH 4.7 (ion exchange)

II $R - SO_3H + B$ $R - SO_3H^{(+)} \cdots\cdots B^-$
At pH 6.7 (partition)

As may be seen from the R_s values, the selected pairs of various homologous compounds are separated very sharply by ion exchange liquid chromatography with a mobile phase containing 40 % methylalcohol at pH 4.7 (see R_s values of compounds 1-2, 9-12, 9-4, etc.).

This chromatographic system obviously offers optimal conditions for HPLC separation of our pyridopyrimidine model substances (Table 1,2).

Essentially the same conclusions may be drawn for the three-ring homologous with different ring sizes (see R_s values of compounds 34-35, 26-27).

That the higher homologous with C_2, C_6 methyl substituents have higher k' values may be a consequence of the hyperconjugational effect of the methyl group, i.e., the strong Lewis base character of the compound Nr 9, 2, 4. The lack of hyperconjugational effect in the methyl derivatives with a saturated A-ring causes reduced retention

(smaller k' values) of this type of compound (Nr 18, 19, 20, 22).

As a proof of the partition mechanism (see equ. 2), the k' value for the same compound are smaller when mobile phase containing 40 % methanol and pH 6.7 was used compared with the k' values are determined using acidic developing solvents when ion exchange mechanism works.

At pH 6.7 increasing the methanol concentration causes the decrease of k' values. In general the resolution is poorer than in case of the ion exchange version.

As shown in Table 5, the log P values of the investigated series of compounds correlate rather well with log k' values, which were generated at pH = 6.7. Much poorer correlation was obtained with the more acidic solvent mixture (pH 5.5, 4.7). This may be taken as another proof of the partition character of the chromatographic process when the base form of the compounds exist.

By contrast, the correlation was found definitely better with the log k' values obtained with the acidic developing mixture when pK values were used as variables (Table 6). This experience gives strong support to the assumption that the protonated form of the compounds moves with an ion exchange mechanism and the retention is controlled mainly by the strength of the base (i.e. the acidity of the ammonium cation).

As shown in Fig. 1, the applied chromatographic system indicates rather high selectivity; the small structural differences are reflected much more by the HPLC

Table 5

Relationship between logK' and logP values

$\log K'_{pH=4,7}$ = 0,513 logP - 0,218	r = 0,671	n = 15	/Table 1/	
$\log K'_{pH=6,7}$ = 0,409 logP - 0,051	r = 0,967	n = 17	/Table 1/	
$\log K'_{pH=4,7}$ = 0,397 logP = 0,184	r = 0,823	n = 8	/Table 2/	
$\log K'_{pH=6,7}$ = 0,254 logP = 0,015	r = 0,849	n = 8	/Table 2/	
$\log K'_{pH=4,7}$ = 0,362 logP + 0,244	r = 0,772	n = 12	/Table 3, saturated rings/	
$\log K'_{pH=6,7}$ = 0,250 logP + 0,174	r = 0,950	n = 12	/Table 3, saturated rings/	

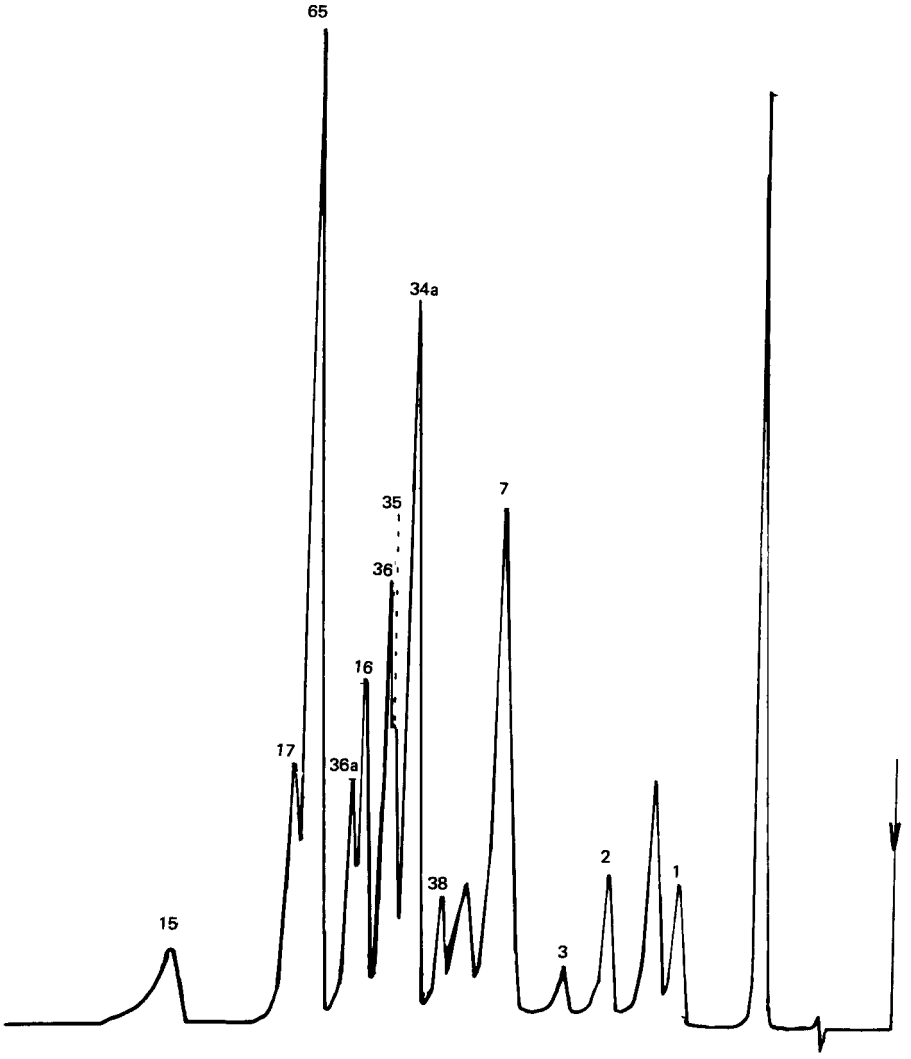


Fig. 1. The separation of mixture of some nitrogen bridged compounds by gradient linear elution technique. /Stationary phase nucleosil 5 SA/mn, mobile phase a 0.1 Mol KH_2PO_4, - b 0.01 Mol KH_2PO_4 in methanol--water 40:60, pH from 3 to 5.5, flow rate 1 ml/min./

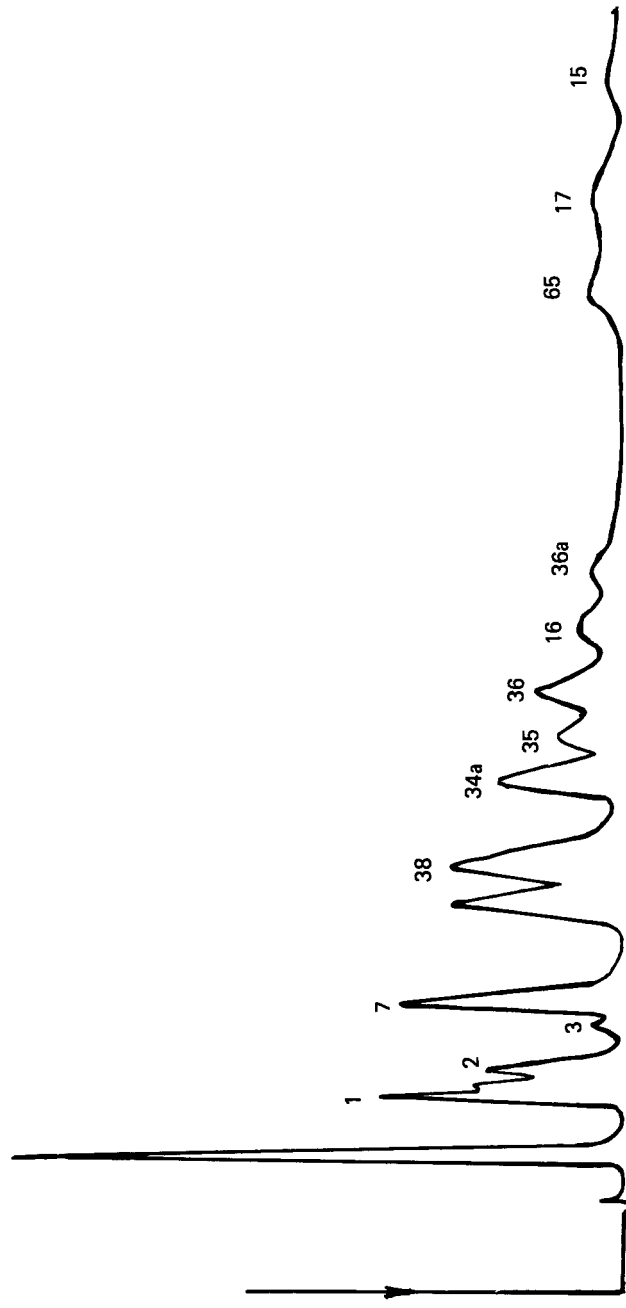


Fig. 2. The separation of mixture of some nitrogen bridged compounds with isocratic elution technique /stationary phase nucleosil 5 SA/mn, mobile phase methanol-water 40:60, pH 5.5, flow rate 1 ml/min./

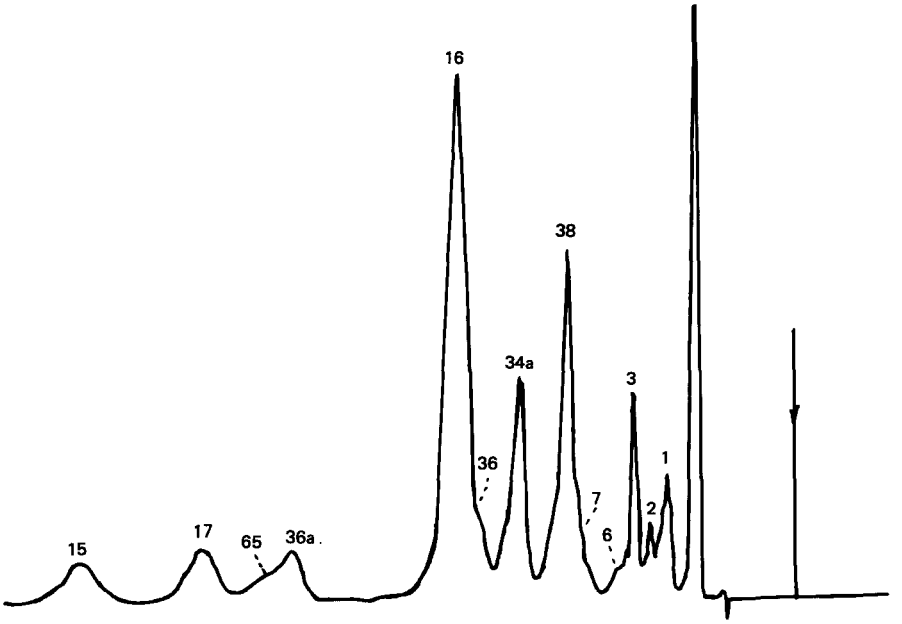


Fig. 3. The separation of mixture of some nitrogen bridged compounds with isocratic elution technique /stationary phase nucleosil 5 SA/mn, mobile phase methanol-water 40:60, pH 6.7, flow rate 1 ml/min./

Table 6

Relationship between logK' and pK values

$\log K'_{pH=6,7} = 0,234\ pK - 0,301$ r = 0,759 n = 11 /Table 3/
$\log K'_{pH=4,7} = 0,312\ pK - 0,435$ r = 0,909 n = 11 /Table 3/

behaviour when the gradient elution technique is used (compare the Figures 1 and 2 and 3).

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DETERMINATION OF GLUTAMINE AND ASPARAGINE BY ISOCRATIC ELUTION REVERSE PHASE LIQUID CHROMATOGRAPHY WITH FLUORESCENT DETECTION

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ABSTRACT

A method was developed specifically for the determination of glutamine and asparagine in the presence or absence of other amino acids. The amino acids were derivatized by o-phthalaldehyde/ 2-mercaptoethanol and separated by isocratic elution with a mobile phase consisting of acetonitrile and sodium acetate buffer. An application of the method for the analysis of glutamine and asparagine in the enzymatic hydrolysate of cottonseed protein is described.

INTRODUCTION

In routine procedure for the determination of amino acid compositions of proteins and peptides, glutamine and asparagine are deamidated to glutamic acid and aspartic acid, respectively, during acid hydrolysis. It has therefore been customary in the analysis of protein hydrolysates to sum glutamine and glutamic acid as Glx and to sum asparagine and aspartic acid as Asx. The recovery of

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glutamine and asparagine in protein hydrolysis is difficult. Equally difficult is the analysis of glutamine and asparagine because there is a lack of standard methods for the identification and quantitation of these amino acids.

Glutamine and asparagine can normally be obtained by enzymatic hydrolysis. After they are separated from the protein, the free glutamine and asparagine can then be analyzed by the use of lithium buffers on ion-exchange resins (1). This ion-exchange method, though useful, is more complicated than desirable unless there is interest in the other amino acids as well, or alternately, if there are no other amino acids present.

Recently high performance liquid chromatography (HPLC) has been widely employed for the analysis of amino acids (2-7). This technique, especially with reversed-phase columns, offers greater efficiency, ease of use, and higher flow rates than the conventional ion-exchange techniques. However, for the complete analysis of protein hydrolysates, the effectiveness of HPLC in terms of reliability and reproducibility is still limited. HPLC is more suitable for the analysis of a few specific amino acids. Most reports deal with the HPLC analysis of all the amino acids, and the information is generally incomplete and insufficient in regard to the analysis of glutamine and asparagine.

We are interested in the analysis of glutamine and asparagine because our research concerns oilseed proteins which are rich in these amino acids. The ability to separate and quantitate these amino acids is very desirable for the characterization of the

oilseed proteins.

In this study we (a) report a fast and reliable method for the analysis of glutamine and asparagine by HPLC, (b) evaluate the use of enzymes in the hydrolysis of protein, and (c) estimate the ratios of glutamine : Glx and of asparagine : Asx in the enzymatic hydrolysate of cottonseed protein.

MATERIALS AND METHODS

Apparatus

Analyses were performed on a Beckman Model 324 gradient liquid chromatograph equipped with a Beckman Model 421 microprocessor-controller and a Krato Model FS970 liquid chromatographic fluorometer. The following fluorometer settings were used for detection: 5 μl flow cell, excitation monochrometer at 330 nm, the emission measured with a 418 nm cut-off filter, time constant of 0.5 sec, and a sensitivity dial setting at 4.7 units.

All sample injections were performed with a Beckman Model 210 sample injector, fitted with a 5 μl loop. A Beckman Ultrasphere ODS column (150 x 4.6 mm; particle size, 5 μ) fitted with a guard column (70 x 4.6 mm) packed with CO:PELL ODS sorbent (particle size, 30-38 μ) (Whatman) was used for chromatographic separations. Chromatographic peaks were recorded on a Beckman Model BD-41 recorder, and integrated by A/D converters in a Hewlett Packard 3345B laboratory automation system. Isocratic elution was used, unless otherwise indicated, in the chromatographic separation, and

the flow rate was 1.0 ml/min. The elution of the amino acids of our interest was complete in 20 min. In the case of protein hydrolysate, the elution was programmed at the end of 20 min to change to 100% acetonitrile in 5 min to wash off the remaining amino acids, followed by changing back to the original solvent in 5 min and then equilibrating for at least 10 min before conducting another injection.

Reagents and Solutions

Amino acids, 2-mercaptoethanol (ME), and o-phthalaldehyde (OPA) were purchased from Sigma Chemical Co. Storage protein of glandless cottonseed flour (Southern Regional Research Center) was prepared according to the method of Zarins and Cherry (8). Viokase was obtained from Viobin Incorporated. All other chemicals were reagent grade.

The o-phthalaldehyde/2-mercaptoethanol derivatizing solution was prepared as follows: o-phthalaldehyde (250 mg) was dissolved in MeOH (6.3 ml). 2-Mercaptoethanol (250 µl) and 0.4 M potassium borate (pH 10.5, 56 ml) were then added. The mixture was flushed with nitrogen and stored in the refrigerator. 2-Mercaptoethanol (100 µl) was added each day to help maintain the reagent strength. The reagent solution was stable for approximately one week.

Acetonitrile was distilled-in-glass grade (Burdick and Jackson) and used without further treatment. High purity water was obtained with a system from Millipore. The acetate buffers were prepared by mixing different ratios of stock solutions of 0.2 M acetic acid and 0.2 M sodium acetate to obtain various pH values and then diluting

with water to desired concentrations. The eluting solvents were prepared by adding acetonitrile to acetate buffers, degassed for 30 min in an ultrasonic bath, and filtered through a 0.5 μm Millipore filter.

Derivatization Procedures

OPA/Me derivatives were prepared as follows: to a solution of 80 nmol amino acid or 400 ug protein hydrolysate in 0.5 ml water was added 0.2 ml OPA/ME solution. After 1 min at room temperature, 0.5 ml 0.1 M potassium phosphate (pH 4.0) was added, followed by 3.0 ml MeOH. The solution was mixed, filtered, and 5 μl injected onto the column.

Viokase-hydrolysis of Protein

The protein sample (30 mg) was suspended in 1.0 ml water, and 1.0 ml of 0.1 N NaOH was added to effect complete dissolution. To this solution, 5.0 ml of 0.04 M tris-(hydroxymethyl)aminomethane (pH 8.2 Tris buffer) in 0.11 M NaCl were added, plus 2.0 ml of dialyzed Viokase suspension (40 mg/ml Tris buffer). A drop of toluene was added and the mixture was incubated at 38°C for 24 hrs. A control was conducted following exactly the same procedure except for omission of the protein sample. At the termination of incubation, enzyme solids were removed by centrifugation, and the pH of the supernatant liquid was adjusted to pH 10.0 before lyophilization. The lyophilized hydrolysate was dissolved in 20.0 ml water and assayed by the HPLC method detailed above.

RESULTS AND DISCUSSION

The use of acetonitrile and sodium acetate buffer as the mobile

phase has been most effective for our purposes. Sometimes, persistent coelutions could be resolved by the use of gradient elutions or by the addition of a third component such as tetrahydrofuran to the acetonitrile-acetate system. However, since simpler and more direct methods were always more reproducible, we kept the elution isocratic when possible and maintained a binary system of acetonitrile and acetate buffer as the mobile phase.

Amino acids reacted instantly with OPA in the presence of ME, but the products were unstable. There are several ways to overcome the lack of stability of the OPA/ME derivatives. The use of ethanethiol to replace mercaptoethanol (7) or the addition of sodium dodecyl sulfate (9) in the derivatization have been claimed to improve product stability and to enhance fluorescent sensitivity. We found neither method satisfactory; ethanethiol was no better than mercaptoethanol and the sodium dodecyl sulfate treatment caused precipitation problems. Instead, the addition of relatively large amounts of MeOH to the OPA/ME products immediately after the products were prepared appeared to effect improved stability and reproducibility. The derivatives of glutamine and asparagine were stable for at least one hour after preparation, whereas those of the others were stable for 10 min.

With the use of acetonitrile-acetate as the mobile phase, the OPA/ME amino acid derivatives were eluted from the reversed-phase column roughly in three groups. As shown in Fig. 1, the first group to come out generally include derivatives of asparagine (Asn), aspartic acid (Asp), serine (Ser), glutamine (Gln), histidine (His),

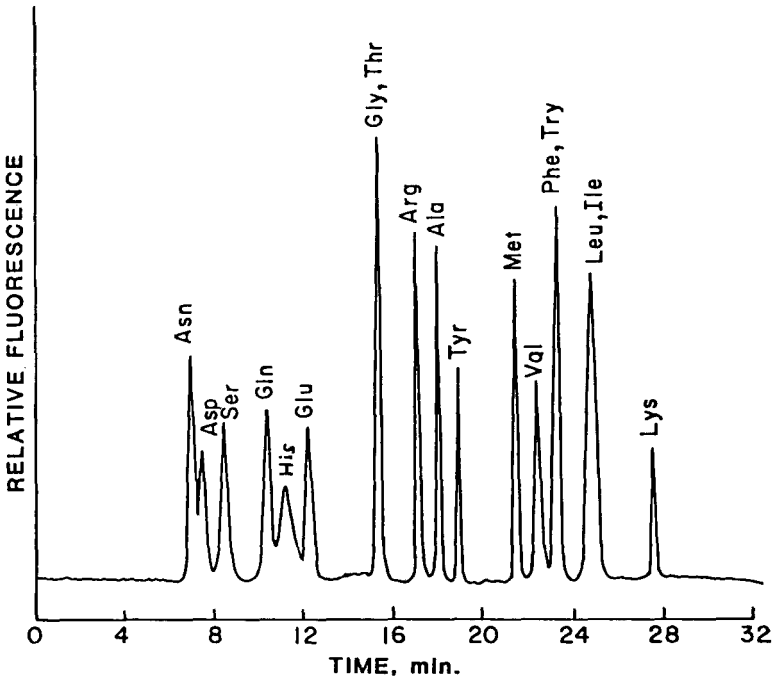

Figure 1. Elution profile of amino acid standards derivatized by the reaction with OPA/ME. Each peak represents 0.1 nmol. Elution conditions: Solvent A, acetonitrile : 0.04 M sodium acetate (pH 4.75), 12 : 88; Solvent B, acetonitrile; gradient program, isocratic at 100% A of 10 min duration, linear step to 50% B in 20 min, isocratic at 50% B of 10 min duration.

and glutamic acid (Glu). In the last group were usually methionine (Met), valine (Val), phenylalanine (Phe), tryptophan (Try), leucine (Leu), isoleucine (Ile), and lysine (Lys). Scattered in the middle were glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala) and tyrosine (Tyr).

We were interested in the analysis of four amino acids (glutamine, asparagine, glutamic acid, and aspartic acid) which,

according to the above mentioned groupings, could be done by dealing with only the first group of derivatives. To achieve the best separation for the six derivatives of the first group, experiments were conducted to investigate effects of acetonitrile and pH in the mobile phase on the retention time. When the derivatives were eluted with solvents of constant pH but various acetonitrile concentrations, the retention time decreased and peaks began to coelute as the percentage of acetonitrile increased. The concentration of acetonitrile at 12% appeared to give the most effective separations; at higher concentrations, column resolution was poor, whereas, at lower concentrations, zone spreading became a problem and column efficiency decreased.

Another series of isocratic elutions was conducted at a constant acetonitrile concentration (12%) in buffers of various pH values (4.35-5.15). All six OPA/ME derivatives in the first group and the derivative of glycine in the second group were investigated. The resulting retention times of these seven derivatives as a function of pH are shown in Fig. 2. Glycine being normally the first to elute in the second group of derivatives, the curve representing glycine served to mark the elution front of the rest of the amino acids. The retention times of asparagine, serine, glutamine, and glycine displayed similar pattern of changes from pH 4.35 to pH 5.40; the curves declined slightly as pH increased from 4.35 to a minimum at about pH 4.60 and then climbed to a maximum at about 5.15 before decreasing again. The OPA/ME derivatives of glutamic acid and aspartic acid were the most sensitive to pH

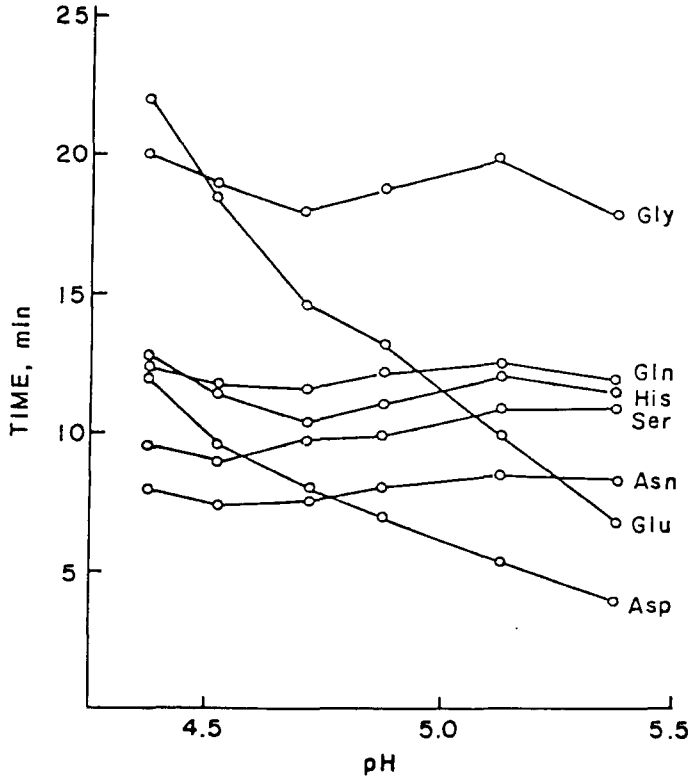

Figure 2. Influence of pH on the retention time of OPA/ME amino acid derivatives. Retention times were obtained by isocratic elutions with solvents consisting of 12% acetonitrile and 88% sodium acetate buffers (0.04 M, pH 4.40-5.40).

changes; the curves of these two amino acids dropped drastically in the pH range of 4.45 to 5.40 crossing over curves of other derivatives. The best separation of these seven derivatives appeared to occur at pH 4.85. A profile of elution under these conditions (12% acetonitrile and pH 4.85) is shown in Fig. 3.

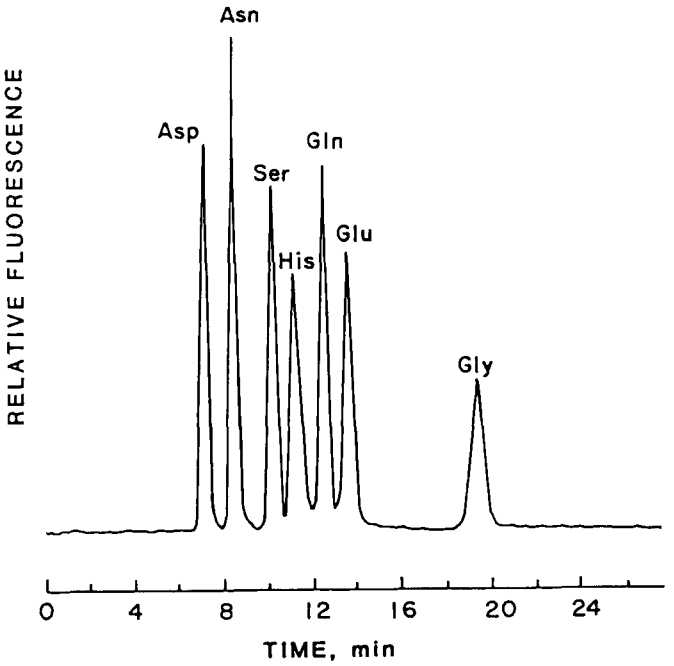

Figure 3. Elution profile of seven amino acid standards derivatized by the reaction with OPA/ME. The mobile phase of the isocratic elution consisted of 12% acetonitrile and 88% sodium acetate buffer (0.04 M, pH 4.85).

Baseline separations for all seven amino acids were obtained. In the concentration range of 50-250 pmol, the OPA/ME derivatives of glutamine, asparagine, glutamic acid, and aspartic acid showed a linearity of response, and the quantitation of these four amino acids was remarkably reliable.

Attempts were made to determine the composition of glutamine and asparagine in the storage protein of cottonseed. The protein was hydrolyzed by various enzyme systems. A multi-step treatment,

based on the method of Hill and Schmidt (10), in which the protein was first hydrolyzed to small peptides by proteases (pepsin or papain) and then to amino acids by aminopeptidase and prolidase was unsatisfactory; the results were inconsistent. The use of Viokase (11), a one step reaction, was found by far the simplest and most consistent in the hydrolysis of cottonseed protein. Fig. 4a shows a front portion of the elution profile of the Viokase-hydrolyzed cottonseed protein as analyzed by the above described HPLC method. The elution of the amino acids of our interest was complete in 20 min and the remaining amino acids were washed off by acetonitrile. Fig. 4b shows another profile of the same protein which had been completely hydrolyzed by acid (6N HCl at 110° for 24 hrs). The peaks of glutamic acid and aspartic acid in Fig. 4b represent the total Glx and Asx, respectively, in the cottonseed protein. Calculations show that, at equivalent amounts of protein under hydrolysis, the sum of glutamine and glutamic acid in the enzyme hydrolysate is smaller than the Glx in the acid hydrolysate. Exactly the same ratio in difference is found between the sum of asparagine and aspartic acid in the enzyme hydrolysate and the Asx in the acid hydrolysate, indicating that the Viokase-catalyzed hydrolysis is incomplete. The value of 62.0% hydrolysis was obtained as an average of three analyses with a standard deviation of 1.88%; this hydrolysis valute was confirmed by the calculation of the ratio of nitrogen recovery between Viokase and acid hydrolysates from conventional ion-exchange analysis. According to Tower et. al (11), most proteins were hydrolyzed by Viokase to 50-80% completion.

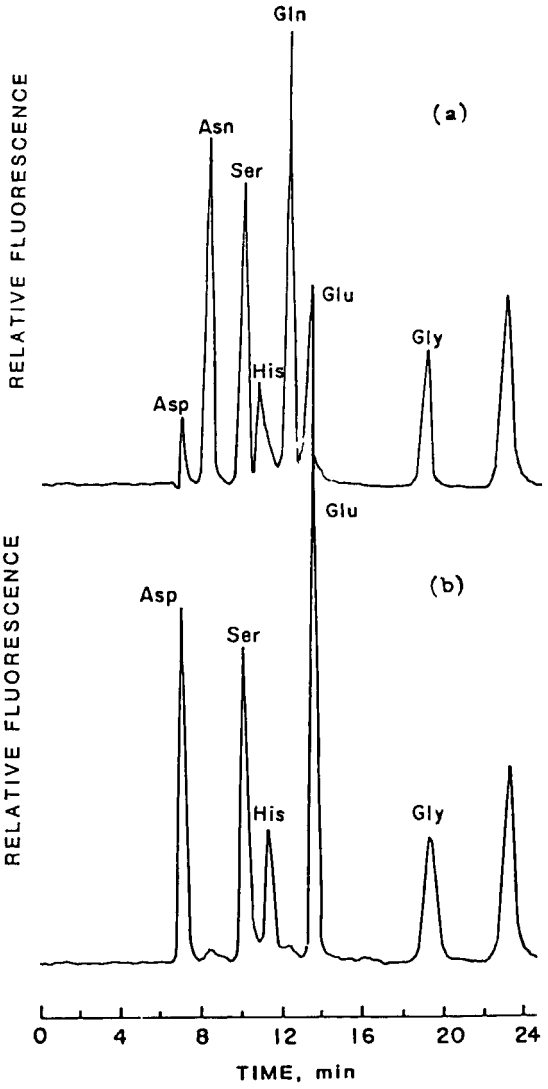

Figure 4. Elution profiles of cottonseed protein which had been (a) Viokase-hydrolyzed and (b) completely hydrolyzed by HCl. The injection of 5 ul OPA/ME derivatized hydrolysate, representing 0.56 ug protein in the case of Viokase-hydrolysis or 0.45 ug protein in the case of acid-hydrolysis, was eluted isocratically for 20 min with the mobile phase consisting of 12% acetonitrile and 88% sodium acetate bufer (0.04 M, pH 4.85), followed by a change to 100% acetonitrile in 5 min to wash off the remaining amino acids.

They argued strongly that the incomplete hydrolysis was not because of the existence of an unhydrolyzable "core" in the protein; they demonstrated that the extent of hydrolysis could be increased to over 90% by dialysing the hydrolysate and reincubating the sac contents several times. Consequently, by using the mean percentage of hydrolysis value, the contents of the various residues found experimentally by enzymatic hydrolysis were corrected to 100% hydrolysis.

According to the calculation method of Tower et al., the glutamine and asparagine contents in cottonseed protein were estimated to be 13.3% and 8.0%, respectively. However, we are not quite satisfied with the generalization of the "coreless" theory in the calculation. Investigations are being conducted in our laboratory for complete enzymatic hydrolysis of oilseed proteins. For the time being we could only report that 62.0% of cottonseed protein was consistently hydrolyzed by the Viokase enzyme system to amino acids in which 55.8% Glx was glutamine and 75.1% Asx was asparagine.

CONCLUTION

We have demonstrated that HPLC technique is very effective in the analysis of glutamine and asparagine. The method can be used in the analysis of protein hydrolysate. When the storage protein of cottonseed was hydrolyzed by the enzyme system Viokase, HPLC analyses showed that, by comparing with the complete hydrolysis with acid, the enzymatic hydrolysis was not complete. The contents of

glutamine and asparagine in cottonseed protein can be estimated by correcting the experimental results to 100% hydrolysis, provided that the protein composition is homogeneous.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TCNB IN POTATOES

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method was developed for the determination of TCNB (tetrachloronitrobenzene), a sprout inhibitor, in potato peels and flesh fortified at levels of 0.16 to 53.5 ppm. TCNB was analyzed on a u Bondapak C_{18} column with UV detection at 210 nm. The mobile phase was acetonitrile-methanol-water (35:35:30) at a flow rate of 1.0 ml/min. Retention time was approximately 10 min. TCNB was extracted by blending for 5 min in acetone. Samples at a level of 1 ppm or higher were directly injected whereas samples below 1 ppm were partitioned into hexane followed by passage through an alumina column. Average recoveries varied from 85.6 to 96.8% with coefficients of variation ranging from 2.18 to 11.68%. A study conducted to test 23 pesticides for possible interferences with TCNB demonstrated that none of them co-chromatographed. The lower limit of detection was 0.08 ppm.

INTRODUCTION

Tetrachloronitrobenzene (TCNB, Fusarex, Tecnazene) is used on potatoes as a sprout inhibitor and as an agent for control of dry rot (Fusarium coeruleum). TCNB has been shown to inhibit sprouting of potatoes up to 11 months. The advantage of TCNB is

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that it does not inhibit wound healing in freshly clamped tubers (1). The recommended application is 1.0 lb of TCNB for every 600 lbs of potatoes (2).

Because of the possibility of sprouting occurring below the application rate and because of the EPA tolerance level of 25 ppm on potatoes, a fast and accurate method for determining TCNB residues is needed. Present methods for the quantification of these residues include polarographic, colorimetric and gas chromatographic. The polarographic method has a very time consuming extraction step whereby the benzene extract must stand for several hours over sodium sulfate (3). There are 3 colorimetric procedures (4-6) in which all are nonspecific. The GC methods (7,8) are good except the extraction and clean-up steps are lengthy.

This paper describes a high-performance liquid chromatographic procedure for determining residue levels of TCNB that overcomes many problems inherent in the other methods.

EXPERIMENTAL

Reagents

All solvents used were HPLC grade obtained from Fisher Scientific Co. Fair Lawn, NJ. The TCNB standard, 99% pure, was obtained from the United States Environmental Protection Agency, Research Triangle Park, NC. The acid alumina, Brockman activity I, 80-200 mesh, was purchased from Fisher Scientific Co. and was used as received.

Liquid Chromatographic System

A model ALC/GPC 244 high-performance liquid chromatograph containing a Model 6000 A pump, a U6K injector and a Model 450 Schoeffel UV detector (Waters Associates, Milford, MA). The detector was set at 210 nm and 0.04 AUFS. A Houston Instruments dual-pen recorder, set at a chart speed of 0.4 in/min, recorded the detector signal.

Column

A Waters Associates 30 cm x 3.9 mm i.d. u Bondapak C_{18} column was used at ambient temperature.

Mobile Phase and Flow Rate

The mobile phase was methanol-acetonitrile-water (35:35:30) with a flow rate of 1.0 ml/min.

Extraction

Fifty g of peel (2-3 mm thick) or flesh were extracted with 100 ml of acetone in a Waring blender, 1 qt jar size, at a high speed for 5 min. The extract was vacuum filtered through Whatman #42 filter paper. The volume was brought to 250 ml using acetone and a 20 ul aliquot was injected into the HPLC. If no TCNB peak was observed, the filtrate was evaporated to approximately 50 ml after which it was tranferred to a liter separatory funnel. One hundred and 30 ml of distilled water was added followed by 1 g of NaCl and 75 ml hexane. The funnel was shaken 2 min and the hexane layer put into another separatory funnel. Fifty ml of hexane was added to the original sample and shaken again for 2 min. The

combined hexane fractions were dried over sodium sulfate. This dried hexane was evaporated to dryness and brought to volume with methanol in a 25 ml volumetric flask.

Sample Clean-up step through alumina. A glass wool plug was placed in the bottom of a 10 ml disposable pipet which had the top 3 cm removed. The column was then dry packed with 4 cm^3 of acid alumina followed by passage of the sample through the column with the 2nd 2ml being collected.

HPLC Analysis

The response curve was determined by taking 0.5, 1.0, 5.0, 10.0 and 20.0 ml aliquots of a TCNB standard (conc 12.8 ppm) and putting each aliquot in a 50 ml volumetric flask and bringing it to volume with methanol. The standards were then passed through an acid alumina column with the 2nd 2 ml being collected. Twenty microliters of each solution were then injected into the HPLC and a curve of detector response vs nanograms of TCNB was plotted. Samples were quantified by comparison of the peak height with that of the standard curve since peak height vs concentration was linear with the range of concentrations used in this study.

Recovery Studies

Both peel and flesh samples (50g) were spiked at levels of 0.16, 0.32, 0.64, 1.07, 6.69 and 53.5 ppm. There were 5 to 6 determinations performed for each spiking level by adding the appropriate concentration of TCNB in liquid form and allowing it to set before extraction. Extractions were performed as described above.

Table 1. Recovery of TCNB Added to Untreated Superior Potatoes

Section of Tuber	No. of Determination	TCNB Added ppm	% Recovery	S.D.
Peel	6	0.16	96.8	4.82
Flesh	6	0.16	93.4	5.82
Peel	5	0.32	89.5	3.00
Flesh	6	0.32	88.4	8.10
Peel	6	0.64	87.7	4.30
Flesh	6	0.64	88.6	4.44
Peel	6	1.07	94.7	7.68
Flesh	6	1.07	89.4	7.77
Peel	6	6.69	89.4	2.96
Flesh	5	6.69	88.3	10.31
Peel	5	53.50	87.3	1.90
Flesh	5	53.50	85.6	1.97

RESULTS AND DISCUSSION

Potatoes were divided into the peel (1st 2-3 mm) and flesh gions. Fifty g portions of each were used in spiking studies and actual samples. Peel and flesh samples were fortified at levels of 0.16, 0.32, 0.64, 1.07, 6.69 and 53.5 ppm. Results of these spiking studies are shown in Table 1. Recoveries were very uniform throughout the different spiking levels with most being 87-90%. Except for the flesh spiked at 6.69 ppm, the coefficients of variation were all below 10%. Most CV% were below 7 % indicating the variation was excellent for a residue method.

Chromatograms of the flesh and peel of fortified potatoes at the 0.32 ppm TCNB level are shown in Figures 1 and Figures 2. TCNB eluted from the column in approximately 10 min with no inter-

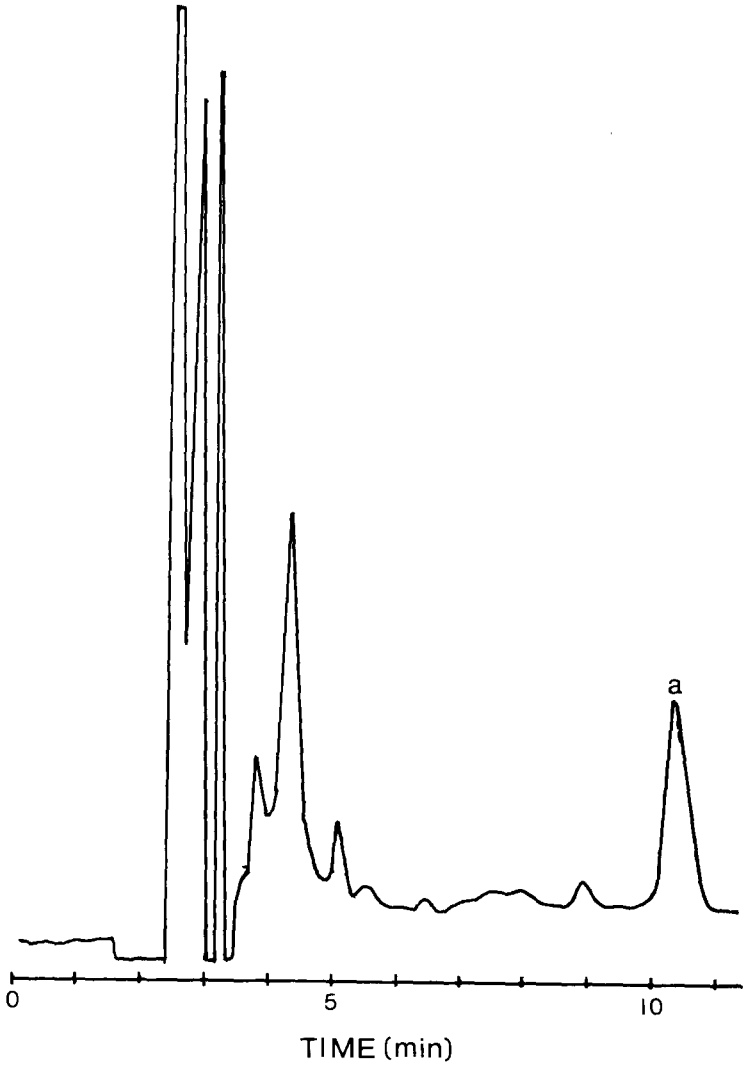

Figure 1. Chromatogram of Superior potato flesh spiked with 0.32 ppm TCNB. Peak (a) TCNB. Chromatographic conditions are given in the text.

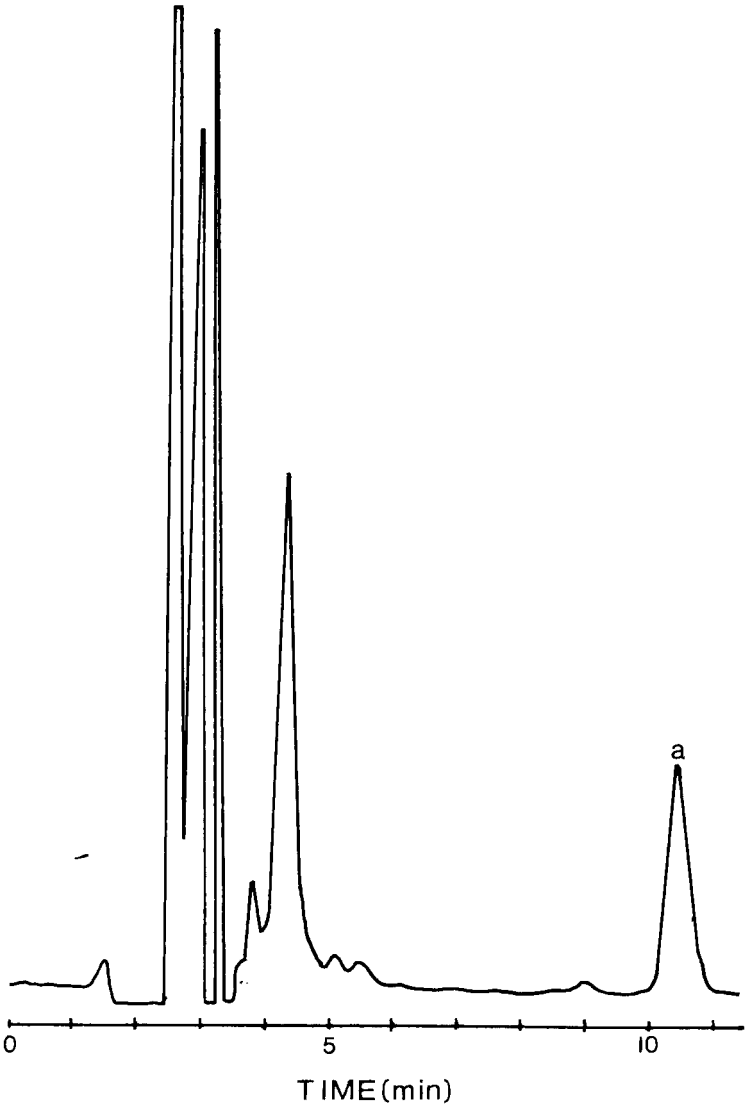

Figure 2. Chromatogram of Superior potato peel spiked with 0.32 ppm TCNB. Peak (a) TCNB. Chromatographic conditions are given in the text.

Table 2. Retention Time of Various Pesticides on C_{18} Relative to TCNB

Pesticide	Relative Retention Time
Guthion	0.49
Carbaryl	0.40
Carbofuran	0.39
PCNB	0.88
Dinoseb	0.19
Dinoseb Acetate	0.68
Maleic Hydrazide	0.21
Chloropropham	0.58
Propham	0.49
PCP	0.21
Amitrole	0.31
Picloram	0.18
Promecarb	0.54
2,4 D Acid	0.18
Monuron	0.39
2,4,5 T	0.18
Dicamba	0.18
Diuron	0.46
Propanize	0.52
Atrazine	0.45
Simazine	0.41
Pirimicarb	0.46
Captan	0.50

ferences. Although the TCNB peak height was low, this added very little to the overall variation of this method which was confirmed by injecting the lowest standard (0.5 ml) 6 consecutive times for which a coefficient of variation of 1.08% was obtained.

The lower limit of detection for this method was determined to be 0.08 ppm. It is doubtful that one could go lower because the blanks begin to show peaks where the TCNB comes off at lower concentrations.

Possible interferences from 23 pesticides were tested by injecting each using the same chromatographic conditions employed for TCNB. As shown in Table 2, none of these pesticides had the same retention time as TCNB.

This HPLC method offers a rapid and precise means of analyzing TCNB in potato flesh and peel, especially at levels equal to or greater than 1 ppm since the samples can be injected directly without cleanup. At concentrations less than 1 ppm a cleanup step must be used like the GC methods (7,8) which then makes the HPLC method valuble as a confirmation procedure.

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CYCLIC MELANOTROPINS. PART VI*. REVERSE PHASE HPLC STUDIES

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ABSTRACT

The chromatographic behavior of 17 cyclic [Cys^4, Cys^{10}]-α-melanocyte stimulating hormone (α-MSH, α-melanotropin) analogs were studied on two reversed phase columns (Altech and Vydac) using several mobile phases. It was observed that analogs which contain a D-amino acid were always eluted earlier than the corresponding L-amino acid-containing analogues. Substitution of penicillamine for cysteine in the 4 position led to a more lipophilic compound as expected, but when penicillamine was substituted in the 10 position, apparently a less lipophilic compound resulted. These observations can be interpreted as a result of a conformational change in the molecule caused by the particular substitution. Furthermore, decreasing the size of the intramolecular disulfide ring led to a decrease in lipophilicity (i.e. retention time). The carba modification of the disulfide bridge had a similar effect as previously seen in oxytocin and vasopressin, that is, a decrease in retention time.

* For Part V see (16).

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INTRODUCTION

High performance liquid chromatography (HPLC) is one of the most powerful methods for the separation and isolation of organic compounds. It has been applied to almost all types of biologically active molecules, of which peptides comprise an important class. Problems in the chromatography of peptides and proteins have been discussed in monographs (1,2) and in many papers devoted to the subject.

Reversed phase high pressure liquid chromatography (RP-HPLC) allows the separation of compounds which differ only slightly in their covalent structure, three-dimensional structure, or other physical-chemical properties. Minor structural modifications often can have unpredictable effects on the retention times of peptides, especially if the modification causes a change in the conformation of the molecule which results in changes of its overall lipophilicity. If, within a series of minimally modified peptide analogs, the introduction of a more lipophilic moiety, such as replacement of H by CH_3 leads to increased retention in RP-HPLC, then (3) the conformations of the analogs are probably similiar. However, if substitution with a more lipophilic substituent leads to decreased retention, then a change in conformation would be predicted.

Several good examples of this behavior have been found in studies of diastereomeric peptides (4-10). For example, two peptides which are identical except for the stereochemistry of one amino acid, should have essentially the same lipophilicity, since

the lipophilicity of the side chains is not altered by a change in stereochemistry. However, in the case of neurohypophysial hormones almost all reported peptides containing one amino acid residue of the D-configuration have longer retention times than the all L-amino acid analogs (5-8, 11). This implied that this substitution lead to a conformational change in the peptide to one of greater lipophilicity (6). It is well known that the conformation of many peptides are strongly solvent dependent (12) and that the conformation can change with the pH or the ionic strength of the solution. Therefore, any comprehensive chromatographic study which desires to examine and understand the behavior of a peptide should utilize a variety of mobile phases as well as stationary phases (13). In this paper we have studied a series of cyclic [$\overline{Cys^4, Cys^{10}}$]-α-MSH analogs which differ only slightly in their overall structure with the aim of understanding how these structural changes are reflected in their chromatographical behavior and have interpreted the results of our investigation in terms of the parameters discussed above.

EXPERIMENTAL

The cyclic melanotropins studied were synthesized at the University of Arizona utilizing the general procedures developed for these peptides in our laboratory (14-17). High performance liquid chromatography was performed on 25 x 0.46 cm I.D. columns, packed with C18 reversed phase material (16 μm (Vydac) or 5 μm (Altech)) using a Spectra Physics SP-8700 liquid chromatograph

equipped with an SP-8400 continuously variable wavelength UV detector (Spectra Physics, Santa Clara, CA, U.S.A.). We used a flow rate of 1 ml/min and 220 nm UV detection to monitor the peptides. Solvents used as mobile phases were of UV grade and were filtered before use through Millipore filters.

RESULTS AND DISCUSSION

We have examined the chromatographic behavior of several cyclic α-MSH analogs which were prepared in our laboratory as part of our examination of conformation-biological activity relationships of this hormone. The cyclic structure of α-MSH (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) was designed based upon the "pseudoisosteric" replacement of methionine-4 and glycine-10 in the native hormone with a disulfide bridge formed via two cysteine residues substituted in these two positions (14). The reverse turn structure imposed by this substitution, encompasses the residues -His(6)-Phe(7)-Arg(8)-Trp(9)-, and is believed to provide the preferred conformation required for biological activity (14-18). Structures of the compounds studied are shown in Fig. 1.

The results of the chromatographic studies utilizing two different C18 column packings (Vydac and Altech) and several mobile phases, are summarized in Table 1 and Fig. 2. It can be clearly seen that the Vydac column has a much greater selectivity and a wider range of retention times for structurally similar compounds. Conversely, the Altech column is more efficient

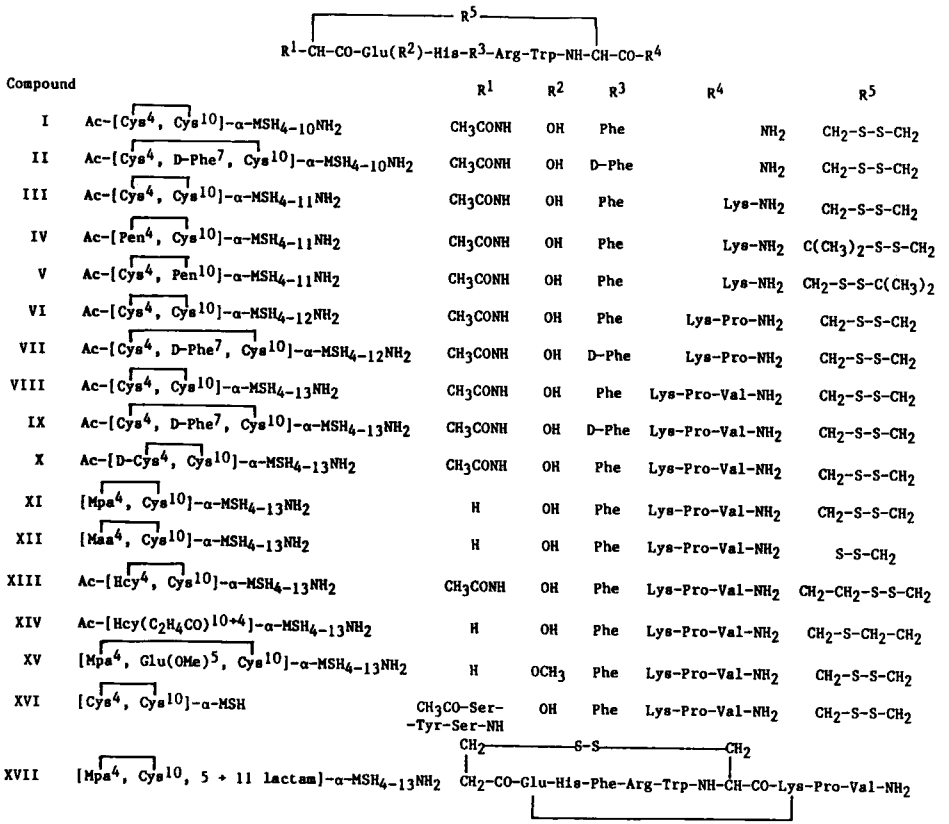

Figure 1: Structures of α-MSH cyclic analogs investigated.

TABLE I

Values of k′ under various conditions of chromatography.

Compound	Column and Conditions[a]			
	A	B	C	D
I	2.80	2.69	2.59	6.41
II	2.07	1.55	1.48	2.90
III	1.73	1.64	1.54	2.72
IV	2.25	2.38	3.07	5.46
V	----	1.30	1.41	2.19
VI	1.89	1.62	1.78	2.97
VII	1.41	1.00	0.90	1.45
VIII	2.75	2.72	4.24	7.14
IX	1.79	1.32	1.41	2.29
X	2.64	2.52	3.02	6.03
XI	3.19	3.25	3.81	8.58
XII	2.93	2.79	3.43	6.65
XIII	3.53	4.83	9.62	18.1
XIV	2.36	1.93	2.25	4.24
XV	5.27	4.96	5.86	14.9
XVI	----	----	4.34	----
XVII	6.60	7.41	9.55	~28.1[b]

a) A: Altech C-18 column, 30% of acetonitrile in 0.5% trifluoroacetic acid.
B: Vydac C-18 column, 21% of acetonitrile in 0.1% trifluoroacetic acid.
C: Vydac C-18 column, 37% of methanol in 0.25 M triethylammonium phosphate pH 2.2.
D: Vydac C-18 column, 15% of acetonitrile in 0.25 M triethylammonium phosphate pH 2.2.

b) Estimated from elution with 20% of acetonitrile.

(i.e. has more theoretical plates) than the Vydac column, which is partially due to the different mesh size of the silica gel beads (5 μm vs. 16 μm, respectively). These two columns also show different responses to the trifluoroacetic acid (TFA) buffer concentration. On both columns increasing the TFA concentration

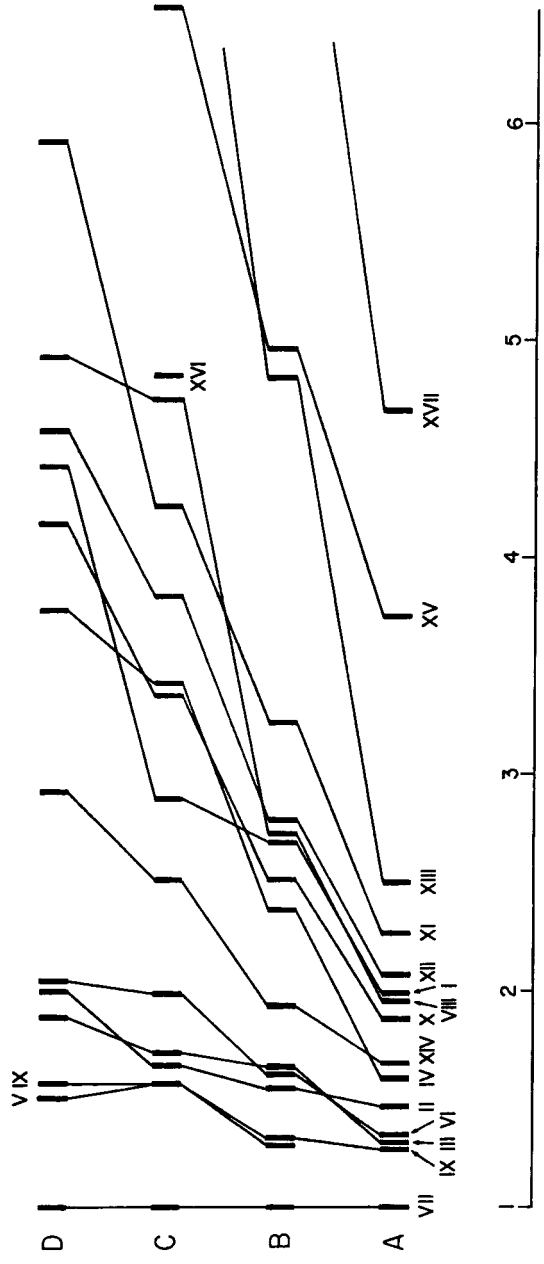

Figure 2: Comparison of relative retention of cyclic α-MSH analogs. Values are calculated relative to k' of compound VII. Columns and conditions A-D are the same as in Table II.

TABLE II

Calculated and Observed Relative Retention Times of Compounds I, III, VI, and VIII.

Compound	Relative Retention Time[a]					
	Calculated[b]		Observed[c]			
	1	2	A	B	C	D
I	0.0	0.0	0.0	0.0	0.0	0.0
III	-1.9	-3.0	-0.7	-1.06	-1.2	-2.5
VI	2.5	0.1	-0.66	-1.08	-0.9	-2.3
VIII	8.4	4.7	-0.05	0.03	1.8	0.5

(a) Numbers are only relative and have no absolute value. A negative number implies that the compound is eluted earlier than compound I, which has a value of 0.

(b) These are calculated for different increment values obtained with various mobile phases (1- 0.1M $NaClO_4$, 0.1% H_3PO_4, gradient of acetonitrile; 2- 0.1 M NaH_2PO_4, 0.1% H_3PO_4, gradient of acetonitrile). The reported values are a sum of increments 20, 21 for additional amino acids, when sequence 4-10 is taken as zero.

(c) Conditions A-D are described in Table I. Values are calculated according to formula: $k'_A/k'_{VII} - k'_I/k'_{VII}$ where k'_A is the value of k' for the given compound (I, III, VI, VII, VIII).

from 0.10% to 0.15% leads to an increase in column efficiency (980 to 1060 theoretical plates in the case of the Altech column, and 504 to 1424 in the case of the Vydac column - in all cases efficiency calculations are based on compound XI). Interestingly, the elution times decrease (about 3.1 times) on the Altech column and increase (about 1.5 times) on the Vydac column as the TFA content increases. The best overall separation of the

compounds studied are achieved in a 0.25 M triethylammonium phosphate (pH 2.2) buffer with acetonitrile as the organic modifier (19). Buffers containing 0.10% trifluoroacetic acid can be used for preparative purifications, but the column is less efficient with the TFA buffer than it is with the phosphate buffer. In addition, prolonged use of 0.10% TFA can lead to a loss of the bonded alkyl chains on the stationary phase (20).

Separations based on the length of the peptide chain are obtained with methanol as the organic modifier in the mobile phase. Cyclic compounds containing the 4-11 (III) and 4-12 (VI) α-MSH sequences are difficult to separate in the trifluoroacetic acid-containing buffer (α = 1.01) as are the 4-10 (I) and 4-13 (VIII) α-MSH sequences (α = 1.01). This problem is overcome with a methanolic triethylammonium phosphate buffer system, which provides excellent separation (α = 1.16 and 1.64, respectively) for these pairs of compounds. Substituting methanol for acetonitrile with the same buffer, the separation is worse (α = 1.09 and 1.11, respectively) (See Fig. 3).

Interestingly, prediction of the elution order for cyclic melanotropins (Table II), based on the amino acid sequence (20, 21), was: Ac-[$\overline{\text{Cys}^4, \text{Cys}^{10}}$]-α-$\text{MSH}_{4-11}$-$\text{NH}_2$, Ac-[$\overline{\text{Cys}^4, \text{Cys}^{10}}$]-α-$\text{MSH}_{4-10}$-$\text{NH}_2$, Ac-[$\overline{\text{Cys}^4, \text{Cys}^{10}}$]-α-$\text{MSH}_{4-12}$-$\text{NH}_2$, and Ac-[$\overline{\text{Cys}^4, \text{Cys}^{10}}$]-α-$\text{MSH}_{4-13}$-$\text{NH}_2$. The observed order, utilizing the best conditions for separation (conditions C) was: Ac-[$\overline{\text{Cys}^4, \text{Cys}^{10}}$]-α-$\text{MSH}_{4-11}$-$\text{NH}_2$, Ac-[$\overline{\text{Cys}^4, \text{Cys}^{10}}$]-α-$\text{MSH}_{4-12}$-$\text{NH}_2$, Ac-[$\overline{\text{Cys}^4, \text{Cys}^{10}}$],

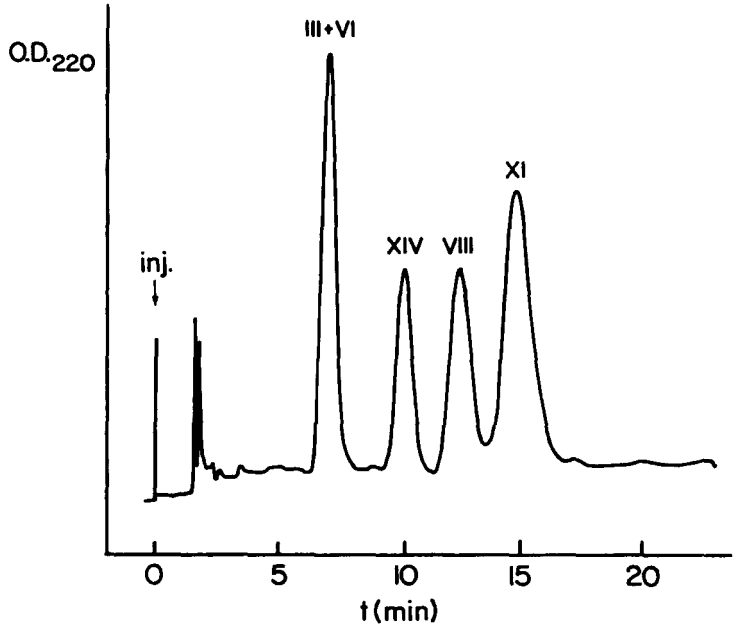

Figure 3: Separation of cyclic analogs III, VI, VIII, XI and XVI. Vydac C-18 column, 2 ml/min, acetonitrile (17% v/v)- 0.1% trifluoroacetic acid (83% v/v).

$\overline{\text{Cys}}^{10}$]-α-MSH$_{4-10}$-NH$_2$, and Ac-[$\overline{\text{Cys}^4}$, $\overline{\text{Cys}}^{10}$]-α-MSH$_{4-13}$-NH$_2$. These results suggested that the effect of proline on the retention time in these reversed phase systems was less than expected. The smaller influence of proline on the retention times of the 4-12 fragment (VI) could be due to its carboxyl-terminal position, since its effect was different from compounds which contain proline inside the peptide chain. A cyclic analog containing the 1-13 sequence of α-MSH, [$\overline{\text{Cys}^4}$, $\overline{\text{Cys}}^{10}$]-α-MSH (XVI), was eluted very close to the 4-13 cyclic analog (VIII), but due to a lack of

material it was only studied in one mobile phase. The relative retention time of the 1-13 sequence versus that of the 4-13 analog was in agreement with the prediction based on amino acid sequence, when the contribution of tyrosine was practically negated by the two serine residues (21, 22).

Interestingly, compounds containing D-phenylalanine in position 7 (D-Phe-7) exhibit very prolonged *in vitro* (23) and *in vivo* (18) biological activities. All D-Phe-7 containing compounds were eluted earlier than the corresponding L-Phe-7 diastereomeric analogs under all the experimental conditions utilized. Interestingly, changing from methanol to acetonitrile led to a very pronounced increase in the difference of retention times. For example, L-Phe-7 and D-Phe-7 analogs with the 4-10 sequence (I and II) had α values corresponding to 1.75 with methanol and 2.21 with acetonitrile, respectively. This difference was less pronounced for analogs containing the 4-13 (VIII and IX) sequence (α = 3.00 for methanol and α = 3.12 for acetonitrile, respectively). On the contrary, a decrease in the difference of retention times is observed in going from a mobile phase containing methanol to one containing acetonitrile when the stereochemistry of cysteine is changed (L-Cys to D-Cys) in the 4 position (VIII and X) (α = 1.40 with methanol and α = 1.18 with acetonitrile, respectively).

Substitution of penicillamine for cysteine in the 4 or 10 position, (compounds IV and V, respectively), should lead to an analog of greater lipophilicity. Indeed, an increase in the

retention time of the compound with penicillamine in position 4 was observed, but with penicillamine in position 10 a decrease in retention time was observed. This suggested that the Pen-10 compound exhibits a change in the overall conformation of the molecule resulting in the inaccessibility of that residue for interaction with the stationary phase.

Substitution of an N-terminal acetylamino group for hydrogen leads to an increase of elution times in buffers with acetonitrile as the organic modifier (compounds VIII and XI, see Fig. 3). In the case of methanol as the organic modifier, the elution order of compounds VIII and XI are reversed. A decrease in the size of the ring by deletion of one methylene group leads to a decrease in retention times (compounds XI and XII) in all systems used. Increasing the size of the ring (by adding a methylene group) leads to an increase in retention times (compounds VIII and XIII), which is especially dramatic on the Vydac column using the triethylammonium phosphate buffer. In both cases the change in the retention time could be predicted from the structural change, that is, both changes occur as a result of the changes in lipophilicity of the substitution. Increasing the size of the ring results in a larger than expected increase in retention time, and apparently, this is probably due to a larger than expected increase in lipophilicity. The longer retention time of the homocysteine-containing compound (XIII) can be explained by greater freedom of the backbone in this analog, resulting from

the increase in the intramolecular ring size. Carba modification of the disulfide bridge in the melanotropins (compound XIV), which were previously studied in the case of oxytocin and vasopressin analogs (24, 25), leads to compounds with shorter retention times upon comparison to the disulfide bridged molecule (XI, see Fig. 3). Similar results were observed in the oxytocin and vasopressin analogs. Methylation of the free carboxylic group of glutamic acid in position 5 (compound XV) increases the retention times as compared to the unesterified peptide. Introduction of a second intramolecular bridge (compound XVIII), caused a similar increase in its relative retention time.

From the data presented, it is clear that even very small structural differences can express themselves by a change in chromatographic behavior. Nonetheless, if careful comparative studies are made, conclusions about conformational similarities can be extracted from the structure-lipophilicity relationships and their expression in the retention time observed for analogs on reverse phase HPLC systems. Further verification of our tentative conclusions will require careful physio-chemical studies of the described compounds. Such studies are in progress in our laboratory.

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FAST ANALYSIS OF TISSUE CATECHOLS USING A SHORT, HIGH-EFFICIENCY (3 μm) LC COLUMN AND AMPEROMETRIC DETECTION

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ABSTRACT

Tissue catecholamines were analyzed in less than 8 minutes per sample, using a 5 cm LC column, packed with a 3 μm particle size reverse phase sorbent, under conditions which permit fast analysis of tissue extracts of up to 70 μl with no loss in resolution or theoretical plate height. The sensitivity of amperometric detection makes this method suitable for analyzing small amounts of catechols in tissues such as rat brain prefrontal cortex.

INTRODUCTION

The formation and function of catecholamines in biological tissues have been the subject of intense investigation which has resulted in the introduction of several approaches to the quantitative determination of catecholamines. The newest of these involves liquid chromatography with electrochemical detection (1-8). Since the application of electrochemical detection (1),

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the single most important development has been the improvement in chromatographic columns.

Until recently, chromatographic columns have measured 25 to 30 cm in length and have been packed with octadecylsilane particles 5 to 10 microns in diameter. For the analysis of catecholamines with such columns, the determination of noradrenaline (NA) has exemplified the conflict between minimal time per analysis and adequate resolution. Fast analyses were obtained only at the price of allowing NA to be eluted with the solvent front (4,6). On the other hand, if the NA was retained long enough to allow adequate resolution, the time per analysis became excessive (5,8).

One partial solution to this speed-resolution conflict was the use of 5-cm columns packed with 3-micron diameter particles (9). However, such columns were also reported to require minute sample injection volumes (less than 5 microliters (9)), thus limiting the quantity of sample that could be applied to the column and, in consequence, the utility of the approach.

These limitations have now been overcome through the use of a 5-cm 3-micron column, under conditions which yield fast, reproducible analyses of six different catecholamines. In these analyses, NA is resolved from both the solvent front and 3,4-dihydroxyphenylalanine (DOPA). The separation requires less than 1100 psi applied pressure and is complete in less than 8 minutes. As much as 70 µl of sample may be injected without loss of either column efficiency or detector linearity.

MATERIALS AND METHODS

The liquid chromatograph (LC) system uses a Milton-Roy mini-pump equipped with a Waters high-pressure filter, a Waters intelligent sample processor (WISP® 710B; Waters Associates, Milford, MA), an amperometric detector (Bioanalytical Systems Inc., W. Lafayette, IN), and a Kipp & Zonen 2-pen recorder (Rainin Instrument Co., Woburn, MA). An in-line 0.5 µm solvent filter (Upchurch Scientific, Seattle, WA) was placed between the injector and the column. The column used for all of the separations was a 5 x 0.5 cm "Little Champ"® column (Regis Chem. Co., Morton Grove, IL) packed with a 3 µm spherical octadecylsilane (ODS) sorbent.

The mobile phase for separation of catechols was 0.1 M monochloroacetic acid (pK = 2.86, buffered to pH 3.0 with 10 N NaOH), containing 0.3 mM Na^+ octylsulfate 0.1 mM EDTA and 1% (v/v) acetonitrile. The mobile phase was pumped at 1.0 ml/min (~1000 psi).

Catechols were extracted from tissues and prepared for LCEC analysis by a previously described procedure (11), with modifications described as follows: tissues were sonically disrupted in 0.4 to 1.0 ml of 0.1 M $HClO_4$ containing 5 mM cysteine (as an anti-oxidant) and 2.7 µM 3,4-dihydroxybenzylamine (DHBA as an internal standard for catechol degradation and recovery). The homogenates were centrifuged at 30,000 x g x 10 min; 300 µl aliquots of the clear supernatant were retained for analysis. Catechols were adsorbed onto miniature alumina columns composed of Eppendorf® pipet tips with a pledget of Pyrex® wool and ~20 mg of acid washed alumina (Crout, 1961).

The 300 µl tissue extract was rapidly adjusted to pH 8.6 by adding 75 µl of 2 M Tris-hydroxymethylaminomethane (TRIS), pH 8.6. The alkaline extract was poured atop the columns which were then centrifuged at 50 x g (500 rpm), to drive the extract through the column. The effluent fraction can be acidified with 25 µl of 8 M $HClO_4$ and saved for the assay of indoles and homovanillic acid (11). The columns were washed with ~400 µl of water was discarded) and eluted into Waters limited volume WISP inserts with 75 µl of 0.1 M monochloroacetic acid (unbuffered). The limited volume inserts were placed directly into WISP vials for injection onto the LC.

The catechol standards were obtained from the Regis Chemical Co. (Morton Grove, IL). Acetonitrile (non-UV) was purchased from Burdick & Jackson (Muskegon, MI). All other reagents were of the highest purity, obtained commerically.

Capacity factors (k') and resolution (Rs) were calculated using the following formulas (12):

$$k' = \frac{\text{Retention Time of Peak - Retention Time Solvent Front}}{\text{Retention Time of Solvent Front}}$$

$$Rs = \frac{2\ (\text{Retention Time A - Retention Time B})}{\text{Peak Width A + Peak Width B}}$$

RESULTS

Preparation of samples for LC on miniature alumina columns has been described previously (11). The current modification of eluting with 0.1 M monochloroacetic acid (MCAA) has resulted in a shorter, simpler preparation. The recovery of several catechols

TABLE 1

Compound	Response (pA/pmol)	R.T. (min)	k'	Recovery From Alumina
noradrenaline	500	2.22	1.92	58.1%
3,4-dihydroxyphenylalanine	442	2.76	2.61	46.4%
adrenaline	421	3.67	3.76	55.0%
3,4-dihydroxybenzylamine	416	4.48	4.76	59.2%
3,4-dihydroxyphenylacetic acid	353	5.48	6.15	43.6%
dopamine	280	7.83	9.07	57.2%

R.T. = retention time
k' = capacity factor

is reproducible, as shown in Table 1. Because a high proportion of the sample can be applied to the 5-cm 3-micron column, more than 35% of the tissue catecholamines can actually be injected onto the column. Additional data relating to the separation and quantitation of catechols are presented in Table 1. The void volume of the columns used in these studies was estimated by multiplying the retention time for the inflection of the solvent front by the flow rate. The capacity factor for noradrenaline, the earliest eluting catechol, was nearly 2.0.

The present method appears to be more sensitive than earlier methods (3,5,8) (see later remarks, under DISCUSSION). The detector response, expressed as pA/pmol, is given in Table 1.

A representative chromatogram is depicted in Figure 1a. To illustrate the applicability of this method to a biological sample, a chromatogram of catecholamines in rat brain prefrontal cortex is also shown (Figure 1b). The retention of NA, in the tissue

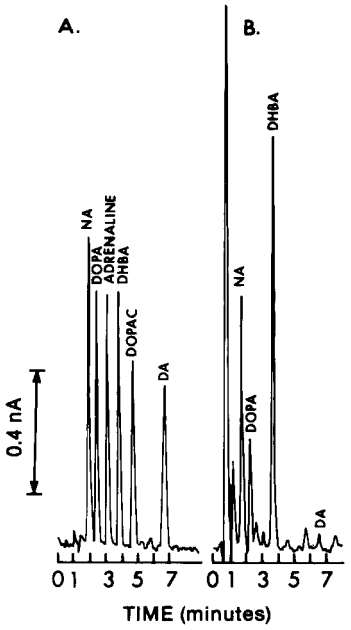

FIGURE 1. Chromatogram of catechols: A. standards (2 pmol each); B. 10 µl of a 75 µl alumina column eluate of rat brain prefrontal cortex from an animal which had been injected with an inhibitor of aromatic amino acid decarboxylase (m-hydroxybenzylhydrazine 0.72 mmol/kg i.p.) 30 minutes earlier.

extract, is sufficiently resolved from the solvent front to permit accurate analysis; the dopamine peak eluted in approximately 7 minutes.

To determine the maximum volume of sample which the column could accept without loss of efficiency, we examined the relationship between injection volume, the height of an experimental theoretical plate (HETP), and resolution (Rs). Figure 2 demonstrates that both HETP and Rs are independent of injection volumes until volumes greater than 70 µl are injected.

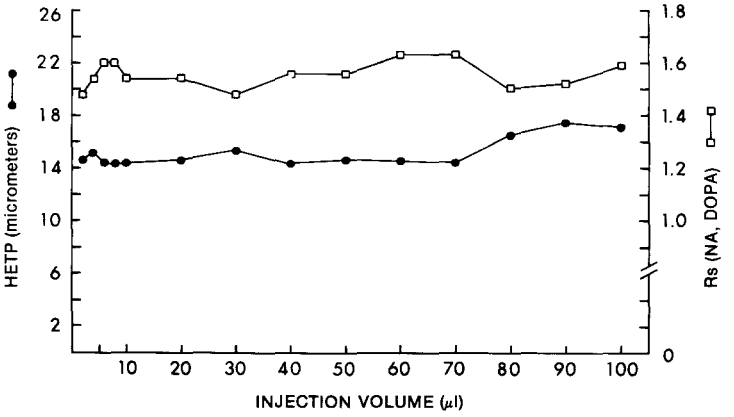

FIGURE 2. The effect of injection volume on theoretical plate height (HETP) and resolution (Rs). A 1 μM solution of catechols was injected in the volumes indicated; HETP and Rs were calculated as described in the text.

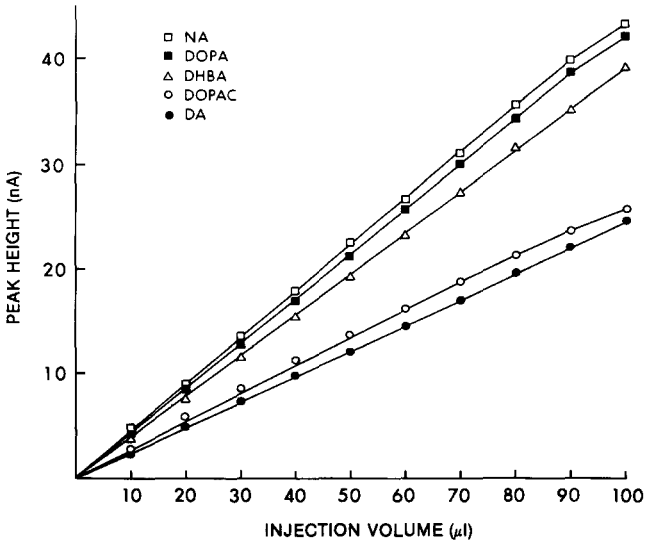

FIGURE 3. Standard curves for catechols as a function of injection volume. A 1 μM solution of catechols was injected at the volumes indicated.

The linearity of the present assay was similarly tested between injection volumes of 2 µl (2 pmol) and 100 µl (100 pmol). Again, detector response was linear for all the catecholamines up to 70 µl (70 pmol) of applied sample.

DISCUSSION

The present method provides conditions for rapid, sensitive, and automated analyses of catecholamines in biological samples. The alumina column step was judged necessary for two reasons: first, alumina elution reduced the number of early eluting peaks which follow the solvent front and obscure the catechol peaks of interest; and second, the preparation eliminated late-eluting peaks (e.g., 5-hydroxytryptamine) which could delay or interfere with the analysis of succeeding samples.

One feature of rapid analysis is an enhanced detector response. However, such enhancement should not be attained by increased oxidizing potential, since it increases both baseline noise and "ghost" peaks. Earlier methods, using longer columns, obtained values of 40 (3), 196 (8), and 300 (5) pA/pmol. However, the 300 pA/pmol response (5) was obtained at the price of an unfavorably high oxidizing potential of 0.9 V. By contrasts, the present method affords a signal of 500 pA/pmol at only 0.7 V oxidizing potential.

The present method also offers adequate resolution of all the catechols examined in less than 8 minutes. Earlier methods have achieved similar resolution, but with retention times of up to 1 hour (8). The present capacity factors, however, are comparable

to those obtained with methods which involve longer retention times (5).

The fast analysis in this case is determined by the combination of the shortness of the column and the high efficiency that results from the 3-micron diameter of the particles. The very small particle size also decreases the loss in column efficiency that typically results from increased, higher-than-optimum flow rates (9,12). Thus the separations shown can be maintained at flow rates even higher than those reported here, yielding these same separations in less than 3 minutes per sample.

FOOTNOTE

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ANALYSIS OF SALICYLAMIDE AND ITS METABOLITES IN BLOOD AND URINE BY HPLC

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ABSTRACT

Sensitive liquid chromatographic assays for salicylamide and its metabolites in urine and plasma were developed to facilitate pharmacokinetic studies of the drug's metabolism. The drug and its hydroxylated metabolite, gentisamide, were extracted and concentrated prior to separation on a small-bore reverse-phase column. Conjugated metabolites were assayed separately using reverse-phase ion-pair chromatography. An accurate method of assay calibration in the absence of pure metabolite standards was developed using radioactively-labelled parent drug. In addition one of the metabolites, salicylamide sulfate, was isolated by ion-pair extraction and purified. A significant species difference in salicylamide metabolism was observed. In the dog the drug is almost exclusively (90%) metabolized to its sulfate conjugate, while in humans the glucuronide conjugates of salicylamide (50%) and gentisamide (15%) predominate over salicylamide sulfate (30%).

INTRODUCTION

Because of its dose-dependent oral bioavailability, salicylamide (SAM) has been a useful drug with which to study first-pass metabolism (1-3). SAM is metabolized by at least three pathways:

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hydroxylation, sulfation and glucuronidation (4,5). We have studied the kinetics and mechanisms of nonlinear SAM metabolism in dogs after oral and i.v. doses of the drug (6,7). Other reported assays (8-12) lacked the sensitivity and specificity required to measure plasma concentrations of SAM and its metabolites in the dog after the administration of small oral doses with low bioavailabilities. We have developed sensitive HPLC assays for SAM and its metabolites in blood and urine which are applicable to dog or human studies.

MATERIALS

Reagents

Methanol, ethyl acetate, acetonitrile and dichloromethane (all HPLC grade) were supplied by Burdick and Jackson Laboratories (Muskegon, MI). Salicylamide and tetrapentylammonium chloride were obtained from Eastman Kodak Co. (Rochester, N.Y.). Salicylic acid methyl amide (N-methylsalicylamide) was purchased from ICN Pharmaceuticals (Plainview, N.Y.). Gentisamide (GAM) (m.p. 214-215°C) was synthesized from gentisic acid (Sigma Chemical Co., St. Louis, MO) using methods described by Bray et al. (13) and Raistrick and Simonart (14). Tetrabutylammonium sulfate (TBA-SO4) was made by mixing tetrabutylammonium hydroxide (40 wt % in water) and tetrabutylammonium hydrogen sulfate (both from Aldrich Chemical Co., Milwaukee, WI) to give a pH of 6.0 in aqueous solution. Phenol reagent solution (2N) was from Fisher Scientific Co (Pittsburg, PA). Carboxyl-^{14}C-salicylamide, 50 mCi/mmole, was custom synthe-

sized by ICN Pharmaceuticals (Irvine, CA). Bacterial β-glucuronidase (Type VII), sulfatase (arylsulfatase Type VI) and myristic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Aquasol® was purchased from New England Nuclear (Boston, MA) and ethyl aminobenzoate (Benzocaine, U.S.P.) was supplied by Merck & Co., Inc. (Rahway, N.J.).

Liquid Chromatography Instrumentation

Mobile phase was delivered by a model A-60-S pump (Eldex Laboratories, Menlo Park, CA) or a model 6000A pump (Waters Assoc., Milford, MA). The injector was a WISP 710B (Waters Assoc.). The column effluent was monitored with a model SF770 detector (Kratos Analytical Instruments, Westwood, N.J.) and its output signal recorded and integrated by a model 3390A integrator (Hewlett-Packard, Avondale, PA).

METHODS

Assay of Salicylamide and Gentisamide

Sample preparation. Frozen plasma samples were thawed at room temperature and gently mixed. A volume of 1.0, 1.5 or 2.0 ml of each sample was added to a 16 mm x 100 mm test tube and the total volume brought to 2.0 ml with water as necessary. Each sample was extracted twice with 6 ml of ethyl acetate and the pooled organic layers were evaporated to dryness under a gentle stream of nitrogen at 40°C. Methanol (20 µl) was added to redissolve the residue in each tube followed by 0.2 to 2.0 ml of mobile phase which contained 8 µg/ml of the internal standard, salicylic acid methylamide

(SAMA). The relative concentration of samples (sample volume/ reconstututed volume) during the extraction was varied from 0.5 to 10 in order to keep peak heights within the range of the calibration curve. Each sample was filtered before injection through a 0.45 micron centrifugal microfilter (#SS009, Schleicher & Schuell, Keene N.H.). Duplicate 40 microliter injections of each sample were made and the plasma concentrations of salicylamide and gentisamide calculated by comparing their average peak height ratios (compound peak height/internal standard peak height) to a calibration curve. The calibration curve was prepared by spiking blank plasma with salicylamide and gentisamide at several concentrations between 0.25 and 5 μg/ml and extracting in the same manner as with unknown samples.

Chromatographic conditions. For the assay of SAM and GAM a 150 mm x 2.1 mm I.D. column packed with RoSiL® Phenyl, 5 micron (Alltech Assoc., Deerfield, IL) was employed. The mobile phase was 10 mM pH 2.2 phosphate buffer in water pumped at a rate of 0.3 ml/min. Column effluent was monitored at 296 nm.

Assay of Sulfate and Glucuronide Conjugates

Sample preparation. Urine samples frozen at -20°C for up to six weeks were thawed and gently mixed. One ml of each urine sample was mixed with 3 ml of methanol, centrifuged for 5 min at 1000 x g and the supernate filtered through a 0.2 micron disposable syringe filter assembly (#4192, Gelman Sciences, Ann Arbor, MI). Duplicate 15 μl injections of each prepared urine sample were made

and the amount of each metabolite was determined by comparing its peak height with a calibration curve prepared from urine samples of known metabolite concentration (see Results and Discussion).

Plasma samples to be assayed for SAM metabolites were concentrated in the following manner. To each 1.0 ml plasma sample was added 0.1 ml of 0.2 M phosphate buffer (pH 5.2) and this aqueous mixture extracted twice with 5 ml of 1:1 hexane/ethyl acetate to remove SAM. After a brief evaporation to remove any residual organic phase, 3.0 ml of acetonitrile containing 8 µg/ml of the internal standard, ethyl aminobenzoate, were added and the samples centrifuged for 5 min at 1000 x g. The supernate was evaporated to dryness under a gentle stream of nitrogen in a water bath maintained at 40°C and the residue was redissolved in 0.3 ml of mobile phase 'A'(see below). After filtration through a 0.45 micron centrifugal microfilter, duplicate 30 µl injections were made of each sample and the concentration of each metabolite determined by comparing its average peak height ratio (compound/internal standard) with a standard curve prepared from urine samples of known metabolite concentration (see Results and Discussion).

Chromatographic conditions. For the conjugates assay a modified Alltech 605 RP column (Alltech Assoc., Deerfield, IL) was employed. The column, packed with 5 micron C18 particles, with an I.D. of 4.6 mm, was cut to a length of 120 mm. A 40 mm x 2.1 mm I.D. precolumn packed with CO:PELL ODS (Whatman, Inc., Clifton, N.J.) was attached directly to the main column. Mobile phase 'A'

was 40% methanol, 12 mM tetrapentylammonium chloride and 10 mM pH 6.2 phosphate buffer. Mobile phase 'B' contained 50% methanol but was otherwise identical to 'A'. The flow rate was 1.0 ml/min. A step gradient from mobile phase 'A' to 'B' 5.0 min after sample injection and a return to 'A' 12.5 min after injection was achieved by a solid state timing device attached to an in-line solvent switching valve (Model 5300 and 5302, Rheodyne, Inc., Cotati, CA). Column effluent was monitored at 230 nm.

Purification of Salicylamide Sulfate (SAMS)

A modification of the method of Mattox (15,16) for the ion-pair extraction of steroid conjugates from aqueous solution was employed in the extraction and purification of salicylamide sulfate from urine. Following a 2-g oral dose of SAM, 200 ml of urine from an adult human male subject was collected and washed with an equal volume of dichloromethane. To the washed urine was added 3.5 g of TBA-SO4 in 25 ml water. The urine was then extracted with 175 ml of dichloromethane. The organic layer was removed, treated with 2.85 g of myristic acid and back extracted with 200 ml of 0.5 M sodium bicarbonate. The aqueous phase was lyophilized and then dissolved in 40 ml of water. Four ml of this solution was injected in aliquots onto the chromatograph described above for assay of SAM conjugates. The effluents under the SAMS peak were collected, pooled and extracted into an equal volume of dichloromethane. This organic phase was washed with water, dried with anhydrous sodium sulfate and evaporated. The resulting crystals of the

tetrabutylammonium salt of salicylamide sulfate had a melting point of 105-110°C. The identity of the purified salt was confirmed by secondary ion mass spectrometry.

RESULTS AND DISCUSSION

Five peaks appeared in chromatograms of human urine and plasma following the oral administration of a 1-g dose of SAM (Figures 1 & 2). Two of these peaks, which appeared only in the plasma, were identified as SAM and GAM by comparison with chromatograms of the pure compounds. To identify the remaining peaks as SAM metabolites, chromatographic fractions containing each of the peaks were collected and subjected to the following treatments: acid hydrolysis (in 2N HCl for 20 hours at 65°C), incubation with bacterial β-glucuronidase (from 1 to 20 hours at 37°C), incubation with sulfatase (from 1 to 20 hrs at 37°C and pH 7.0) and colorimetric determination of gentisic acid derivatives (17). The earliest fraction, identified as salicylamide glucuronide (SAMG), was converted to SAM by glucuronidase or acid hydrolysis, gave no color in the gentisic acid test and was unaffected by treatment with sulfatase. The next fraction was identified as gentisamide glucuronide (GAMG) because it was converted to GAM by glucuronidase or acid hydrolysis, gave a positive gentisic acid test and was unaffected by treatment with sulfatase. The final eluting fraction, identified as salicylamide sulfate (SAMS), was converted to SAM by either sulfatase or acid hydrolysis but not glucuronidase and gave a negative gentisic acid test. Although SAM and GAM were

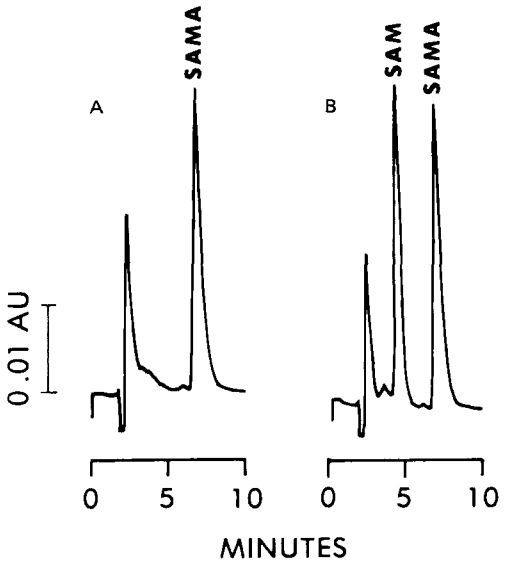

FIGURE 1 - SALICYLAMIDE ASSAY. Chromatograms of dog plasma extract before (A) and 9 min after (B) a 5 mg/kg oral dose of salicylamide (SAM concentration=1.0 µg/ml).

separated under the conditions used to assay the conjugated metabolites, they were assayed separately under conditions which provided greater sensitivity. Using a phenyl rather than a C-18 bonded phase column greatly improved the peak shape of salicylamide and therefore the sensitivity of the assay. Measuring the absorbance of SAM at the secondary maximum of 296 nm rather than at a lower wavelength significantly reduced the level of background interference and thus further increased assay sensitivity.

Calibration curves for SAM and GAM were derived from the pure compounds but the difficulty in obtaining metabolites of known purity and stability led to the development of an alternative

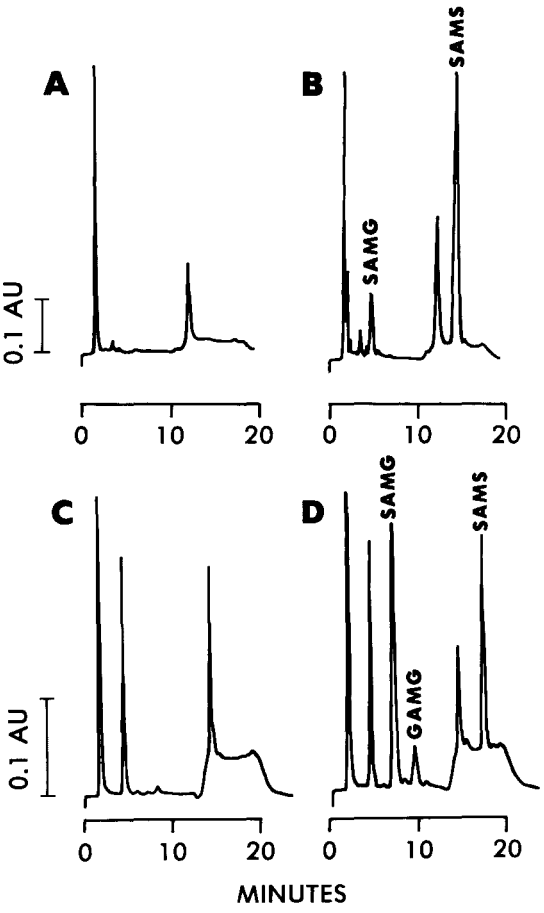

FIGURE 2 - METABOLITE ASSAY. Chromatographs of dog urine collected before (A) and for 320 min after (B) a 40 mg/kg oral dose of salicylamide and human urine collected before (C) and for 240 min after (D) a 10 mg/kg oral dose of salicylamide.

method for quantitation of GAMG, SAMG and SAMS. An adult male mongrel dog was given an oral dose of 200 mg SAM labelled with 40 microcuries of ^{14}C-salicylamide. Urine collected from this animal during the next 2 hrs was subjected to the assay for SAM metabolites and fractions of the HPLC eluent were collected. The amount of radioactivity under each metabolite peak was determined by counting each 1 ml fraction in 10 ml of Aquasol on a Beckman LS 9800 scintillation counter. The amount of each metabolite in this urine sample was calculated by dividing the amount of radioactivity collected under each peak by the known specific radioactivity of the administered drug. A calibration curve for the metabolite assay was prepared by diluting this urine sample with blank urine. Standard urine samples were stored at -20°C with no loss of metabolites for 3 months at which time the calibration procedure was repeated. The accuracy of this method was confirmed by using the assay to measure a 0.1 mg/ml solution of the purified tetrabutylammonium salt of SAMS. The urine-calibrated metabolite assay gave an average value of 0.104 mg/ml (± 0.01, n=4) for this solution.

Although the lowest point on the calibration curve for the SAM/GAM assay corresponds to a plasma concentration of 75 ng/ml, the assay is capable of measuring plasma levels of 10 ng/ml if the calibration curve is extended. Within-run coefficients of variation for this assay were $\leq$ 3% (n=10) over the range of the calibration curve (0.075 to 16 μg/ml) and inter-assay variation was less than 10% (n=6). For the conjugated metabolite assay the

TABLE 1

Salicylamide metabolite recovery in urine of dog and man

Metabolite	Species and Dose[a]			
	Dog, 10 mg/kg n=5	Dog, 20 mg/kg n=13	Human, 10 mg/kg n=2	Human, 10-20 mg/kg[b] n=4
SAMG	12 ± 7[c]	10 ± 7	51	51 ± 6
GAMG	0	0	14	15 ± 1.3
SAMSO4	88 ± 24	90 ± 15	34	32 ± 4

[a] Data from seven adult male mongrel dogs, weight 21-27 kg, and one adult male human subject, weight 100 kg. Dogs received dose via gastric tube in a vehicle of 8 ml propylene glycol and 2 ml ethanol, followed by 100 ml water. Human subject ingested the powdered drug followed by 250 ml water.

[b] Data of Morris and Levy (12) from three human subjects.

[c] Values shown (mean ± S.D.) are expressed as % of recovered dose in urine collected for at least 4 hours after the indicated oral dose of SAM.

coefficients of variation over the range of the calibration curve (0.07 to 0.3 mg/ml for SAMG and 1.0 to 4.0 mg/ml for SAMS) were 5% (SAMG, n=6) amd 7% (SAMS, n=6) within runs and 6.5% (both conjugates, n=7) between runs. Calibration curves for both assays were linear with intercepts near zero.

Table 1 compares the urinary recovery of SAM metabolites found in dogs and humans after the oral administration of SAM. These

data indicate that at the dose levels studied there are significant differences in metabolism between species. Most striking is the absence of hydroxylation in the dog. Neither gentisamide nor its conjugates are present in dog plasma or urine even at doses of 40 mg/kg SAM. In contrast, humans metabolize a significant portion of orally administered SAM by hydroxylation to GAM. It is also apparent from our data that the conjugated metabolites are formed to a different extent in the two species. In humans, salicylamide glucuronide is the major metabolite, accounting for just over half the urinary metabolites while in the dog salicylamide sulfate accounts for about 90% of the recovered metabolites.

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SIMULTANEOUS HPLC DETERMINATION OF OXCARBAZEPINE, CARBAMAZEPINE AND THEIR METABOLITES IN SERUM.

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ABSTRACT

We propose a simple procedure for the simultaneous determination of the anticonvulsants oxcarbazepine, carbamazepine and three of their metabolites (10-hydroxy-10,11-dihydro-carbamazepine, trans-10,11-dihydroxy-10,11-dihydro-carbamazepine and 10,11-epoxy-carbamazepine) in serum or plasma. The alkalinized sample is extracted with ethyl acetate. The extract is evaporated to dryness and taken up with the mobile phase. An aliquot is injected into the liquid chromatograph and eluted with water/methanol/acetonitrile (55/40/5, by vol.) on a 5-μm C-18 reversed-phase column. Eluent is monitored at 254 nm. No interference by other anticonvulsants or by endogenous constituents from the sample is observed. Owing to its good precision, specificity, sensitivity, and selectivity, this method is well adapted to the therapeutic monitoring of oxcarbazepine or carbamazepine treated patients, as well as for pharmacokinetic studies.

INTRODUCTION

Carbamazepine (CBZ) is an effective anticonvulsant drug mainly used against psychomotor and generalized tonic-clonic seizures. Oxcarbazepine (OCZ), the 10,11-dihydro-10-keto derivative of CBZ, has shown anticonvulsant properties in animals and humans (1) and is now under clinical evaluation. In man, CBZ is partially metabolized to 10,11-epoxy-carbamazepine (ECBZ), iminostilbene, and trans-10,11-dihydroxy-10,11-dihydro-carbamazepine (DHCBZ) (2,3).

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OCZ is extensively converted into 10-hydroxy-10,11-dihydro-carbamazepine (HCBZ), which is its main metabolite in serum, and into DHCBZ (4-6).

The clinical interest of management of CBZ serum levels is now well established (2). Determination of its metabolites is also of interest in therapeutic monitoring and, more evidently, in pharmacokinetic studies. The clinical usefulness of the quantitation of OCZ and/or its metabolites in serum is under evaluation. Yet, pharmacokinetic studies needs for determination of both parent drug and metabolites. Our aim was to develop a single method for the determination of OCZ, CBZ and their respective metabolites in serum.

EXPERIMENTAL

Reagents and Standards

Ethyl acetate R.G., methanol R.G., acetonitrile R.G. and NaOH 1 mol/l were obtained from Merck (Darmstadt, GFR). Water was deionised and distilled in a glass apparatus. CBZ, ECBZ, DHCBZ, OCZ, HCBZ, and 9-hydroxymethyl-10-carbamoylacridane were kindly supplied by Ciba-Geigy (Basel, Switzerland).

Extraction solvent: ethyl acetate containing 0.5 mg 9-hydroxymethyl-10-carbamoylacridane per liter, as internal standard. This solution is stable at 4°C for at least 4 months.

Mobile phase: water/methanol/acetonitrile (55/40/5, by vol.) degassed by helium sparging.

Apparatus

The liquid chromatograph consisted of a Pye-Unicam 4010 dual piston pump (Cambridge, UK), a Rheodyne 7125 injection valve with a 500- µl loop (Berkeley, CA, USA), a fixed wavelength LKB Uvicord SII 2338 detector (Bromma, Sweden), and a Kipp and Zonen BD 40 recorder (Delft, The Netherlands).

Analysis were performed on a reversed-phase 5-μm spherical C-18 Resolve column (150 mm x 3.9 mm id) (Waters Ass., Milford, MA, USA).

Operating Conditions

Mobile phase flow rate: 0.9 ml/min; temperature: ambient; detector wavelength : 254 nm, time constant: 2 s, sensitivity: 0.01 and 0.05 A full scale.

Procedure

Into a 10-ml stoppered glass centrifuge tube, pipette 500 μl of serum or plasma, add 50 μl of NaOH 1 mol/l and mix. Add 2.50 ml of extraction solvent. Shake on a rotary mixer for 5 min at 20 rpm and centrifuge. With a 1-ml Eppendorf pipette, transfer 2 ml of the organic layer into a 10-ml conical glass centrifuge tube. Evaporate to dryness at 50°C under a stream of nitrogen. Add 100 μl of mobile phase to the dry residue, vortex and inject 40 μl.

Quantitation

Each drug or metabolite was quantitated by measuring the ratio of its peak height to that of the internal standard, and by comparing with the ratio obtained for a calibration serum analysed under identical conditions.

The calibration serum was prepared by mixing 99.4 volumes of a drug-free human serum with 0.6 volume of a fresh ethanolic solution of CBZ, HCBZ, DHCBZ, OCZ, and ECBZ at 2000, 2000, 1000, 500, and 500 mg/l, respectively. Frozen aliquots of this serum are stable for at least 4 months.

RESULTS

Figure 1 shows the chromatograms of a drug-free serum, the calibration serum, and sera from patients treated with OCZ and CBZ, respectively. Retention times are 4.2 (DHCBZ), 5.2 (HCBZ), 6.1 (ECBZ), 7.6 (OCZ), 9.2 (internal standard), and 13.8 min (CBZ).

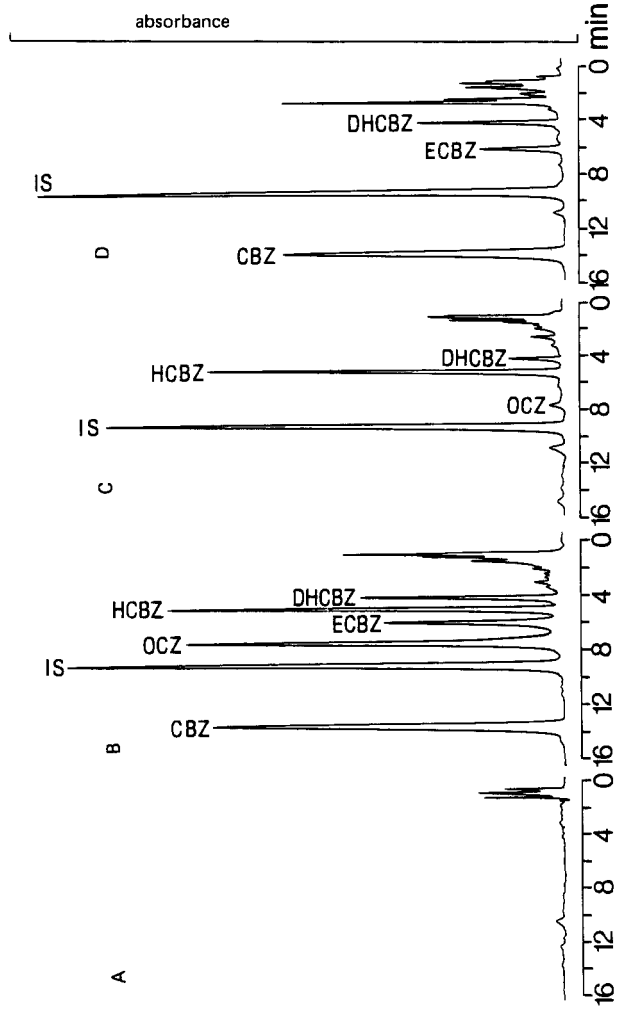

FIGURE 1. Chromatograms of (A) a drug-free serum extracted without internal standard; (B) the calibration serum containing DHCBZ (6.0 mg/l), HCBZ (12.0 mg/l), ECBZ (3.0 mg/l), OCZ (3.0 mg/l), and CBZ (12.0 mg/l); (C) a serum from a patient treated with OCZ; (D) a serum from a patient treated with CBZ.
Sensitivity: 0.01 A full scale, except for CBZ (0.05 A). IS: internal standard.

TABLE 1

Precision, Detection Limit and Linearity for CBZ, OCZ and their Metabolites in Serum.

	within-run precision CV% (*)		detection limit (**)	linearity (***) range	slope	intercept	S_{yx}
CBZ	2.2% (2N=40)	(8.50)	0.05	0.2-25	0.2694	0.0130	0.0901
ECBZ	4.6% (2N=40)	(1.16)	0.1	0.2-10	0.1077	-0.0054	0.0214
DHCBZ	12.5% (2N=78)	(1.83)	0.2	0.5-20	0.0643	-0.0061	0.0230
OCZ	5.6% (2N=28)	(2.60)	0.05	0.2-20	0.2170	0.0679	0.1647
HCBZ	2.2% (2N=40)	(12.9)	0.2	0.2-30	0.0605	0.0062	0.0080

(*) estimated from duplicates of patients'sera analysed under routine conditions, with the exception of OCZ, for which precision is estimated from calibration or control sera.
The mean levels (mg/l) of all these sera are in parentheses.

(**) for a signal to baseline noise ratio of 3 (in mg/l)

(***) the calibration curve regression is estimated over the levels range indicated (6 points, mg/l). All correlation coefficients are more than 0.996. S_{yx} : standard error of estimate.

Salicylate, theophylline, caffeine, ethosuximide, primidone, phenobarbital, phenytoin, and iminostilbene elute at 1.8, 2.0, 2.6, 2.6, 3.4, 4.6, 10.3, and 10.4 min, respectively. Valproic acid is not detected. None of these compounds interfere. Table 1 gives precision, detection limit and linearity for the five compounds analysed.

DISCUSSION

In order to achieve simultaneous determination of OCZ, CBZ and three of their metabolites without interference by other anticonvulsants or endogenous constituents from the sample, we carefully selected the mobile phase, the column packing and the extraction procedure.

TABLE 2

Mean Serum Levels ± SD (mg/l) found in OCZ or CBZ treated Patients.

	OCZ treated patients (N=20)	CBZ treated patients (N=20)
CBZ	—	8.50 ± 2.78
ECBZ	—	1.16 ± 0.54
DHCBZ	1.15 ± 1.14	2.50 ± 1.35
OCZ	0.18 ± 0.15	—
HCBZ	12.87 ± 7.37	—

The 5-μm C-18 stationary phase was chosen for its capacity to adequately resolve the compounds analysed (resolution from 1.63 to 6.13; plate heights from 0.070 to 0.038 mm). The excellent separation of the 5 compounds and of possible interfering drugs such as phenobarbital and phenytoin was also obtained by recourse to a ternary mobile phase which is composed of commonly used solvents, avoiding column equilibration with mobile phase additives.

The sample is alkalinized to decrease the recovery of phenobarbital and phenytoin, high concentrations of which might cause quantitation problems. The following extraction solvents were tried: ethyl acetate, methyl-isobutylketone, dichloromethane, ethyl ether, di-isopropylether, and several mixtures of these solvents. Methyl-isobutylketone and dichloromethane yield clean chromatograms and do not extract phenobarbital and phenytoin. However, lesser extraction efficiency of the compounds to be analysed was observed. Overall best results were obtained with ethyl acetate. This solvent was first proposed by Dörhöfer (7), whose extraction procedure was applied here with some modifications.

Our procedure provides two advantages over published determinations of OCZ (5-8): 1°) interference by phenobarbital, phenytoin and other anticonvulsants is not encountered, 2°) CBZ and its metabolites may also be simultaneously assayed.

Preliminary results of OCZ, CBZ and metabolites quantitation in serum of chronically treated patients are given in table 2.

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SPECIFIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AMPICILLIN IN BULKS, INJECTABLES, CAPSULES & ORAL SUSPENSIONS BY REVERSE-PHASE ION-PAIR CHROMATOGRAPHY

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ABSTRACT

A rapid, specific, stability-indicating high-performance liquid chromatographic (HPLC) method has been developed for the assay of Ampicillin in Ampicillin Trihydrate bulk, capsules and oral suspensions and Sodium Ampicillin bulk and injectables. The assay is specific for Ampicillin in the presence of possible contaminants; Penicillin V, Phenylglycine, and 6-Aminopenicillanic Acid (6-APA); the degradation product, Penicilloic Acid of Ampicillin; and all excipients present in the formulations assayed. Ampicillin, Ampicillin formulations, and formulation excipient blends were force-degraded to further demonstrate specificity.

The assay is precise, accurate, linear over the range 50% to 125% of expected Ampicillin sample level, and stability-indicating toward the described thermal, acid, base, aqueous, and light degradations.

The procedure employs an ion-pairing eluent with UV detection at 254 nm. Ampicillin Trihydrate and Sodium Ampicillin bulk are stable in assay diluent for six hours allowing the use of automatic HPLC injectors for unattended analysis. One set of HPLC parameters can assay bulks and formulations.

INTRODUCTION

D(-)-α-aminobenzylpenicillin, Ampicillin, is a semi-synthetic penicillin with activity against both gram-positive and gram-negative

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bacteria. It is available in injectable, capsule, and oral suspension forms.

Although the iodometric titration assay (1) will differentiate between intact penicillin nucleus and degradation products containing open β-lactam structure, it is still subject to interference from penicillin precursors or polymers of ampicillin (2-3). Other methods of analysis include colorimetry, microbiology (4), and non-aqueous titration. These methods also lack specificity. A higher degree of specificity can be achieved using high-pressure liquid chromatography. Anion-exchange columns have been used to assay ampicillin in nitrofurantoin (5) and in pharmaceutical preparations (6). With the advent of microparticulate HPLC columns, greater efficiency and resolution can be achieved. The literature contains references to reverse-phase HPLC systems for chromatographing ampicillin (7), investigating impurities of ampicillin (8), analyzing ampicillin in body fluids (9-10), separation of ampicillin from epicillin (11) and polymers of ampicillin (12), and quantitative analysis of ampicillin (13).

Ampicillin possesses both carboxylic acid and primary amine functionalities. Therefore, reverse-phase ion-pairing techniques can be used to chromatograph ampicillin. Heptane sulfonic acid has been used to ion-pair the primary amine and analyze ampicillin in human urine (14) and to investigate ampicillin degradation products (15). Tetrabutylammonium hydroxide has been used to ion-pair the carboxylic acid and separate ampicillin from its penicilloic acid (16).

Our purpose was to develop a specific HPLC system based on the reverse-phase ion-pairing technique using dodecyl sodium sulfate to analyze ampicillin in commercial drug forms.

EXPERIMENTAL

Instrumentation

The HPLC system consisted of a Waters 6000A pump operated at 3.0 ml/min., Model 440 fixed wavelength UV detector equipped to monitor 254 and 280 nm, WISP Model 710B autosampler programmed to inject 25-µl, and a 30 cm x 3.9 mm µ-Bondapak C-18 column (Waters Associates, Inc., Milford, Mass.). A Varichrom Variable Wavelength UV detector was used to monitor a third wavelength (Varian Associates, Palo Alto, CA.). The chromatograms were recorded on a Model 7100B dual-channel recorder and a Model 7127A single-channel recorder (Hewlett-Packard, Avondale, PA.). The assay wavelength was 254 nm. Integration was performed by a Model 3354 laboratory automation computer equipped with a 2:1 voltage divider (Hewlett-Packard, Avondale, PA.).

Reagents and Materials

Acetonitrile was UV grade, glass distilled (Burdick and Jackson, Lab., Inc., Muskegon, Mich.). Formic acid 88% w/w (Mallinckrodt Chem. Works, St. Louis, MO.), dodecyl sodium sulfate (Eastman Kodak, Rochester, N.Y.), and o-o´-biphenol (Aldrich Chem., Co., Inc., Milwaukee, Wisc.) were reagent grade. All aqueous reagents were prepared with water purified by reverse-osmosis (Millipore Corp., Bedford, Mass.). Ampicillin Trihydrate, Sodium Ampicillin, Penicillin V, Phenylglycine, 6-APA, and Penicilloic Acid of Ampicillin were high quality reference materials. Ampicillin bulks and formulations were of pharmaceutical quality.

Operating Parameters

The assay of Ampicillin was performed at ambient temperature. Detection was at 254 nm (0.1 AUFS). The flow rate was 3.0 ml/min.

Injection volume was 25-µl. The Ampicillin retention time was approximately 6.5 min.

Mobile Phase — 0.035M Dodecyl Sodium Sulfate/2.0M Formic Acid Stock Solution

10.1 g of Dodecyl Sodium Sulfate and 87 ml of formic acid were added to a 1-liter volumetric flask. The contents were dissolved in and diluted to volume with water.

Mobile Phase — 100 ml of 0.035M Dodecyl Sodium Sulfate/2.0M formic acid stock solution and 350 ml of acetonitrile were added to a 1-liter volumetric flask containing approximately 400 ml of water. The resulting solution was diluted to volume with water and mixed. The mobile phase was filtered through 0.45 micron filter paper. The proportion of acetonitrile may be modified in the range 30% to 38% to obtain the desired Ampicillin retention time. Increasing the proportion of acetonitrile will decrease retention time, decreasing the proportion of acetonitrile will increase retention time.

Standard and Sample Diluent — 35:65, acetonitrile:water.

Sample Diluent for Ampicillin/Probenecid Oral Suspensions — USP 1% pH 6.0 phosphate buffer.

System Suitability

A solution of Ampicillin Trihydrate reference material was prepared at 2.5 mg Ampicillin/ml. 25-µl was injected at 0.1 AUFS. The retention time of Ampicillin was between 4.5 and 8.5 minutes. If the Ampicillin retention time was not between 4.5 and 8.5 minutes, then new mobile phases would have been prepared, making the appropriate modifications in the acetonitrile concentration, until re-injection of the Ampicillin test solution produced an Ampicillin retention time between 4.5 and 8.5 minutes.

The column efficiency, based on the Ampicillin peak, was greater than 2100 plates per column as determined using the following formula:

$$\text{Efficiency (plates/column)} = 5.54 \left(\frac{\text{Retention Time}}{\text{Peak Width at Half Height}} \right)^2$$

where the retention time and peak width at half height were in the same units. If the efficiency was inadequate, then the column would have been replaced and the system suitability re-done.

Standard Preparation

Ampicillin Trihydrate in-house reference standard (assigned potency 862 mcg ampicillin activity/mg versus USP Ampicillin Trihydrate Standard Lot No. H-RD at 832 mcg/mg) was used 'as is'. Approximately 146 mg was accurately weighed into a 50-ml volumetric flask. The standard was dissolved in and diluted to volume with standard diluent. The solution was stable for six hours.

Internal Monitor Stock Solution

A solution of o-o´-biphenol was prepared in standard diluent at 0.8 mg/ml. An appropriate aliquot was pipetted into standards and samples prior to diluting to volume to produce a final concentration of 0.08 mg/ml.

Standardization & Injection Sequence

A standard was injected at the beginning of the assay, after every six samples, and at the end of the assay. Each sample was injected once. Each sample was calculated using the average response factor of the standard injections that 'bracket' it.

Sample Preparation

Ampicillin Trihydrate Bulk Powder — approximately 146 mg of bulk powder was accurately weighed into a 50-ml volumetric flask. The sample was dissolved in and diluted to volume with sample diluent.

TABLE 1

Preparation of Sodium Ampicillin Injectables

Activity/Vial From Label (mg)	Appropriate Vol. Flask (ml)	Further Vol. Dilution
125	50	None
250	100	None
500	200	None
1000	100	25.0/100.0
2000	200	25.0/100.0
10000	200	10.0/200.0

Sodium Ampicillin Bulk Powder

Approximately 130 mg of bulk powder was accurately weighed into a 50-ml volumetric flask. The sample was dissolved in and diluted to volume with sample diluent.

Sodium Ampicillin Injectables

Using small portions of sample diluent, the contents of one vial were completely transferred to an appropriate volumetric flask. The sample was dissolved in and diluted to volume with sample diluent. Using the same sample diluent, further volumetric dilutions were made to achieve an acceptable final concentration as indicated in Table 1.

Ampicillin Trihydrate Capsules

The contents of ten capsules were combined. A portion of the combined Ampicillin Trihydrate capsule contents, equivalent to 125 mg of Ampicillin activity, was accurately weighed into a 50-ml volumetric flask. The sample was diluted to the neck of the flask with sample diluent. With occasional swirling, the sample was sonicated for five minutes until all particles were finely suspended. The sample was diluted to volume with sample diluent and mixed. The sample was filtered through 0.6 micron filter paper placed in a disc filter holder.

TABLE 2

Preparation of Multiple Dose Ampicillin Trihydrate Oral Suspensions (5 ml dose)

Activity from Label (mg/5 ml)	Appropriate Volumetric Flask (ml)
125	50
250	100
500	200

Multiple Dose Ampicillin Trihydrate Oral Suspensions (5 ml dose)

125 mg/5 ml, 250/5 ml, 500 mg/5 ml Ampicillin Activity — using a burette, the contents of the bottle were reconstituted with water as per label directions. The bottle was mechanically shaken in a horizontal position for 25 minutes. The bottle was inverted several times just prior to sampling to evenly resuspend all particles. Using a 5.0-ml glass syringe fitted with a 13-gauge needle, sufficient suspension was withdrawn to wet the syringe. The suspension was expelled back into the bottle. Slightly more than a 5.0-ml dose was withdrawn from the bottle. The syringe was inverted and brought to 5.0-ml expelling all air bubbles. The 5.0-ml dose was ejected into the appropriate volumetric flask (as indicated in Table 2) already one-half full of sample diluent. The sample was diluted to volume with sample diluent and mixed. The sample was sonicated five minutes and filtered through 0.6 micron filter paper placed in a disc filter holder.

Single Dose Ampicillin Trihydrate Oral Suspensions

125 mg/bottle, 250 mg/bottle, 500 mg/bottle Ampicillin Activity — using a glass syringe, the contents of the bottle were reconstituted with water as per label directions. The bottle was mechanically shaken in a horizontal position for 25 minutes. The bottle was inverted several

TABLE 3

Preparation of Single Dose Ampicillin Trihydrate Oral Suspensions

Activity/Bottle From Label	Appropriate Volumetric Flask (ml)
125	50
250	100
500	200

times just prior to sampling to evenly re-suspend all particles. The entire contents were drained into the appropriate volumetric flask (as indicated in Table 3) for 30 seconds. The sample was diluted to volume with sample diluent and mixed. The sample was sonicated five minutes and filtered through 0.6 micron filter paper placed in a disc filter holder.

Single Dose Ampicillin Trihydrate/Probenecid Oral Suspension

3.5 g/bottle Ampicillin Activity. Using a burette, the contents of the bottle were reconstituted as per label directions. The bottle was mechanically shaken in a horizontal position for 25 minutes. The bottle was inverted several times just prior to sampling to evenly re-suspend all particles. The entire contents were drained into a 1-liter volumetric flask for 30 seconds. The sample was diluted to volume with 1% pH 6.0 phosphate buffer and mixed. A 75.00-ml aliquot was transferred to a 100-ml volumetric flask and diluted to volume with 1% pH 6.0 phosphate buffer. The sample was filtered through filter paper discarding the first 10 ml of filtrate.

Calculations — Ampicillin Response Factor

$$F = \frac{W_{STD} \times P_{STD}}{A_{STD} \times 50 \text{ ml} \times 1000 \text{ mcg/mg}}$$

TABLE 4

DF Values For Injectables

Ampicillin Potency (mg/vial)	DF
125	50
250	100
500	200
1000	400
2000	800
10000	4000

Ampicillin Trihydrate & Sodium Ampicillin Bulks

The potency in mcg/mg was calculated using the following formula:

$$P_{SAMP} = \frac{F \times A_{SAMP} \times 50 \text{ ml} \times 1000 \text{ mcg/mg}}{W_{SAMP}}$$

Sodium Ampicillin Injectables

The potency in mg/vial was calculated using the following formula:

$$V_{SAMP} = F \times A_{SAMP} \times DF$$

Ampicillin Trihydrate Capsules

The potency in mg/average capsule weight was calculated using the following formula:

$$C_{SAMP} = F \times A_{SAMP} \times \frac{ACW}{W_{SAMP}} \times 50 \text{ ml}$$

Ampicillin Trihydrate Oral Suspensions

The potency in mg/5 ml dose or mg/bottle was calculated using the following formula:

$$OS_{SAMP} = F \times A_{SAMP} \times DF$$

TABLE 5

DF Values For Ampicillin Trihydrate Oral Suspensions

Ampicillin Potency (mg/5 ml dose) or (mg/bottle)	DF
125	50
250	100
500	200
3500	1333

Where: F = Ampicillin standard response factor

P_{SAMP} = Ampicillin bulk potency, mcg/mg

P_{STD} = Ampicillin standard potency, mcg/mg

A_{SAMP} = Ampicillin peak area of sample

A_{STD} = Ampicillin peak area of standard

W_{STD} = Standard weight, mg

W_{SAMP} = Sample weight, mg

V_{SAMP} = Ampicillin potency, mg/vial

C_{SAMP} = Ampicillin potency, mg/average capsule weight

ACW = Average capsule weight

OS_{SAMP} = Ampicillin potency, mg/5 ml dose or mg/bottle

DF = Dilution Factor

Specificity

The specificity of the method was determined by injecting precursors of Ampicillin, a degradation product (Penicilloic Acid of Ampicillin), and all Ampicillin Trihydrate capsule and oral suspension excipients. The specificity of the assay was further demonstrated by force degrading both Ampicillin Trihydrate and Sodium Ampicillin Bulk under heat, acidic, basic, aqueous, and UV light conditions. Actual Ampicillin injectable, capsule, and oral suspension formulations as well as excipient placebo blends were

force degraded under heat, aqueous, and UV light conditions. These degraded samples were chromatographed to check for visible interferences. Three detection wavelengths (254, 280, and either 227, 233, or 241 nm) were monitored for these degradation samples. Ampicillin peak height ratios, among the three wavelengths, for these degradation samples were compared to the peak height ratios of an undegraded sample of Ampicillin. On a given day and instrument, the peak ratios of undegraded and degraded Ampicillin should be different if interfering degradation products with different absorptivities than Ampicillin are present. The absorptivity of Ampicillin at 280 nm is very poor. Monitoring 280 nm was a method of determining whether any interfering degradation products were produced.

Recovery Studies

Ampicillin Trihydrate was spiked into two different capsule excipient blends at the 50%, 100%, and 150% levels. Ampicillin Trihydrate was spiked into two different oral suspension excipient blends at the 50%, 100%, and 150% levels.

The precursors of Ampicillin; Penicillin V, Phenylglycine, and 6-APA and the degradation product, Penicilloic Acid of Ampicillin were completely resolved from Ampicillin (Fig. 1). None of the capsule excipients or oral suspension excipients interfered with Ampicillin (Fig. 2).

Ampicillin Trihydrate and Sodium Ampicillin Bulk were degraded thermally, in 0.01N HCl, in 0.01N KOH, in water, and by accelerated light (3×10^{16} photons/sec/cm^2 at 254 nm). Elevated temperatures were used for the acid and aqueous degradations to facilitate otherwise slow degradation.

Sodium Ampicillin injectables were degraded thermally, by accelerated light, and upon reconstitution with water. Elevated temperature was

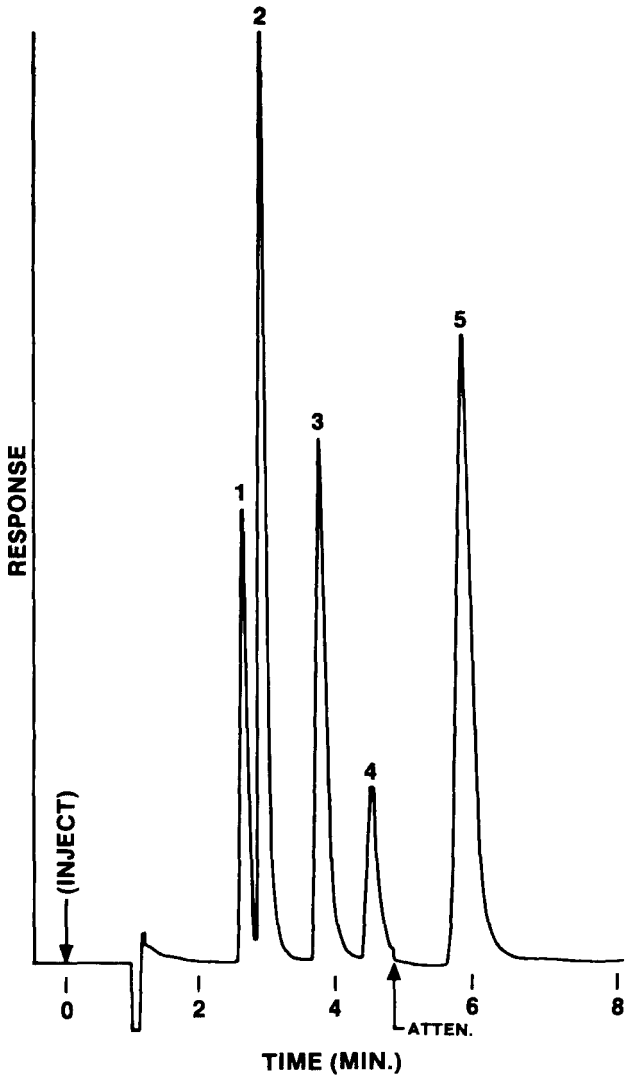

Figure 1 — Separation of Phenylglycine (1), 6-APA (2), Penicillin V (3), Penicilloic Acid of Ampicillin (4), and Ampicillin (5).

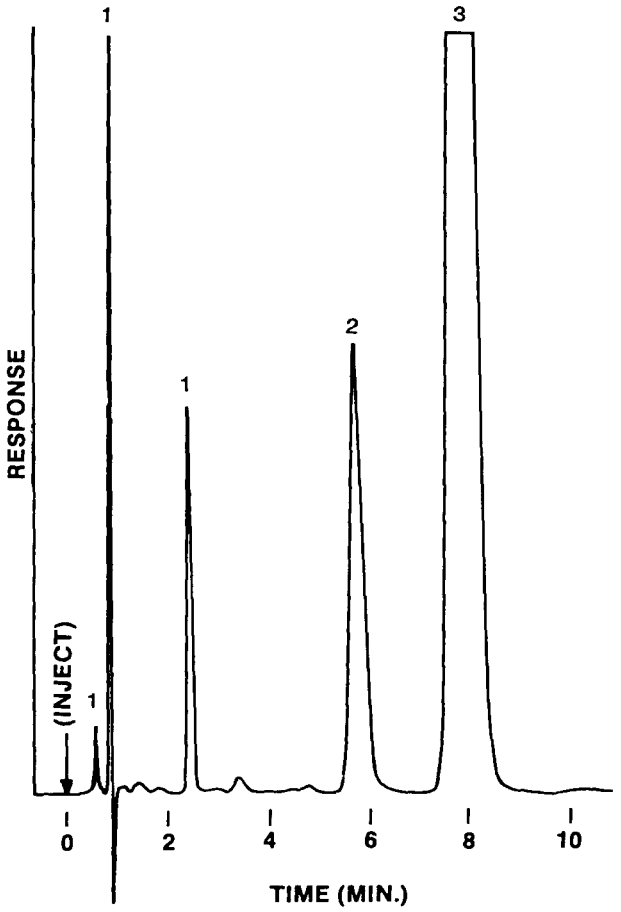

Figure 2 — Chromatogram of an authentic Ampicillin Oral Suspension. Key: 1 = Excipients; 2 = Ampicillin; 3 = Probenecid.

used for the aqueous degradation. The degradations were performed in the injectable vial to allow any interferences from the container to form.

Ampicillin Trihydrate capsule contents, the intact capsule, and a placebo blend were degraded thermally, by accelerated light, and in water. Elevated temperature was used for the aqueous degradation.

Ampicillin Trihydrate oral suspension blend and the intact oral suspension bottle were degraded thermally and by accelerated light. The oral suspension blend was degraded in water with elevated temperature.

Approximately 15-50% degradation was targeted for, although in some cases more or less degradation was produced.

No visibly interfering degradation products were produced in any of the aforementioned forced degradations of drug substance or placebo blends. The stress conditions were purposely chosen to be more severe than any conditions to which the product may be subjected. Many unknown degradation peaks were produced.

The peak height ratios, among the three wavelengths monitored, for degraded Ampicillin compared to undegraded Ampicillin were very consistent. This is one confirmation of the stability-indicating nature of the assay. At 280 nm, where Ampicillin's absorptivity is very low, no interfering degradation peaks appeared.

Forced degradation studies produced an appreciable degradation of Ampicillin (15-50%), the possibility of finding an internal standard that will be absolutely free of potential interferences from the degradation products of Ampicillin or the excipients present in the drug forms is doubtful.

We used an internal monitor, a compound that elutes in a zone free from interference in undegraded drug forms (Fig. 3). The internal

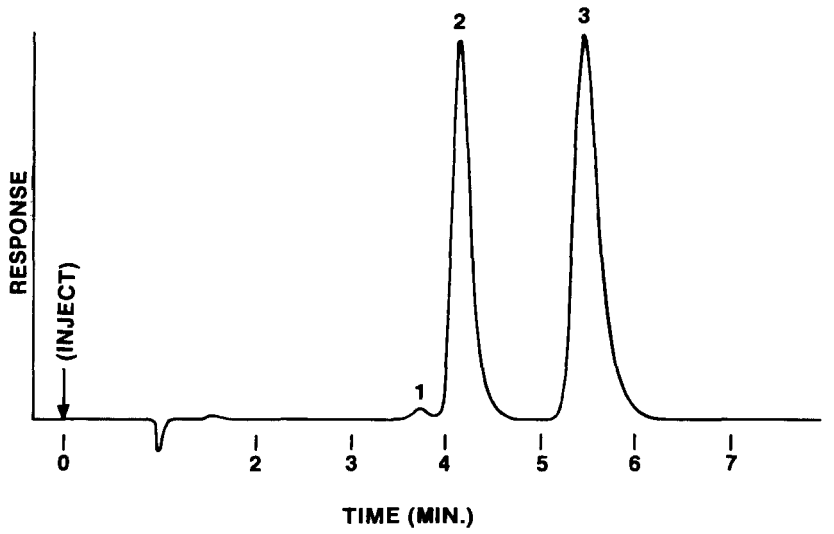

Figure 3 — Chromatogram of Sodium Ampicillin and internal monitor. Key: 1 = Penicilloic Acid of Ampicillin, 2 = o-o´-biphenol (internal monitor) 3 = Ampicillin.

monitor served to monitor the injection volume of the autoinjector. Due to its potential of having an interference in degraded drug forms, it was not and cannot be used as an internal standard to calculate assay results.

Six injections of one preparation of both Ampicillin Trihydrate and Sodium Ampicillin were made at the 2.5 mg/ml level. The reproducibility of injection of Ampicillin Trihydrate bulk (2 RSD = 1.2%) and Sodium Ampicillin bulk (2 RSD = 1.1%) was found to be excellent.

Both Ampicillin Trihydrate and Sodium Ampicillin were injected in triplicate at 1.25, 1.90, 2.50, and 3.10 mg/ml. The levels were chosen to correspond to 50%, 75%, 100%, and 125% of expected sample level.

Linearity of response versus Ampicillin concentration for Ampicillin Trihydrate was found to be linear in the range tested

TABLE 6

Linearity of Ampicillin Trihydrate & Sodium Ampicillin

Compound	Concentration Range Tested mg/ml	Correlation Coefficient	Percent Deviation From Origin, %
Ampicillin Trihydrate	1.23-3.09	0.99986	—0.6
Sodium Ampicillin	1.28-3.09	0.9985	—0.7

TABLE 7

Calculated Biases For Ampicillin Trihydrate & Sodium Ampicillin Over The Range of 50% to 125% of Sample Level

Compound	Actual Conc. mg/ml	Calculated Conc. mg/ml	% Deviation
Ampicillin Trihydrate	3.089	3.101	0.4%
	1.870	1.877	0.4%
	1.232	1.230	—0.2%
Sodium Ampicillin	3.093	3.044	—1.6%
	1.908	1.907	<0.1%
	1.276	1.235	—3.2%

TABLE 8

Ampicillin Recovery From Capsule Blends

Blend No.	Spike Added mg/ml	Spike Recovered mg/ml	% Recovery
1	3.563	3.520	98.7
1	2.484	2.486	100.1
1	1.298	1.290	99.4
2	3.791	3.782	99.8
2	2.514	2.517	100.1
2	1.267	1.270	100.2

(correlation coefficient = 0.99986) and the percent deviation from the origin was extremely small (intercept x 100/response at standarization level). Linearity of response versus Ampicillin concentration for Sodium Ampicillin was linear (correlation coefficient = 0.9985) and the percent deviation from the origin was extremely small (Table 6).

Using single-point standardization at the 100% Ampicillin level, the biases calculated for Ampicillin Trihydrate at the 50, 75, and 125% levels was not greater than 1% (relative). The biases for Sodium Ampicillin were not more than 2% (relative) except for the 50% level which was —3.2%. This was not considered a serious bias especially at the 50% level. Refer to Table 7.

Based on the data in Tables 6 and 7, single-point standardization may be used.

Table 8 shows the accuracy of the procedure for two different capsule excipient blends. Recovery in all cases at three different Ampicillin levels was >98%.

Table 9 shows the accuracy of the procedure for two different oral suspension excipient blends. Recovery in all cases at three different Ampicillin levels was >98%.

TABLE 9

Ampicillin Recovery From Oral Suspension Blends

Blend No.	Spike Added mg/ml	Spike Recovered mg/ml	% Recovery
1	3.782	3.778	99.9
1	2.502	2.512	100.4
1	1.257	1.269	101.0
2	3.742	3.748	100.2
2	2.494	2.466	98.9
2	1.259	1.249	99.2

TABLE 10

Overall Procedural Variability

Sample No.	Ampicillin Trihydrate Bulk, mcg/mg	Sodium Ampicillin Bulk, mcg/mg	Injectable 250 mg/vial	Capsule 250 mg/cap	Capsule 500 mg/cap
1	830	887	247	240	473
2	832	883	250	236	472
3	828	880	242	237	472
4	826	889	237	233	473
5	826	883	244	234	473
6	825	889	245	235	473
Average	828	885	244	236	473
RSD (%)	0.3	0.4	1.8	1.0	0.1

Sample No.	Oral Suspension 125 mg/5 ml	Oral Suspension 250 mg/5 ml	Oral Suspension 500 mg/5 ml	Oral Suspension 3.50 g/bottle
1	118	244	496	3.48
2	120	239	496	3.54
3	120	242	487	3.71
4	118	231	482	3.55
5	117	237	491	3.53
6	120	237	475	3.54
Average	119	238	488	3.56
RSD (%)	1.1	1.9	1.7	2.2

Six different preparations of Ampicillin Trihydrate bulk, Sodium Ampicillin bulk, Sodium Ampicillin injectables, two lots of Ampicillin Trihydrate capsules, and four lots of Ampicillin Trihydrate oral suspensions were assayed by this procedure.

Table 10 shows the overall procedural variability of the method for all the dosage forms studied. As the sample handling became more involved, the RSD increased.

TABLE 11

Ampicillin Stability in 1% pH 6.0 Phosphate Buffer

Time (hr.)	% Ampicillin Trihydrate Remaining	Time (hr.)	% Sodium Ampicillin Remaining
0	100.0	0	100.0
2.8	98.9	2.0	99.5
3.8	98.0	3.0	98.9
4.9	96.6	4.0	97.4
5.9	95.7	5.1	97.2
6.9	95.1	6.1	96.0
8.0	93.1	7.1	94.5
9.0	92.8	8.2	92.8
10.0	92.0	9.2	89.8

TABLE 12

Ampicillin Stability in 35:65, Acetonitrile:Water

Time (hr.)	% Ampicillin Trihydrate Remaining	% Sodium Ampicillin Remaining
0	100.0	100.0
0.4	100.7	100.3
1.0	100.2	99.7
1.9	100.0	99.7
2.3	99.6	99.6
2.9	99.5	99.6
3.3	99.4	99.9
3.8	99.6	99.0
4.2	99.1	99.6
4.8	99.3	99.3
5.2	99.2	99.7
5.8	99.1	99.4
6.1	98.8	98.8
6.7	99.9	98.9
7.1	99.3	98.2

TABLE 13

Automated, Unattended Assays

LABORATORY 1			LABORATORY 2		
Time (hr.)	Ampicillin Trihydrate Lot 1, mcg/mg	Sodium Ampicillin Lot 1, mcg/mg	Time (hr.)	Ampicillin Trihydrate Lot 2, mcg/mg	Sodium Ampicillin Lot 2, mcg/mg
0	855	902	0	821	873
0.4	861	905	0.4	821	874
0.9	858	901	0.8	821	872
1.3	856	902	1.4	821	876
1.9	857	901	1.8	822	873
2.3	854	900	2.2	819	876
2.9	854	901	2.8	821	875
3.2	853	904	3.1	819	873
3.8	852	894	3.5	823	871
4.2	847	898	4.1	822	874
4.8	846	894	4.5	824	872
5.2	845	896	4.9	822	872
5.7	852	902	5.4	821	879
6.0	849	896	5.8	828	881
6.6	866	904	6.2	818	890
7.0	860	898	6.8	797	882
			7.2	829	870
			7.6	817	870
Average	854	900		820	874
RSD (%)	0.6	0.4		0.8	0.5

The precision of the bulk, capsule and oral suspension results (125, 250, 500 mg/5 ml) also depends on uniformity of blend as well as the HPLC method. The precision of the injectable and oral suspension (3.50 g/bottle) results depend on the uniformity of blend and fill weight as well as the HPLC method.

Tables 11 and 12 show the stability of both Ampicillin Trihydrate and Sodium Ampicillin in two proposed assay diluents.

Ampicillin is stable in 35:65, acetonitrile:water for approximately six hours (<1% degradation). The degradation is very slow and an Ampicillin standard prepared at the same time as the samples

TABLE 14

Comparison of HPLC versus Iodometric Assay For
Ampicillin Trihydrate & Sodium Ampicillin

	Ampicillin Trihydrate mcg/mg Ampicillin		Sodium Ampicillin mcg/mg Ampicillin	
Lot	HPLC[a]	Iodometric[a]	HPLC[b]	Iodometric[c]
1	848	856	872	872
2	850	834	890	879
3	822	805	886	886
4	854	838	885	874
5	840	864	884	886
6	854	846	893	876
7	854	850	905	903
8	853	846	881	889
9	854	854	899	896
10	858	847	894	878
11	838	843	890	886
12	846	849	894	892
13	856	848	900	891
14	841	835	897	889
15	848	846	896	886
16	800	836	882	890
17	839	856	869	876
18	844	846	879	844
19	848	842	879	889
20	827	842	877	886
Average	844	844	887	883

a Average of duplicate preparations.

b Average of five preparations.

c Average of four preparations.

will exhibit the same degradation. Therefore, if samples are calculated versus standard injections that 'bracket' those samples, no apparent loss will be evident and automated, unattended assays may be performed for up to six hours. Table 13 shows the assay results of two different laboratories using the standard 'bracketing' technique.

Table 14 shows the comparison of the HPLC method to the iodometric method for twenty lots of Ampicillin Trihydrate and twenty lots of

Sodium Ampicillin. There was no significant difference between the means at the 95% confidence level as determined by the null hypothesis for material of this quality.

CONCLUSION

The described HPLC procedure has proven to be applicable to various Ampicillin formulations. One set of HPLC parameters successfully assayed all the formulations tested; separated precursors, degradation products, and formulation excipients; and showed good agreement with the iodometric assay. An assay diluent in which Ampicillin is stable was found. This will allow for the full benefits of the new autosampling equipment and be amenable to quality control operations.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(6), 1267-1271 (1984)

LC CALENDAR

1984

APRIL 29 - MAY 3: Analyical Applications of Supercritical Fluids - Supercritical Fluid Technol Symposium, at the meeting of the AOCS, Dallas, TX. Contact: Dr. J. W. King, CPC Internat'l, Moffett Tech Center, Argo, IL, 60501, USA.

MAY 17: Symposium: Therapeutic Drug Monitoring & Toxicology for the 80's: Clinical & Instrumental Perspectives, Farmington, CT, sponsored by the UConn Medical School & AAAC Connecticut Valley Chapter. Contact: Dr. Steven H. Wong, Dept. of Lab. Med., UConn Medical School, Farmington, CT, 06032, USA.

MAY 20 - 26: 8th Intl. Symposium on Column Liquid Chromatography, New York Statler Hotel, New York City. Contact: Prof. Cs. Horvath, Yale University, Dept. of Chem. Eng., P. O. Box 2159, Yale Stn., New Haven, CT, 06520, USA.

MAY 24 - 29: American Associatio for the Advancement of Science - Annual Meeting & Exhibit, New York City. Contact: AAAS Meetings Dept., 10th Floor, 1101 Vermont Avenue, NW, Washington, DC, 20005, USA.

JUNE 3-5: International Symposium on LCEC and Voltammetry, Indianapolis Hyatt Regency Hotel, Indianapolis, IN. Contact: The 1984 LCEC Symposium, P. O. Box 2206, West Lafayette, IN, 47906, USA.

JUNE 10 - 13: Annual Meeting of the Institute of Food Technologists, Anaheim, CA. Contact: IFT, 221 N. LaSalle Street, Suite 2120, Chicago, IL, 60601, USA.

JUNE 10-14: 14th Northeast Regional ACS Meeting, sponsored by the Western Connecticut and New Haven Sections, at Fairfield University, Fairfield, CT. Contact:D. L. Swanson, American Cyanamid Co., Stamford, CT, USA.

JUNE 18-20: Second International Conference on Chromatography & Mass Spectrometry in Biomedical Sciences, sponsored by the Italian Group for Mass Spectrometry in Biochemistry & Medicine, Milan, Italy. Contact: Dr. A. Frigerio, via Eustachi 36, I-20129 Milan, Italy, or Dr. H. Milon, P. O. Box 88, CH-1814 La Tour-de-Peilz, Switzerland.

JUNE 18-21: Symposium on Liquid Chromatography in the Biological Sciences, Ronneby, Sweden, sponsored by The Swedish Academy of Pharmaceutical Sciences. Contact: Swedish Academy of Pharmaceutical Sciences, P. O. Box 1136, S-111 81 Stockholm, Sweden.

JUNE 19 - 23: SPI/SPE Plastics Show & Conf. East, Philadelphia, PA. Contact: SPE, 14 Fairfield Drive, Brookfield Center, CT, 06805, USA.

JUNE 21 - 22: Conference on Quantitative Characterization of Plastics & Rubber, McMaster University, Hamilton, Ont., Canada. Contact: John Vlachopoulos, Dept. of Chem. Eng., McMaster University, Hamilton, Ont., L8S 4L7, Canada.

JULY 1 - 7: 12th International Carbohydrate Symposium, Jaarbeurs Congress Centre, Utrecht, The Netherlands. Contact: J. F. G. Vliengenthart, Dept. of Bio-Organic Chem., State Univ. of Utrecht, P. O. Box 5055, NL-3502JB, Utrecht, The Netherlands.

JULY 9 - 13: 10th International Conference on Organic Coatings Science & Technol., Athens, Greece. Contact: V. Patsis, Materials Research Lab, CSB 209, State Univ. of NY, New Paltz, NY, 12561, USA.

JULY 15 - 20: International Conference on Ion Exchange, Cambridge College, UK. Contact: Conference Committee, IEX-84, Soc. Chem. Ind., 14 Belgrave Square, London, SWIX 8PS, UK.

AUGUST 21 - 24: 24th Int'l Conf on Analytical Chem. in Development, Sri Lanka. Contact: Secretary, Organizing Committee, Centre for Anal. Chem R & D, Dept. of Chem., University of Colombo, P. O. Box 1490, Colombo 3, Sri Lanka.

AUGUST 26-31: National ACS Meeting, Philadelphia, PA. Contact: Meetings, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

SEPTEMBER 10-14: Advances in Liquid Chromatography, including the 4th Annual American-Eastern European Symposium on LC and the Int'l Symposium on TLC with Special Emphasis on Overpressured Layer Chromatography, sponsored by the Hungarian Academy of Sciences' Chromatography Committee & Biological Research Center and the Hungarian Chemical Society, in Szeged, Hungary. Contact: Dr. H.

Kalasz, Dept. of Pharmacology, Semmelweis University of Medicine, P.O.Box 370, H-1445 Budapest, Hungary, or Dr. E. Tyihak, Research Inst. for Plant Protection, P.O.Box 102, H-1525 Budapest, Hungary.

SEPTEMBER 16 - 21: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Marriott Hotel, Philadelphia, PA. Contact: D. B. Chase, DuPont Co., Experimental Station 328, Wilmington, DE, 19898, USA.

OCTOBER 1-5: 15th Int'l. Sympos. on Chromatography, Nurenberg, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, D-6000 Frankfurt Main, West Germany.

OCTOBER 8 - 10: ASTM Committee E-19 on Chromatography, St. Louis Sheraton Hotel, St. Louis, MO. Contact: F. M. Rabel, Whatman, Inc., 9 Bridewell Place, Clifton, NJ, 07014, USA.

OCTOBER 24 - 26: Third Workshop/Symposium on LC/MS and MS/MS, Montreux, Switzerland. Contact: R. W. Frei, Dept. of Anal. Chem., Free University, De Boelelaan 1083, NL-1081 HV Amsterdam, The Netherlands.

OCTOBER 28 - NOVEMBER 1: 2nd International Congress on Computers in Science, Washington, DC. Contact: S. R. Heller, EPA, PM-218, Washington, DC, 20460, USA.

NOVEMBER 13 - 16: 23rd Eastern Analytical Symposium, New York Penta Hotel, New York City. Contact: S. D. Klein, Merck & Co., P. O. Box 2000/R80l-106, rahway, NJ, 07065, USA.

DECEMBER 10-12: "TLC/HPTLC-84: Expanding Horizons in TLC," Sheraton-University City, Philadelphia, PA. Contact: J. C. Touchstone, University of Pennsylvania, Dept. OB-GYN, 3400 Spruce Street, Philadelphia, PA.

DECEMBER 16-21: International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii, sponsored by the Chemical Inst. of Canada, Chemical Soc. of Japan, and the American Chem. Soc. Contact: PAC CHEM '84, International Activities Office, American Chem. Soc., 1155 Sixteenth St., NW, Washington, DC, 20036, USA.

1985

FEBRUARY 11-14: Polymer 85, Int'l Symposium on Characterization and Analysis of Polymers, Monash University, Melbourne, Australia, sponsored by the Polymer Div., Royal Australian Chemical Inst. Contact: Polymer 85, RACI, 191 Royal Parade, Parkville Victoria 3052, Australia.

APRIL 15 - 17: Second International Symposium on Instrumental TLC, Wurzburg, West Germany. Contact: H. M. Stahr, 1636 College Veterinary Medicine, Iowa State University, Ames, IA, 50011, USA.

APRIL 28 - MAY 3: 189th National ACS Meeting, Miami Beach. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

JUNE 24 - 28: 59th Colloid & Surface Science Symposium, Clarkson College of Technology, Potsdam, NY. Contact: J. P. Kratohvil, Institute of Colloid & Surface Science, Clarkson College of Technology, Potsdam, NY, 13676, USA.

JULY 1-5: Ninth International Symposium on Column Liquid Chromatography, sponsored by the Chromatography Discussion Group and by the Royal Society of Chemistry's Chromatography & Electrophoresis Group, Edinburgh, Scotland. Contact: Prof. J. H. Knox, 9th ISCLC Secretariat, 26 Albany Street, Edinburgh, EH1 3QH, Great Britain.

JULY 4: 4th International Flavor Conference, Greece. Contact: C. J. Mussinan, IFF R&D, 1515 Highway 36, Union Beach, NJ, 07735, USA.

SEPTEMBER 8-13: 190th National ACS Meeting, Chicago. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA

SEPTEMBER 29 - OCTOBER 4: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Marriott Hotel, Philadelphia, PA. Contact: T. Rains, NBS, Center for Analytical Chemistry, Chemistry B-222, Washington, DC, 20234, USA.

1986

APRIL 6-11: 191st National Am. Chem. Soc. Mtng., Atlantic City, NJ. Contact: A. T. Winstead, ACS, 1155 16th Streeet, NW, Washington, DC, 20036, USA.

SEPTEMBER 7-12: 192nd National Am. Chem. Soc. Mtng., Anaheim, Calif. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA

1987

APRIL 5-10: 193rd National Am. Chem. Soc. Mtng., Denver, Colo. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

AUGUST 30 - SEPTEMBER 4: 194th National Am. Chem. Soc. Mtng., New Orleans, LA. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

1988

JUNE 5 - 11: 3rd Chemical Congress of the North Americanmn Continent, Toronto, Ont., Canada. Contact: A. T. Winstead, ACS, 1155 Sixteenth St, NW, Washington, DC, 20036, USA.

SEPTEMBER 25 - 30: 196th ACS National Meeting, Los Angeles, CA. Contact: A. T. Winstead, ACS, 1155 Sixteenth Street, NW, Washington, DC, 20036, USA.

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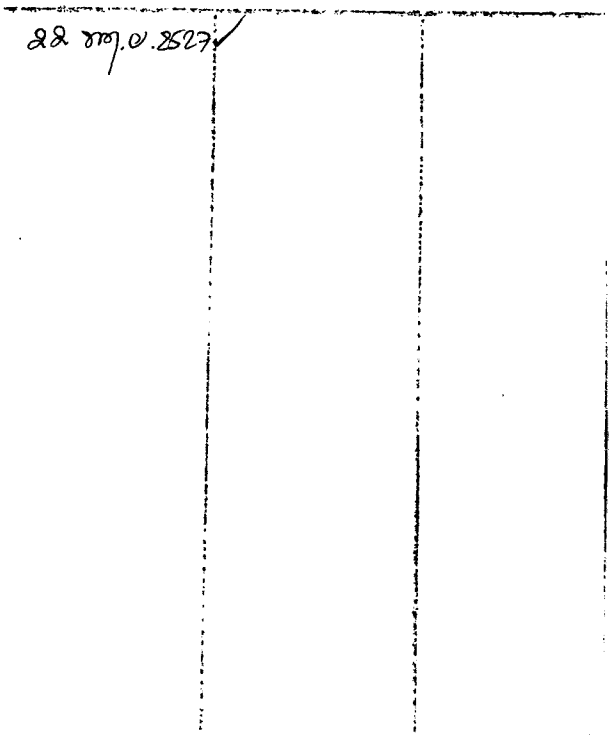

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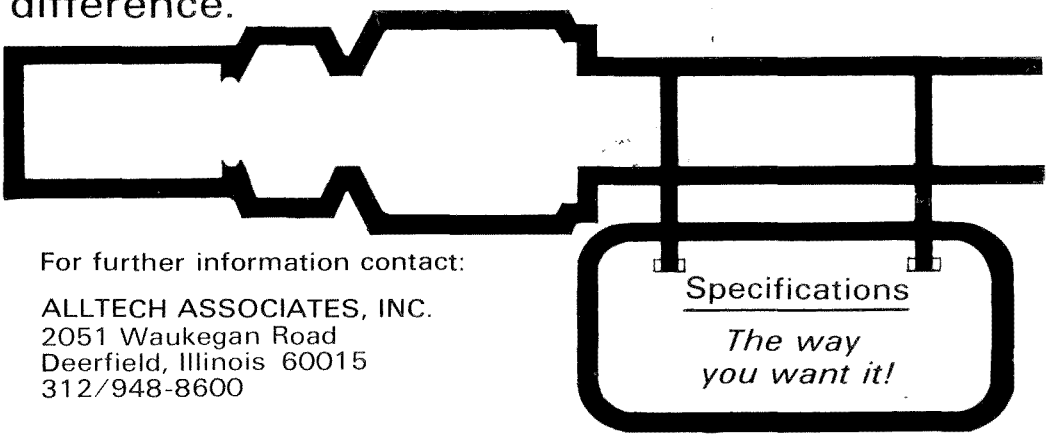

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