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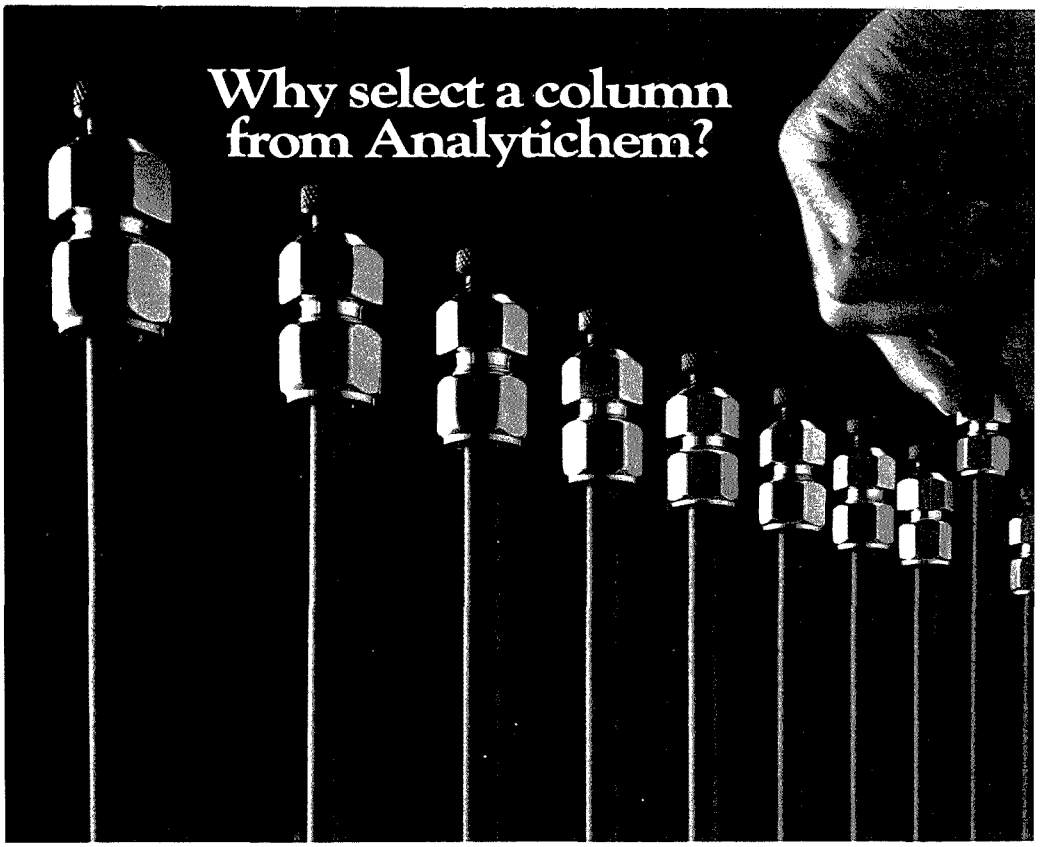

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Special Section on Thin-Layer Chromatography

Regular Contributed Papers

THIN-LAYER CHROMATOGRAPHY

Edited by

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Fairfield, Connecticut

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(7), 1273-1288 (1984)

EFFECTS OF DIFFERENT STATIONARY PHASES AND SURFACTANT OR CYCLODEXTRIN SPRAY REAGENTS ON THE FLUORESCENCE DENSITOMETRY OF POLYCYCLIC AROMATIC HYDROCARBONS AND DANSYLATED AMINO ACIDS

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*Contribution from: Department of Chemistry
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ABSTRACT

The detectable luminescence of twelve dansyl amino acids and four polycyclic aromatic hydrocarbons (PAH's) spotted on five common TLC stationary phases was evaluated. The detectable luminescence varied appreciable for compounds associated with different stationary phases. The use of surfactant and cyclodextrin spray reagents caused luminescence enhancements on some stationary phases but not others. The reagents did not affect all compounds to the same degree indicating that qualitative information could be obtained in some cases. The largest luminescence increase for a compound spotted on silica gel was for pyrene (i.e., 47-fold) sprayed with sodium cholate. The degree to which the plates were dried also affected the luminescence intensity. Possible reasons for the observed effects are discussed.

INTRODUCTION

The detection and quantitation of luminescent compounds in TLC is an important and highly sensitive technique (1). Stationary phase and spray reagent effects can appreciably alter the luminescence behavior of a variety of compounds. Unfortunately there are few analytical studies which consider these effects

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(2-4). Fluorescent probes have been effectively used to examine the surface characteristics of derivatized silica gels (5, 6) and a number of interesting studies have been done on materials used to induce room-temperature phosphorescence on supports such as filter paper and silica gel (7-13).

Solutions of micellar aggregates and cyclodextrin molecules have recently been used to enhance the fluorescence of a variety of compounds (14-16). There have also been reports on the use of micellar mobile phases to improve luminescence detection in LC (17, 18). As yet there have been no systematic studies on the use of surfactant and cyclodextrin spray reagents to enhance the scanning densitometric determination of luminescent compounds on a variety of TLC supports. In this study the luminescence characteristics of several dansyl amino acids and polycyclic aromatic hydrocarbons (PAH's) on five common TLC supports are examined. The modifications of the luminescent intensity of these compounds using different spray reagents are evaluated and discussed.

EXPERIMENTAL

Materials. High purity α,β and γ cyclodextrin was obtained from Advanced Separation Technologies, Inc., 37 Leslie Court, Whippany, NJ 07981. Sodium dodecyl sulfate (SDS) from Bio Rad, cetyltrimethylammonium chloride (CTAC) from Fisher, sodium cholate from Sigma and Zwittergent 3-12 (a zwitterionic surfactant) from Calbiochem were used as received. Silica gel (K6), reverse phase (KC18), alumina (K3) and cellulose (K2) TLC plates were obtained from Whatman. Polyamide -6 TLC plates were obtained

from Brinkmann. HPLC grade water and methanol were obtained from Burdick & Jackson. A Shimadzu CS-910 scanning densitometer coupled to a C-R2AX data station was used for all determinations.

Methods. One microliter of 10^{-3}M solutions of twelve dansyl amino acids and four polycyclic aromatic hydrocarbons (Table 1) was individually spotted (Drummond micropipet) on five types of TLC support (i.e., silica gel, alumina, C_{18}-reversed phase, cellulose and polyamide). The spots were scanned and quantitated with a Shimadzu CS-910 densitometer in the fluorescence mode. The excitation wavelength was 256 nm for the PAH's and 320 nm for the dansyl amino acids. A "UV-D_2" cut-off filter was placed in front of the detector. The chromatographic plates were then sprayed with a 1% solution of either SDS, CTAC, sodium cholate, Zwittergent or β-cyclodextrin for 10 seconds at a distance of 2 feet. The spots were then scanned at 2 minutes (wet plates) and 75 minutes (dry plates) after spraying. Blanks (i.e., prespotted plates sprayed with water containing no reagent) were run in all cases.

Spotting reproducibility was ±2.3% as determined from the analysis of 10 identical experiments. The variation in fluorescence caused by plate to plate inconsistencies (within a given lot) was ±3.2%.

RESULTS AND DISCUSSION

A compound's fluorescence can vary tremendously when in contact with different TLC matrices. Consequently, stationary phase effects on luminescence detection and quantitation can be appreciable. Figure 1 illustrates the magnitude of this effect

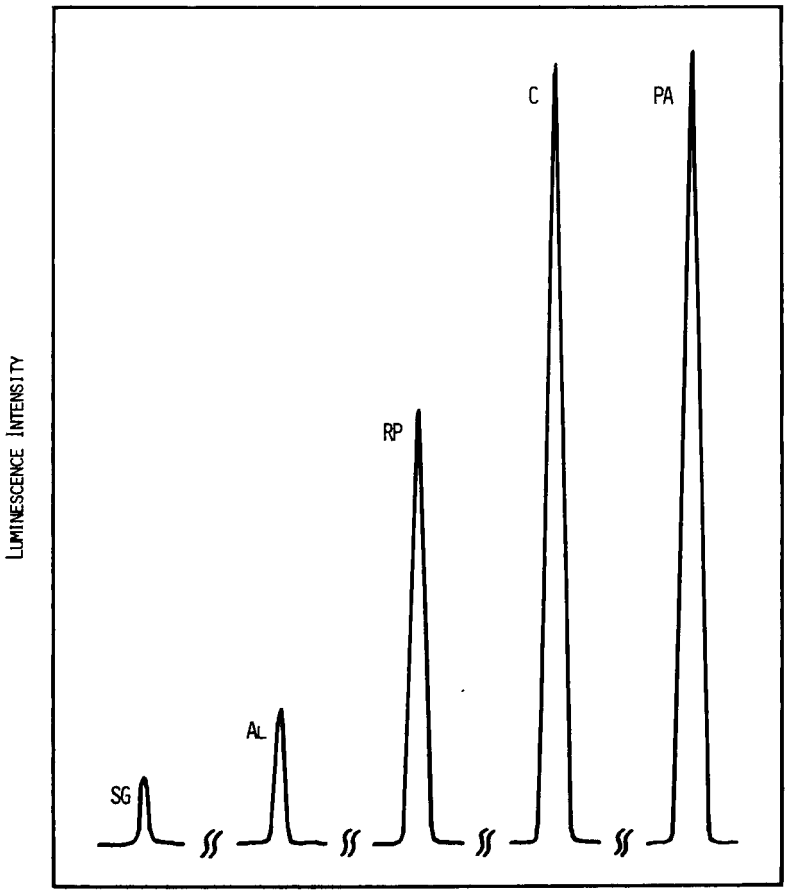

Figure 1. A comparison of the stationary phase effect on the detectable luminescence of one nanogram of dansyl glycine spotted on each plate. SG = silica gel, Al = alumina, RP = dimethyloctadecylsilanated reversed phase, C = cellulose, PA = polyamide. The detectable luminescence was 18 to 25 greater on polyamide and cellulose then on silica gel. The detectable luminescence on C_{18} reversed phase was 10 to 15 times greater than that on silica gel while alumina rarely produced more than a 2-fold increase over silica gel.

for dansyl glycine. Analogous results were obtained for the other compounds in this study. The magnitude of detectable luminescence varied on different stationary phases as follows: cellulose ≈ polyamide > C_{18}-reversed phase > alumina > silica gel. The detectable luminescence of dansyl amino acids on cellulose or polyamide plates was as much as 25 times greater than on silica gel. The detectable luminescence on alumina was only slightly better than on silica gel (about 2-fold) while it was as much as 15 times greater on C_{18} reversed phase plates. Possible reasons for these effects will be discussed at the end of this section.

The effect of surfactant and cyclodextrin spray reagents on the luminescence of dansyl amino acids and PAH's varies considerably for different stationary phases. In fact, significant enhancements were only observed on silica gel and alumina (Tables I and II). The spray reagent effect on luminescence on reversed phase, cellulose and polyamide plates was much less pronounced and was as likely to cause modest decreases in luminescence as increases (Tables III, IV and V). It is apparent that the analytical usefulness of these particular spray reagents is mainly limited to silica gel and alumina. It is also interesting that the stationary phases on which these reagents produce their greatest effect are those which seem to "inhibit" the fluorescence of dansyl amino acids and PAHs the most (i.e., silica gel and alumina, in Figure I).

A closer look at the data in Tables I and II reveals several interesting trends as well as significant differences in spray reagents. The usefulness of these reagents in lowering detection limits and increasing the linear dynamic range is illustrated in

Table I. A List of the Fluorescence Enhancements for a Variety of Dansylated Amino Acids and Polycyclic Aromatic Hydrocarbons (on Silica Gel) Resulting from Surfactant or Cyclodextrin Spray Reagents.[a]

	Spray Reagent									
	β-Cyclodextrin		CTAC		SDS		Sodium Cholate		Zwittergent	
Compound[b]	wet[c]	dry[d]	wet[c]	dry[d]	wet[c]	dry[d]	wet[c]	dry[d]	wet[c]	dry[d]
α-aminobuteric acid	2.2	5.4	9.9	5.8	2.6	1.9	3.1	1.5	1.9	1.0
norleucine	1.9	4.0	8.9	4.2	3.1	2.2	3.4	1.4	2.4	1.2
leucine	2.5	3.9	7.8	4.5	2.2	2.0	7.2	1.1	10	1.5
norvaline	3.0	4.9	9.3	5.1	3.0	2.0	4.7	1.9	2.9	1.3
phenylalanine	4.7	5.4	9.2	5.9	3.1	1.8	8.0	1.0	3.7	1.3
methionine	2.4	4.7	8.1	5.7	1.9	1.0	3.5	1.0	1.2	1.1
glutamic acid	3.1	7.0	12	5.7	4.9	1.3	4.7	1.0	2.5	1.0
serine	2.1	6.0	9.3	5.0	2.8	1.3	6.1	1.1	3.1	1.4
glycine	5.0	8.4	11	5.6	1.3	2.5	3.1	1.3	4.0	1.2
threonine	2.4	5.2	10	5.7	3.7	1.0	4.0	1.2	2.7	1.3
tryptophan	2.9	5.7	13	7.4			7.3	1.4	2.1	1.2
aspartic acid	3.4	5.5	12	5.7	5.7	1.0	3.6	1.5	1.2	1.1
pyrene	19	16	20	6.9	27	7.0	47	27	47	5.8
benzanthracene	2.1	14	4.5	3.4	7.6	3.1	10	6.2	10	4.7
benzo (α) pyrene	1.9	5.5	7.5	3.4	5.4	2.0	6.5	5.0	6.5	5.1
benzo (e) pyrene	1.0	6.4	10	3.0	5.9	2.3	5.1	3.0	6.6	5.0

[a] The spray reagents consisted of 1% of the surfactant or cyclodextrin in water. The enhancements listed are taken as the fluorescence peak area (after spraying) divided by the peak area of the dry, unsprayed fluorescent spot.

[b] All listed amino acids are dansylated, therefore the fluorescence enhancement is for the attached fluorophor.

[c] Fluorescence was measured two minutes after spraying.

[d] Fluorescence was measured 75 minutes after spraying.

Table II. A List of Average Fluorescence Enhancements for Twelve Dansyl Amino Acids and Individual Enhancements for the Polycyclic Aromatic Hydrocarbons on Alumina.[a]

	Spray Reagent									
	β-cyclodextrin		CTAC		SDS		Sodium Cholate		Zwittergent	
Compound	wet[b]	dry[c]	wet[b]	dry[c]	wet[b]	dry[c]	wet[b]	dry[c]	wet[b]	dry[c]
dansylamino acids	2.3	3.4	1.0	3.4	3.7	1.9	3.4	0.8	3.9	0.8
pyrene	20	8.9	5.6	4.9	39	2.1	30	15	27	12
benzanthracene	6.0	4.2	7.9	2.8	9.1	2.3	9.5	5.1	9.9	4.8
benzo (α) pyrene	3.1	3.0	4.5	2.9	14	3.1	7.0	4.3	5.8	4.0
benzo (e) pyrene	0.5	2.0	4.0	2.5	4.2	2.0	5.2	3.3	5.2	3.7

[a]The spray reagents consisted of 1% of the surfactant or cyclodextrin in water. The enhancements listed are taken as the fluorescence peak area (after spraying) divided by the peak area of the dry, unsprayed fluorescent spot.

[b]Fluorescence was measured two minutes after spraying.

[c]Fluorescence was measured 75 minutes after spraying.

Table III. Average Fluorescence Changes for Twelve Dansyl Amino Acids and Four Polycyclic Aromatic Hydrocarbons (PAH) on C_{18}-Reversed Phase.[a]

	Spray Reagents									
	β-cyclodextrin		CTAC		SDS		Sodium Cholate		Zwittergent	
Compounds	wet[b]	dry[c]	wet[b]	dry[c]	wet[b]	dry[c]	wet[b]	dry[b]	wet[b]	dry[c]
Dansyl amino acids	0.6	1.0	1.6	2.2	0.6	1.2	1.2	1.7	1.2	1.3
PAH	0.8	1.0	0.8	0.8	1.2	1.0	1.3	1.1	1.1	1.0

[a]The spray reagents consisted of 1% of the surfactant or cyclodextrin in water. The enhancements listed are taken as the fluorescence peak area (after spraying) divided by the peak area of the dry, unsprayed fluorescent spot.

[b]Fluorescence was measured two minutes after spraying.

[c]Fluorescence was measured 75 minutes after spraying.

Table IV. Average Fluorescence Changes for Twelve Dansyl Amino Acids and Four Polycyclic Aromatic Hydrocarbons (PAH) on Cellulose.

	Spray Reagents									
	β-cyclodextrin		CTAC		SDS		Sodium Cholate		Zwittergent	
Compounds	wet[b]	dry[c]	wet[b]	dry[c]	wet[b]	dry[c]	wet[b]	dry[c]	wet[b]	dry[c]
Dansyl amino acids	0.8	1.9	0.5	1.8	0.1	0.3	0.2	0.4	0.3	0.8
PAH	1.2	1.9	1.2	2.0	1.4	2.2	1.0	1.1	1.1	1.0

[a]The spray reagents consisted of 1% of the surfactant or cyclodextrin in water. The enhancements listed are taken as the fluorescence peak area (after spraying) divided by the peak area of the dry, unsprayed fluorescent spot.

[b]Fluorescence was measured two minutes after spraying.

[c]Fluorescence was measured 75 minutes after spraying.

Table V. Average Fluorescence Changes for[a] Twelve Dansyl Amino Acids and Four Polycyclic Aromatic Hydrocarbons (PAH) on Polyamide.

	Spray Reagent									
	ß-cyclodextrin		CTAC		SDS		Sodium Cholate		Zwittergent	
Compound	wet[b]	dry[c]	wet[b]	dry[c]	wet[b]	dry[c]	wet[b]	dry[c]	wet[b]	dry[c]
Dansyl amino acid	0.5	1.0	0.3	0.8	0.8	1.0	0.5	0.7	0.8	0.6
PAH	1.7	1.5	1.3	0.9	2.1	1.2	1.2	1.1	1.0	0.9

[a]The spray reagents consisted of 1% of the surfactant or cyclodextrin in water. The enhancements listed are taken as the fluorescence peak area (after spraying) idvided by the peak area of the dry, unsprayed fluorescent spot.

[b]Fluorescence was measured two minutes after spraying.

[c]Fluorescence was measured 75 minutes after spraying.

Figure 2 for dansyl glycine. β-Cyclodextrin and CTAC have a much greater effect on the luminescence of the dansyl amino acids than the other surfactants (Table I). It is interesting that CTAC has its greatest effect when the plate is freshly sprayed (i.e., still wet) whereas β-cyclodextrin has a greater effect when dry. Nearly all of the surfactant spray reagents produce a greater effect on silica gel and alumina when freshly sprayed. This could be a result of the presence of aggregational structures (e.g., micelles or bilayers) in the wet media. A given spray reagent tends to affect all the dansylated amino acids by about the same amount (for a given stationary phase) although there are small variations (Tables I-V). The polycyclic aromatic hydrocarbons (PAH) tended to show greater variation on silica gel and alumina but very little on the other stationary phases. Pyrene gave the largest fluorescence enhancements of any compound tested (Figure 3). Enhancements of 20 times or more (Table I, II and Figure 3) were not unusual for any freshly sprayed chromatogram. Sodium cholate produced the greatest overall luminescence increases for the PAH's although β-cyclodextrin and Zwittergent were also effective. On the other hand, CTAC was the most effective reagent for the dansyl amino acids, closely followed by β-cyclodextrin. Sodium cholate and Zwittergent produced little effect on the dansyl amino acids in the dry state. It seems that there is a certain amount of reagent selectivity in the luminescence enhancements of different compounds. This could be potentially useful in the identification of certain substances.

A complete elucidation of the mechanism(s) through which the luminescence is controlled and reasons for the variations in

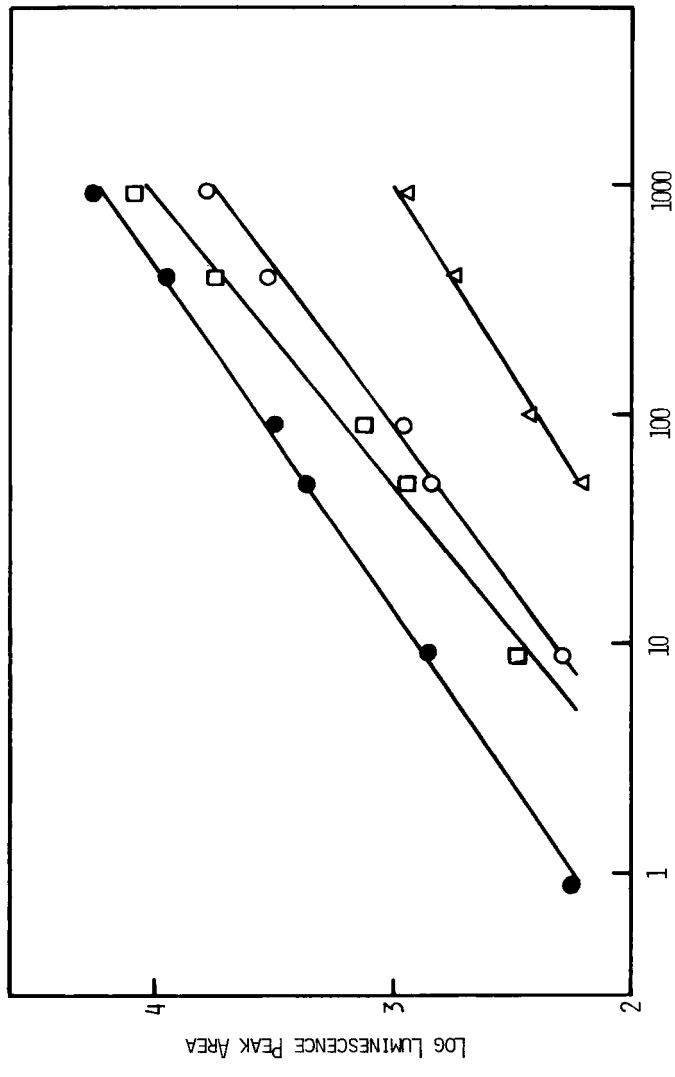

Figure 2. Plots showing the effect of different spray reagents on the linear dynamic range of dansyl glycine on silica gel. Δ = no spray reagent, O = CTAC sprayed and dried, □ = β-cyclodextrin sprayed and dried, O = CTAC freshly sprayed (i.e., the luminescence was measured 2 min. after spraying while it was still wet).

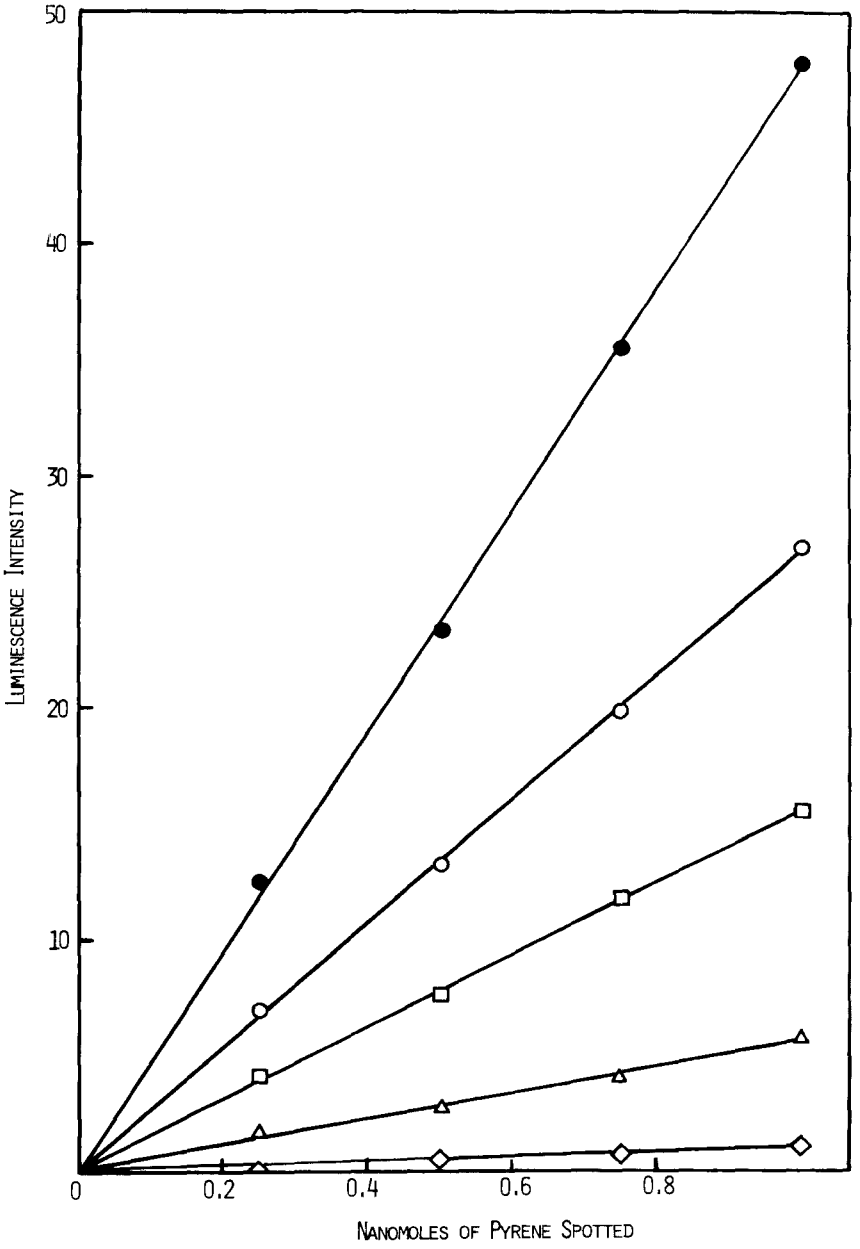

Figure 3. Plots showing the effect of different spray reagents on the calibration curve of pyrene on silica gel. ◇ = no spray reagent, Δ = Zwittergent sprayed and dried, □ = β -cyclodextrin sprayed and dried, 0 = sodium cholate sprayed and dried, 0 = sodium cholate freshly sprayed.

it are beyond the scope of this paper. However, some general statements can be made concerning the role of the stationary phase and spray reagents in luminescence detection on TLC plates. Firstly, the observed enhancements due to the spray reagents were not the result of increased solubilization of the fluorophor on the surface of the stationary phase or to internal reflections from a liquid surface layer. Spray "reagent" blanks (e.g., pure water, H_2O/methanol, etc) consistently caused reduced luminescence on all stationary phases. Secondly, when luminescent compounds are spotted on the more strongly adsorbing media (i.e., silica gel and alumina) they produce substantially less fluorescence (Figure 1). The spray reagents, however, produce their greatest effects on these same media. It has been reported that strong absorption can increase the nonradiative rate constant of pyrene (19) which leads to a decrease in fluorescence. It was further reported that the addition of certain compounds (such as long chain alcohols or glycerol) would preferentially interact with the strong adsorption sites thereby allowing pyrene "to be adsorbed in areas of weaker interactions" (19). This tended to diminish the probability of static quenching of tightly bound pyrene as well as enhance the possibility of dynamic eximer formation (due to the greater mobility of pyrene (19)). Certainly many of these phenomena are possible in the present system as all of the spray reagents strongly adsorb to silica gel. In the case of β-cyclodextrin, formation of stable inclusion complexes with the fluorophor would be another important factor (4). Furthermore, it is well known that micellar aggregates can enhance fluorescence (15,16) and this could be a factor in the freshly sprayed chro-

matograms. The aggregational structure would be destroyed upon drying, however. Spray reagents have essentially no effect on luminescence of compounds spotted on C_{18}-reversed phase plates. In this case the adsorption sites have been silanized and the relatively nonpolar environment of any existing aggregates is no better than that which already exists on the stationary phase. Surfactants also adsorb somewhat to cellulose and polyamide. However, if the environment provided by the surfactant or cyclodextrin is less hospitable to the luminescent specie than the stationary phase, then one observes decreased luminescence (Tables II, IV and V).

ACKNOWLEDGEMENT

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DETERMINATION OF SOLVATION EFFECTS IN LIQUID ADSORPTION CHROMATOGRAPHY WITH MIXED MOBILE PHASES

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ABSTRACT

A model of chromatographic process, involving formation of multimolecular solute-solvent solvates in the mobile phase, is discussed. This model leads to an equation describing dependence of the capacity ratio upon mobile phase composition. The TLC data for some solutes chromatographed in n-heptane+chloroform on silica gel are interpreted by means of the above equation.

INTRODUCTION

Beginning with the works of Ościk (1) and Snyder (2) almost twenty years ago, theoretical studies in a continuing stream have examined means of evaluating main effects in liquid adsorption chromatography with mixed mobile phases. Reviews of these efforts (3-6) show the extent of these investigations. The main principle of simple chromatographic models is competitive character of adsorption process

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occuring at liquid/solid interface (2-6). Moreover, these models can incorporate additional assumptions - treating such effects as surface heterogeneity of the adsorbent, differences in molecular sizes of solute and solvent molecules, changes of surface phase composition upon mobile phase composition, nonspecific and specific molecular interactions in the surface and bulk solutions.

In many chromatographic systems, in which specific solute-solvent and solvent-solvent interactions are possible, e.g., hydrogen bonding between solute and solvent molecules, the association effects play an important role (7-13). A most general description of liquid adsorption chromatography with mixed mobile phases, involving solute-solvent and solvent-solvent association, has been presented in the previous paper (9). In the case of model assuming formation of double associates in the mobile phase, a simple equation has been proposed to describe dependence of the capacity ratio upon mobile phase composition (7,8). This equation has been used to interpret HPLC (10,12) and TLC (13) data. This interpretation gives answer to the question: what type of association in the bulk solution is dominant, solute-solvent or solvent-solvent association.

In this article we will study a model of chromatographic process, involving formation of multimolecular solute-solvent complexes (solvates) in the mobile phase. This model leads to an equation, which will be examined by using TLC data of some solutes chromatographed in n-heptane+chloroform on silica gel.

THEORY

Let us consider liquid adsorption chromatography with mixed mobile phase, in which strong specific interactions between solute and solvent molecules cause the formation of mixed complexes (solvates). Further assumptions are as follows:

(a) the initial solution is devided into two phases, the surface phase and the mobile phase,

(b) adsorption has a competitive character,

(c) molecules of solute and solvents have different sizes and a spherical shape,

(d) the total number of moles of all solvents in the surface phase is constant and independent upon the presence of solute molecules because of their infinitely low concentration,

(e) the adsorbent surface is supposed to be homogeneous,

(f) the surface phase is ideal and monomolecular,

(g) the solvates are formed in the mobile phase.

Assumptions (a)-(e) are frequently used in the theory of liquid adsorption and liquid adsorption chromatography (6,14,15). The remaining assumptions, (f) and (g), are reasonable for adsorbent surfaces interacting strongly with the solute molecules. In this case the solute-adsorbent interactions are predominant over specific solute-solvent interactions in the solution. As a consequence the solvates break down in the potential field generated by the adsorbent surface. Therefore, there are only monomers in the surface phase.

Let s denotes s-th solute chromatographed in multicomponent eluent, in which solvents are numbered successively beginning from the most efficient eluting solvent to the weakest solvent. Thus, 1-st solvent is the most efficient one. Similarly as in the previous paper (9) the chromatographic process may be represented by quasi-chemical reactions describing competitive character of solute adsorption and solvation phenomenon in the mobile phase. Thus, the solute adsorption is represented by the phase-exchange reversible reaction (6,9):

$$s_{(m)} + r\ 1_{(s)} \rightleftharpoons s_{(s)} + r\ 1_{(m)} \qquad (1)$$

where the subscripts (m) and (s) refer to the mobile and surface phases, respectively, s and 1 denote molecules of the s-th solute and 1-st solvent, and r is the ratio of molecular sizes of the s-th solute and 1-st solvent. The solvation phenomenon, occuring in the mobile phase, may be represented by the following reaction:

$$s_{(m)} + q\ 1_{(m)} \rightleftharpoons 1_q s_{(m)} \qquad (2)$$

where $1_q s_{(m)}$ denotes (q+1)-molecular complex in the mobile phase composed of one solute molecule and q molecules of 1-st solvent. The equilibrium constants describing reactions 1 and 2 may be expressed as follows:

$$K_{s1} = (y_s/x_s)\cdot(x_1/y_1)^r \qquad (3)$$

and

$$C_q = z_q/[\, x_s(x_1)^q] \qquad (4)$$

where y_s and y_1 are mole fractions of the s-th solute and 1-st solvent in the surface phase, x_s and x_1 are mole fractions of the s-th solute and 1-st solvent in the mobile phase, and z_q is the mole fraction of the solvate $1_q s$ in the mobile phase defined as the ratio of the number of solvates $1_q s$ to the total number of molecules in the mobile phase. The mole fractions y_s , y_1 , x_s and x_1 define concentrations of unassociated molecules of the s-th solute and 1-st solvent.

The total mole fractions of the s-th solute and solvents in the mobile phase are expressed by :

$$x_s^o = x_s + \sum_{q=1}^{p} z_q = x_s \left[1 + \sum_{q=1}^{p} C_q (x_1)^q \right] \qquad (5)$$

$$x_1^o = x_1 + \sum_{q=1}^{p} q\, z_q = x_1 + x_s \sum_{q=1}^{p} q\, C_q (x_1)^q \approx x_1 \qquad (6)$$

$$x_i^o = x_i \quad \text{for} \quad i > 1 \qquad (7)$$

Since the mole fraction x_s is infinitely low in comparison to the solvent concentration (c.f., assumption d), the total mole fraction x_1^o is in a good approximation equal to x_1 . According to the assumption (g) the surface phase contains only monomers of solute and solvents. Therefore, the total mole fractions y_s^o and y_1^o are equal to y_s and y_1 , respectively. The distribution coefficient k_s , defined as the ratio of the total mole fractions of the s-th solute in the surface and mobile phases, respectively, i.e.,

$$k_s = y_s^o / x_s^o \ , \qquad (8)$$

in the case of the model under considerations assumes the following form:

$$k_s = y_s/x_s^o = (y_s/x_s)\left[1 + \sum_{q=1}^{p} C_q (x_1)^q\right]^{-1} \qquad (9)$$

Taking into account the proportionality between distribution coefficient k_s and capacity ratio k'_s, we have:

$$k'_s = \beta\, k_s = \beta\, (y_s/x_s)\left[1 + \sum_{q=1}^{p} C_q (x_1)^q\right]^{-1} \qquad (10)$$

where β is characteristic for a given adsorbent and independent upon the eluent nature in a good approximation (2). The ratio y_s/x_s may be determined from equation 3 and then equation 10 assumes the following form:

$$k'_s = \beta\, K_{s1}\, (y_1/x_1)^r \left[1 + \sum_{q=1}^{p} C_q (x_1)^q\right]^{-1} \qquad (11)$$

In the case of one-component eluent (solvent 1), x_1 is equal to unity and the capacity ratio of the s-th solute chromatographed in the pure solvent 1 is expressed as follows:

$$k'_{s1} = \beta\, K_{s1} \left(1 + \sum_{q=1}^{p} C_q\right)^{-1} \qquad (12)$$

Thus, if solvent forms associates with the solute, the capacity ratio of the solute chromatographed in this pure solvent depends on the phase-exchange equilibrium constant K_{s1} and association (solvation) constants C_1 , C_2 ,..., C_p .

Combining equations 11 and 12 we have:

$$k'_s = k'_{s1} (y_1/x_1)^r \frac{1 + \sum_{q=1}^{p} C_q}{1 + \sum_{q=1}^{p} C_q (x_1)^q} \qquad (13)$$

Equation 11 and its another form, equation 13, describe dependence of the capacity ratio upon mobile phase composition for the eluent, in which 1-st solvent can form different multimolecular solvates $1_q s$ for $q=1,2,\ldots,p$. In many systems one type of solvates is dominant, e.g., (q+1)-molecular solvates; then, equations 11 and 13 assume simpler forms:

$$k'_s = \beta K_{s1} (y_1/x_1)^r [1 + C_q (x_1)^q]^{-1} \qquad (14)$$

$$k'_s = k'_{s1} (y_1/x_1)^r \frac{1 + C_q}{1 + C_q (x_1)^q} \qquad (15)$$

Another simplifications of equations 11 , 13 , 14 and 15 are possible for $r=1$ (identical molecular sizes of solute and solvent molecules) and/or $y_1=1$ (this assumption is valid for the whole concentration region except the low concentrations of x_1 and for very strong adsorption of 1-st solvent in comparison to adsorption of other solvents). The mole fraction y_1 may be determined from the excess adsorption data measured for 1-st solvent or calculated by means of the theoretical isotherm equations (6).

The equilibrium constants K_{s1} and C_q may be evaluated from the chromatographic data by using another forms of equations 11 and 14 :

$$(y_1/x_1)^r(k'_s)^{-1} = \sum_{q=0}^{p} C'_q(x_1)^q \qquad (16)$$

$$(y_1/x_1)^r(k'_s)^{-1} = C'_o + C'_1(x_1)^q \qquad (17)$$

where

$$C'_o = (\beta K_{s1})^{-1} \qquad (18)$$

$$C'_q = C_q/(\beta K_{s1}) \text{ for } q = 1,2,\ldots,p \qquad (19)$$

For r=1 and y_1=1 equations 16 and 17 become:

$$(k'_s x_1)^{-1} = \sum_{q=0}^{p} C'_q(x_1)^q \qquad (20)$$

$$(k'_s x_1)^{-1} = C'_o + C'_1(x_1)^q \qquad (21)$$

According to equation 21 the dependence $(k'_s x_1)^{-1}$ vs. $(x_1)^q$ is linear. Equation 21 with q = 1 has been examined by using HPLC and TLC data (10,12,13); for many chromatographic systems it gives a good representation. It means that assumption of double associates in the mobile phase gives frequently satisfactory results. In this paper we shall examine equation 21 for $q > 1$ by using TLC data.

EXPERIMENTAL

Chemicals

Chloroform and n-heptane (puriss. grade) were obtained from Polskie Odczynniki Chemiczne (Gliwice, Poland). The silica gel (type 100) was from E.Merck (Darmstadt, FRG).

TLC measurements

The TLC measurements were made under thermostated conditions at 293K. Silica gel was the adsorbent and chloroform+n-heptane

was the eluent. The adsorbent layers were 0.3 mm thick and were activated for 2h at 408K. The chromatograms were developed by the ascending technique to a distance of 16 cm. The spots were visualized using the universal reagent (16). The R_F-values have been measured for : p-nitroaniline, o-nitroaniline, 2-nitro-p-toluidine, 4-nitro-p-toluidine, 2,3-dihydroxynaphthalene and o-dinitrobenzene. The R_F-values were converted into R_M-values using the well-known equation:

$$R_M = \log [(1 - R_F)/R_F] \qquad (22)$$

However, the capacity ratio was calculated as follows:

$$k'_s = 10^{-R_M} \qquad (23)$$

The experimental dependence k'_s vs. x_1 for different solutes chromatographed in chloroform+n-heptane was examined by using equation 21 .

RESULTS AND DISCUSSION

In the paper (17) the TLC data for many solutes of different chemical structure were measured by using the following binary mobile phases: methanol, ethanol, n-propanol, n-butanol with benzene, ethyl acetate, acetone and ethylmethylketone with toluene, ethyl acetate and chloroform with n-heptane.

Analysis of TLC data for solutes chromatographed in alcohol+benzene and ketone+toluene shown that effects connected with association of 1-st solvent are dominant. However, for many solutes chromatographed in ethyl acetate

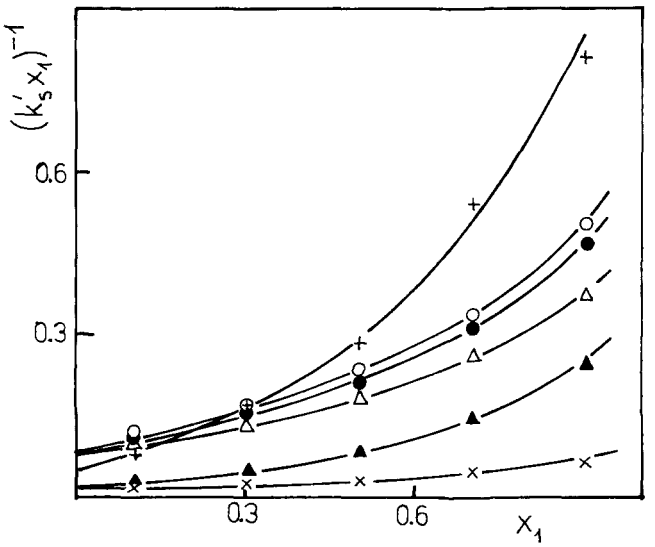

FIGURE 1 . Dependence $(k'_s x_1)^{-1}$ vs. x_1 for six solutes chromatographed in chloroform+n-heptane on silica gel. Solutes: p-nitroaniline (▲), o-nitroaniline (○), 2-nitro-p-toluidine (●), 4-nitro-p-toluidine (△), 2,3-dihydroxynaphthalene (×) and o-dinitrobenzene (+).

and chloroform with n-heptane the solvation effects play an important role. For majority solutes chromatographed in these eluents the model assuming formation of double associates in the mobile phase gives a good representation of TLC data in question (17). However, this model (equation 21 with q=1) is not suitable to interpret the TLC data presented in the experimental part. Experimental points does not fulfil equation 21 with q=1 (see Figure 1). Plotting these data according to equation 21 with q=2 , we observe a good linear behaviour of $(k'_s x_1)^{-1}$ vs.$(x_1)^2$;Fig.2 . It follows from Figure 2 that the model assuming formation

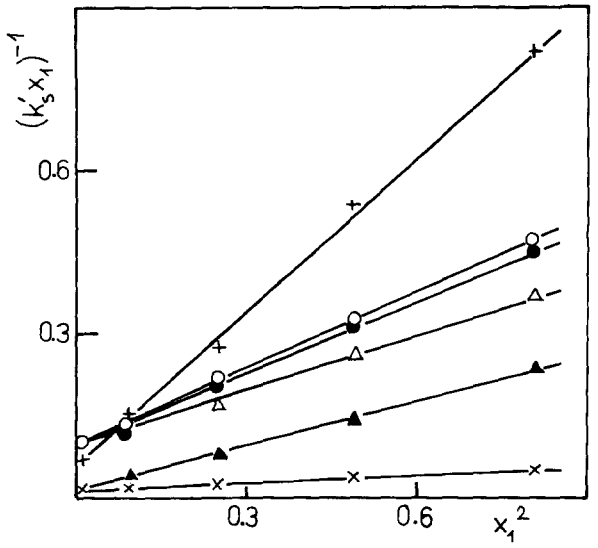

FIGURE 2 . Dependence $(k'_s\ x_1)^{-1}$ vs. $(x_1)^2$ for the solutes presented in Figure 1.

of solvates (one solute molecule + two solvent molecules) in the mobile phase gives a reasonable representation of TLC systems in question.

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THE CORRELATIONS BETWEEN CAPACITY FACTORS k′ AND R_M VALUES IN LIQUID COLUMN AND THIN - LAYER CHROMATOGRAPHY

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ABSTRACT

The purpose of this studie being carried out is to define some regularities between Thin-layer and Liquid Column Chromatography; i.e. between experimental obtained and theotetical calculated R_M-values and experimental obtained logk′-values for some non-active organic substances in mixed binary solvents systems. It was studied the kind these correlations change with changing mobile phase compositions. The present paper is a certain aspect of new studies on possibilities of the use Thin-layer Chromatography as a pilot technique for Liquid Column Chromatography (1,2). In a study on the optimization of chromatographic process it is necessary to define the correlations between R_M-values which characterize the retention in TLC and k′-values which characterize the retention in in LCC.

In the paper the changes of these correlations with the changes of mobile phase compositions are studied. The conformity between theoretical calculated and experimental obtained R_M-values of chromatographed substances for different mobile phase compositions are investigated. A good

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agreement between theoretical and experimental data makes possible to foresee on the basis of the correlations between logk'- and R_M-values the retention in column chromatography.

INTRODUCTION

Considering the wide application of chromatography it is necessary to find relations between the parameters obtain by different chromatographic techniques. Advantages of Thin-layer Chromatography cause that it can be used as a pilot technique for Liquid Column Chromatography.

The possibility of theoretical calculations of R_M-values makes possible to use these data in HPLC conditions. In this purpose it is necessary to define the correlations between R_M and logk'-values of chromatographed substances for a given mobile phase system. From the study it appears that the simple theoretical dependence R_M=logk' is not exactly conformable to experimental data. It is caused by different conditions of the chromatographic process on a plate and in a chromatographic column; i.e. a presence of a gas phase in a chamber, a different stationary phase profile on a plate and in a column, a different mobile phase flow-rate, etc. (3,4).

In the paper the graphical correlations between R_M and logk'-values for some substances in three different mobile phase systems have been shown. The changes of these correlations with the changes of a mobile phase type and its composition are studied.

EXPERIMENTAL

Experimental R_M-values of investigated substances were determined by ascending adsorption thin-layer chromatography.

Silica gel 60 (Merck) was used as an adsorbent. The layer tickness was 0.25 mm. Plates were activated for 2h at 135°C. Chromatograms were developed in thermostatic conditions at 21 ± 0.5 °C in saturated chambers.

As the mobile phases were used pure isooctane, hexane, cyclohexane and chloroform, and their binary mixtures of compositions φ_1 : 0.1 ; 0.3 ; 0.5 ; 0.7 ; 0.9 (φ_1 was a volume fraction of a stronger mobile phase component). As a chromatographed substances were used naphtalene and its methyl derivatives and polycyclic aromatic hydrocarbons. These substances were visualized on chromatograms in iodine pairs or in UV light.

For LCC experiments was used Pay Unicam LC 20 Liquid Chromatograph. Merck Lichrosorb Si 60 of 0.5 μm particle diameter was used as an adsorbent. The same as in TLC studies substances and binary mixtures were used.

RESULTS AND DISCUSSION

Experimental R_M-values of chromatographed substances were obtained from TLC studies. Ościk's thermodynamic theory of adsorption from multicomponent systems was used as the basis of our investigations (5-7). The equation resulting from it makes us possible to calculate theoretical R_M-values for studied substances :

$$R_{M1,2} = \varphi_1 \Delta R_{M1,2} + (\varphi_1^s - \varphi_1)(\Delta R_{M1,2} + A_z) + R_{M2} + Y \qquad (1)$$

where $R_{M1,2}$, R_{M1}, R_{M2} are R_M-values of a given solute in a mixed mobile phase "1+2" and in pure solvents "1" and "2" respectively; $\Delta R_{M1,2} = R_{M1} - R_{M2}$ is the difference

of the R_M-values of a solute in pure solvents "1" and "2"; φ_1 is a volume fraction of a stronger "1" component of a mobile phase; the value A_z represents molecular interactions between molecules of the chromatographed substance and these of the components of a solvent ; Y represents the influence of the kind of the chromatographed substance and components of a binary mobile phase on the parameters discussed. In the case of ideal solvents this value is equal to zero or it is negligible small in the case of non-ideal solvents.

From experimental data logk'-values of chromatographed substances were calculated.

Fig.1a,b shows the graphical relations between theoretical calculated on the base of eq.1 and experimental obtained R_M-values for chosen substances for given mobile phase compositions for two different mixed phases. In all cases a good agreement of theoretical and experimental values of function $R_M = f(\varphi_1)$ has been obtained. Eq.1 describes satisfactory experimental data for mixed phases composed of type N or A solvents and for type N(B) substances(8). This results from the fact that systems of this type are conformable to the theoretical assumptions of this equation, e.g. insignificant and non-specific interactions between solvent and solute molecules.

In the case of type N + N solvents, e.g. isooctane-hexane or hexane-cyclohexane, the plots of $R_M = f(\varphi_1)$ relationships have flat shapes (Fig.1a). That means that the increase of φ_1-value not influences considerably on

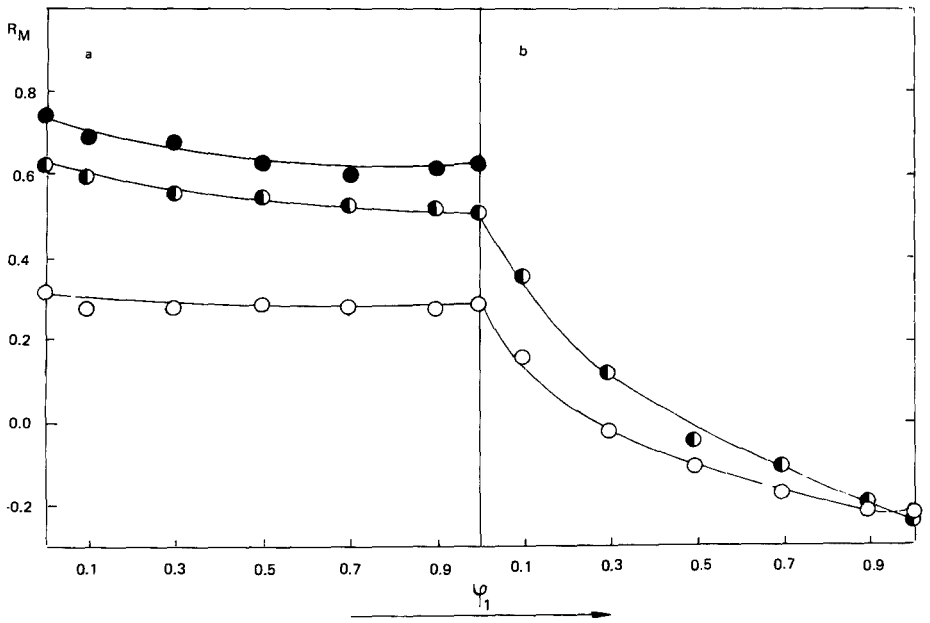


Fig.1

The graphical relationships between theoretical (lines) and experimental (circles) R_M-values and the composition of the mobile binary solvents phase;

(a) the mobile phase: isooctane - hexane,

(b) the mobile phase: hexane - chloroform,

○ 1,5-dimethylnaphtalene,

◐ phenantrene,

● fluoranthene.

the retention of chromatographed substances in this kind of mobile phase systems. Both of these mobile phases are mixtures of non-active solvents which non-specific interact with investigated substances, and that the increase of R_M-value not changes the elution strenght of a given mobile phase.

The different situation is in the case of hexane-chloroform mobile phase(type N +A). The curves illustrated $R_M = f(\varphi_1)$ relationships for this system have steep shapes (Fig.1b). It is comprehensible when we notice that the more active component of a mobile phase (chloroform) is able to arise stronger intermolecular interactions with regard to its electron-acceptor properities. The increase of chloroform amount in a mobile phase from $\varphi_1 = 0.0$ to 0.3 influences considerably on R_M-values of chromatographed substances. The R_M-values not change so considerably with a further increase of φ_1-values.
In the range of low φ_1-values (0.0 - 0.3) there are a continuous increase of chloroform amount in an adsorption phase and that effects on the retention of chromatographed substances (9).

On the base of experimental obtained logk'-values and experimental obtained and theoretical calculated R_M-values of studied substances the correlations $\log k' = f(R_M)$ are illustrated graphical for given mobile phase compositions. From the theoretical equation $\log k' = R_M$ it seems that these plots ought to be straight-lines with slopes equal to unity and that they are independent on the kind and the composition

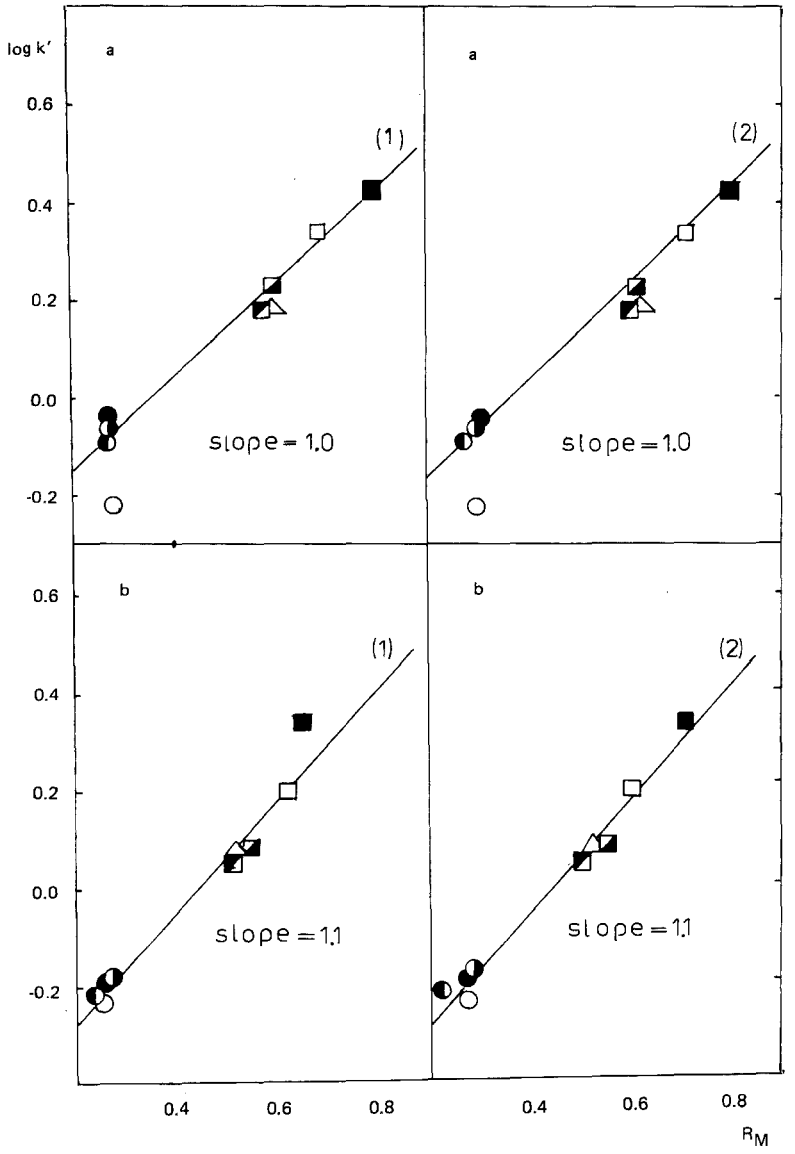
of a mobile phase. From experimental data it arises that though these graphical relationships are straight-lines the slopes of them are not only different from unity but are different for various mobile phase compositions as well.

Fig.2 and 3 show the graphical relationships between experimental logk'-values and theoretical and experimental R_M-values for chromatographed substances for hexane - cyclohexane and isooctane - hexane systems and for given mobile phase compositions. These correlations are straight-lines of near unity slopes. From the comparison of graphical calculated slopes it seems that they change insignificantly with mobile phase compositions. In practice they receive similar values in a narrow range for both mobile phases. The oscilations of slope values result rather from experimental errors then from changes in mobile phase properities resulting from the changes in its composition.

The analisis of the plots on Fig.2 and 3 shows that R_M-values for naphtalene and its methyl derivatives are near the same in both mixed mobile phases irrespective of the site of the substituting groups in the naphtalene molecule. This results from small values of the adsorption energies of these groups on silica gel (10).

In the case of polycyclic aromatic hydrocarbons the differences in R_M-values result from the fact that interactions between solvent and solute molecules are negligible and a higher influence of the structural effects of these molecules can be observed.

It can be noticed from Fig.2 and 3 that the correlations $\log k' = f(R_M)$ are different for two groups of chromatographed


log k'
a
(1)
slope = 1.0
a
(2)
slope = 1.0
b
(1)
slope = 1.1
b
(2)
slope = 1.1
0.6
0.4
0.2
0.0
-0.2
0.4
0.6
0.8
R_M

substances; i.e. for naphtalene and its methyl derivatives and for polycyclic aromatic hydrocarbons. The distribution of these correlations can be explained by structural effects of these organic compounds. To study this problem more precisely is necessary to gather much more experimental data.

A different situation is in the case of hexane - chloroform system (N + A type). Fig.4 shows the correlations between logk' and R_M-values for chosen mobile phase concentrations for this system. The distinct changes of the line slopes with changing the mobile phase composition are observed. The straight-lines become more steep while the amount of chloroform (φ_1) in a mobile phase increases, e.g. the increas of the mobile phase elution strenght influences on the shapes of the straight-lines illustrated graphical the relationships $\log k' = f(R_M)$ (9). For this system hexane - chloroform, (N + A type) the separation of these correlations for two groups of compounds is not observed.

Fig.2

The correlations between experimental logk'-values and experimental (line 1) and theoretical (line 2) R_M-values with using isooctane - hexane as the mobile phase;

(a) $\varphi_1 = 0.1$; (b) $\varphi_1 = 0.7$;

○ naphtalene, ◐ β-methylnaphtalene, ◑ 1,3-dimethylnaphtalene, ● 1.5-dimethylnaphtalene, ◩ anthracene, ◪ pyrene, ■ chrysene, □ fluoranthene, △ phenantrene.

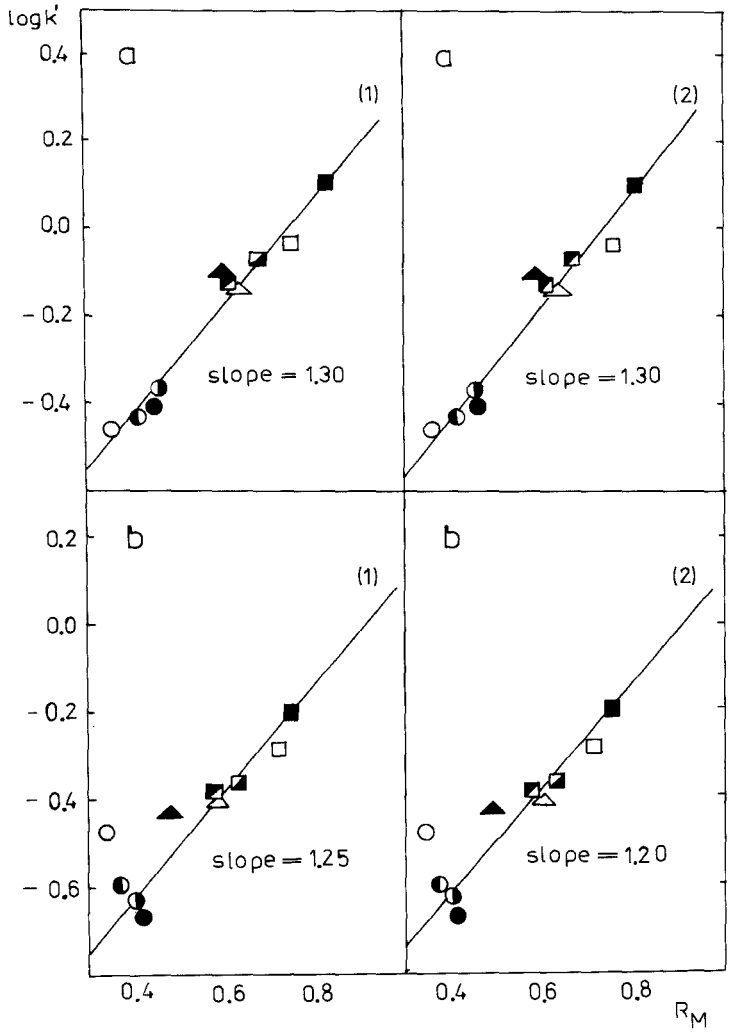


Fig.3

The correlations between experimental logk'-values and experimental (line 1) and theoretical (line 2) R_M-values with using hexane - cyclohexane as the mobile phase ;

(a) $\varphi_1 = 0.1$; (b) $\varphi_1 = 0.3$;

○ naphtalene, ◐ β-methylnaphtalene, ◑ 1,3-dimethylnaphtalene,

● 1,5-dimethylnaphtalene, ◩ anthracene, ◪ pyrene,

■ chrysene, □ fluoranthene, △ phenantrene, ▲ fluorene.

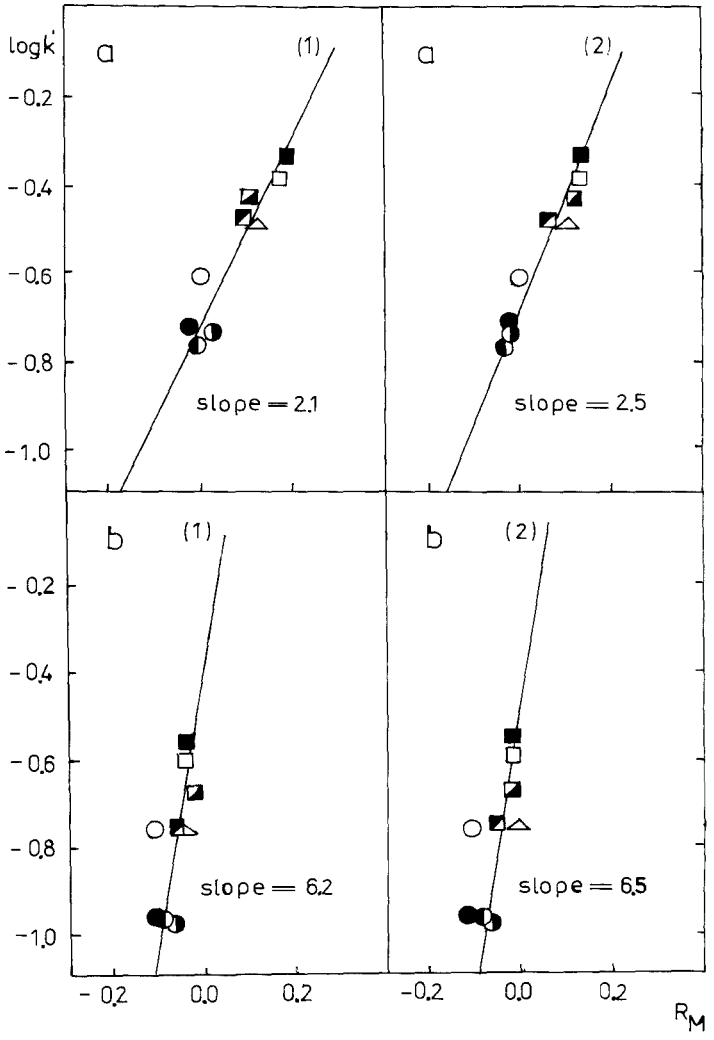


Fig.4

The correlations between experimental logk'-values and experimental (line 1) and theoretical (line 2) R_M-values with using hexane - chloroform as the mobile phase ; (a) $\varphi_1 = 0.3$; (b) $\varphi_1 = 0.5$; the remaining explanations as above

From the data presented in this paper it appears that the correlations between logk' and R_M-values for chromatographed substances are straight-lines and, as it was noticed, their shapes depend on the kind and the composition of a mobile phase.

The above considerations sygnalizes the possibility of the use TLC-data, experimental obtained or theoretical calculated on the base of the eq.1, in more complex and time- wasting chromatographic technique as Liquid Column Chromatography, and that TLC can be used as a pilot technique for more precise studies.

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SEPARATION OF EICOSANOIDS BY REVERSE-PHASE THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

A method is described for separating eicosanoids including prostaglandins, their metabolites, and HETE. This was accomplished using commercially available reverse-phase thin-layer chromatographic plates and 0.0025M phosphoric acid-acetonitrile (52:48, v/v) containing 0.2M sodium chloride as a mobile phase. The use of reverse-phase thin-layer chromatography offers an alternative means of separating eicosanoids over traditional silica gel.

INTRODUCTION

Silica gel thin-layer chromatography (TLC), since its introduction in 1956 by Stahl (1) has traditionally been used as both a preparative and analytical technique. Green and Samuelsson (2) first afforded silica gel TLC to separate prostaglandins. Since then, impregnation of the silica with silver (3) or iron (4) and numerous solvent systems (5-10) have been extended to separate the ever increasing number of arachidonic acid metabolites. Today, revisions are occurring to include the lipoxygenase products (11-13). The commercial availability in the late 1970's of n-alkyl bonded silica gel TLC plates has proven to be of much value in the analysis of substances with only minor structural

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differences (14,15). Although it is doubtful, due to the number of metabolites, that any one 20 x 20 cm TLC plate can effect complete separation of all eicosanoids in one dimension, reverse-phase TLC offers advantages over traditional silica TLC. This paper describes the first separation of eicosanoids by reverse-phase TLC using octadecyl bonded silica.

MATERIALS AND METHODS

All solvents were high performance liquid chromatography (HPLC) grade obtained from Fischer Scientific. Sodium chloride (NaCl) and phosphomolybdic acid were ACS grade (Fischer). Stock solutions of individual or mixtures of eicosanoids (Upjohn) were dissolved to a final concentration of 1 mg/ml in acetonitrile. A Drummond 10 ul Wiretrol was used to spot the TLC plates with 7.5-10 ul of each solution. Reverse-phase C_{18} plates (Whatman KC_{18}), 20 x 20 cm x 250 um, with a preadsorbant strip were used without prior activation. They were ascendingly developed in an unlined glass tank until the solvent had moved 0.5 cm from the top edge of the plate, dried, and developed again to the same distance. Development took approximately 75 minutes each time. The mobile phase consisted of 0.0025M phosphoric acid-acetonitrile (52:48, v/v). Addition of 0.2M NaCl to the mobile phase prevented disruption of the bonded layer by the water rich mobile phase as reported previously (16). Arachidonic acid and eicosanoids were visualized by spraying the plates with a saturated solution of phosphomolybdic acid in methanol.

RESULTS AND DISCUSSION

The resolution of arachidonic acid with its most common products by reverse-phase TLC is illustrated in Figure 1. As seen by the Rf values given in Table 1, there is separation of all the major biological metabolites. Although PGA_2 and PGB_2 comigrate; these are thought not to be formed enzymatically but by decomposition of PGE_2 in acid or alkaline environments respectively (12).

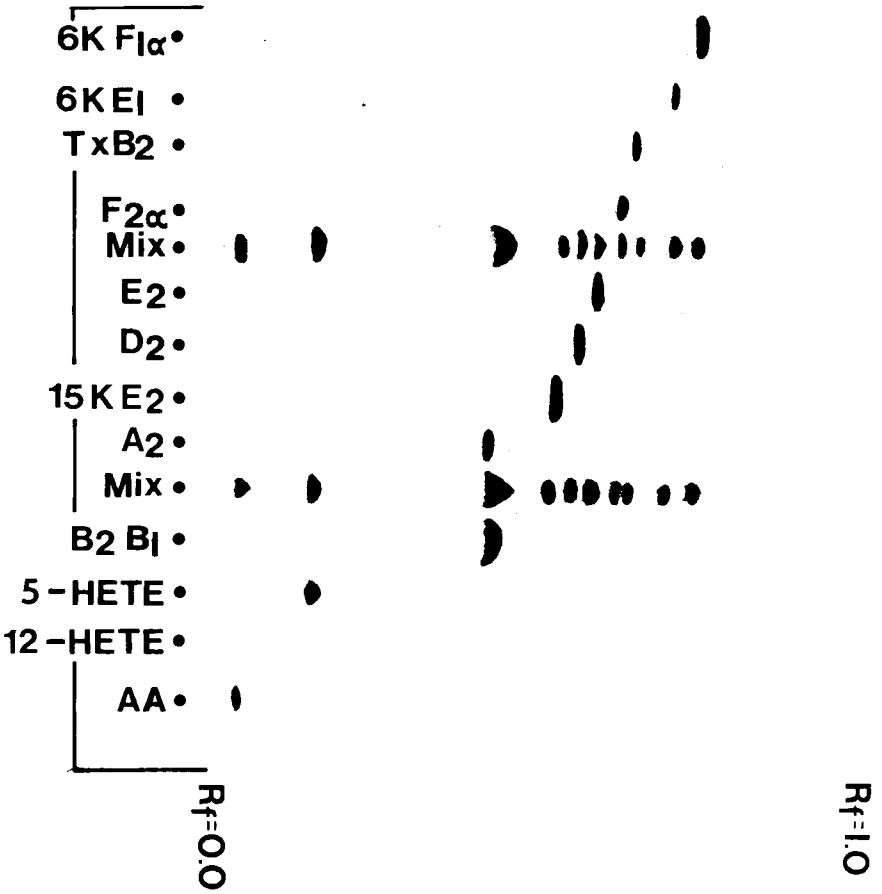

FIGURE 1: Reverse-phase TLC plate illustrating the separation of arachidonic acid and major metabolites. Solvent system: 0.0025M phosphoric acid-acetonitrile (52:48, v/v) containing 0.2M NaCl.

Table 2 gives the Rf values of other eicosanoids and metabolites, mostly plasma metabolites.

Reverse-phase TLC also offers a degree of separation of prostaglandins which differ only in their degree of unsaturation as shown in Figure 2 and by the Rf values given in Tables 1 and 2. Separation of $PGF_{2\alpha}$ or PGE_2, with 2 alkene groups, from $PGF_{1\alpha}$ and PGE_1, respectively, containing a single carbon-carbon double bond,

TABLE 1
Rf Values of Arachidonic Acid and Common Eicosanoid Derivatives

Compound	Rf
6-keto $PGF_{1\alpha}$	.72
6-keto PGE_1	.68
TxB_2	.61
$PGF_{2\alpha}$	.59
PGE_2	.55
PGD_2	.53
15-keto PGE_2	.49
PGA_2	.43
PGB_2	.43
5-HETE	.16
12-HETE	.13
AA	.04

TABLE 2
Rf Values of Other Eicosanoids and Metabolites

Compound	Rf
6,15-Diketo $PGF_{1\alpha}$	.67
6,15-Diketo 13,14 Dihydro $PGF_{1\alpha}$	.65
$PGF_{1\alpha}$	.57
15-keto $PGF_{2\alpha}$	.54
PGE_1	.53
13,14-Dihydro 15-keto $PGF_{2\alpha}$	.50
13,14-Dihydro 15-keto PGE_2	.47
PGB_1	.43

becomes important when TLC is used as a preparative step before radioimmunoassay. In the radioimmunoassay of prostaglandins, there is little cross-reactivity between prostaglandins having ring substituents, i.e. $PGF_{2\alpha}$ with PGE_2 or PGD_2, but a high cross-reactivity between prostaglandins having the same structure except for their number of double bonds (17). In contrast, in silica gel TLC, there is comigration of monoenoic and bisenoic prostaglandins, therefore iron or silver impregnation of the silica gel is used to overcome this (3,4).

The mobile phase, a one phase solution, in this reverse-phase TLC separation of arachidonic acid and 19 eicosanoids gives separation of all compounds except PGE_1 with PGD_2 and PGB_1, PGB_2 and PGA_2. Reverse-phase TLC is used in complex mixtures because

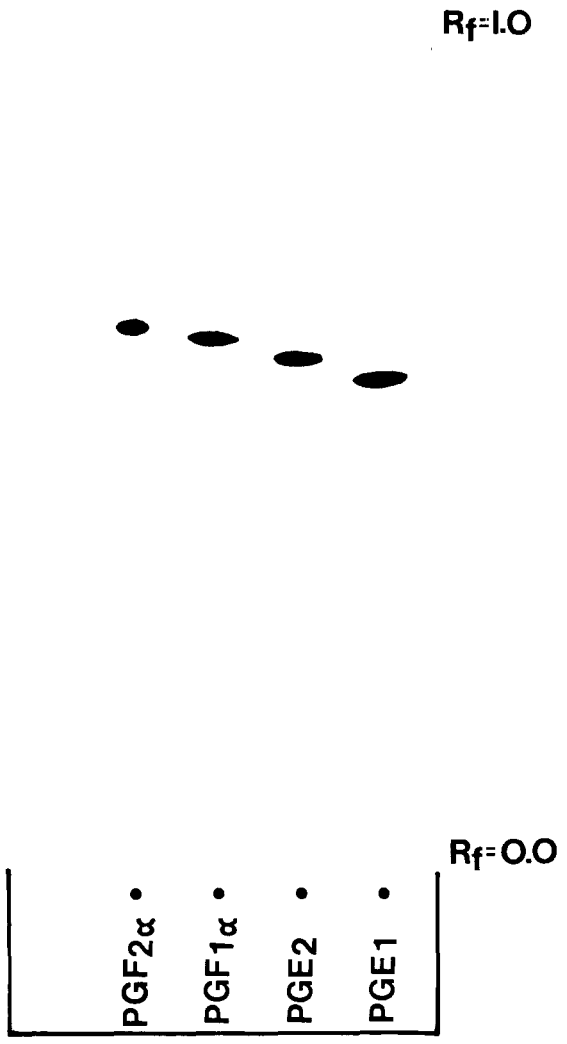

FIGURE 2: Migration of monoenoic versus bisenoic prostaglandins on reverse-phase TLC plate. Solvent system: 0.0025M phosphoric acid-acetonitrile (52:48, v/v) containing 0.2M NaCl.

the mobile phase is a two solvent system that can easily be adjusted to achieve separation (16). Additionally, ion-pairing reagents or pH adjustments can be exploited to maximize separation. Ionic suppression of the carboxyl group was used in this mobile phase to increase the hydrophobicity of the eicosanoids, hence increasing their affinity for the stationary, C18 phase.

In contrast, the mobile phase used in normal phase TLC of eicosanoids can be the organic phase of a two phase system with the water content depending on the amount of acid, solvents, and temperature at which equilibration occurs or a one phase system with little or no water. Since water represents a weak solvent in reverse-phase TLC, little change in separation is seen in high humidity conditions; in sharp contrast to normal phase TLC (16). Therefore, activation of the plate by heating is not necessary in reverse-phase TLC; a step required for most normal TLC plates (12).

More importantly, reverse-phase TLC, whether used as a primary or secondary method, combined with normal-phase TLC, would further aid the confirmation of compound identity and help to avoid comigration of eicosanoids as experienced in the past with 6-keto $PGF_{1\alpha}$ and PGE_2 (13).

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THIN LAYER CHROMATOGRAPHIC STUDIES OF 30 ORGANIC ACIDS ON CALCIUM SULPHATE

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ABSTRACT

Thin layer chromatographic behaviour of 30 organic acids on coatings of calcium sulphate and calcium sulphate containing charcoal, p-dimethylaminobenzaldehyde, flyash, silica gel G etc. has been studied. Farm chemicals: plant growth regulators (benzoic, cinnamic, gallic, β-naphthalene acetic, β-naphthoxy acetic and indole-3-acetic acids) and herbicides (phenoxy acetic acid) have been separated from one another and from several other organic acids.

INTRODUCTION

Thin layer chromatography is a versatile technique for the separation of organic acids. Several new coating materials (1,2) have been discovered and tested for separation. The older material, silica gel-coated glass plates developed in mixed solvent systems have widely been used for the separation of amino acids, benzoic acid, substituted benzoic acids and sorbic acids from fruit beverages, citric acid

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cycle intermediates and lactic acid, esters of p-hydroxy benzoic acid, phosphoric acid etc. Ion-exchange thin layer chromatography has been used for the separation of tryptophan from an aminoacylase-N-acetyltryptophan reaction mixture (1). Most phenyl-thiohydantoin (PTH) and methylthiohydantoin (MTH) amino acid derivatives could also be separated from one another by this method. Thin layers of silica gel (3) containing silver oxide have been used for the separation of substituted benzoic, phthalic, maleic and fumaric acids. Thin layers of silica gel G F_{254} containing cellulose MN_{300} F_{254} (4) have been used for the separation of food preservatives. In our previous publications (5-8) it has been shown that papers impregnated with calcium carbonate/ calcium sulphate have a great separation potential for organic acids. Therefore, now an attempt has been made to test the separation potential of calcium sulphate/calcium sulphate containing p-dimethylamino-benzaldehyde, methyl orange, starch, activated char-coal, calcium carbonate, flyash, silica gel G etc. coated glass plates. The results obtained are des-cribed in this paper.

EXPERIMENTAL

Apparatus and Materials: A Stahl apparatus with a universal applicator (adjustable thickness of the applied layers from 0-2.0 mm) (made in India), glass plates (20x4 cm), glass jars (25x5 cm) and tempera-ture controlled electric oven were used.

Activated charcoal and acetone (E. Merck, India), ammonium vanadate (Riedel, Germany), benzene (Reechem, India), bromophenol blue and calcium sulphate dihydrate (S.M. Chemicals, India), carbontetrachloride, silica gel G and 1,4-dioxane (Glaxo Laboratories, India), p-dimethylaminobenzaldehyde (BDH, India), starch (NCL, India) of analytical grade and flyash 100-200 mesh (Thermal Power Station, Kasimpur, U.P., India) were used.

Flyash was dried at 100° in an electric oven before use. The principal ingredients (9,10) of flyash are silica, alumina and iron oxides. Lime and carbon are present in minor proportions. The actual composition of the flyash depends on the variety of coal used and degree of burning.

Aqueous or ethanolic solutions (0.1N) of the test materials were used. In case it was not possible to prepare 0.1N solutions a saturated solution was used.

Preparation of Plates: A slurry of calcium sulphate (I) obtained by mixing calcium sulphate (30 g) with distilled water (D W) (70 ml), was applied on the glass plates with the help of the applicator so that the thickness of calcium sulphate slurry would be 0.75 mm. The plates were first allowed to dry at room temperature and then in a temperature controlled electric oven at 110° for 1 hr. The plates of silica gel G (II) were also prepared by the same procedure using the slurry of silica gel G made by mixing 48 g of it with 100 ml of distilled water.

The procedure described above was also used to make plates of the following coatings.

Coating III: Calcium sulphate (30 g) + activated charcoal (0.3 g) + D W (70 ml).

Coating IV: Calcium sulphate (30 g) + activated charcoal (0.6 g) + D W (70 ml).

Coating V: Calcium sulphate (30 g) + activated charcoal (1.5 g) + D W (70 ml).

Coating VI: Calcium sulphate (30 g) + calcium carbonate (0.15 g) + D W (70 ml).

Coating VII: Calcium sulphate (30 g) + calcium carbonate (0.3 g) + D W (70 ml).

Coating VIII: Calcium sulphate (30 g) + calcium carbonate (0.6 g) + D W (70 ml).

Coating IX: Calcium sulphate (30 g) + p-dimethyl-aminobenzaldehyde (p-DAB) (1 ml of 10%) + D W (70 ml).

Coating X: Calcium sulphate (30 g) + flyash (0.3 g) + D W (70 ml).

Coating XI: Calcium sulphate (30 g) + flyash (0.6 g) + D W (70 ml).

Coating XII: Calcium sulphate (30 g) + flyash (1.5 g) + D W (70 ml).

Coating XIII: Calcium sulphate (15 g) + silica gel G (15 g) + D W (70 ml).

Coating XIV: Calcium sulphate (30 g) + silica gel G (0.3 g) + D W (70 ml).

Coating XV: Calcium sulphate (30 g) + silica gel G (1.5 g) + D W (70 ml).

Coating XVI: Calcium sulphate (30 g) + silica gel G (3.0 g) + D W (70 ml).

Coating XVII: Calcium sulphate (30 g) + silica gel G (4.5 g) + D W (70 ml).

Coating XVIII: Calcium sulphate (30 g) + starch (0.6 g) + D W (70 ml).

Coating XIX: Calcium sulphate (30 g) + starch (1.5 g) + D W (70 ml).

Coating XX: Calcium sulphate (30 g) + starch (3.0 g) + D W (70 ml).

Coating XXI: Silica gel G (12 g) + activated charcoal (0.5 g) + D W (25 ml).

Coating XXII: Silica gel G (12 g) + ammonium vanadate (0.5 g) + D W (25 ml).

Coating XXIII: Silica gel G (12 g) + bromophenol blue (0.5 g) + D W (25 ml).

Coating XXIV: Silica gel G (12 g) + calcium nitrate (3.0 g) + D W (25 ml).

Coating XXV: Silica gel G (12 g) + copper sulphate (1 ml of 0.1M) + D W (25 ml).

Coating XXVI: Silica gel G (12 g) + cresol red (0.5 g) + D W (25 ml).

Coating XXVII: Silica gel G (12 g) + p-DAB (1 ml of 10%) + D W (25 ml).

Coating XXVIII: Silica gel G (12 g) + flyash (0.5 g) + D W (25 ml).

Coating XXIX: Silica gel G (12 g) + resorcinol (1 ml of 10%) + D W (25 ml).

Coating XXX: Silica gel G (12 g) + thymol blue (0.5 g) + D W (25 ml).

In all cases slurry was made as mentioned above except p-DAB and resorcinol. In these cases first the slurry was made and then ethanolic solution of p-DAB or resorcinol was added to it. After a thorough mixing the slurry so obtained was coated on the plates.

Spotting of Test Solution: Test solution was spotted on the plate with the help of a fine capillary. The plates were kept at room temperature (30°) for 15 min for the removal of solvent and then developed in a solvent system. For tailing, the front limit (RI) and the rear limit (RT) were measured while for other acids R_f values were taken as usual.

$$R_f = \frac{\text{Distance travelled by substance (cm)}}{\text{Distance travelled by solvent front (10 cm)}}$$

Test Solutions and their Detection: The acids on the plates were detected by the reported procedure summarized below:

(1) Alanine, (2) arginine HCl and (3) l-aspartic acids were detected by 1% aqueous ninhydrin solution; (4) acetic, (5) adipic, (6) ascorbic, (7) barbituric, (8) benzoic, (9) cinnamic, (10) citraconic, (11) citric, (12) formic, (13) fumaric, (14) gallic, (15) hippuric, (16) indole-3-acetic, (17) malic, (18) maleic, (19) β-naphthaleneacetic, (20) β-naphthoxy-acetic, (21) nicotinic, (22) oxalic, (23) oxaloacetic, (24) phenoxyacetic, (25) quinic, (26) salicylic, (27) sulphamic, (28) tartaric, (29) trans-aconitic

and (30) trichloroacetic acids were detected by 1% ethanolic alkaline bromophenol blue solution.

RESULTS

Various separations of the acids under study on different coatings are possible, some of them are summarized below. R_f values are given in parentheses that follow the number of the acid, marked in the experimental section.

Coating I: 9(0), 19(0) and 20(0) from 1(1.0), 2(1.0), 4(1.0), 5(0.8), 6(1.0), 7(1.0), 8(1.0), 10(1.0), 11 (1.0), 12(1.0), 13(0.85), 14(1.0), 15(0.85), 17(1.0), 18(1.0), 21(1.0), 22(1.0), 23(1.0), 24(1.0), 25(1.0), 27(1.0), 28(1.0), 29(1.0) and 30(1.0).

Coating II: 2(0.6) from 3(0.15), 4(0-1), 5(0-2.5), 6(0-2), 7(0-1.5), 8(0-2), 9(0-1), 10(0-2), 11(0-3.5), 12(0-1), 13(0-3.3), 15(0-0.7), 16(0-2), 17(0-1), 18(0-1), 19(0-0.5), 20(0), 21(0-1.5), 22(0-4.5), 23(0-2.5), 24(0-4), 25(0-0.5), 26(0-1), 27(0-1), 28(0-1), 29(0-1) and 30(0-1).

Coating III: 2(1.0), 3(1.0), 6(1.0), 27(1.0), 28(0.9) and 30(0.9) from 7(0-6), 8(0-6), 16(0-5), 19(0) and 20(0-2.5).

Coating IV: 22(1.0), 27(1.0) and 30(0.8) from 7(0-3), 8(0-3), 9(0-2), 10(0-3), 15(0-2), 16(0-5), 19(0-1), 20(0-1), 23(0-3) and 26(0-3.5).

Coating V: 3(0-7), 22(1.0) and 27(1.0) from 7(0-2), 8(0-3), 9(0), 10(0-2.5), 11(0-3.5), 13(0-3), 14(0-2.5),

15(0-1), 16(0), 18(0-3), 19(0), 20(0), 21(0-3), 23(0-1), 24(0-3), 26(0-2) and 29(0-2.5).

Coating VI: 1(1.0) and 2(1.0) from 3(0), 4(0), 7(0-2), 9(0-2), 10(0-2), 12(0), 15(0-3), 16(0-4), 18(0-2), 19(0), 20(0-2),21(0-4), 22(0-4), 23(0), 24(0-3), 25(0-5), 26(0-5), 29(0-4) and 30(0-2).

Coating IX: 9(0), 19(0) and 20(0) from 1(1.0), 2(0.9), 3(1.0), 4(1.0), 5(1.0), 6(1.0), 7(1.0), 8(1.0), 10(1.0), 11(1.0), 12(1.0), 13(0.8), 14(1.0), 15(1.0), 17(1.0), 18(1.0), 21(1.0), 22(1.0), 23(1.0), 24(0.65), 25(1.0), 27(1.0), 28(1.0), 29(1.0), and 30(1.0).

Coating X: 16(0-5) and 24(0.7) from 6(1.0), 10(1.0), 11(1.0), 14(1.0), 15(1.0), 17(1.0), 18(1.0), 22(1.0), 25(1.0), 27(1.0), 28(1.0), 29(1.0) and 30(1.0).

Coating XIII: 1(0.85), 2(0.4) and 3(0.7) from 4(0), 5(0-2), 6(0-1), 7(0), 8(0-0.5), 9(0), 10(0), 11(0), 12(0), 13(0), 15(0), 16(0), 17(0), 18(0), 19(0), 20(0), 21(0), 22(0), 23(0), 24(0), 25(0), 27(0), 28(0), 29(0) and 30(0).

Coating XIV: 9(0) and 19(0) from 1(1.0), 2(1.0), 3(1.0), 5(0.9), 6(1.0), 7(1.0), 8(0.8), 10(1.0), 11(1.0), 13(0.9), 15(0.8), 17(0.8), 18(5-10), 21(6-10), 22(1.0), 23(1.0), 25(1.0), 27(1.0), 28(1.0), 29(1.0) and 30(1.0).

Coating XVIII: 19(0) from 6(1.0), 11(1.0) and 21(1.0).

Coating XXI: 1(0.85) from 4(0-1), 5(0-1), 6(0-1.5), 7(0-1), 8(0-1), 9(0-0.5), 10(0-1), 11(0-1.5), 12(0-1),

13(0-3), 15(0-1), 16(0-2), 17(0-1), 18(0-1), 19(0), 20(0-0.5), 21(0), 22(0), 23(0), 24(0-0.5), 26(0-1), 27(0-1), 28(0-1), 29(0-1) and 30(0-1).

Coating XXIII: 1(0.9) and 2(0.5) from 4(0-1.5), 6(0-1.5), 19(0), 21(0-2), 22(0-1), 25(0-1) and 26(0-2).

Coating XXIV: 1(0.7) and 2(0.6) from 4(0-1), 6(0-1), 19(0), 21(0-2), 22(0-1), 25(0-1) and 26(0-2).

Coating XXV: 1(0.8) and 2(0.45) from 4(0-1), 5(0-1.5), 6(0-1.5), 7(0), 8(0-1), 9(0-1.5), 10(0-1), 11(0-1.5), 12(0-0.5), 13(0-2), 15(0), 16(0-1.5), 17(0-1), 18(0-1.5), 19(0), 20(0), 21(0-1), 22(0-2), 23(0-1.5), 24(0-2), 25(0-1.5), 26(0-2.5), 27(0-2), 28(0-2), 29(0-2.5) and 30(0-2).

Coating XXVI: 1(0.8), 2(0.4) from 4(0-1), 6(0-1), 19(0), 21(0-1), 22(0-1) and 25(0-0.5).

Coating XXVII: 1(0.85), 2(0.4) and 3(1.0) from 4(0-1), 5(0-1.5), 6(0-2), 7(0), 8(0-1), 9(0-0.8), 10(0-0.8), 11(0-1.5), 12(0-0.7), 13(0-2), 15(0), 16(0-1.5), 17(0-1), 18(0-1), 19(0-0.4), 20(0-0.5), 21(0-1), 22(0-1), 23(0-1), 24(0-1), 25(0-0.8), 26(0-1.7), 27(0-1.8), 28(0-1.8), 29(0-1) and 30(0-1.3).

Coating XXVIII: 1(1.0) and 2(0.8) from 4(0), 5(0), 6(0), 7(0), 8(0), 9(0), 10(0), 11(0), 12(0), 13(0), 15(0), 16(0), 17(0), 18(0), 19(0), 20(0), 21(0), 22(0), 23(0), 24(0), 25(0), 26(0), 28(0), 29(0) and 30(0).

Coating XXIX: 1(1.0) and 2(0.65) from 4(0-1), 5(0-1), 6(0), 7(0), 8(0-1), 9(0-1), 10(0-1), 11(0-1), 12(0-1), 13(0-2.5), 15(0), 16(0-1), 17(0-1), 18(0-1), 19(0-1), 20(0), 21(0-1), 22(0-2), 23(0-1.5), 24(0-1), 25(0-1), 26(0-1), 27(0-2), 28(0-2), 29(0-1) and 30(0-1).

Coating XXX: 1(0.9) and 2(0.55) from 4(0-1), 6(0-1), 14(0-1.5) and 21(0-1.5).

Separations achieved are recorded in tables 1, 2, 3 and 4.

DISCUSSION

Organic acids are naturally occurring materials that exist in different parts of the plants and animals. Some of the organic acids (11) are used as herbicides and plant growth regulators and they drain into water generally during monsoon period. Some acids (11) are toxic/mutagenic/carcinogenic to human beings, animals as well as aquatic organisms. Therefore, there is a growing interest in the development of new and inexpensive methods of the analysis of organic acids.

It seems that silica gel G has a very high adsorption capacity for acids. Therefore, most of the acids either have very low R_f values or they tail. Charcoal and flyash show the same behaviour. Flyash is an inexpensive and easily available material. Papers (9,10) describing its utility for the purification of water have been published. Calcium sulphate is a good coating material for the separation

TABLE 1: Separations Achieved on Plates Coated with Calcium Sulphate in Distilled Water.

Sl. No.	Acid	Separated from
1.	Cinnamic(0)	Adipic(0.9), alanine(1.0), ascorbic(1.0), barbituric(6-10), citraconic(1.0), citric(1.0), fumaric(0.85), hippuric(1.0), malic(1.0), maleic(1.0), nicotinic(1.0), oxalic(1.0), oxaloacetic(1.0), sulphamic(1.0), tartaric(1.0), and trans-aconitic(1.0) acids.
2.	β -Naphthaleneacetic(0)	Adipic(1.0), alanine(1.0), ascorbic(1.0), barbituric(1.0), citraconic(1.0), citric(1.0), formic(1.0), fumaric(0.85), gallic(1.0), hippuric(1.0), malic(1.0), maleic(1.0), nicotinic(1.0), oxalic(1.0), oxaloacetic(1.0), quinic(1.0), sulphamic(1.0), tartaric(1.0), trans-aconitic(1.0), and trichloroacetic(1.0) acids.
3.	β -Naphthoxyacetic(0)	Adipic(1.0), alanine(1.0), arginine HCl(0.9), ascorbic(1.0), barbituric(0.9), citraconic(1.0), citric(1.0), gallic(0.8), hippuric(0.8), maleic(1.0), nicotinic (0.8), oxalic(1.0), oxaloacetic (1.0), quinic(0.9), sulphamic (1.0), tartaric(1.0), trans-aconitic(1.0) and trichloroacetic (1.0) acids.

TABLE 2: Separations Achieved on Plates Coated with Calcium Sulphate in Organic Solvents.

Sl. No.	Acid	Separated from	Solvent system
1.	Benzoic(1.0)	Alanine(0), arginine HCl(0), ascorbic(0), l-aspartic(0), barbituric(0), citraconic(0), citric(0), formic(0), fumaric(0), gallic(0), hippuric(0), indole-3-acetic(0-5), malic(0), maleic(0), nicotinic(0), oxalic(0), oxaloacetic(0), quinic(0), sulphamic(0), tartaric(0), and trans-aconitic(0) acids.	Benzene
2.	Cinnamic(1.0)	Acetic(0), adipic(0), ascorbic(0), barbituric(0), citraconic(0-3), citric(0), formic(0), fumaric(0), gallic(0), hippuric(0), malic(0), maleic(0), nicotinic(0), oxalic(0), oxaloacetic(0), quinic(0), sulphamic(0), tartaric(0), and trans-aconitic(0) acids.	Benzene

continued

TABLE 2: continued

Sl. No.	Acid	Separated from	Solvent system
3.	β-Naphthalene-acetic(1.0)	Acetic(0), adipic(0), alanine(0), arginine HCl(0), ascorbic(0), l-aspartic(0), barbituric(0), citraconic(0), citric(0), formic(0), fumaric(0), gallic(0), hippuric(0), indole-3-acetic(0), malic(0), maleic(0), nicotinic(0), oxalic(0), oxaloacetic(0), quinic(0), tartaric(0), and trans-aconitic(0) acids.	Benzene
4.	Acetic(0)	Adipic(1.0), ascorbic (1.0), barbituric(1.0), benzoic(1.0), cinnamic (1.0), citraconic(1.0), indole-3-acetic(1.0), malic(1.0), β-naphthoxy-acetic(1.0), and salicylic (1.0) acids.	1,4-Dioxane
5.	Formic(0)	Adipic(1.0), ascorbic (1.0), barbituric(1.0), benzoic(1.0), cinnamic (1.0), citraconic(1.0), indole-3-acetic(1.0), malic(1.0), β-naphthoxy-acetic(1.0), and salicylic(1.0) acids.	1,4-Dioxane

TABLE 3: Separations Achieved on Plates Coated with Calcium Sulphate Containing Other Materials in Distilled Water

Sl. No.	Acid	Separated from	Material coated
1.	Cinnamic(0)	Adipic(0.8), ascorbic (0.8), barbituric(1.0), benzoic(0.65), citraconic(0.9), citric(1.0), formic(1.0), fumaric (0.8), gallic(0.9), hippuric(0.8), malic (0.9), maleic(0.9), nicotinic(1.0), oxalic (1.0), oxaloacetic(0.9), phenoxyacetic(0.65), quinic(0.9), sulphamic (1.0), tartaric(0.9), trans-aconitic(1.0) and trichloroacetic(0.9) acids.	Calcium sulphate (30 g) + p-DAB (1 ml of 10%)
2.	β-Naphthaleneacetic(0)	Acetic(0.9), adipic (0.75), ascorbic(1.0), barbituric(0.9), benzoic(1.0), citraconic (1.0), citric(1.0), formic(1.0), fumaric (0.8), gallic(0.9), hippuric(1.0), malic (0.9), maleic(1.0),	Calcium sulphate (30 g) + p-DAB (1 ml of 10%)

continued

TABLE 3: continued

Sl. No.	Acid	Separated from	Material coated
	β-Naphthalene-acetic(0)	nicotinic(1.0), oxalic (1.0), oxaloacetic(1.0), phenoxyacetic(0.9), quinic(1.0), sulphamic (1.0), tartaric(1.0), trans-aconitic(1.0) and trichloroacetic(1.0) acids.	
3.	β-Naphthoxy-acetic(0)	Adipic(0.9), ascorbic (0.9), barbituric(0.8), benzoic(0.8), citraconic(0.9), citric(0.7), fumaric(0.8), hippuric (0.8), malic(0.7), maleic(0.9), oxalic (0.9), oxaloacetic(0.7), phenoxyacetic(0.7), quinic(0.9), sulphamic (0.9), tartaric(0.7) and trans-aconitic(0.7) acids.	Calcium sulphate (30 g) + p-DAB (1 ml of 10%)
4.	Indole-3-acetic(0)	Ascorbic(0.7), malic (1.0), maleic(1.0), sulphamic(1.0), oxalic (1.0) and quinic(0.9) acids.	Calcium sulphate + 1% flyash

continued

TABLE 3: continued

Sl. No.	Acid	Separated from	Material coated
5.	Oxalic(1.0)	Indole-3-acetic(0-2.5) and phenoxyacetic (0-2.5) acids.	Calcium sulphate + 5% charcoal
6.	Alanine(0.9)	Arginine HCl (0.6) and l-aspartic(0.7) acids.	Calcium sulphate + silica gel G (1:1)

of organic acids because most of the acids move in the form of single spot, three acids remain at the point of application and only two acids, aspartic and salicylic, tail (coating I). The separation potential of calcium sulphate can be further enhanced by mixing it with other materials such as activated charcoal, calcium carbonate, flyash and p-DAB (coatings III-XX). Some important separations achieved are discussed below.

Results recorded in tables 1, 2, 3 and 4 indicate that plant growth regulators (naturally occurring inhibitors) benzoic and cinnamic acids are separated from alanine, barbituric, citric, fumaric, gallic, hippuric, indole-3-acetic and trans-aconitic acids

TABLE 4: Separations Achieved on Plates Coated with Silica gel G (12 g) Containing p-DAB (1 ml of 10%) in Distilled Water

Sl. No.	Acid	Separated from
1.	Alanine(0.85)	Acetic(0), adipic(0), ascorbic(0), l-aspartic(1.0), benzoic(0), cinnamic(0), citraconic(0), citric(0), formic(0), fumaric(0), hippuric(0), indole-3-acetic(0), malic(0), maleic(0), β-naphthaleneacetic(0), β-naphthoxyacetic (0), nicotinic(0), oxalic(0), oxaloacetic(0), phenoxyacetic(0), quinic(0), salicylic(0), sulphamic(0), tartaric(0), and trans-aconitic(0) acids.
2.	Arginine HCl (0.6)	Acetic(0), adipic(0), ascorbic(0), l-aspartic(1.0), benzoic(0), cinnamic(0), citraconic(0), citric(0), hippuric(0), indole-3-acetic(0), malic(0), maleic(0), β-naphthaleneacetic(0), β-naphthoxyacetic(0), nicotinic(0), oxalic(0), oxaloacetic(0), phenoxyacetic(0), quinic(0), salicylic(0), sulphamic(0), tartaric(0), trans-aconitic(0) and trichloroacetic(0) acids.

continued

TABLE 4: continued

Sl. No.	Acid	Separated from
3.	l-Aspartic (1.0)	Benzoic(0), cinnamic(0), citraconic(0), citric(0), formic(0), hippuric(0), indole-3-acetic(0), malic(0), maleic(0), β-naphthaleneacetic(0), β-naphthoxyacetic (0), nicotinic(0), oxalic(0), oxaloacetic(0), phenoxyacetic(0), quinic(0), salicylic(0), sulphamic(0), tartaric(0), trans-aconitic(0) and trichloroacetic(0) acids.

etc. on calcium sulphate coating in benzene; plant growth regulators (auxins) β-naphthaleneacetic and β-naphthoxyacetic acids are separated from alanine, benzoic, ascorbic, citric, fumaric, gallic, hippuric, sulphamic acids etc. on calcium sulphate containing p-DAB coating in distilled water and indole-3-acetic acid is separated from ascorbic, maleic, sulphamic, oxalic acids on calcium sulphate containing flyash coating in distilled water; herbicide, phenoxyacetic acid is separated from cinnamic, β-naphthaleneacetic, β-naphthoxyacetic acids on calcium sulphate containing p-DAB coating in distilled water; aminoacids, arginine HCl, alanine and aspartic acid are separated from benzoic, cinnamic, citric, fumaric, indole-3-acetic, β-naphthaleneacetic, β-naphthoxyacetic, phenoxyacetic, salicylic, oxalic acids etc. on silica gel G containing p-DAB coating in distilled water.

These results suggest the possible use of the above coatings in separation and identification of several organic acids.

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TLC SEPARATION OF SOME METAL IONS ON IMPREGNATED LAYERS

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ABSTRACT

8-Hydroxy quinoline and dibenzoyl methane have been tried as impregnants for working out TLC separation schemes for a large number of metal ions in group of five or six on silica gel 'G' plates. Dibenzoyl methane impregnation also offers a method for the removal of iron from the silica gel 'G'.

INTRODUCTION

A survey of literature (1,2) shows that the separation of metal ions has been attempted by many workers by paper chromatography and thin layer chromatography, but little attention has been paid to the impregnation technique for the separation of metal ions on thin layers. Srivastava and Coworkers (3,4) have used this technique by employing different complexing agents for improving the separation of some metal ions on silica gel thin layers. The present paper is an extension of this work where 8-OH-quinoline and dibenzoyl methane have been tried as impregnants for improving the TLC separation of metal ions of interest.

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EXPERIMENTAL

All the chemicals used were of Analar grade. The silica gel 'G' of SISCO make was used as an adsorbent. The TLC plates (Thickness 0.5 mm) were prepared by spreading a slurry of a mixture of 50 g. of silica gel 'G' and a definite amount of impregnant in 100 ml of a mixture of ethanol and water (50:50). The plates were dried for 24 hours at a constant temperature of 60±1°C. The metal salts (0.1% w/v solution in water) were applied to the layers using glass capillary and the chromatograms were developed at a constant temperature of 32±1°C. After development of the plates, spots were visualised by spraying first with a solution of 0.25% alizarin in ethanol and then with a solution of 0.1% of dithizone in chloroform.

RESULTS

An exmination of the hRf values in Table 1, shows that all the metal ions of interest can not be separated in a single run either on plain silica gel plate or on impregnated plate. On the basis of the hRf values observed on the impregnated plate, these metal ions have been put in the following groups for a satisfactory separation.

Metal ions separated with 8-OH quinoline impregnation.

1. Th(IV), Pb(II), Mg(II), Fe(III), V(V)
2. Mg(II), UO_2(II), Cd(II), Ni(II), Pd(II)
3. Pb(II), Mn(II), Cd(II), Ni(II), Cu(II)
4. Th(IV), Mn(II), UO_2(II), Cd(II), Co(II), Cu(II).

Metal ions separated with dibenzoylmethane impregnation.

1. V(V), Pb(II), Co(II), Zn(II), Cu(II), Fe(III)
2. Th(IV), UO_2(II), Mg(II), Cd(II), Ni(II), Cu(II)
3. Th(IV), Pb(II), Mn(II), Co(II), Zn(II), Cu(II).

TABLE 1

Metal Ion	Taken as	Solvent System: BuOH-EtOAc-HOAc 40:10:10 hRf		Solvent System: EtOAc-HCOOH-H_2O-Pyridine 30:10:10:5 hRf	
		Plain	Impregnated*	Plain	Impregnated **
Pb(II)	Nitrate	17	21	25	18
Mn(II)	Acetate	32	38	21	23
Cd(II)	Acetate	54 LT	63	30	41
Ni(II)	Acetate	38	75	56	73
Zn(II)	Acetate	54 MT	77	37	52
Fe(III)	Nitrate	57 LT	80	16	100
Cu(II)	Acetate	43	85	74	81
Co(II)	Nitrate	37 MT	76	27	34
Mg(II)	Acetate	22	29	22	24
UO_2(II)	Nitrate	49	49	30	14
Th(IV)	Nitrate	02	02	21	04
V(V)	Sod.Salt	87	88	07	03
Pd(II)	Chloride	85	87	-	-

hRf values reported are the mean of two or more identical runs, 10 cm development.

Impregnated* with 8-OH-quinoline,
Impregnated** with dibenzoyl methane.

DISCUSSION

Thus it is apparent that by the selection of the impregnant, it is possible to separate satisfactorily all the metal ions under study. Thus suitable pentanary and hexanary separation schemes of analytical importance have been worked out. It is worthwhile to mention that the hRf values on impregnated plate in both the cases are higher than on silica gel 'G' thin layer plates. This suggests that the complex formed by interaction of the metal ion with the impregnant is more soluble than the free metal ion in the solvent system employed. Thus it may be concluded that the movement of different metals on impregnated plates is governed by complex formation between the

impregnant and the metal ion and by their differential solubility in the developing system.

Moreover, the use of dibenzoylmethane as an impregnant offers a method for the removal of iron from silica gel 'G' which can be accomplished in the following manner :

The silica gel is treated with a trace amount (0.2%) of dibenzoylmethane in - EtOAc - HCOOH - H_2O - Pyridine (30:10:10:5) and heating it at 80°C for two minutes. After this the slurry is allowed to stand for 2-3 hours, the iron present in silica gel forms a soluble complex with dibenzoylmethane and is removed as a red colored floating layer.

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THIN-LAYER CHROMATOGRAPHY OF METAL IONS IN OXALIC ACID - OXALATE SYSTEMS

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ABSTRACT

The adsorption behaviour of 48 metal ions has been studied in Oxalic acid - Oxalate systems using silica gel - G layers. The effect of p_H on R_f values was also investigated. A plot of -log Ksp Vs. R_M and R_f shows the dramatic behaviour of Hg^{2+} ion in 0.1 M Potassium Oxalate + 0.1 M Ammonium Oxalate (1:1) system. A number of interesting separations have been achieved e.g., Fe^{3+} - Ti^{4+}, Zr^{4+} - Ti^{4+}, Ce^{4+} - La^{3+}, Zr^{4+} - Th^{4+}, Te^{4+} - Se^{4+} and Zr^{4+} - Y^{3+} - La^{3+}. Ti^{4+} and Nb^{5+} were separated from a mixture of number of ions.

INTRODUCTION

Thin layer chromatography offers an interesting method for the separation of metal ions in microgram quantities. The separation potential of this technique is greatly enhanced if complexation is used. Considering this aspect of the problem, we have tried to use solvents containing Oxalic acid and Potassium or Ammonium Oxalate solutions as eluants using silica gel-G thin layers as

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the stationary phase. As a result of the change in p_H and the concentration of the complexing Oxalate anion, some very interesting separations have been achieved which are presented in the following article.

MATERIALS AND METHODS

Apparatus

Toshniwal (made in India) TLC apparatus was used for the preparation of thin layers on glass plates (20x3 cm). The plates were developed in glass jars (20x6 cm). p_H meter (Toshniwal, India) was used for p_H determination.

Reagents

Silica gel-G, Potassium Oxalate, Ammonium Oxalate and Oxalic acid used were all of analytical grade from B.D.H., England. Other reagents were of AnalaR grade.

Test solutions and Detectors

Test solutions were generally prepared in 0.1 M metal chlorides, sulphates or nitrates. A little amount of the corresponding acid is added to prevent hydrolysis. Conventional spot test reagents were used for detection perposes.

Preparation of Silica gel-G plates

The slurry used was prepared by mixing silica gel-G in D.M.W. in the ratio of 1:3 with constant shaking for about 5 min. This slurry was immediately coated on clean glass plates with the help of an applicator and uniform thin layers ($\sim$ 0.15 mm Thick) were obtained. The plates were first allowed to dry at room temperature and then in an electric oven at 100 $\pm$ 5°C for 2 hrs. These were then stored in an oven at room temperature.

Procedure

Test solutions (1 or 2 spots) were placed on the dry silica gel-G plates with the help of thin glass capillaries. After drying the spots, plates were developed in a chosen solvent and the ascent was fixed at 11 cm in all cases. R_T and R_L values were measured as usual after detection.

Solvent systems

In all, seven solvent systems were used. Their composition and p_H values are given in Table 1.

TABLE 1.

p_H METRIC STUDIES IN OXALIC ACID - OXALATE MEDIA

Solvent	Composition	p_H
S_1	0.1M Oxalic acid	0.7
S_2	0.1M Potassium oxalate + 0.1M Oxalic acid(1:1)	2.2
S_3	0.1M Ammonium oxalate + 0.1M Oxalic acid (1:1)	2.2
S_4	0.1M Potassium oxalate + 0.1M Ammonium oxalate + 0.1M Oxalic acid (1:1:1)	3.2
S_5	0.1M Potassium oxalate + Ammonium oxalate(1:1)	6.8
S_6	0.1M Ammonium oxalate	7.3
S_7	0.1M Potassium oxalate	7.6

RESULTS

The plots given in the figures are only for those metal ions which give compact spots. A number of separations of analytical importance have actually been realised as given in Table 2.

DISCUSSION

It is surprising that very few studies on silica gel-G thin layers have been done using aqueous Oxalate solutions as eluants. The results in Table 3 show that it is possible to obtain some very interesting separations using these eluants. Thus Ti^{4+} can be separated from Fe^{3+}, Al^{3+}, Th^{4+}, In^{3+}, UO_2^{2+} and La^{3+}. These separations are very neat and the spots are well separated. Ti^{4+} has a R_f value of almost zero and the strong adsorption is due to its high charge. Zr^{4+} can be separated from Y^{3+}, La^{3+} and Th^{4+}. Zr^{4+} has a R_f value 0.0. probably because it is precipitated as confirmed by the results summarized in Table 3.

The R_f values of 48 cations in solvent systems are summarized in Figures 1-4. An analysis of the results tabulated in these figures show that it is possible for one to seperate Ti^{4+}, Ag^{+},

TABLE 2.

SEPARATIONS ACHIEVED EXPERIMENTALLY (40 min)

Solvent System	Separations achieved, metal ion (R_T-R_L)			
S_1	Ti^{4+}	(0.00-0.20) -	Fe^{3+}	(0.88-1.00)
	Ti^{4+}	(0.00-0.19) -	Al^{3+}	(0.90-1.00)
	Pb^{2+}	(0.00-0.00) -	Tl^{+}	(0.90-1.00)
S_2	Ti^{4+}	(0.00-0.30) -	Th^{4+}	(0.80-1.00)
	Ti^{4+}	(0.00-0.40) -	In^{3+}	(0.85-1.00)
	Ti^{4+}	(0.00-0.30) -	UO_2^{2+}	(0.85-1.00)
	Ti^{4+}	(0.00-0.35) -	VO^{2+}	(0.90-1.00)
	Ti^{4+}	(0.00-0.30) -	Zr^{4+}	(0.80-1.00)
	Ti^{4+}	(0.00-0.35) -	Cu^{2+}	(0.90-1.00)
	Ti^{4+}	(0.00-0.30) -	Cd^{2+}	(0.95-1.00)
	Ti^{4+}	(0.00-0.25) -	Be^{2+}	(0.80-1.00)
	Bi^{3+}	(0.00-0.00) -	Tl^{+}	(0.65-1.00)
	Ti^{4+}	(0.00-0.30) -	Co^{2+}	(0.90-1.00)
	Ti^{4+}	(0.00-0.25) -	Ni^{2+}	(0.95-1.00)
	Ti^{4+}	(0.00-0.20) -	Pd^{2+}	(0.85-1.00)
	Ti^{4+}	(0.00-0.18) -	Mg^{2+}	(0.80-1.00)
	Ti^{4+}	(0.00-0.25) -	A mixture of Th^{4+}, UO_2^{2+}, VO^{2+}, Co^{2+}, Ni^{2+}, Zr^{4+}, Pd^{2+}, Mg^{2+}, Cu^{2+}, Cd^{2+} and Be^{2+}	
S_4	Pb^{2+}	(0.00-0.28) -	Cd^{2+}	(0.90-1.00)
	Ti^{4+}	(0.00-0.30) -	La^{3+}	(0.90-1.00)

(continued)

TABLE 2 (continued)

Solvent System	Separations achieved, metal ion (R_T - R_L)			
	Fe^{3+}	(0.50-0.70) -	Mn^{2+}	(0.90-1.00)
	Pb^{2+}	(0.00-0.30) -	Mn^{2+}	(0.85-1.00)
	Ce^{4+}	(0.00-0.27) -	Pr^{3+}	(0.80-1.00)
	Ce^{4+}	(0.00-0.25) -	La^{3+}	(0.83-1.00)
	Ce^{4+}	(0.00-0.08) -	Sm^{3+}	(0.50-0.85)
	Nb^{5+}	(0.00-0.10) -	VO^{2+}	(0.92-1.00)
	Nb^{5+}	(0.00-0.07) -	Se^{4+}	(0.85-1.00)
	Nb^{5+}	(0.00-0.08) -	Mo^{6+}	(0.85-1.00)
	Nb^{5+}	(0.00-0.07) -	Cr^{3+}	(0.82-1.00)
	Nb^{5+}	(0.00-0.00) -	Zn^{2+}	(0.78-1.00)
	Nb^{5+}	(0.00-0.00) -	Mn^{2+}	(0.80-1.00)
	Nb^{5+}	(0.00-0.08) -	In^{3+}	(0.80-1.00)
	Nb^{5+}	(0.00-0.08) -	Ir^{3+}	(0.80-1.00)
	Nb^{5+}	(0.00-0.08) -	Cd^{2+}	(0.85-1.00)
	Nb^{5+}	(0.00-0.08) -	Pd^{2+}	(0.85-1.00)
	Nb^{5+}	(0.00-0.10) -	Pr^{3+}	(0.82-1.00)
	Nb^{5+}	(0.00-0.08) -	A mixture of VO^{2+}, Cd^{2+}, Pr^{3+}, In^{3+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+}, and Cr^{3+}	
S_6	Zr^{4+}	(0.00-0.08) -	Y^{3+}	(0.50-0.60)-
			La^{3+}	(0.85-0.95)
	Te^{4+}	(0.00-0.20) -	Se^{4+}	(0.85-1.00)
	Zr^{4+}	(0.00-0.00) -	Th^{4+}	(0.70-0.92)
	Fe^{3+}	(0.06-0.16) -	Ni^{2+}	(0.80-1.00)
	Fe^{3+}	(0.10-0.20) -	Co^{2+}	(0.87-1.00)

TABLE 3.

PRECIPITATION OF CATIONS WITH THE SOLVENT SYSTEMS USED

Solvent	Cations + Solvent	
	Cations which precipitate	Cations which do not precipitate
S_1	Hg_2^{2+}, Pb^{2+}, Ag^{+}, Zr^{4+}, Te^{4+}, Th^{4+}, Co^{2+}, La^{3+}, Cu^{2+}, Bi^{3+}, Hg^{2+}	Tl^{+}, UO_2^{2+}, In^{3+}, Mn^{2+}, Fe^{3+}, Ni^{2+}, Se^{4+}, Cd^{2+}, Al^{3+}, VO^{2+}, Be^{2+}, Pd^{2+}, Ba^{2+}, Ti^{4+}, Ir^{3+}, Pt^{4+}, Sn^{2+}, Sr^{2+}, Ga^{3+}, Cr^{3+}, Mo^{6+}, Au^{3+}, Sn^{4+}, Zn^{2+}, W^{6+}, Sb^{3+}, Nb^{5+}, Mg^{2+}
S_2	Hg_2^{2+}, Pb^{2+}, Zr^{4+}, Te^{4+}, Ba^{2+}, Th^{4+}, Co^{2+}, La^{3+}, Cu^{2+}, Bi^{3+}, Ag^{+}, Sr^{2+}	Tl^{+}, UO_2^{2+}, In^{3+}, Mn^{2+}, Fe^{3+}, Ni^{2+}, Se^{4+}, Cd^{2+}, Al^{3+}, VO^{2+}, Be^{2+}, Pd^{2+}, Ti^{4+}, Ir^{3+}, Pt^{4+}, Sn^{2+}, Ga^{3+}, Cr^{3+}, Mo^{6+}, Au^{3+}, Sn^{4+}, Zn^{2+}, W^{6+}, Sb^{3+}, Nb^{5+}, Mg^{2+}
S_3	Hg_2^{2+}, Pb^{2+}, Zr^{4+}, Te^{4+}, Th^{4+}, La^{3+}, Al^{3+}, Cu^{2+}, Bi^{3+}, Ag^{+}, Ba^{2+}, Sr^{2+}	Ti^{4+}, Tl^{+}, UO_2^{2+}, In^{3+}, Fe^{3+}, Ni^{2+}, Se^{4+}, Cd^{2+}, VO^{2+}, Be^{2+}, Pd^{2+}, Mn^{2+}, Ir^{3+}, Pt^{4+}, Sn^{2+}, Ga^{3+}, Cr^{3+}, Mo^{6+}, Au^{3+}, Sn^{4+}, Zn^{2+}, W^{6+}, Sb^{3+}, Nb^{5+}, Al^{3+}, Mg^{2+}
S_4	Pb^{2+}, Hg_2^{2+}, Zr^{4+}, Te^{4+}, Th^{4+}, Co^{2+}, La^{3+}, Bi^{3+}, Ag^{+}, Ba^{2+}, Sr^{2+},	Nb^{5+}, Tl^{+}, UO_2^{2+}, In^{3+}, Fe^{3+}, Ni^{2+}, Se^{4+}, Cd^{2+}, Al^{3+}, Cu^{2+}, Be^{2+}, Ga^{3+}, Mg^{2+}, Pd^{2+}, Ti^{4+}, Mn^{2+}, Ir^{3+}, Pt^{4+}, Sn^{2+}, Cr^{3+}, Mo^{6+}, Au^{3+}, Sn^{4+}, Zn^{2+}, W^{6+}, Sb^{3+}
S_5	Hg^{2+}, Pb^{2+}, Zr^{4+}, Th^{4+}, Co^{2+}, La^{3+}, Bi^{3+}, Ag^{+}, Ba^{2+}, Sr^{2+}	Sb^{3+}, Ti^{4+}, Nb^{5+}, Te^{4+}, Tl^{+}, UO_2^{2+}, In^{3+}, Fe^{3+}, Ni^{2+}, Se^{4+}, Cd^{2+}, Al^{3+}, VO^{2+}, Cu^{2+}, Be^{2+}, Pd^{2+}, Mn^{2+}, Ir^{3+}, Mg^{2+}, Pt^{4+}, Sn^{2+}, Ga^{3+}, Cr^{3+}, Mo^{6+}, Au^{3+}, Sn^{4+}, Zn^{2+}, W^{6+}

(continued)

TABLE 3 (continued)

Solvent	Cations + Solvent	
	Cations which precipitate	Cations which do not precipitate
S_6	Ag^+, Pb^{2+}, Ba^{2+}, Sr^{2+}, Zr^{4+}, Ba^{2+}, Th^{4+}, Co^{2+}, La^{3+}, Bi^{3+}, Hg_2^{2+}	Ti^{4+}, Nb^{5+}, Te^{4+}, Tl^+, UO_2^{2+}, Cu^{2+}, Fe^{3+}, Ni^{2+}, Se^{4+}, Cd^{2+}, Al^{3+}, VO^{2+}, Be^{2+}, Pd^{2+}, Mn^{2+}, Ir^{3+}, Pt^{4+}, Sn^{2+}, Sb^{3+}, Mg^{2+}, Ga^{3+}, Cr^{3+}, Mo^{6+}, Au^{3+}, Sn^{4+}, Zn^{2+}, W^{6+}
S_7	Hg_2^{2+}, Pb^{2+}, Ba^{2+}, Sr^{2+}, Zr^{4+}, Th^{4+}, Co^{2+}, La^{3+}, Bi^{3+}, Ag^+, Ba^{2+}	Fe^{3+}, Ti^{4+}, Nb^{5+}, W^{6+}, Te^{4+}, Tl^+, Sb^{3+}, UO_2^{2+}, In^{3+}, Ni^{2+}, Se^{4+}, Cd^{2+}, Al^{3+}, VO^{2+}, Cu^{2+}, Be^{2+}, Pd^{2+}, Mn^{2+}, Ir^{3+}, Cr^{3+}, Ga^{3+}, Pt^{4+}, Sn^{2+}, Mo^{6+}, Au^{3+}, Sn^{4+}, Zn^{2+}, Mg^{2+}

Sn^{2+}, Pb^{2+}, Sb^{3+} and Ce^{4+} from numerous metal ions. In addition to the separations already achieved and presented in Table 2. Other separations are also possible which are analytically very important e.g., separation of Ce^{4+} from Pr^{3+}, Sm^{3+}, Nd^{3+}, La^{3+} and Ba^{2+} ; separation of Y^{3+} from Sr^{2+} and of Tl^+ from Pb^{2+} . Nb^{5+} can also be separated from numerous metal ions such as Zr^{4+}, Y^{3+}, Sr^{2+}, Se^{4+}, Ga^{3+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, VO^{2+} Cr^{3+}, Mo^{6+}, Bi^{3+}, Pb^{2+}, Pd^{2+}, Cd^{2} In^{3+}, La^{3+}, Pr^{3+}, Nd^{3+}, Sm^{3+}, Ir^{3+}, Mg^{2+} and Tl^+ (See Figs. 3 and 4)

In order to understand the chromatographic behaviour of the ions, the R_f values were determined at different p_H values and the results are plotted in Figs. 5a and 5b. This behaviour may be divided into the following types:

(i) Those ions whose R_f values are high and independent of p_H i.e., Co^{2+}, Ni^{2+}, Mn^{2+}, VO^{2+}, Be^{2+}, Cd^{2+}, Nd^{3+}, Sm^{3+}, Ir^{3+} and Pt^{4+} .

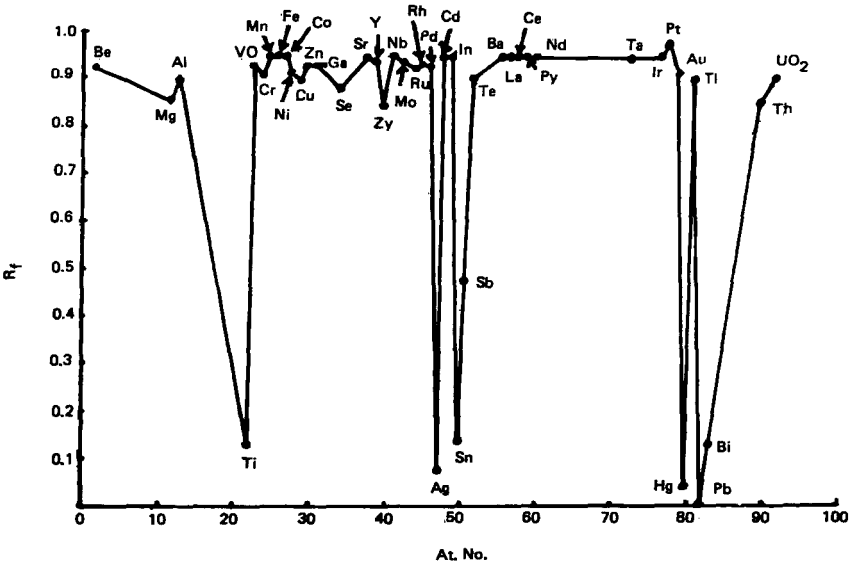

FIG. 1 A Plot of R_f vs Atomic Number in 0.1M Oxalic Acid

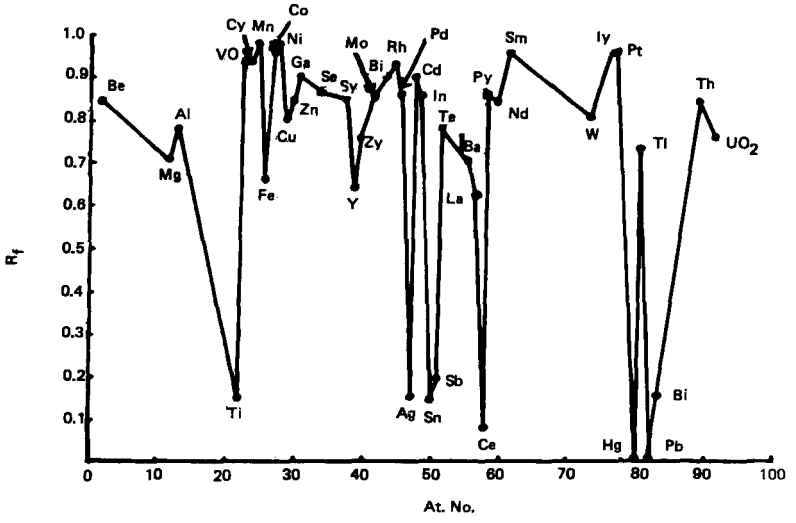

FIG. 2 A Plot of R_f vs Atomic Number in 0.1M Potassium Oxalate + 0.1M Oxalic Acid (1:1)

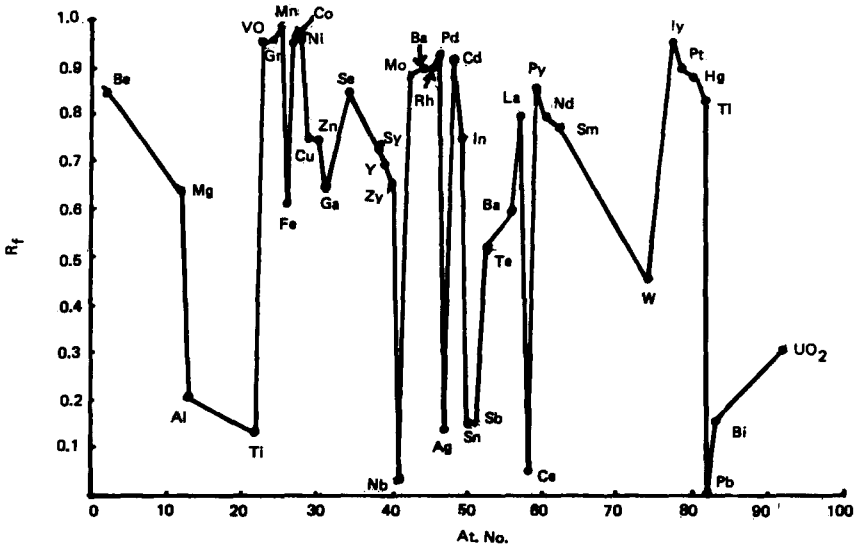

FIG. 3 A Plot of R_f vs Atomic Number in 0.1M Potassium Oxalate + 0.1M Ammonium Oxalate + 0.1M Oxalic Acid (1:1:1)

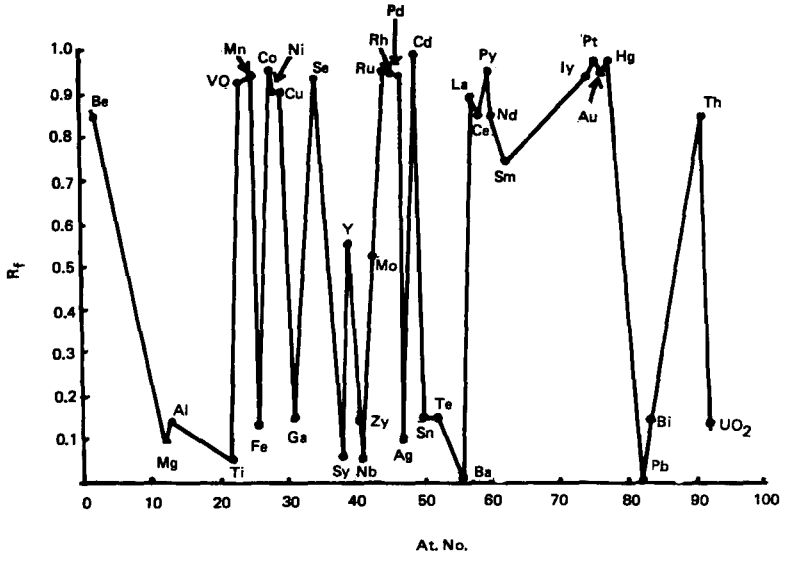

FIG. 4 A Plot of R_f vs Atomic Number in 0.1M Ammonium Oxalate

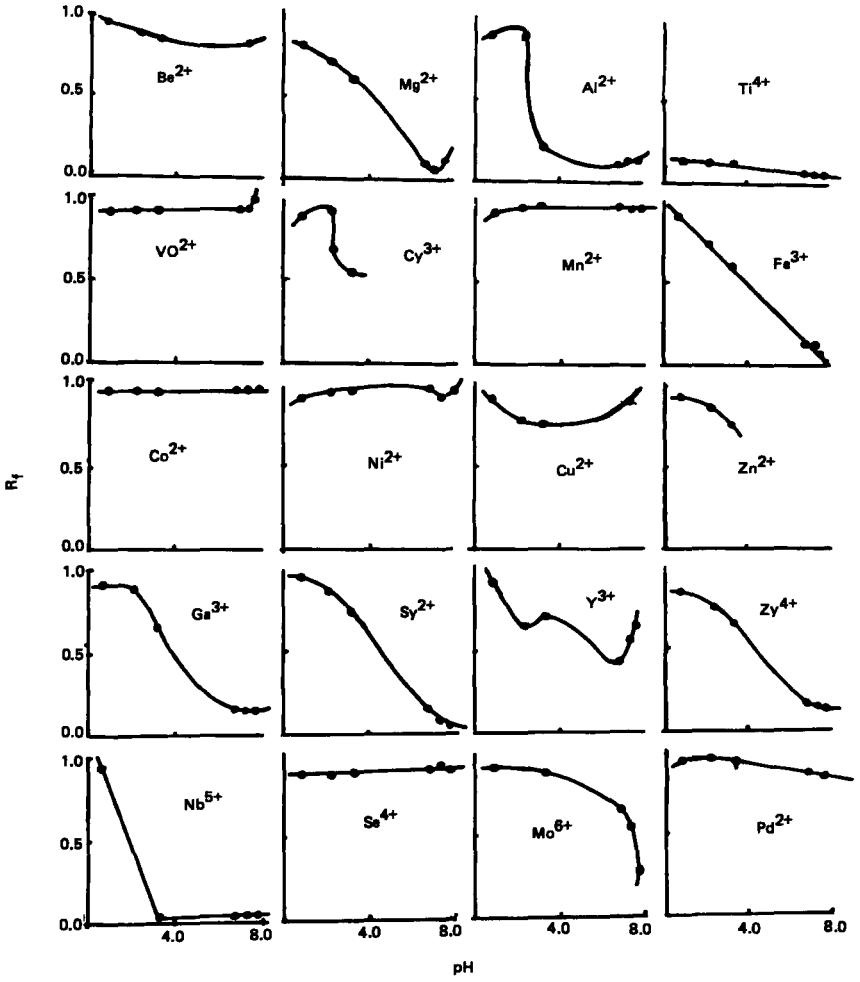

FIG. 5A Plots of R_f vs pH for Various Metal Ions

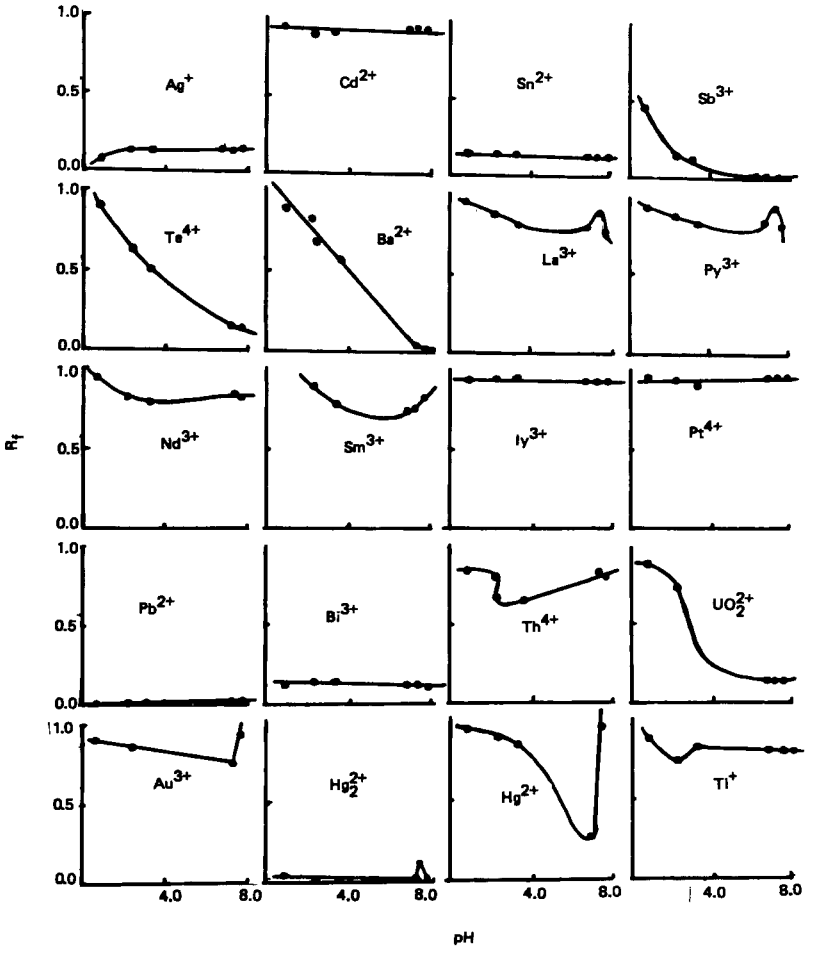

FIG. 5B Plots of R_f vs pH for Various Metal Ions

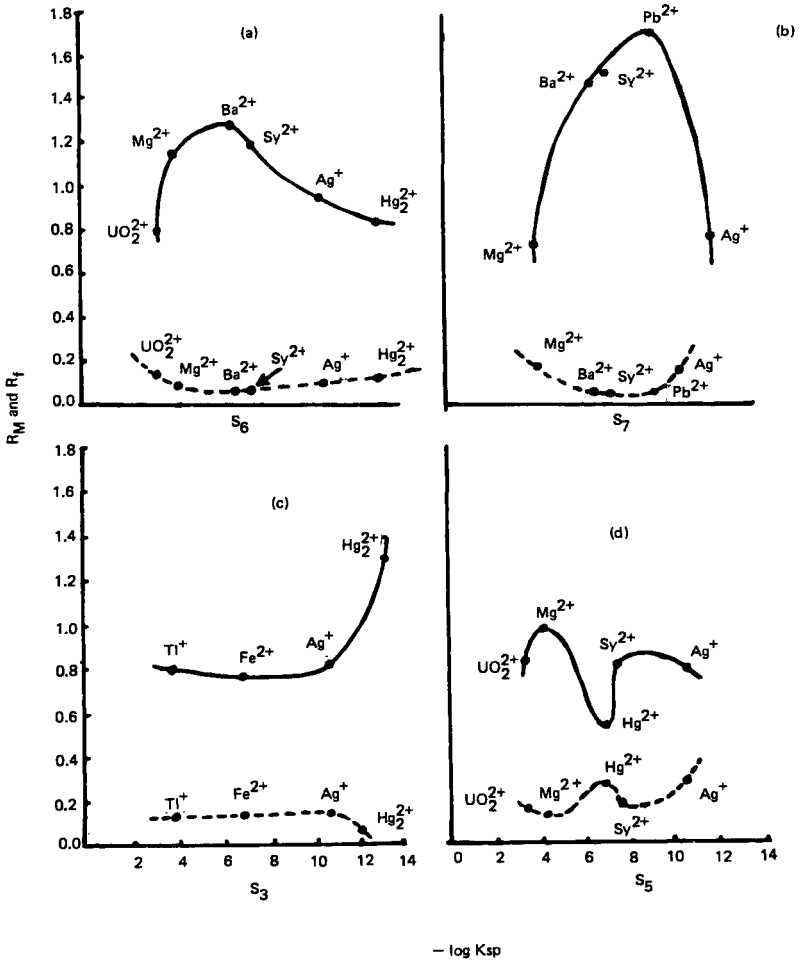

FIG. 6A, B, C, D Plots of -log Ksp vs R_M and R_f of Various Metal Ions

The high values are due to the fact that the oxalates of these ions do not precipitate. The change in R_f value is very small because as the ions do not precipitate, hence change in the oxalate ion concentration or change in H^+ concentration does not affect the mobility of these ions.

(ii) The ions whose R_f values are low but independentof p_H e.g., Ag^+, Sn^{2+}, Bi^{3+}, Hg_2^{2+}, Ti^{4+} and Pb^{2+}. Out of these, all the

ions precipitate in the various solvent systems studied except Sn^{2+} and Ti^{4+}. It therefore appears that the low R_f values of these metal ions are due to precipitation and in case of Sn^{2+} and Ti^{4+}, the low R_f value is probably due to the specific interaction of these ions with the silica gel-G.

(iii) The third category is of those ions whose R_f decresses almost linearly with increase in pH. These ions are Ga^{3+}, Sr^{2+}, Fe^{3+}, Mg^{2+}, Zr^{4+}, Te^{4+} and Ba^{2+}. A reference to Table 3 shows that as the p_H is increased, they slowly precipitate and hence there is a decrease in their R_f.

A plot of -log Ksp Vs. R_M and R_f for all those ions whose R_f is less than 0.5 is shown in Fig. 6a, b, c, d. The results show that as the solubility decreases initially there is an increase in the R_M values, as expected. However, on a further decrease in the solubility, there is a decrease in R_M value and the curve passes through a maximum in Figs. 6a and b and even in Fig. 6c there is a tendency towards a maximum. In fig.6d there is only one point i.e., Hg^{2+} which does not fall in line with this trend. The explanation is, that there are two interactions which are in operation i.e., the solubility effect and the complex formation effect. The decrease in R_f with decrease is solubility is expected. However, the increase in R_f with decrease in solubility is due toccomplex formation. Thus even though, the solubility in an aqueous system decreases, there is an increase in solubility in the complex forming oxalic acid systems.

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TLC SEPARATIONS OF AMINO ACIDS ON SILICA GEL IMPREGNATED LAYERS

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ABSTRACT

Copper sulphate and polyamide were tried as impregnants for improving the separation of twenty amino acids on silica gel 'G' layers using a new solvent system MeOH-BuOAc-AcOH-Pyridine(20:20:10:5). Tables are presented to illustrate the improvement in resolution of amino acids on silica gel plates.

INTRODUCTION

Literature survey reveals many TLC systems for the separation of amino acids[1,2]. The use of pyridinium tungstoarsenate for TLC separation of amino acids was reported by Srivastava and coworkers[3]. Recently a comparison of amino acids separation on different layers was reported by Sleckmann and Sherma[4]. The present paper deals with the use of copper sulphate and polyamide mixed silica gel 'G' layers for satisfactory separation of amino acids.

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EXPERIMENTAL

The plates of 0.5 mm thickness were prepared by spreading a slurry of a mixture of silica gel 'G' (SISCO make) and 0.25 % solution of copper sulphate in distilled water. Polyamide mixed layers were made by first homogenising 50g silica gel and 10g of polyamide and then making a slurry with distilled water. The plates were dried at a constant temperature of 60°C for 24 hours.

The aq. solution of amino acids was applied to the layer by glass capillary and the chromatograms developed at 17 ± 1°C with MeOH-BuOAc-AcOH-Pyridine (20:20:10:5). After development the plates were sprayed with 0.1 % ninhydrin in acetone and heated at a temperature of 60°C for one hour.

RESULTS AND DISCUSSION

Table 1 gives hR_f values of amino acids on plain and impregnated silica gel. Tables 2-4 give resolution of amino acids on plain and impregnated layers. The resolution values (R) were calculated by dividing the distance between spot centres by the sum of radius of the two spots. A value of R more than 1.5 was considered as completely resolved and 'R' was placed in the table. A zero value of 'R' indicates that resolution was not possible either due to the tailing of the spot or due to same hR_f value.

It is worthwhile to mention that the hR_f values on impregnated plate is governed not only by the solubility of the product of interaction of amino acid and impregnant in the solvent system employed[5], but also by the adsorption behaviour of the inter-

TABLE 1

hR_f^* Values for Amino Acids

Amino Acid	A	B	C
l-leucine (Leu)	65	63	71
d,l-isoleucine (Ile)	66	72	81
d,l-tryptophane (try)	63	68	75
d,l-methionine (Met)	64	64	72
d,l-valine (Val)	64	60	77
l-lysine·HCl (Lys)	16_T	12	33
l-histidine·HCl (His)	22_T	20	39
d,l-β phenyl alanine (Phe)	64	65	82
d,l-threonine (Thr)	50	51	67
d,l-alanine (Ala)	46	45	64
d,l-serine (Ser)	40	43	56
l-tyrosine (Tyr)	58	61	71
l-glutamic acid (Glu)	41	48	58
d,l-aspartic acid (Asp)	28	25	44
l-arginine·HCl (Arg)	24_T	19	39
glycine (Gly)	36	46	49
l-proline (Pro)	37	36	58
l-cysteine·HCl (Cys)	20_T	17	29
d,l-2 amino butyric Acid (Aba)	51	54	61
l-ornithine·HCl (Ont)	27_T	23	35

* = The values are average of two as more identical runs, 10 cm in 35 minutes. T = Tailing, A = hR_f values on plain silica gel, B = hR_f values on copper sulphate impregnated plate, C = hR_f values on polyamide mixed layers.

TABLE 2

Resolution Data on Silica Gel 'G' Plate

	Ont	Aba	Cys	Pro	Gly	Arg	Asp	Glu	Tyr	Ser	Ala	Thr	Phe	His	Lys	Val	Met	Try	Ile
Leu	R	R	R	R	R	R	R	R	R	R	R	R	0.25	R	R	0.22	0.25	0.5	0.22
Ile	R	R	R	R	R	R	R	R	R	R	R	R	0.5	R	R	0.44	0.5	1.0	
Try	R	R	R	R	R	R	R	R	R	R	R	R	0.0	R	R	0.0	0.0		
Met	R	R	R	R	R	R	R	R	R	R	R	R	0.0	R	R	0.0			
Val	R	R	R	R	R	R	R	R	R	R	R	R	0.0	R	R				
Lys	0.0	R	0.0	R	R	0.0	R	R	R	R	R	R	R	0.0					
His	0.0	R	0.0	R	R	0.0	R	R	R	R	R	R	R						
Phe	R	R	R	R	R	R	R	R	R	R	R	R							
Thr	R	0.22	R	R	R	R	R	R	R	R	R								
Ala	R	1.1	R	R	R	R	R	1.25	R	1.33									
Ser	R	R	R	0.66	0.88	R	R	0.22	R										
Tyr	R	R	R	R	R	R	R	R											
Glue	R	R	R	1.0	1.25	R	R												
Asp	0.22	R	R	R	R	0.88													
Arg	0.0	R	0.0	R	R														
Gly	R	R	R	0.25															
Pro	R	R	R																
Cys	0.0	R																	
Aba	R																		

TABLE 3

Resolution Data on Copper Sulphate Impregnated Plate

	Ont	Aba	Cys	Pro	Gly	Arg	Asp	Glu	Tyr	Ser	Ala	Thr	Phe	His	Lys	Val	Met	Try	Ile
Leu	R	R	R	R	R	R	R	R	R	R	R	R	0.40	R	R	R	0.80	0.80	R
Ile	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
Try	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R		
Met	R	R	R	R	R	R	R	R	R	R	R	R	0.5	R	R	R			
Val	R	R	R	R	R	R	R	R	0.40	R	R	R	R	R	R				
Lys	R	R	R	R	R	R	R	R	R	R	R	R	R	R					
His	1.0	R	1.0	R	R	0.40	R	R	R	R	R	R	R						
Phe	R	R	R	R	R	R	R	R	R	R	R	R							
Thr	R	1.2	R	R	R	R	R	R	R	R	R								
Ala	R	R	R	R	R	R	R	1.20	R	0.80									
Ser	R	R	R	R	1.20	R	R	R	R										
Tyr	R	R	R	R	R	R	R	R											
Glu	R	R	R	R	R	R	R												
Asp	0.66	R	R	R	R	R													
Arg	R	R	0.80	R	R														
Gly	R	R	R	R															
Pro	R	R	R																
Cys	R	R																	
Aba	R																		

TABLE 4

Resolution Data on Polyamide Mixed Silica Gel Layers

	Ont	Aba	Cys	Pro	Gly	Arg	Asp	Glu	Tyr	Ser	Ala	Thr	Phe	His	Lys	Val	Met	Try	Ile
Leu	R	R	R	R	R	R	R	R	1.43	R	R	R	R	R	R	0.31	1.33	0.3	R
Ile	R	R	R	R	R	R	R	R	R	R	R	R	0.21	R	R	1.14	R	R	
Try	R	R	R	R	R	R	R	R	1.0	R	R	R	R	R	R	0.50	0.86		
Met	R	R	R	R	R	R	R	R	0.31	R	R	R	R	R	R	1.45			
Val	R	R	R	R	R	R	R	R	R	R	R	R	1.25	R	R				
Lys	0.57	R	R	1.14	R	R	R	R	R	R	R	R	R	R					
His	1.14	R	R	R	R	0.0	R	R	R	R	R	R	R						
Phe	R	R	R	R	R	R	R	R	R	R	R	R							
Thr	R	R	R	R	R	R	R	R	1.14	R	0.86								
Ala	R	0.75	R	R	R	R	R	R	R	R									
Ser	R	1.43	R	0.57	R	R	R	0.57	R										
Tys	R	R	R	R	R	R	R	R											
Glu	R	0.75	R	0.0	R	R	R												
Asp	R	R	R	R	1.43	R													
Arg	1.14	R	R	R	R														
Gly	R	R	R	R															
Pro	R	0.86	R																
Cys	R	R																	
Aba	R																		

action product. It is apparent from tables 3 and 4 that by selection of impregnant a satisfactory separation of amino acids can be achieved. The reported solvent system requires 35 minutes for 10 cm run and thus offers a rapid separation of amino acids on impregnated silica gel layers.

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DETERMINATION OF 4-EPI-MECLOCYCLINE, A TETRACYCLINE ANALOG, IN CREAM FORMULATION BY HPLC AND HPTLC

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ABSTRACT

An unidentified peak has been observed in the chromatograms of thermally stressed meclocycline sulfosalicylate creams by high performance liquid chromatography (HPLC). The unknown has been isolated by preparative HPLC and by high-performance thin layer chromatographic (HPTLC) methods and identified to be a reversible isomer, the 4-epimer, of meclocycline, a tetracycline analog. The identification has been accomplished by matching the ultraviolet, mass, and circular dichroism spectra of the unknown with those of the synthetic epimer. The conversion of meclocycline to the 4-epimer has been achieved in glacial acetic acid and the product purified by HPTLC. The 4-epimer has been found to be easily converted to the original meclocycline in hot ethanol. The data indicate the epimerization not to be the favored reaction, but rather an equilibrium state which favors the intact meclocycline.

INTRODUCTION

The current marketed cream formulation of the antibiotic, meclocycline sulfosalicylate (1), (I, Fig. 1), is recommended for the topical treatment of acne. Meclocycline base is a 7-chloro-6-methylene-5-hydroxy-derivative of tetracycline. Although no degradation products of meclocycline have been previously reported, a small unknown peak has been observed in

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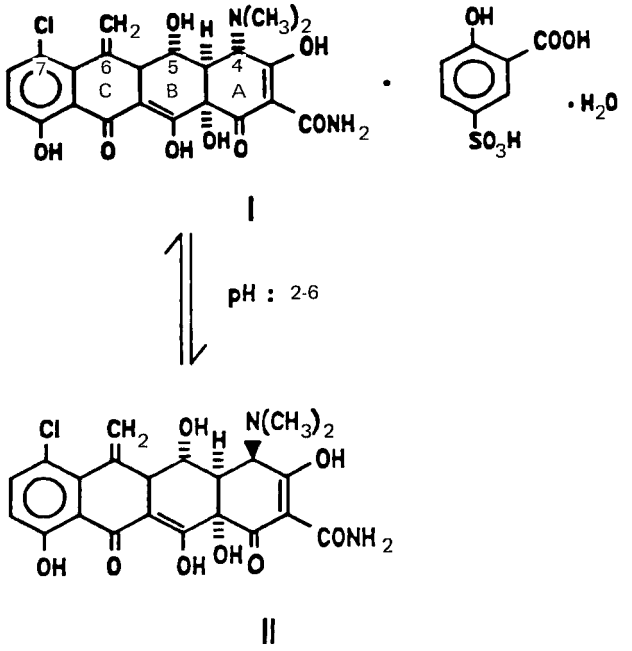

Figure 1: Structures of Meclocycline Sulfosalicylate (I) and its 4-epimer (II).

the chromatograms of some temperature-stressed cream samples by a recently developed high-performance liquid chromatography (HPLC) assay method(2).

In general, the major degradative pathways for tetracyclines have been reported to be epimerization, dehydration, hydrolysis and oxidation(3,4). However, only epimerization and dehydration are most commonly encountered, especially under the relatively mild environmental conditions to which pharmaceutical preparations are normally subjected. The absence of the C-6 hydroxyl group in meclocycline precludes the dehydration and concomitant aromatization to the anhydro-derivative which occurs so readily in most tetracycline analogs. This would suggest epimerization as the only significant degradative route for meclocycline.

Initially, the separation of tetracyclines and their epimers was accomplished by partition chromatography and differential crystallization(5). Several thin layer chromatographic (TLC) methods of separation have also been applied to tetracyclines and their degradation products(6-11). Most of the above techniques were either not applicable to meclocycline and its epimer or were too cumbersome to give reliable and reproducible results. One exception was the TLC system employing edetic acid-treated silica gel plates and a developing solvent consisting of methyl ethyl ketone saturated with McIlvaine's buffer(9). Several HPLC systems developed for tetracyclines and their breakdown products have been extensively reviewed (12). However, it was found that the best HPLC system for meclocycline was based on a reversed-phase Vydac column as reported for a few tetracycline analogs(13). Using this column and a mobile phase of tetrahydrofuran in ammonium edetate(2), an occasional unknown peak was observed in chromatograms during stability analyses of aged meclocycline sulfosalicylate creams.

This paper describes how the suspected epimer was first synthesized and purified by high-performance TLC (HPTLC) from raw material, and then isolated from temperature-stressed cream formulation by HPLC and HPTLC and identified by ultraviolet, mass, nmr and circular dichroism spectroscopy.

EXPERIMENTAL

Materials

Meclocycline sulfosalicylate (I) and methacycline, 6-methylene-5-hydroxytetracycline (Pfizer, Inc., New York, NY) were used in all experiments as received. For the cream samples, a 1% (w/w) formulation of meclocycline sulfosalicylate cream(1) was used. Tetrahydrofuran (Burdick and Jackson Labs Inc., Muskegon, MI) was HPLC grade and (ethylenedinitrilo)-tetraacetic acid (edetic acid) (J.T. Baker Chemical Co.,

Phillipsburg, NJ) and all the other chemicals and solvents were analytical reagent grade.

High Performance Liquid Chromatography

Both analytical and preparative HPLC work were performed on a Waters Associates M224 liquid chromatograph equipped with M6000A pumping system and M440 UV detector operated at 340 nm (Waters Associates, Milford, MA). A syringe-loaded injection valve with either a 10 µl loop (Valco Valve, Valco Instrument Co., Houston, TX) or alternatively a 2 ml loop (Waters Associates Model U6K Universal Injector, Milford, MA) was used. The columns were packed with octadecylsilane material bonded to microparticulate silica gel (10 µm), Vydac 201 TP Reversed Phase (25 cm x 3.2 mm I.D.) for analytical work and Vydac 201 Reversed Phase (25 cm x 10 mm I.D.) for preparative work (The Separations Group, Hesperia, CA). The 0.001 M ammonium edetate needed for the mobile phase was prepared by mixing 0.6 g of edetic acid with 2 ml of methanol and dissolving in 15 ml of concentrated ammonia. Approximately 1800 ml of water were added, and the pH was adjusted to precisely 6.6 with glacial acetic acid. Water was added to make a final volume of two liters. The solution was filtered through a fluoropore membrane filter (0.5 µm) and mixed with tetrahydrofuran in a ratio of 85:15 (v/v) for analytical and 87:13 (v/v) for preparative work as the mobile phase. The flow rates were 0.8 ml/min for analytical and 3.5 ml/min for preparative columns. Columns were periodically purged with 95% ethyl alcohol (Form III) (Sargent-Welch Sci., Co., Skokie, IL) for maximum performance. A 10-mV strip chart recorder was employed at a chart speed of 0.5 cm/min. Samples were dissolved in methanol and diluted with mobile phase before injection.

Thin Layer Chromatography

The TLC system consisted of high-performance silica gel plates, LHP-K, 10 x 10 cm (0.2 mm) with a preadsorbent layer

for sample application (Whatman Inc., Clifton, NJ). The plates were presoaked in 0.1 M disodium edetate solution for 1 hr and activated by drying with a warm air-dryer for a minimum of 1 hr. The preadsorbent area was left above the soaking solution. The developing solvent was prepared by saturating methyl ethyl ketone (99%) with McIlvaine's buffer - a dibasic sodium phosphate (0.2 M) and citric acid (0.1 M) buffer - adjusted to pH 4.7. All the visualizations were done by direct observation under long wavelength UV light.

Spectroscopic Instrumentation

NMR spectra were recorded on a Varian FT 80A 80 MHz instrument in the Fourier transform mode in d_7-dimethylformamide using tetramethylsilane as internal reference at ambient temperature. The chemical shift values were recorded in ppm and were calculated from cps data. UV spectra were recorded with a double beam Perkin Elmer 554 Spectrophotometer in 1-cm quartz cells at ambient temperature using the HPLC mobile phase as solvent for sample preparation and in the reference cell. Mass spectra were obtained by solid probe technique on a Finnegan 1015-D mass spectrometer with a 6100 Data System. The spectrum of each compound was obtained at an ionizing energy of 70eV. Circular dichroism spectra of the samples were determined in a 1-cm path length cell at ambient temperature using a Cary-60 Spectropolarimeter equipped with a Cary-6001 circular dichroism accessory.

Preparation of Mixture of I and 4-Epi-I (II)

A solution of I (200 mg, 0.28 mmoles) in 100 ml of glacial acetic acid was heated on a steam bath for 2 hr. The acid was evaporated using a stream of nitrogen. The residue was reconstituted in 10 ml of methanol and kept refrigerated overnight. This procedure caused some I to precipitate, and a filtrate enriched with the product (II) was achieved. The product content of the filtrate was 30-40% of the total

mixture, based on area % quantitation by HPLC. ^{1}H NMR (DMF-d_7),δ2.56 (S, 6H, $(CH_3)_2N$ for I), 2.69 (S, 6H, $(CH_3)_2N$ for II), 4.03 (S, 1H, H-4 for I), 4.21 (S, 1H, H-4 for II), 4.70-4.85 (m, 4H, methylene H for I and II), 5.60-5.68 (m, 4H, aromatic H for I and II). The mixture was used in the next step of separation by HPLC and HPTLC.

Isolation of 4-Epi-I(II) from Mixture of I and II by HPLC

An aliquot of the filtrate (mixture of I and II in methanol) was mixed with mobile phase (1:1, v/v) and chromatographed on a preparative reversed-phase column using the mobile phase of tetrahydrofuran in edetate buffer at pH 6.6 (87:13 v/v). The peaks corresponding to I and II were collected and reanalyzed on an analytical column for isomer purity. Both collections were shown to be over 99% pure based on area % quantitation by HPLC. The UV spectra of the collected peaks in the mobile phase (2.46×10^{-5} M for I and 4.78×10^{-5} M for II) showed λmax at 238 nm (E_{238} 26000), 280 nm (E_{280} 19500) and 350 nm (E_{350} 14200) for I and 235 nm (E_{235} 12600), 270 nm, shoulder (E_{270} 8200) and 370 nm (E_{370} 6700) for II. The HPLC collections were freeze-dried and the residues were extracted with methanol. However, the methanol extract showed excessive amount of ammonium edetate which interfered in the mass spectral and nmr determinations of the product. Ethanol extracted less of ammonium edetate but some heating was required in the dissolution, and this resulted in almost complete reversal of the product to I.

Isolation of 4-Epi-I(II) from Mixture of I and II by HPTLC

An aliquot of the filtrate (mixture of I and II in methanol) was streaked on the preadsorbent layer of HPTLC plate (pre-treated with disodium edetate). The plate was developed in methyl ethyl ketone saturated with McIlvaine's buffer at pH 4.7. Two readily discernible bands for I and II were developed with R_f values of 0.24 and 0.09, respectively. The small amount

of methacycline impurity (R_f ~ 0.19) coeluted with I. Visualization was by long wavelength UV light. The silica gel bands were scraped off the plates, mixed with methanol, centrifuged and the extracts evaporated to dryness. Although the edetate salt was present again, this was not a major problem, since disodium edetate is not very soluble in methanol (ammonium edetate is soluble in methanol). However, methanol extracted from the silica gel some extra material which interfered with both mass spectral and nmr determinations. When the developing solvent was used instead of methanol for the extraction, the yellow crystalline product from band II was shown to be over 90% pure II by HPLC area % integration. The rest of the material was I, either converted from II during preparation or from tailing of I on the plate. Band I was shown to be over 99% pure I. MS for both I and II: m/z 476 (M^+, isotopic cluster of 476:478 in ratio of approximately 3:1). CD for I: λ^t 260 nm ($[\theta]^t$ -3.8 x 10^4), $\lambda^p max$ 290 nm ($[\theta]^p$ 2.2 x 10^4), λ^t 330 nm ($[\theta]^t$ -1.0 x 10^4). CD for II: λ^p 245 nm ($[\theta]^p$ 0.4 x 10^4), $\lambda^p max$ 280 nm ($[\theta]^p$ 0.8 x 10^4), $\lambda^t max$ 305 nm ($[\theta]^t$ -1.1 x 10^4).

Isolation of 4-Epi-I(II) from Aged Pharmaceutical Preparations

In order to isolate the unknown decomposition product from aged cream preparations of I, a significantly stressed formulation if I (about 4% epimerization) was selected and extracted with methanol. The yellow methanol extract was streaked directly onto the preadsorbent area of the high-performance TLC plate (pretreated with disodium edetate solution). Two sharp bands were observed using methyl ethyl ketone saturated with McIlvaine's buffer at pH 4.7 for development (R_f ~0.20 for I and R_f ~0.09 for II). The small amount of methacycline impurity (R_f ~0.19) coeluted with I. The bands corresponding to compounds I and II were scraped off the plate, extracted

with developing solvent, centrifuged and the extracts evaporated to dryness. By using HPLC area % integration, the band of I was shown to be over 99% pure and the band of II was 73-88% pure. The rest of the band II was again I, either converted from II during preparation or from residual tailing of I on the same plate. The MS and CD spectra of band II exhibited peaks similar to those observed in the MS and CD spectra of synthetic II, isolated from mixture of I and II.

RESULTS AND DISCUSSION

The amount of degradation of I in cream formulations, shown by HPLC, was quite minimal as might be expected for solid suspensions. Stability data for representative aged samples showed less than 1% of the product in HPLC chromatograms even after a storage of a year and a half at room temperature. Typical chromatograms for a standard I and a cream sample of I with the suspected 4-epimer (II) present are shown in Fig. 2. The system gave a baseline separation for I and II, peaks B and C, respectively, and for methacycline, peak A, an impurity always present in raw material but never present at more than 3% of the total weight. The retention times were about 5 minutes for methacycline, 7 minutes for II and 8.5 minutes for I. The retention times for I and impurities could be easily shifted by varying the relative amount of tetrahydrofuran in the mobile phase. It was preferred, however, to keep the retention time of I in the range of 5.5 and 8.5 minutes.

The 4-epimer of I, an inactive and nontoxic drug substance (II), was being considered the most likely candidate for the unknown peak because of the following facts. The pH of the cream preparations of I is about 3, which is favorable, based on the chemistry of tetracyclines, to the formation of 4-epimer(5). Also, the 4-epimers of tetracyclines have been shown to elute before the parent compounds in reverse phase

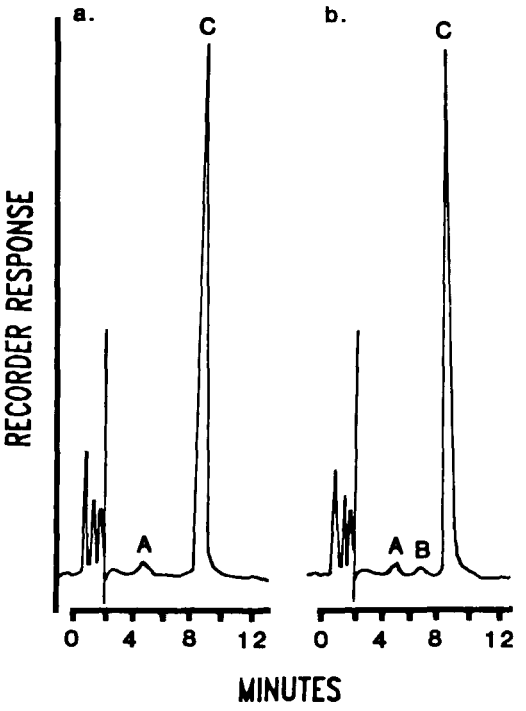

Figure 2: Chromatograms of meclocycline sulfosalicylate (I) standard (a) and a cream sample of I, aged for 30 days at 50° (b). Peak A: methacycline, Peak B: 4-epi-I, Peak C: I. Conditions: mobile phase, 0.001 M ammonium edetate, pH 6.6-tetrahydrofuran (85:15, v/v), pH adjusted with glacial acetic acid; column, Vydac 201 TP Reversed Phase, 25 cm x 3.2 mm I.D.; flow rate, 0.8 ml/min; detector, UV at 340 nm.

HPLC systems (13). However, no such epimer of I was available for testing, and no previous methods for its preparation were known either. Therefore, a synthetic scheme for preparation of 4-epi-I had to be devised. The synthesis was based on the fact that saturated solutions of tetracyclines in glacial acetic acid have been shown to equilibrate within 24 hours(5). The most favorable conditions for the C-4 epimerization of I were

found to be a saturated solution of I in hot glacial acetic acid heated on a steam bath for 2 hr. After this time, equilibrium was reached with the conversion of 20-30% of I. Higher temperatures and longer heating times did not produce more of the suspected epimer. However, a filtrate containing 30-40% of II could be achieved by partial precipitation of I. A typical chromatogram of such an equilibrium mixture is shown in Fig. 3a. A comparison of the NMR spectra in d_7-dimethylformamide for I and the equilibrium mixture showed the mixture to have new peaks with typical chemical shifts to lower field of the C-4 proton (from 4.03 ppm to 4.21 ppm) and the dimethyl protons of the C-4 amino group (from 2.56 ppm to 2.69 ppm) relative to I, indicative of an epimer. Similar trends in the chemical shifts have been found in NMR spectra of tetracycline and its 4-epimer(14,15).

Since an HPLC method effecting separation and quantitation of I and its breakdown product existed, and the respective preparative column was available, preparative HPLC separation of the product from I was attempted. This method gave at least 99% pure product relative to I, based on area % quantitation by HPLC (Fig. 3b), but further clean-up from the mobile phase proved difficult. One difficulty encountered was the fact that depending on reaction conditions, the product could be converted back to I. When the purified product was heated in ethanol on steam bath for half an hour, almost complete reversal (~98%) back to I was observed by HPLC. However, good UV spectra of both the product and I were recorded directly on the HPLC collections (Fig. 4). They were very similar and showed typical absorbance patterns reported for 4-epimer and the parent compound of other tetracyclines(5). The absence of absorbance in the 400-500 nm region was strong evidence, in addition to the structural features, that the product was not an anhydro-derivative of I, which should exhibit strong absorbance in that

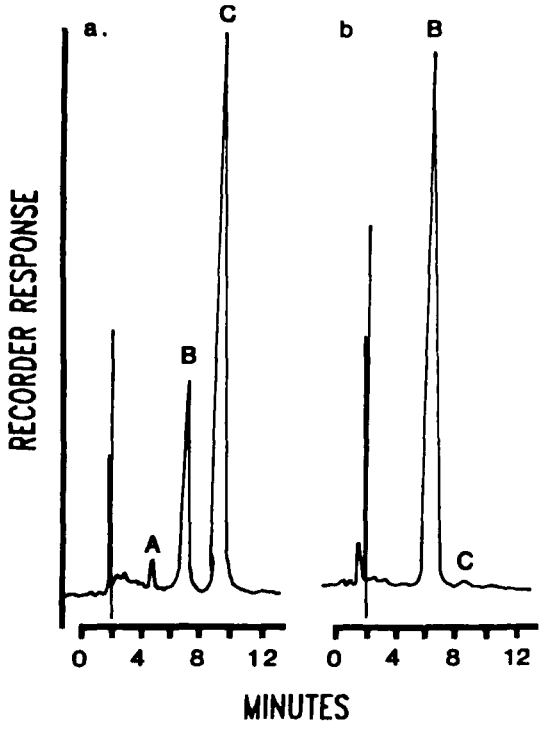

Figure 3: Chromatograms of mixture produced by heating I in glacial acetic acid for 2 hr on steam bath (a) and 4-epi-I after preparative HPLC separation from the above mixture, (b) Peak Identification and chromatographic conditions as in Fig. 2.

region(5). Also, the 4-epimers of tetracycline analogs, in general, have been shown to have less UV absorbance in the 250-300 nm region than the parent compound(5). This is to be expected since C-4 epimerization changes the conformation of the ß-dicarbonyl system of ring A which contributes to the strong absorption at around 260 nm.

Several TLC methods for tetracyclines and their breakdown products reported in the literatuae were tried, but only one was applicable to the resolution of I and its 4-epimer (9). This

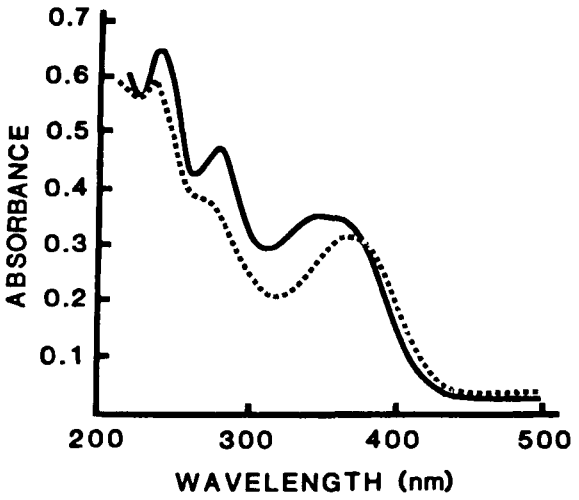

Figure 4: Ultraviolet spectra of I (–) and 4-epi-I (--) in the HPLC mobile phase (0.001 M ammonium edetate, pH 6.6-tetra hydrofuran, 87:13, v/v pH adjusted with glacial acetic acid) collected by preparative HPLC. Concentrations were 16.6 µg/ml for I and 15.1 µg/ml for 4-epi-I.

method prescribed silica gel plates soaked in disodium edetate (0.1 M) for 1 hr and activated by drying overnight at room temperature. To shorten the time for analysis, the activation was modified to drying the plate with a warm-air dryer for a minimum of 1 hr and to using high-performance TLC plates with a preadsorbent application layer instead of standard silica gel plates. Development of the plate in methyl ethyl ketone saturated with McIlvaine's buffer at pH 4.7 gave a very efficient resolution of I (R_f ~ 0.24) and II (R_f ~ 0.09).

It has been documented that the circular dichroism (CD) curves of tetracycline and 4-epitetracycline show a large difference in molar ellipticity between the two epimers at 262 nm(16). The CD-data for I and II demonstrated the same (Fig. 5). The large negative molar ellipticity of I, due to

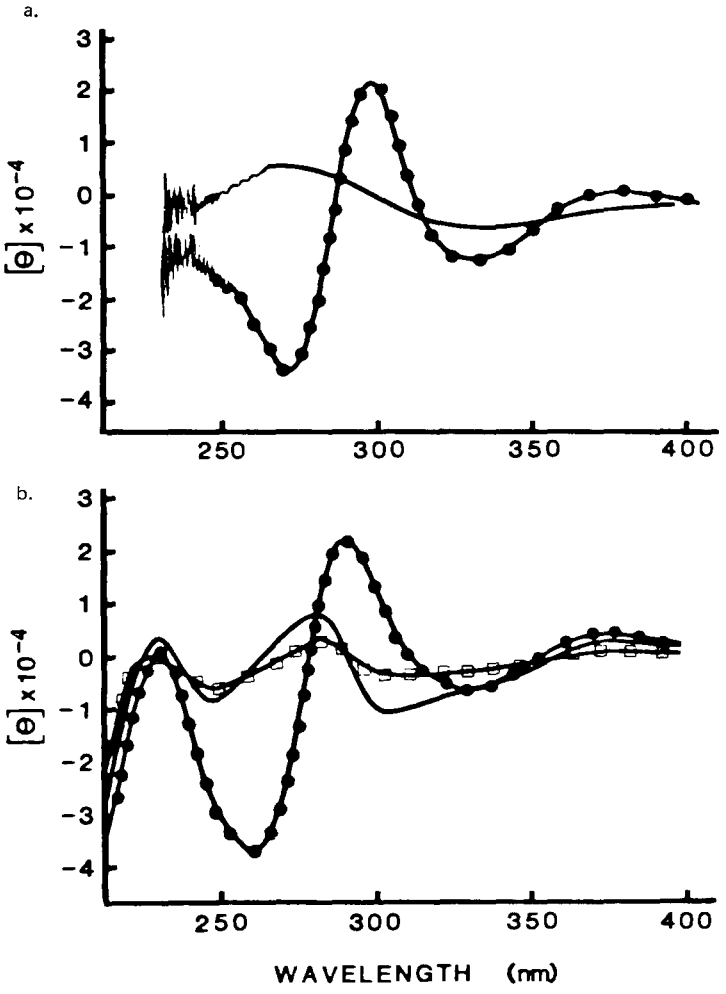

Figure 5: Molar ellipticities (Θ) of I (●) and synthetic 4-epi-I (-) in the HPLC mobile phase (the same as in Fig. 4) (a) and I (●), synthetic 4-epi-I (-) and the 4-epi-I isolated from aged MCSS cream (□) in methanolic-hydrochloric acid (0.01 N) (b) as function of wavelength. Concentrations were 28 µg/ml for I and for 4-epi-I.

the A-ring band at around 260 nm, was almost nonexistent in both the synthetic 4-epimer and in the 4-epimer isolated from the formulation of I.

The overall data conclusively point to the presence of 4-epimer of I as the major degradation product in the pharmaceutical formulations of I under the stress conditions. In addition, the data suggest that the epimerization at C-4 is not the favored reaction, but rather an equilibrium state which favors the intact meclocycline.

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INSTRUMENTAL HPTLC OF AFLATOXINS: FLUORESCENCE ENHANCEMENT BY CORN FREE FATTY ACIDS

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ABSTRACT

Greater than 100% recoveries using instrumental HPTLC were observed for aflatoxin (AFT) analysis in spiked corn samples. I_2 detection of spots over-laying AFT B_1 and B_2 were identified by GLC as C_{16}-C_{18} free fatty acids (FFA). These FFA were found to enchance the fluorescence of AFT B_1 from 13.7% to 35.7% greater than controls resulting in >100% recoveries. The inclusion of glacial acetic acid in the TLC mobile phase resulted in an increased mobility of the FFA which eliminated the positive interference on AFT fluorescence. Recoveries using the modified developing solvent then gave values in acceptable ranges.

INTRODUCTION

During the development of a method to analyze for aflatoxins in small samples of corn and corn dusts (1.0 g-0.01 g), a positive error in B_1 and B_2 values was consistently observed during aflatoxin (AFT) spiking and recovery studies using instrumental HPTLC with fluorescence detection.

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An actual example would consist of three 1.0 g samples of ground corn spiked with 50 ppb AFT B_1 and 10 ppb AFT B_2. After appropriate extraction and clean-up according to Zennie et al. (1), the AFT 's were quantified on HPTLC plates. Recovery would range from 56 to 83 ppb for B_1 and 12 and 14 ppb for B_2. These results show a 112 to 166% recovery for B_1 and a 120 to 140% recovery for B_2.

The most obvious cause for the increased fluorescence enhancement would be corn naturally contaminated with AFT; this however was ruled out by analyzing the unspiked corn and finding no discernable amounts of AFT 's. Inspection of the thin-layer plate under long-wave UV light revealed plainly visible AFT spots from both AFT standards and AFT spiked samples with no obvious overlapping or interferring fluorescent background material at or near the AFT 's. However, when the AFT areas were marked with pencil under UV light and then the plate placed in an I_2 tank, large areas which directly overlaid the AFT spots became visible. These were large tear shaped spots which had R_f values slightly ahead of AFT B_1 and B_2, but which tailed over the AFT B_1 and B_2 areas. Removal of this interference for accurate AFT determination is warranted.

EXPERIMENTAL

Column chromatography and preparatory TLC of corn extracts free of AFT resulted in the isolation of the interferring spot. The IR spectrum of the isolated material (neat on NaCl plates) gave absorptions at 1720 cm^{-1} and a large broad band between 3400 cm^{-1}

and 2500 cm^{-1} which are suggestive of a carboxylic acid. Additional strong absorptions at 2920 cm^{-1} and 2850 cm^{-1} and another at 720 cm^{-1} denoted a long chain fatty acid. A stretching vibration at 3010 cm^{-1} revealed unsaturation. The material was esterified using BF_3 in methanol and run on GLC and positively identified as a typical mixture of long chain fatty acids seen in corn, i.e., palmitic, stearic, oleic, linoleic, and linolenic acid.

All HPTLC experiments were performed using 10 cm x 10 cm. HPTLC plates (E. Merck) developed in an unlined tank. The developing solvents for all experiments were either solvent A (8% acetone in chloroform) or solvent B (chloroform, acetone, glacial acetic acid [92+8+1]). The fluorescence enhancement studies and the free fatty acid (FFA) R_f determinations, were done by over-spotting the AFT on various concentrations of FFA. All AFT measurements were done on a Camag variable wave-length densitometer in fluorescence mode with a Hewlett Packard 3390A reporting integrator. Light source was a mercury lamp with excitation at 365 nm using a 400 nm cut off filter. Scanning slit width was 5 mm x 0.3 mm with a 0.5 mm/sec scan speed.

Samples of ground corn (1.0 g) were extracted with 25 ml $CHCL_3$, 1.0 g celite and 1 ml H_2O in a 125 ml Erlenmeyer flask with 30 min of shaking. This method of extraction results in approximately 10 mg of extractables for 1.0 g corn.

For the AFT recovery experiment an extract from 1.0 g of AFT-free corn was cleaned-up by flash column chromatography (FCC) (3) using diethyl ether. The fraction which normally contains AFT is eluted off with 20% acetone in chloroform and brought to dryness

under N_2. To this residue 200 µl of benzene-acetonitrile (98+2) is added and then 5 µl of this solution is spotted on a HPTLC plate and then over-spotted with various concentrations of AFT B_1 and B_2. The plate was then developed with either solvent A or solvent B.

RESULTS AND DISCUSSION

Corn oil contains 1.5 to 4.0% FFA (4). Assuming that none of the FFA are separated from the AFT during the clean-up procedure, then the final dilution used for spotting (here in our case 200 µl for 1.0 g samples) would contain 0.75-2.0 µg/µl of FFA. Thus a 5 µl spot could contain 3.75-10.0 µg of FFA; a concentration well within the range of interference.

Experiments with the isolated free fatty acid mixture showed that the R_f values are strongly concentration dependent (Table 1). The higher R_f's are produced by the greater concentration of the FFA's presumably because the material occurring in the front of the spot deactivates the silica gel as migration proceeds and the following material encounters a less polar absorbant (2).

Attempts to exclude completely the FFA during the clean up procedure with FCC by mobile phase modification were unsuccessful. However it was found that a decrease in the amount of FFA was observed in the AFT fraction if acetone-chloroform mixtures (i.e. 20% A/C) were used to remove the AFT from the column. The CB clean-up procedure (5) which uses chloroform-methanol (97+3) to remove AFT from the clean-up column had significantly higher FFA in the AFT fraction than the FCC clean-up procedure as revealed by I_2.

TABLE 1

R_f Values of Corn Free Fatty Acids

of Differing Concentrations

Concentration of FFA (μg/spot)	R_f value[a]	R_f value[b]
1.4	0.35	0.49
3.7	0.35	0.49
7.4	0.39	0.50
22.0	0.44	0.54
36.6	0.48	0.56
73.3	0.54	0.60
220.0	0.63	0.68
366.5	0.66	0.69
1.5 ng - AFT B_1	0.35	0.33
0.3 ng - AFT B_2	0.26	0.28

[a] Mobile phase: 8% acetone in chloroform.

[b] Mobile phase: 8% acetone + 1% glacial acetic acid in chloroform.

However, the FCC clean-up procedure still gave recoveries of AFT greater than 100%. Using ammonia, diethylamine or other basic additiives to both the column mobile phase and the TLC eluent to retard migration of the FFA were rejected because of the possibility of AFT degradation (6).

The problem was solved by the addition of 1% glacial acetic acid (GAA) to the TLC solvent. Mobile phase B caused the FFA to migrate significantly ahead of the AFT B_1 and B_2 with no overlapping and streaking with R_f values averaging 0.24 and 0.29 greater than AFT B_1 and B_2 respectively (Table 1). FFA in concentration ranges usually seen in AFT analysis gave an R_f

difference of 0.16 and 0.21 greater for AFT B_1 and B_2. The GAA addition also significantly reduced the FFA R_f differences due to concentration. Presumably the GAA causes complete protonation of the carboxylic acid portion of the FFA and eliminates any partial ionization which would contribute to the streaking. It also would decrease the polarity of the FFA thus giving them greater mobility through the silica gel and consequently a higher R_f.

Fluorescence enchancement by the FFA was also concentration dependent (Table 2). Even though higher concentrations of the FFA have a greater R_f difference compared to AFT-B_1 and B_2 as did the lower concentrations of FFA, they significantly enhanced the fluorescence to a greater degree than the lower concentrations of FFA. Apparently the tailing of the FFA in the higher concentrations was sufficient to over lay AFT B_1 and B_2. Using various concentrations of FFA's and over spotting with 1.5 ng of AFT B_1 and 0.3 ng B_2 mixture, the optimum concentration range of 1.33 µg to 7.33 µg of FFA was found to give the closest R_f's to AFT B_1 and B_2 using mobile phase A as HPTLC developing solvent (Table 1). The fluorescent enhancement produced at these concentrations averaged about 13.7% for AFT B_1 and 16.1% for AFT B_2. However larger concentrations of the FFA's (36.5 to 366.5 µg) produced an average of 35.7% enhancement for AFT B_1 and 28.6% for B_2.

Recovery results using actual corn extracts with a FCC clean-up procedure using a HPTLC solvent with and without GAA are depicted in Table 3. The average recovery without the GAA addition for B_1 and B_2 are 126.5% and 120.3%. With GAA addition to the mobile phase the average recovery for AFT B_1 is 98.75% and for AFT

TABLE 2

% Enhancement of Fluorescence* of 1.5 ng AFT B_1 and 0.3 ng AFT B_2 by Corn FFA

FFA Concentration µg/spot	AFT - B_1	AFT - B_2
1.4	10%	14.3%
3.7	15%	20.4
7.4	16.2%	13.6
22.0	30.2%	22.9
36.6	33.9%	25.9
73.3	29.9%	24.0
220.0	39.5%	26.6
366.5	39.6	37.9

*Measured in triplicate using HPTLC plates.

TABLE 3

Recoveries* of AFT B_1 and B_2 in Spiked 1.0 g Corn Extracts

Spiked AFT B_1, B_2 in ppb	Recovered[a] ppb B_1, B_2	Recovered[b] ppb B_1, B_2
12, 2.4	15.6, 2.8 (130, 117)	11.7, 2.3 (97.5, 95.8)
20, 4.0	24.5, 4.3 (122.5, 107.5)	19.5, 3.9 (97.5, 97.5)
60, 12.0	74.9, 13.4 (125, 111.7)	62.4, 12.2 (104, 101.7)
100, 20.0	129.0, 29.4 (129, 147)	88.4, 13.7 (88.4, 68.5)
200, 40.0	259.2, 46.2 (129.6, 115.5)	213, 34.7 (106.5, 86.7)
500, 100	612.2, 123.0 (122.4, 123)	493, 99.3 (98.6, 99.3)

*Measured in triplicate and averaged. % Recoveries in parenthesis.

[a]Mobile phase: 8% acetone in chloroform.

[b]Mobile phase: Chloroform, acetone, glacial acetic acid (92+8+1).

B_2 91.6%. If the unexplained low value for the 20 ppb B_2 is dropped then the recovery averages 96.2.

For the AFT range used (12-500 ppb for B_1 and 2.4-100 ppb for B_2) an average difference of 27.75% for B_1 and 28.7% for B_2 in recovery was observed for the 2 different mobile phases. Obviously mobile phase B gave recoveries much closer to the actual values.

The prevention of the FFA interference in HPTLC analysis becomes even more significant for mold infected corn and older stored corn (1 yr old). This is because an increased level of FFA is observed due to the hydrolysis of corn triglycerides by fungi, bacteria, heat, seed damage, and moisture (3,7). In particular, high levels of FFA have been attributed to Aspergillus flavus contamination of cotton seed, coconut oil, and chocolate seed (8,9,10). These results should be interpreted only for the HPTLC plates and the developing solvent used. However an inspection of any developed TLC chromatograms used for corn AFT analysis with over-lapping I_2 positive spots could reveal FFA not removed during the clean-up procedure.

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ANALYSIS OF PCB'S BY THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

A screening thin layer chromatography (TLC) technique has been developed that can be done in the field. Quantitative analysis by TLC densitometry can be done in a laboratory (or field van). A densitometer is needed for quantitative work by TLC. An Apple computer may be added to use as a computational device. Semi-quantitative results may be obtained by visual comparisons. Confirmations may be made by silver halide visualization on TLC. Dehalogenation with sodium biphenyl, chlorination with antimony pentachloride, or mass spectral confirmation may be used. Quantitative analysis is possible of the derivatized positive samples.

INTRODUCTION

EPA's concern has prompted a rash of analyses for PCB's. PCB's may be analyzed by sophisticated and expensive instrumentation or by simple TLC analysis. PCB's (polychlorinated biphenyls) are very commonly encountered organic chemicals; the chlorine content varies from <20% to 70% or more. Commercial trade

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names are Arochlor® or Pyranol®. They have been used as transformer oils, capacitor dielectric fluids, paint vehicles, pesticide extenders, and plasticizers for resins. They end up in solid foods, milk and chewing gum by indirect and direct routes.

Gas chromatographic (GC) analyses have been used to analyze PCB's since 1956.[1] Methods have been improved[2-10] as they were used and applied to new matrices.[11-13] Reviews of methods and instrumentation[14,15] have been published. EPA funded a study on method standardization.[16]

Levine[17] et al, compared cleanup methods and found that sulfuric acid extraction was the optimum cleanup for transformer oils. In this work acid cleanup was compared to SEP PAK® C^{18} cleanup and GLC compared with TLC quantitation.

EXPERIMENTAL REAGENTS AND APPARATUS

Thin layer plates - Whatman C^{18} reverse phase with fluorescent indicator (Excitation 254 nm), Whatman, Inc., Clifton, NJ; thin layer plates - Merck silica normal phase with fluorescent indicator (Excitation 254 nm), Brinkmann Instruments, Des Plaines, Illinois; 8% $AgNO_3$ in silica gel plates, Analtech Inc., Wilmington, Delaware; separatory funnels (250

ml); calibrated tubes, 1-15 ml with Teflon® stopcocks, Kimball Glass Company, Vineland, New Jersey; disposable pipettes, Fisher Scientific Company, Pittsburgh, Pennsylvania; PCB standards, EPA Research Triangle North Carolina; microliter syringes, Hamilton, Las Vegas, Nevada; concentrator tubes, Research Specialty, Los Angeles, California; dimethylformamide, acetonitrile, pet ether,and methanol were redistilled in glass; Milli Q water, Millipore Systems, Downers Grove, Illinois; sodium chloride reagent, Fisher Scientific Company, Pittsburgh, Pennsylvania, short wave length UV light (254 mm), UV Products, San Gabriel, California; $AgNO_3$ spray, $AgNO_3$, Fisher Scientific Company; sprayer, Kontes Glass Company, Vineland, New Jersey; TLC 800 Scanner, Kontes Glass Company; gas chromatograph with Ni63 detector, model 270, Packard Instrument Company, Downer's Grove, Illinois; recorders, Linear Instrument - P. J. Cobert Associates, St. Louis, Missouri. GLC columns used were 50% 3% OV-17 and 50% 3% OV-1; on Gas Chrom Q 100-120 mesh, six foot glass columns, 3 mm I.D. were used. Column temperature used was 200°C or 225°C; SEP PAK® (C^{18}) cartridges, Waters Associates, Milford, Massachusetts.

METHODS

Dimethyl formamide (DMF) was compared with acetonitrile (ACN) as an extraction solvent in terms of the amount of PCB recovered and cleanliness of sample extracts. A modification of the Mills[18] method for pesticide analysis was used. One half ml of oil, 50 ml of pet ether, 50 ml of DMF or ACN were mixed and pet ether separated and discarded. Fifty milliliter of salt water was added to the residual solvent used in the extraction and the PCB's partitioned into pet ether. Concentration was done under nitrogen on a steam bath at approximately 40°C. The samples were analyzed by thin layer chromatography (TLC) on normal and reverse phase, using 1% acetone in 99% Heptane and 95/5/1 + 1/2% NaCl, methanol water and ammonium hydroxide, respectively as developing solvents. TLC on normal phase TLC plates with $AgNO_3$ in the solid phase allows direct visualization on the plate by short wave UV irradiation.

Gas chromatography was done by injecting aliquots of concentrated extracts on the mixed phase columns with the Packard Ni^{63} electron capture detector. The column temperature was 225°C, injector, 250°C and detector, 280°C. The columns usually lasted 6 months

or more. Difficult samples required lowering column temperatures to 200°C. The analysis schemes are shown in figure two.

Antimony Pentachloride Chlorination[19]

An extract which was cleaned up by extraction, partition, (the cleanup by SEP PAK® is shown in figure one) or sulfuric acid reaction, is placed in a Teflon® lined screw-capped test tube (Bakelite plastic cap). 100 µl $SbCl_5$ is added carefully to test tube (10 ml) and the cap screwed on. Teflon tape is wrapped around the tube to seal it. The tube is heated to 150°C for two hours. The contents are removed to concentrated HCL in a separatory funnel. The acid layer when clear is extracted two times with Benzene. The Benzene is washed with $NaHCO_3$ to remove excess HCL and $SbOCl_3$. The benzene extract was concentrated and analyzed by TLC or GC as above. Temperatures of 240°C - 250°C were used to increase the analysis rate of derivatized samples.

Comparison of Sulfuric Acid[17] and SEP Pak® Cleanups

Concentrated acid was used to contact the extracts and removed by aqueous washing and dehydration with Na_2SO_4. SEP PAK® C^{18} columns were washed with methanol

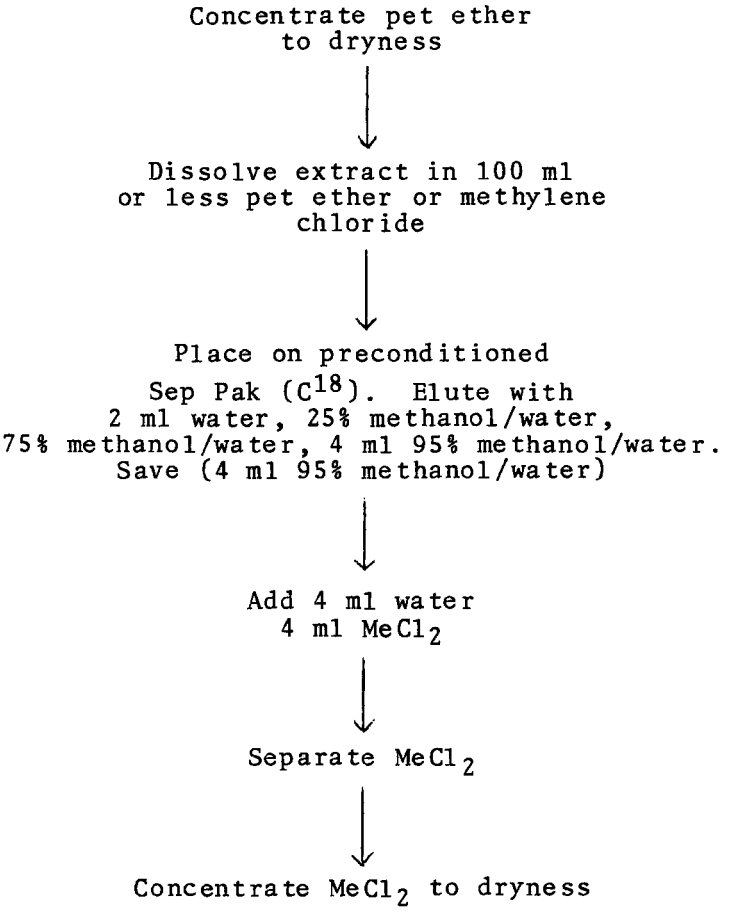

FIGURE 1
Sep Pak® Cleanup

(MeOH) and water and vacuum dried. Extracts after concentration and redissolving were placed on the SEP PAK® column in 100 µl of methylene chloride or pet ether and eluted with 2 ml 25% MeOH/HOH, 2 ml 50% MeOH/HOH water, 4 ml MeOH/HOH (95-5). The last eluate

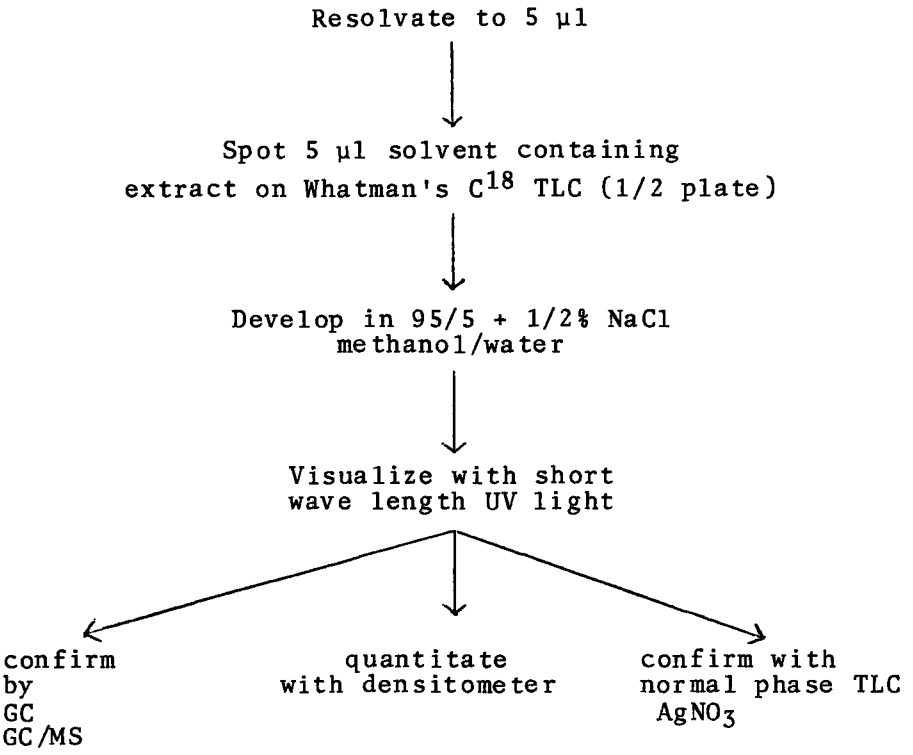

FIGURE 2
PCB Analysis

was mixed with 4 ml water, 4 ml $MeCl_2$, shaken and the $MeCl_2$ layer removed. The extracts desolvated, redissolved in isooctane and analyzed by TLC or GLC. Quantitation by GC was done by triangulation of the area of peaks matching the Arochlor analyte closest in pattern to the analyte. TLC quantitation was done by area measurement of the TLC bands under UV irradiation with a Kontes 800 scanner. Concentrations of 5 samples

TABLE I

Thin Layer Chromatography

Rf's, PCB's and PBB

Compound	Normal Phase RF	Reverse Phase RF
Arochlor 1248	.6	.7
Arochlor 1254	.5	.6
Arochlor 1260	.4	.5
PolyBromo Biphenyl	.3	.4
	Heptane Solvent	95% Ethanol Solvent

Sensitivity 0.1 µg Fluorescent Quenching

Sensitivity 0.5 µg $AgNO_3$ conversion

TABLE II

Analysis PCB's Thin Layer Chromatography

Densitometry with Camag Densitometer

	Band Area		Slope factor Area/ng
1 µg	70 x 10	700	600
2 µg	126 x 10	1260	630
3 µg	170 x 10	1700	560
4 µg	210 x 10	2100	520

Sensitivity less than 10 ng PCB

Repeatability ± 5%

TABLE III

Arochlor Concentrations as Arochlor 1260

TLC Sulfuric Acid Cleanup	TLC Sep Pak Cleanup	GLC Sep Pak Cleanup
11	10	15
4	4	6
41	40	40
54	55	53
17	18	14

of contaminated transformer oil were compared using TLC, GLC analysis with sulfuric acid and SEP PAK C^{18} cleanups.

RESULTS

The Rf's of PCB's are shown in Table I. The densitometric results obtained with the Camag densitometer are shown in Table II. Comparative analysis of transformer oils are shown in Table III.

CONCLUSION

TLC may be used to rapidly and inexpensively screen samples of transformer oils for PCB's. Quantitative analysis may be done by TLC densitometry or GC electron capture analysis. Cleanup steps using SEP PAK® C^{18} or sulfuric acid cleanups are very beneficial for improving the quality of the extracts for chemistry rapid analysis. Confirmation may be made by silver nitrate reaction on normal phase silica TLC, GLC, GC/MS. Antimony pentachloride derivatization converts PCB's to decachlorobiphenyl making confirmation and analysis more simple--one compound to analyze--and more sensitive.

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REGULAR CONTRIBUTED PAPERS

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(7), 1405-1424 (1984)

GENERAL APPROACH FOR THE ESTIMATION OF OCTANOL/WATER PARTITION COEFFICIENT BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

General approach for the relation of reversed-phase high-performance liquid chromatographic retention behaviour to the hydrophobic properties of drugs was developed. Hydrophobicity of compounds was characterized by the logarithm of 1-octanol/water partition coefficient (log $P_{o/w}$). Reversed-phase retention times of 26 model compounds (log $P_{o/w}$ values of them ranged from -1.22 to 3.84) were measured using 3 to 5 different mixture of acetonitrile and water as eluent and the logarithm of the capacity ratios (log k') was calculated.

The linear relationship between the log k' values and the percentage of the acetonitrile in the eluent was tested for each molecule. The slope and the intercept (log k'_o) of these straight lines were

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calculated and introduced to the following regression equation:

$$\log P_{o/w} = a \text{ 'slope'} + b \log k'_o + c$$

a, b and c were constants obtained by least squares method. Correlation coefficient of the equation was 0.95. The quotient of the values of a and b gave the percentage of the acetonitrile in the eluent (48%) by which the chromatographic partition system became the best model of the 1-octanol/water partition system. The proposed method provides the possibility of estimating log $P_{o/w}$ values for compounds which can not be eluted by one isocratic eluent composition.

INTRODUCTION

Hydrophobic properties of new biologically active agents characterized by the logarithm of 1-octanol/water partition coefficients (log $P_{o/w}$) are widely used in quantitative structure-activity relationship (QSAR) investigations [1,2]. The traditional shake-flask method for determining log $P_{o/w}$ has many disadvantages, such as the relatively large amount of material required, changes in partition process due to impurities of solute, the tedious and time consuming measurements of the concentration of compounds in both phases. Therefore, this method is often replaced by different chromatographic procedures, for example by thin-

layer chromatography (TLC) [3,4], reversed-phase high-performance liquid chromatography (RP-HPLC) [5,6,7,8,9,10], and by gas-liquid chromatography (GLC) [11,12].

All attempts to use RP-HPLC for determining $\log P_{o/w}$ are based on eq. 1.

$$\log k' = \log P + \log V_s/V_m \qquad (1)$$

where k' is the capacity ratio calculated from the retention time (t_R) and dead time (t_o) according to $(t_R - t_o)/t_o$; P is the partition coefficient of the compound in the given chromatographic partition system; V_s/V_m is the ratio of the mobile and the stationary phase volumes.

If a Collander [13] type relationship (eq. 2) holds between the log P values measured in two different partition systems, eq. 3 can be set up:

$$\log P_{2/w} = a_1 \log P_{3/w} + b_1 \qquad (2)$$

$$\log P_{o/w} = a_2 \log k' + b_2 \qquad (3)$$

Subscript w indicates water and subscript 2 and 3 refer to two different non-aqueous solvents in the partition system. a_1, b_1, a_2 and b_2 are constants obtained by the least squares method. Leo [14] showed the limitations of the validity of eq. 2, namely, that the plot $\log P_{2/w}$ vs. $\log P_{3/w}$ is linear only if solvents 2 and 3 similar in character, or if the compounds examined are structurally related.

The validity of eq. 3 was proved only when one of the above requirements is met. In this point of view papers dealing with this subject can be devided into two groups. In the first approach structurally closely related compounds were investigated in a reversed-phase chromatographic system using methanol, acetone and acetonitrile as organic modifier in the eluent. For example McCall [5], Carlson et al. [7], Guerra et al. [8] have already published good correlations between log $P_{o/w}$ and log k' for phenols, anilines, benzene and nitroimidazole derivatives. In these cases the similarity of the chromatographic partition system to the 1-octanol/water partition system is not necessary for obtaining good correlation.

In the second approach the chromatographic partition system is the same as 1-octanol/water partition system, namely, 1-octanol coated column and 1-octanol saturated water as eluent were used for log k' determination. In such cases good correlation between log $P_{o/w}$ and log k' values for structurally different compounds was published by Unger et al. [8] Mirrlees et al. [9]. This latter approach seems to be more general from the theoretical point of view, although it has practical disadvantages. For example, chromatographic conditions cannot be optimized for a

wide series of compounds and the reproduction of the same 1-octanol-coated column seems to be difficult.

In this work the chance of the good correlation between RP-HPLC retention data and the log $P_{o/w}$ values of structurally heterogeneous compound set using conventional RP-18 column and acetonitrile - water mixture as eluent was investigated. The hydrophobicity range of the model compounds was wide (log $P_{o/w}$ values ranged from -1.22 to 3.87). As they cannot be eluted using the same eluent composition microcomputer analysis of the HPLC retention data of drugs as a function of the eluent composition has been also carried out.

THEORY

Horváth et al. [15] described the retention processes of an unionized solute on an apolar stationary phase adapting the solvophobic theory. According to their model the interaction between the solute and the stationary phase can be regarded as a reversible association of the solute molecules (S) with the hydrophobic ligands (L) at the surface. The strength of the interaction between S and L is also influenced by the property of the eluent which forces the solute to associate.

The experimentally found linear relationship between the log k' and the percentage of the organic phase in the eluent (OP%) can be described by eq. 4.

$$\log k' = a_3 \, OP\% + b_3 \qquad (4)$$

where b_3 can be considered as $\log k'_0$, the logarithm of the capacity ratio at zero percentage of the organic phase in the eluent. The physical meaning of a_3 (the 'slope' of the straight line) is the change of log k' caused by 1% addition of the organic phase to the eluent. The plot of eq. 4 is linear only in a given range of OP% values [16], therefore this relationship should be experimentally tested.

Eq. 4 provides the possibility of calculating the log k' values belonging to optional percentage of the organic phase in the eluent. In this way by the help of eq. 4 the applicability of eq. 5 can be extended to those cases when log k' values of the compounds cannot be measured at the same chromatographic circumstances. Combining eq. 3 and eq. 4 together one obtains eq. 5.

$$\log P_{o/w} = a_2 \text{ 'slope' } OP\% + a_2 \log k'_0 + b_2 \qquad (5)$$

Testing the validity of eq. 5 OP% values should be considered constant for all of the investigated compounds. One cannot know in advance that value of OP%. In order to estimate the best eluent composition

by which 1-octanol/water partition system can be chromatographically modelled eq. 5 was set up as a regression equation with two independent variable (eq. 6).

$$\log P_{o/w} = a_4 \text{ 'slope'} + b_4 \log k'_o + c_4 \quad (6)$$

The regression coefficients a_4, b_4 and c_4 can be calculated using the least squares method. As $a_4=a_2$OP% and $b_4=a_2$, the quotient of the a_4 and b_4 will show the optimum eluent composition by which the best correlation can be found between log $P_{o/w}$ and RP-HPLC retention data.

In the case of closely related compounds the 'slope' values can be considered constant [17], which means that the straight lines described by eq. 4 are parallel, thus log k' values measured at any concentration of the organic phase will be directly proportional to log $P_{o/w}$ values of compounds. We should be careful, however, when using log k' values measured at a certain OP% value without knowing the 'slope' values of the compounds, because if the 'slope' values are not the same for all compounds tested or does not show high correlation to log k'_o values different ranks of log k' values can be obtained at different OP% values. In this case one can not know the chromatographic conditions at which the rank of log k'

values is the same as the rank of log $P_{o/w}$ values of the compounds. Therefore eq. 6 can be suggested to examine relationship of the log $P_{o/w}$ and retention data for different types of compounds.

Correlation coefficient and the standard error of the estimate of eq. 6 will show the theoretical limit of the generalization of log $P_{o/w}$ estimation using HPLC retention data measured on RP-18 column and acetonitrile-water mixture as eluent. One can obtain the optimum composition of methanol-water, acetone-water and any other mixture as well using the measured 'slope' and log k'_o data obtained by the respective mixture and calculating a_4 and b_4 coefficients in eq. 6.

MATERIALS AND METHODS

26 model compounds were tested. The compounds were selected according to the following criteria:

1. Log $P_{o/w}$ values of the compound is available in the literature.
2. The compound is in a neutral (unionized) form between pH 2 and pH 7. This is necessary in order to avoid ion pairs during chromatographic measurements because ion pair formation can influence the partition processes of the compounds. On

the other hand, log $P_{o/w}$ values in general refer to the neutral molecules.

3. The compounds possess a wide range of log $P_{o/w}$ values.

The selected compounds and their log $P_{o/w}$ values are shown in Table 1. Compounds were obtained from the Semmelweis University Medical School. Most of the compounds are listed in the 6th Edition of Hungarian Pharmacopea and they comply with its requirements regarding purity.

Log $P_{o/w}$ values were available from Hansch and Leo's compilation [18]. If one compound had more than one different log $P_{o/w}$ value, that value measured by Hansch or Fujita and their co-workers was used in the correlation analysis. In certain cases great discrepancies could be observed among the log $P_{o/w}$ values determined in different laboratories, as be seen in Fig. 1. This circumstance may increase the error of our results.

Log k' values of the model compounds were measured by using 3 to 5 different percentages of acetonitrile in the eluent. The experimental conditions of the measurements are summarized in Table 2.

The percentage of the acetonitrile in the eluent (OP%) was increased by 5% steps from 5% to

TABLE 1.

Compounds Investigated and their log $P_{o/w}$ Values [18].

Compound	log $P_{o/w}$
Resorcin	0.80
Sulphadimidine	0.32
Sulphamethoxypyridazine	0.40
Barbital	0.65
Phenobarbital	1.42
Chloramphenicol	1.14
Salicylamide	1.28
Phenacetin	1.58
Vanillin	1.37
Benzaldehyde	1.45
Acetanilide	1.16
Nicotinamide	-0.57
Benzoic acid	1.87
Salicylic acid	2.25
Acetylsalicylic acid	1.23
Coffein	-0.07
Hydrochlorothiazide	-0.07
Cortexolone	2.46
Dexamethasone	1.99
Desoxycortone	2.88
Sulphaguanidine	-1.22
Isoniazide	-1.14
Methylsalicylate	2.46
Hydrocortisone	1.61
Progesterone	3.87
Testosterone	3.31

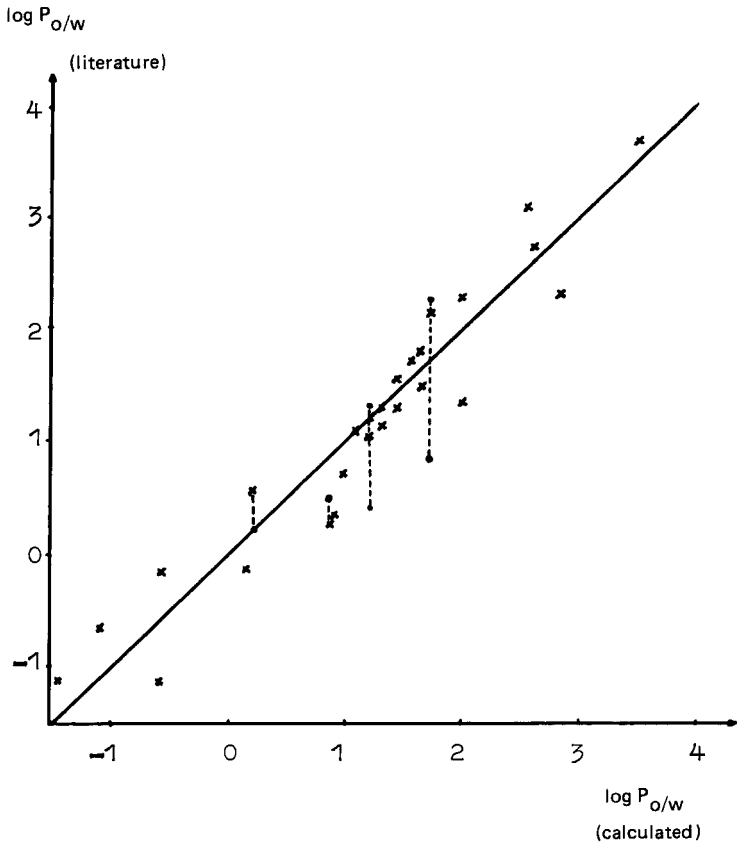

FIGURE 1.

Plot of the Calculated and the Literature log $P_{o/w}$ Values According to eq. 7.

The sign x means the values used in the calculation and the sign o----o shows the value of log $P_{o/w}$ from different laboratories.

TABLE 2.

Experimental Conditions of the HPLC Measurements

Column:* RP-18 LiChrosorb, 250 mm, ID 4.6, dp 10 μ

Injector:* Rheodyne Model 7010 Sample Injection Valve

Detector:* ISCO Model 226 Absorbance Monitor

Detection: 254 nm

Pump: Labormim Liquopump Model 312

Integrator: Chinoin Digint Model 24

Recorder: Endim Model 621.01

Temperature: 22 °C ± 2 °C

Pressure: 30-50 bar

Eluent: 5 to 90 % acetonitrile (Reanal, purified for HPLC) and 0.05 M KH_2PO_4 buffer (pH=4.6)**

Dead time determination: $NaNO_3$ (Reanal)

Calculations:*** Apple II+ microcomputer

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* Instruments were kindly provided by Chromatronix Inc.

** For the measurements of acidic compounds 1-2 drops of 85% H_3PO_4 were added to the eluent (pH=2) in order to get symmetrical peaks.

*** The computer was kindly provided by Dime's Group Inc.

90%. The pH was 4.6 inorder to avoid the ionization of solutes. This pH value was selected on the basis of pK_a values for weak basic, neutral and weak acidic model compounds. The pH change of the eluent from 4.6 to 2 in case of acidic compounds (salicylic acid, acetyl salicylic acid, benzoic acid) was necessary considering their pK_a values. The peak symmetry and the shortest retention time indicated that the compounds were in the neutral form during the chromatographic procedure and the speed of the partition process was much higher than the rate of elution. Dead time determination using $NaNO_3$ (Reanal) was carried out at each measurement of the t_R value. An appropriate amount of $NaNO_3$ was added to each injected solution and log k' values were calculated from the respective dead time and the retention time values. The linearity of OP% vs. log k' plot was tested by the least squares method for each compound.

All calculations including the processing of the measured retention data and least squares analysis were carried out with an Apple II+ microcomputer using a program written by us in BASIC language. Log k' values of all compounds measured at different percentages of acetonitrile are stored in data files, which can be continuously enlarged and easily used in correlation analysis.

RESULTS AND DISCUSSION

Table 3. shows the most important parameters of the log k' - OP% relationships: the 'slope' values, the log k'_o values and the correlation coefficients for each of the model compounds. Log k' values of each compound were measured by using that percentage of acetonitrile by which t_R value fell between $2t_o$ and $3t_o$ covering at least 15% range of the organic phase concentration.

Data points belonging to very low retention times (log k'<-0.60) were omitted from the calculations. These data points usually decreased significantly the value of the correlation coefficients.

Substituting the 'slope' and the log k'_o values thus obtained into eq. 6 the following parameters were calculated:

$$\log P_{o/w} = 90.23 \text{ 'slope'} + 1.854 \log k'_o + 1.911 \quad (7)$$

$$n=26 \quad R=0.949 \quad s=0.413 \quad F=104.7 \quad F_{(2,24\ p\ 0.95)}=3.4$$

where n is the number of compounds, R is the multiple correlation coefficient, s is the residual error, and F is the Fischer-test value. The relationship described by eq. 7 is significant according to the R and F values. Comparing the calculated and the known log $P_{o/w}$ values of each compound none of them can be considered a significant outlier. Although the resi-

TABLE 3.

The Measured Coefficients of eq. 4

Compound	slope	log k'_o	cor.coef.
Resorcin	−0.0150	0.259	0.990
Sulphadimidine	−0.0280	0.854	0.997
Sulphamethoxypyridazine	−0.0285	0.892	0.990
Barbital	−0.0402	1.063	0.981
Phenobarbital	−0.0319	1.341	0.999
Chloramphenicol	−0.0414	1.625	0.997
Salicylamide	−0.0255	0.871	0.984
Phenacetin	−0.0226	1.002	0.981
Vanillin	−0.0244	0.866	0.999
Benzaldehyde	−0.0303	1.575	0.999
Acetanilide	−0.0270	1.021	0.991
Nicotinamide	−0.0382	0.251	0.941
Benzoic acid	−0.0284	1.252	0.987
Salicylic acid	−0.0301	1.425	0.988
Acetylsalicylic acid	−0.0272	1.077	0.974
Coffein	−0.0299	0.552	0.979
Hydrochlorothiazide	−0.0456	0.887	0.912
Cortexolone	−0.0138	0.757	0.993
Dexamethasone	−0.0139	0.568	0.997
Desoxycortone	−0.0147	1.120	0.990
Sulphaguanidine	−0.0272	0.011	0.932
Isoniazide	−0.0382	0.060	0.947
Methylsalicylate	−0.0244	1.727	0.997
Hydrocortisone	−0.0129	0.436	0.991
Progesterone	−0.0192	1.831	0.995
Testosterone	−0.0143	1.085	0.998

dual error is ten times as high as the usual error of log $P_{o/w}$ determination by the traditional shake-flask method, it is not too high in view of the deviation of the log $P_{o/w}$ values obtained from different laboratories (Fig. 1).

The intercorrelation of the two independent variables in eq. 7 is very low, therefore both variables are significant in the equation. The correlation matrix of variables is given in Table 4.

The quotient of the regression coefficients of the 'slope' and the log k'_o values gives 48.6 . It means, that the same relationship between log $P_{o/w}$ and log k' values according to eq. 3 could have been obtained if log k' values belonging to 48% acetonitrile-water mixture as eluent were used. It also means that the chromatographic partition system containing 48% acetonitrile and 52% water mixture and RP-18 column shows the greatest similarity to the 1-octanol-water partition system.

In those cases when the hydrophobicity range of the investigated compounds is wide and one cannot measure log k' values for each compound using the proposed mixture as eluent eq. 6 is suggested for estimating log $P_{o/w}$ values. When the 'slope' values are nearly the same for all of the investigated

TABLE 4.

Correlation Matrix of Variables Used in eq. 7.

	log $P_{o/w}$	log k'_o	'slope'
log $P_{o/w}$	1.000	0.683	0.616
log k'_o	0.683	1.000	-0.062
'slope'	0.616	-0.062	1.000

compounds, or the 'slope' and the log k'_o shows intercorrelation, then the log $P_{o/w}$ and log k' values measured at any eluent mixture will show significant relationship.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(7), 1425-1432 (1984)

LIQUID CHROMATOGRAPHIC BEHAVIOR OF SELECTED AZA-ARENES AND POTENTIAL METABOLITES

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ABSTRACT

Reversed phase HPLC was applied to analyses of selected aza-arenes and potential metabolites (5,6-benzoquinoline, 5,6-benzoquinoline-N-oxide, and the N-methyliodide salt of 5,6-benzoquinoline). Naphthalene and anthracene were employed as reference materials. Water-methanol and ammonium phosphate-methanol mobile phases were used with several commercial octadecyl reversed phase columns and a column using laboratory synthesized ODS stationary phase. Chromatographic behavior of the hydrocarbon reference compounds were excellent on all packings with either mobile phase. Benzoquinoline and derived materials were more difficult analytical subjects; water-methanol mobile phases proved unsuitable giving very long retention times with unacceptable efficiencies. Ammonium phosphate containing mobile phases were more appropriate for the nitrogen containing materials especially when used with end capped or polymeric end capped stationary phases.

INTRODUCTION

Liquid chromatography has emerged as the method of choice for analysis of polynuclear aromatic hydrocarbon (PAH) carcinogens and their metabolites(1). Normally reversed phase columns are used with mobile phases consisting of water-methanol, water-acetonitrile, or water-tetrahydrofurane mixtures. Under these

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conditions chromatographic performance is usually excellent with theoretical plate heights near two particle diameters for well packed columns. There appears to be little difference between analytical efficiency for PAH and their metabolites. Elution of metabolites between the column void volume and the substrate retention volume is an added advantage during metabolism studies (metabolism is usually accompanied by gains in polarity).

Aza-arenes (NPAH) and their metabolites appear to be more difficult analytical subjects than PAH since the parent compounds and their metabolites are subject to protonation. In addition, metabolism of NPAH might produce metabolites with a partial or full positive charge localized on the nitrogen atom (amine oxide or N - methyl salt). As a necessary prelude to an investigation of the metabolism and biological actions of NPAH the liquid chromatographic behavior of a mixture of 5,6-benzoquinoline (BQ), 5,6-benzoquinoline-N-oxide (BQNO), and the N-methyl iodide derivative of 5,6-benzoquinoline (BQMeI) on several reversed phase stationary materials was studied.

METHODS AND MATERIALS

A Perkin-Elmer 3B liquid chromatograph with column temperature control was used in these studies. Chromatography columns (5 μ ODS, 4.6 X 250 mm) were purchased from commercial vendors (Alltech, Houston, TX; Laboratory Data Control, Riviera Beach, FL; Perkin-Elmer, Norwalk, CT; Supelco, Bellefonte, PA), or packed using techniques previously described(2). End capped

ODS packing material was synthesized by reaction of octadecyl bonded silica with trimethylsilyl chloride in distilled carbon tetrachloride for two hours at room temperature, followed by filtering, washing (carbon tetrachloride, methanol, methylene chloride) and drying overnight at room temperature. Polymeric ODS packings were prepared by reaction of silica gel (Analtech, Newark, DE, 10 μ silica gel without binder) with octadecyltrichlorosilane and end capping according to a published procedure (3).

Model metabolites were prepared from 5,6-benzoquinoline (Aldrich Chemical, Milwaukee, WI). The amine oxides was synthesized by oxidation of the NPAH with m-chloroperbenzoic acid at room temperature in methylene chloride for 2 hours. Methyl iodide and BQ were heated in benzene to 100°C in a sealed tube for 4 hours and precipitated BQ methiodide recrystallized from methanol-benzene.

RESULTS AND DISCUSSION

Although reversed phase columns with water-organic solvent mobile phases are well suited for analysis of PAH and their metabolites it was immediately obvious that these conditions were not appropriate for NPAH and model metabolites. While naphthalene and anthracene gave excellent peaks under these conditions BQ showed considerable evidence of non-ideal behavior (Fig. 1). In general the number of theoretical plates for BQ was no more than 100 for 25 cm columns. At the same time plate height counts

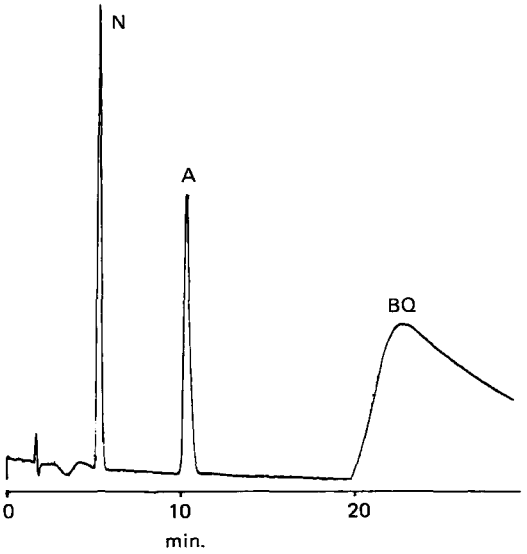

Figure 1. HPLC of anthracene (A), BQ, BQNO, BQMeI, and naphthalene (N) on Spherisorb ODS (4.6 X 150 mm), 7:3 methanol:water mobile phase at 1 ml/min.

for naphthalene and anthracene were excellent. The model amine-oxide and methiodide metabolites appeared to be completely retained and did not elute from the column.

It is well known that bonded phase HPLC packings have many unreacted silanols that can act as ion exchange groups(4). The first modification of conditions was to change the mobile phases to mixtures of 0.1 M ammonium phosphate (pH 5.0) and methanol. As evidenced in Fig. 2, the chromatographic behavior of BQ was greatly improved. Furthermore BQ amine oxide (ret. time = 3.9 min) appears in the chromatogram. The quaternary methyl iodide salt was still completely retained under these conditions.

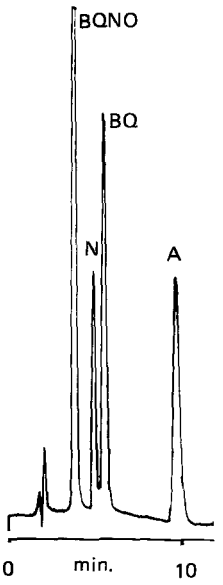

Figure 2. HPLC of mixture in Fig. 1 on Spherisorb ODS (4.6 X 150 mm), 7:3 methanol:0.1 M ammonium phosphate (pH 5.0) mobile phase at 1 ml/min.

The next change in experimental conditions was to end cap a sample of Spherisorb ODS with trimethylsilyl chloride to decrease the number of available silanol groups. The chromatogram resulting from analysis of a mixture of anthracene, BQ, BQNO, BQMeI, and naphthalene is displayed in Fig. 3. For the first time BQMeI appears in the chromatogram (ret. time = ca. 8.6 min), although with unsatisfactory efficiency. In addition, BQNO elutes earlier on the end-capped column (2.8 min) than on the previous one (3.9 min), and the relative retention of BQ and naphthalene are reversed. These data suggest that there are fewer non-ideal

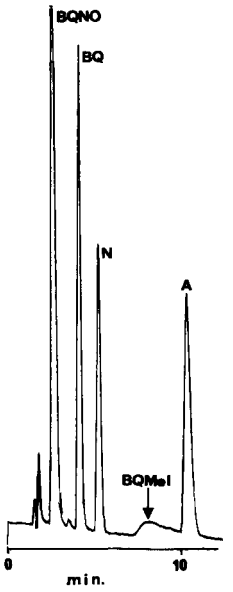

Figure 3. HPLC of mixture in Fig. 1 on Sperisorb ODS end-capped with trimethylsilyl chloride (4.6 X 150 mm) mobile phase in Fig. 2.

solute-stationary phase interactions with the end-capped column. It also is clear that the fully charged methyliodide is the most difficult analytical subject in this group of compounds. The order of elution of the NPAH materials by a purely hydrophobic mechanism should be BQMeI<BQNO<BQ because of their relative polarities.

A number of commercial reversed phase columns and a polymeric ODS column prepared in this laboratory were examined. While there were considerable differences in chromatographic behavior of BQ, its amine oxide and methyliodide salt no column

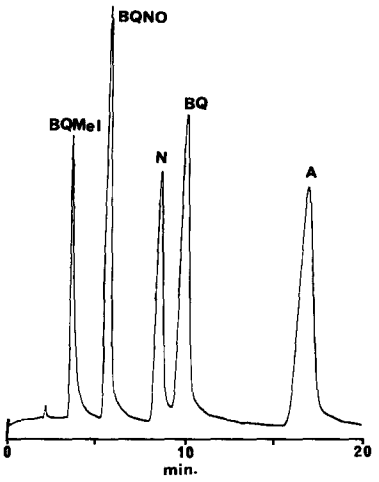

Figure 4. HPLC of mixture in Fig. 1 on semi-preparative column (10 X 200 mm), 1:1 methanol:0.1 M ammonium phosphate (pH 5.0) mobile phase at 5 ml/min.

tested allowed chromatography of the fully charged methyl derivative with complete success. Columns with polymeric coatings appeared to perform best and gave the expected order of elution for BQMeI, BQNO, and BQ although peaks derived from BQMeI characteristically tailed to some degree. Under ideal conditions tailing of the methiodide salt is sufficiently suppressed to allow successful analytical or preparative analyses to be made. Fig.4 illustrates the chromatography of the mixture of PAH, BQ, BQNO, and BQMeI on a semi-preparative column prepared in the laboratory.

The mixture of BQ, BQNO, BQMeI and the two PAH appears to be an excellent probe for column evaluation.

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REVERSE PHASE HPLC DETERMINATION OF 5,6-DIHYDRO-5-AZACYTIDINE IN BIOLOGICAL FLUIDS

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ABSTRACT

A sensitive and specific reverse phase HPLC method which allows measurement of the new antitumor agent 5,6-dihydro-5-azacytidine (DHAC) in biological fluids at concentrations as low as 50 ng/ml (2×10^{-7} M) has been developed. After addition of 5'-chloro-5'-deoxy-5,6-dihydro-5-azacytidine as an internal standard, sequential ultrafiltration, boronate gel affinity chromatography and cation exchange chromatography are employed to isolate DHAC from plasma or urine. DHAC is then reacted with N,N-dimethylformamide diethylacetal to form a dimethylaminomethylene derivative with enhanced UV detectability (λ_{max} = 264 nm, log ε = 4.3) and better retention on a reverse phase column. Isocratic separation is then accomplished on a fully loaded and end-capped ODS column with 0.050 M formic acid in 20% acetonitrile/water. This assay has been used to determine the plasma pharmacokinetics of DHAC in rats given a single i.v. bolus dose of 50 mg/kg. Analysis of the drug in human plasma indicates that this method is suitable for determining DHAC disposition and pharmacokinetics in human subjects.

INTRODUCTION

5-Azacytidine (5-AC, Figure 1) was synthesized in 1963 (1) and has since been found to be clinically useful in the treatment

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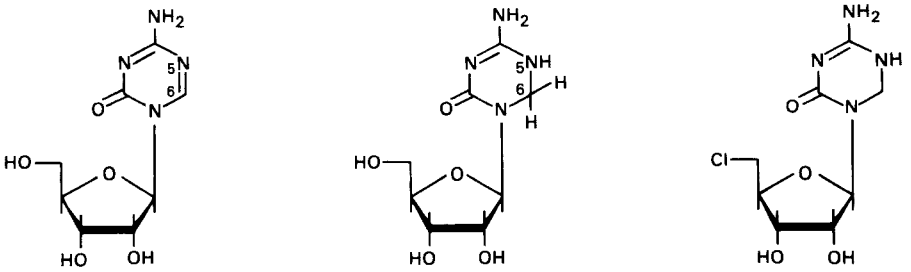


FIGURE 1. Structure of 5-AC, DHAC and the Internal Standard 5'-Cl-DHAC.

of acute myelocytic leukemia (2,3). Unfortunately, when this drug is given as a bolus injection, severe nausea and vomiting occur. These dose-limiting toxicities can be reduced or eliminated by administering 5-AC slowly via constant infusion (4,5). However, 5-AC decomposes in aqueous solution via opening of the triazine ring to produce compounds with limited antitumor activity (6). This aqueous instability makes strict dosage control for infusional therapy very difficult to achieve. To circumvent this problem the hydrolytically susceptible 5,6-imino double bond of 5-AC was reduced to produce 5,6-dihydro-5-azacytidine (DHAC, Figure 1) (7). This reduced nucleoside is not susceptible to hydrolytic attack, has good solubility, and is stable in aqueous solutions for weeks over a broad pH range.

DHAC is currently undergoing Phase I clinical trial. This potential new antitumor agent shows a broad spectrum of activity in murine model tumor systems. Good reproducible activity has been demonstrated against murine L1210 and P388 leukemias. In the L1210 system maximum activity of DHAC was observed at a dose level which was 33 times the optimum dose of 5-AC. The antitumor efficacy of DHAC at this particular dose was about 80% of that shown by 5-AC (7). Screening in solid tumor models has also

shown activity against the human MX-1 mammary xenograft, the murine CD8F mammary and the subcutaneously implanted colon 38 tumors (8).

Like 5-AC, DHAC is cell-cycle-specific with S-phase cells being the most sensitive (9). This provides a rational basis for infusional therapy over an extended time period. This also means that although the administered dose of DHAC will be relatively large, it will be administered over the course of 24 or more hours. Thus, because of this potentially extended schedule, determination of DHAC pharmacokinetics in humans will require both a sensitive and specific analytical method to measure this compound in plasma and other physiological fluids. The following report details the development of a reverse phase HPLC assay for DHAC and demonstrates its suitability for use in animal and human pharamacokinetic studies.

MATERIALS

DHAC (4-Amino-5,6-dihydro-1-β-D-ribofuranosyl-1,3,5-triazin-2(1H)-one, NSC 264880) was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI, and used as received. The drug (Lot AP-02-03) was supplied as its hydrochloride salt and was greater than 99% pure. Hexamethylene-phosphoramide (HMPA), N,N-dimethylformamide diethylacetal (DMF-DEA) and thionyl chloride were obtained from Aldrich Chemical Co. (Milwaukee, WI). Both HMPA and DMF-DEA were vacuum distilled from CaH_2. N,N-Dimethylformamide (DMF, "distilled in glass" grade, Burdick and Jackson, Muskegon, MI) was mixed with benzene (J.T. Baker Chemical Co., Phillipsburg, NJ) and water in a 125:15:6 ratio by weight and the water removed by azeotropic distillation. The DMF fraction was then redistilled from CaH_2 at reduced pressure. Pyridine (J.T. Baker) was redistilled from potassium hydroxide. All purified reagents and solvents were stored over activated 4 A molecular sieves (4-8 mesh, Aldrich)

under argon. Acetonitrile and methanol were HPLC grade (Fischer Scientific Co., Fairlawn, NJ) solvents, and together with distilled water were filtered through the appropriate 0.45 μm solvent-resistant filters (Millipore Corp., Bedford, MA) before mixing to make the mobile phase. Acetic acid, ammonium acetate (Mallinckrodt, Paris, KY), ammonium hydroxide (J.T. Baker), ammonium phosphate, formic acid (MCB Manufacturing Chemists, Inc., Gibbstown, NJ) and phosphoric acid (Fisher) were employed in making buffers.

The internal standard, 5'-chloro-5'-deoxy-5,6-dihydro-5-azacytidine (5'-Cl-DHAC, Figure 1), was synthesized by reacting DHAC with thionylchloride. DHAC hydrochloride (200 mg, 0.71 mmol) was added to a solution of thionylchloride (0.3 ml, 2.5 mmol) in 2.0 ml HMPA. This reaction mixture was stirred for 16 hr at room temperature and then maintained at 4°C for 24 hr. The solution was then diluted with 18 ml H_2O and applied directly to a 5 x 6 cm cation exchange column in the H^+ form (Dowex AG 50W-X8, 100-120 mesh; Bio-Rad, Richmond, CA). The column was washed with 1.0 ℓ H_2O and the product eluted with an equal volume of 1N NH_4OH. The eluate was evaporated at 45°C to yield a solid residue, which showed a major spot (R_f = 0.43) and a minor spot (R_f = 0.89) after TLC on silica gel with 5% 1N NH_4OH in 1:1 $CHCl_3$:CH_3OH with visualization at 254 nm (DHAC R_f = 0.18). A portion of this product was purified further by semi-preparative HPLC on a 10μm μBondapak C_{18} column (7.8 mm x 300 mm; Waters Associates, Milford, MA) using a mobile phase of 6% methanol in pH 5 0.010 M $(NH_4)_2PO_4$ buffer (v/v) at a flow rate of 2.0 ml/min. 5'-Cl-DHAC was monitored by UV detection at 234 nm and the appropriate fractions were collected. Buffer was removed by passing the HPLC eluate through an 8 x 70 mm cation exchange column in the NH_4^+ form. The column was washed with 30 ml H_2O and the product eluted with 30 ml pH 10 NH_4OH. Evaporation produced a crystalline residue, mp 204° (decomposition); NMR (D_2O) δ5.80 (d,1H,

$J_{1',2'}$ = 6Hz), 4.74 (s,2H), 4.34 (m,1H,C_4'-H), 4.26 (m,2H, C_2'-H and C_3'-H), 3.87 (m, 2H,C_5'-H); UV λ_{max} 0.01 M K_3PO_4, pH = 7.1) 231 nm, log ε = 3.67; GC/MS (tetrakis-trimethylsilyl derivative) m/z (rel intensity) 552 ($M^{\ddagger}$, 7.9), 551 (M-H, 15), 537 (M-CH_3,8.2) 517 (M-Cl,18), 388 (13), 294 (s-H,10), 287 (b^+30, 11), 257 (b,100) 217 (69), 73 (81); MS (FAB,Xe) 267 (^{37}Cl MH^+, 37), 265 (^{35}Cl MH^+, 100).

METHODS

Proton NMR spectra were recorded on a Varian XL-200 spectrometer (Varian Associates, Palo Alto, CA). Chemical shifts are reported in δ ppm downfield from deuterated sodium 3-(trimethylsilyl)proprionate which was used as an internal standard in D_2O solutions. A Beckman Model 34 spectrophotometer (Beckman Instruments, Irvine, CA) was used to obtain UV spectra. Mass spectra were obtained on a VG Micromass 7070E mass spectrometer (VG Analytical, Altrincham, England) operated at an accelerating voltage of 6kV and dynamic resolution of 2000. For gas chromatography-mass spectrometry (GC/MS) analyses, nucleosides were derivatized on a microscale with 2:1 acetonitrile:bis(trimethylsilyl)-trifluoroacetamide (Aldrich) at room temperature. Separations were effected on a 1.83 m x 2 mm i.d. glass column packed with 3% OV-17 on 120/140 mesh Gas-Chrom Q (Applied Science Laboratories, State College, PA). The Hewlett-Packard 5710A gas chromatograph (Hewlett-Packard, Avondale, PA) was interfaced to the mass spectrometer via a single stage glass jet separator and was temperature programmed from 210° to 250° at 4°/min. GC/MS operating conditions were: GC injector, 250°; jet separator and transfer lines, 240°; ion source, 250°; and scan speed, 2 sec/decade. Positive ion fast atom bombardment (FAB) mass spectra were obtained by using a FAB source at ambient temperature. Ionization was effected by a beam of xenon atoms derived by neutralizing xenon ions accelerated through 8.6-9.4 kV. Glycerol was used

as the sample matrix and spectra were acquired at a scan speed of 10 sec/decade under the control of a VG 2035 data system.

Separations were accomplished at ambient temperature (20°C) on a 5-μm Spherisorb S5 ODS II column (Regis Chemical Co., Morton Grove, IL) using a mobile phase of 0.050 M formic acid in 20% CH_3CN/H_2O (v/v) at a flow rate of 1.5 ml/min. The analytical column was preceded by a column inlet filter with a replaceable 2-μm element. The remainder of the HPLC system was comprised of a Waters Associates Model 6000A solvent delivery system, a U6K U6K injector and a LC-85 variable wavelength detector (Perkin-Elmer, Norwalk, CT). Injections of standards and unknown samples (50 μl) were made using a 100 μl Waters gas tight syringe. Peak areas and heights were simultaneously determined on a SP4100 computing and recording integrator (Spectra-Physics, Santa Clara, CA).

Standards were made by adding 80 μl of 5'-Cl-DHAC internal standard solution (4.2 μg) to 1.0 ml aliquots of plasma in a 3-ml capacity glass conical centrifuge tube and then by spiking with the required volume of DHAC standard solution. Pooled rat plasma and outdated human plasma were used for these spiked standards. For biological samples, 1.1 ml of plasma was transferred to a 15-ml capacity glass conical centrifuge tube and centrifuged for 10 min at approximately 1000 x g on a Dynac table top centrifuge (Clay Adams, Becton Dickinson and Co., Parsippany, NJ). Exactly 1.0 ml of this plasma was transferred to a 3-ml capacity glass centrifuge tube and internal standard was added as above. All samples were briefly vortexed and transferred to a MPS-1 micro-partion system equipped with a YMP membrane (Amicon Corp., Danvers, MA) for ultrafiltration by centrifugation at 1145 x g for 40 min. Half a milliliter of the resulting ultrafiltrate was mixed with 5 ml 0.25 M NH_4OAc (pH 8.8); and this solution was transferred to an 8 x 40 mm phenylboronate affinity column (Affi-Gel 601, Bio-Rad), previously equilibrated with 10 ml of the same

buffer. The column was washed with an additional 10 ml of NH_4OAc buffer, and then the DHAC and internal standard were eluted with 10 ml 0.1 M formic acid. This eluate was directly applied to an 8 x 15 mm Dowex 50W-X8 cation exchange column in the NH_4^+ form. The ion exchange column was washed with 15 ml distilled water and the nucleosides were eluted with 2 x 7.5 ml pH 10 NH_4OH solution (approximately 30 mM). This eluate was evaporated to dryness and the resulting residue transferred to a smaller (i.e., 10 or 15 ml) flask with about 2 ml water. This solution was likewise evaporated to dryness. An 8 mm stirring bar was added to the flask, and the residue was dried in vacuo for 10 min. A hot air blower (Model 202, Oster Corp., Milwaukee, WI) was used to warm the flask for 2 min to ensure complete drying.

Two hundred microliters DMF and 100 µl 2M DMF-DEA in pyridine were added to the residue under argon. This solution was briefly stirred and then maintained at room temperature for 30 min. Excess reagents were then removed in vacuo (and trapped in a dry ice cold trap) while the solution was stirred and gently heated with a hot air blower. Heating was continued for 3 min after appearance of an apparently dry residue to ensure complete removal of all reagents. The residue was dissolved in 0.50 ml water and stored at 4°C until analysis.

For study of DHAC pharmacokinetics in rats, the drug was dissolved in 0.9% NaCl solution at a concentration of 50 mg/ml. Male Sprague-Dawley rats, weighing 260-300 g (Taconic Farms, Germantown, NY) were anesthetized with ether and a 50 mg/kg dose of DHAC was given as a single bolus injection via the tail vein. Animals were sacrificed under ether anesthesia at varying timed intervals after DHAC injection and blood from each animal's inferior vena cava was collected in separate heparinized glass tubes kept on ice. Plasma was obtained by centrifugation at 1000 x g for 10 min and was then frozen until analysis.

Recoveries of DHAC and 5'-Cl-DHAC from rat plasma were determined by comparison of the absolute HPLC peak area of standards

derivatized directly to that of comparable spiked plasma samples derivatized after workup. Ultrafiltration and protein precipitation were compared by 3 replicate analyses using each method of the same pooled plasma from a rat receiving 50 mg/kg DHAC. For deproteination by methanol precipitation, 3 x 1 ml methanol was added to each sample with shaking for 30 sec following each addition. The resulting suspension was centrifuged at 400 x g for 15 min. Isopropranol (10 ml) was added to the supernatant and this solution was evaporated to dryness under reduced pressure. The residue was dissolved in 5 ml 0.25 m NH_4OAC, pH = 8.8, and extracted with 5 ml chloroform. The aqueous layer was then removed and filtered through a 0.45 μm Millex-HA filter unit before undergoing affinity and ion exchange chromatography.

The peak area or peak height ratio of DHAC to 5'-Cl-DHAC internal standard was computed for each spiked standard and plotted against DHAC concentration to generate a calibration curve (Figure 2). A calculator least squares program (TI-55-II, Texas Instruments, Dallas, TX) was used to define the best straight line through each set of standard points. A blank was also run each time a calibration curve was constructed. Initial pharmacokinetic parameters for DHAC were calculated from the rat plasma concentration (C_p)-time curve by the method of residuals (10). The experimental data points were then fit to the biexponential function representing a two-compartment open model ($C = Ae^{-\alpha t} + Be^{-\beta t}$) by using MLAB, an on-line computer modeling laboratory utilizing an interactive, non-linear least squares program (11). Based on observed assay characteristics, each data point was weighted by $1/C_p^2$.

RESULTS

Both DHAC and the 5'-Cl-DHAC internal standard could be efficiently isolated from plasma and urine and derivatized repro-

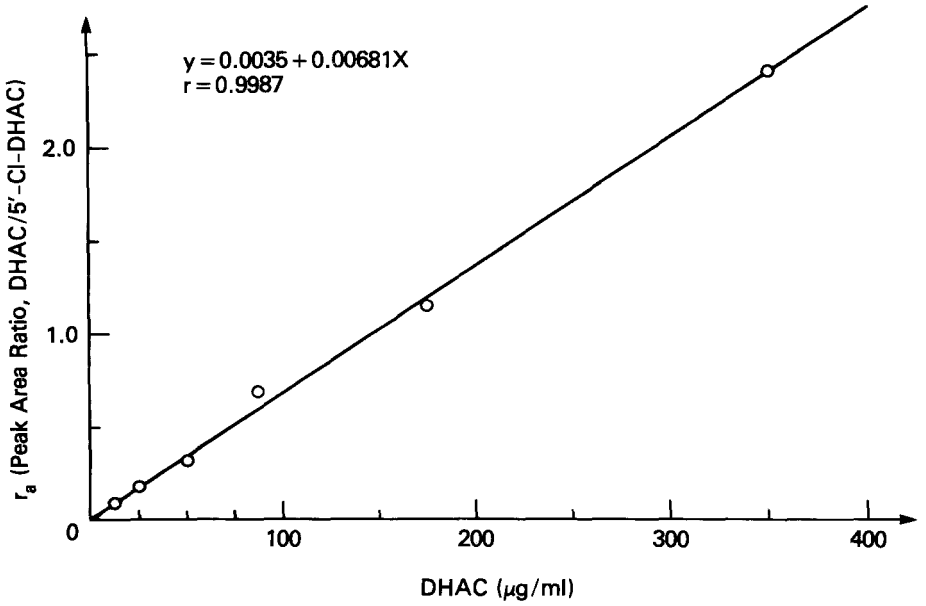


FIGURE 2. Typical Calibration Curve for Rat Plasma.

ducibly at the concentrations expected in biological samples. For DHAC spiked at concentrations of 0.1 to 10.0 μg/ml in human plasma, a recovery of 92 ± 10% (n = 5) was observed. Higher recoveries were found at the higher concentrations of DHAC with almost complete recovery (> 98%) at concentrations greater than 2.5 μg/ml. For 5'-Cl-DHAC spiked at 3.3 μg/ml plasma, 82 ± 10% (n = 6) could be recovered by the multi-step isolation procedure. Conversion to the dimethylaminomethylene (DMAM) derivative was quite reproducible since the variance in the HPLC peak areas of directly derivatized 5'-Cl-DHAC internal standard was less than 3% (n = 6). Both ultrafiltration and methanol precipitation were compared to determine DHAC protein binding and to assess their suitability for removal of these plasma proteins. No protein binding was observed since the quantitative results from the ultrafiltered and precipitated samples were equivalent; however,

greater DHAC recoveries and HPLC responses were seen after ultrafiltration. Linear and reproducible calibration curves could be constructed from spiked standards over a wide concentration range for all biological fluids examined (Figure 2):

rat plasma: $y = 0.0184 + 0.0134x$, 1.0-50 µg/ml ($r = 0.9972$)
rat plasma: $y = 0.0035 + 0.0068x$, 12.5-350 µg/ml ($r = 0.9987$)
human plasma: $y = 0.0310 + 0.3870x$, 0.07-1.4 µg/ml ($r = 0.9996$)
human urine: $y = 0.0125 + 0.0064x$, 50-500 µg/ml ($r = 0.9998$)

(Since the amount of internal standard added varied according to the concentration range examined, the slopes of the above calibration curves are different.)

HPLC analysis of derivatized samples isolated from 1.0 ml aliquots of rat plasma from animals given a bolus injection of 50 mg/kg DHAC resulted in chromatograms similar to Figure 3. The selectivity of the derivatization procedure and the resulting shift of the DHAC λ_{max} to 264 nm minimized interferences and allowed reliable peak integration. The limit of quantitation was found to be about 50 ng/ml ($< 2 \times 10^{-7}$ M) for both human and rat plasma using 1.0 ml spiked samples; the limit of detection was about half this amount. A limit of detection was not determined for urine since high concentrations of DHAC were anticipated and no interferences were encountered in sample blanks.

DISCUSSION

HPLC was chosen as a method of analysis for DHAC because of the potential problems that would have been encountered had the other applicable analytical technique, gas chromatography, been employed. Nucleosides require derivatization for vapor phase analysis (12). Silylation is probably the most widely employed method to form volatile nucleoside derivatives (13). However, silylation of DHAC results in at least partial aromatization of the base to form the corresponding derivative of 5-AC (14).

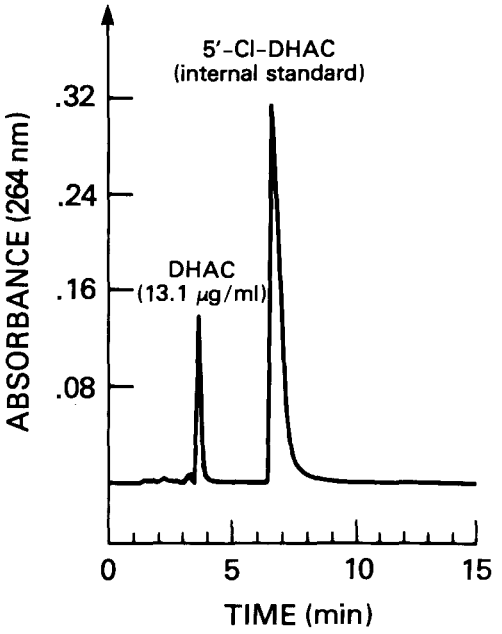


FIGURE 3. Representative HPLC Analysis of DHAC in Rat Plasma. HPLC conditions are as described in Methods. A male Sprague-Dawley rat was intravenously administered a single bolus 50 mg/kg dose of DHAC. The animal was sacrificed 63 min after drug injection and the plasma analyzed.

Since 5-AC may be a metabolite of DHAC (7), a method which does not equivocably differentiate between the two compounds is ruled out. Permethylation, an alternate technique for making volatile derivatives of nucleosides, is not straightforward with cytidine derivatives and gives multiple products (15,16). This too would be a problem since adequate sensitivity to define plasma pharmacokinetics completely is a concern.

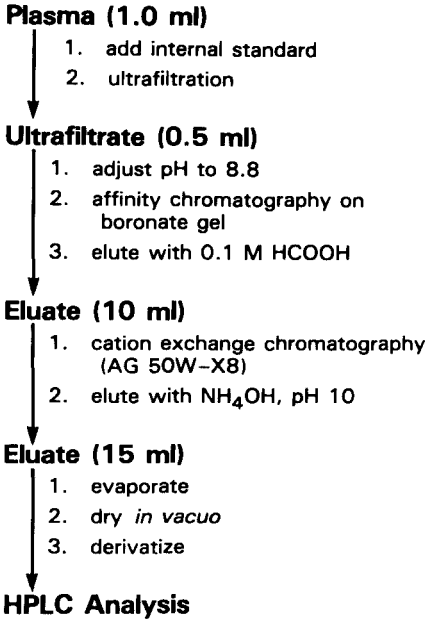
Determination of DHAC by HPLC appeared more promising since many procedures for nucleoside analysis were available (17,18). However, problems still existed. One was the isolation and con-

centration of the drug from a complex biological matrix such as plasma or urine where it was a trace component. A second problem involved enhancing the detectability of DHAC since it had a relatively low UV absorbance at a wavelength where interferences were common. This meant consideration had to be given to derivatization. As will be seen, these two problems were interrelated since derivatization was not efficient unless the DHAC was isolated from the biological matrix in an anhydrous state. Thus it was necessary to employ the rather extensive isolation procedure outlined in Scheme I.

Removal of proteins and lipids from biological samples is essential to protect the analytical column in reverse phase HPLC, especially if large sample volumes are to be injected, as is the case for the numerous samples usually encountered in a pharmacokinetics study. We found ultrafiltration of biological samples by centrifugation in a MPS-1 micropartition system to be a rapid and simple method of deproteinization. Recoveries of both DHAC and the internal standard from spiked plasma were actually greater than after protein removal by methanol precipitation. This indicated plasma protein binding was minimal and ultrafiltration could be used reliably. Plasma ultrafiltrate, however, was not amenable to direct chromatographic analysis without further cleanup.

A two-step column chromatography procedure was found to be necessary to isolate DHAC from the remaining biological matrix so that it could be reliably derivatized and analyzed. Affinity chromatography using an immobilized phenylboronic acid, which selectively binds ribosides via the 2',3'-*cis*-diol moiety was employed as the first step. This procedure was a modification of that reported by Gehrke *et al.* for the isolation of ribonucleosides from urine (18). Even though the nucleosides were eluted from the affinity column with 0.1 N formic acid, residual ammonium acetate from the initial buffer was hard to eliminate and hindered the subsequent derivatization. Partly for this reason and partly

DHAC ISOLATION PROCEDURE



because chromatographic interferences still existed, a second step employing cation exchange chromatography was employed. DHAC could be bound to the cation exchange resin at the pH of the affinity column eluant, so this step followed directly in line. After elution with ammonium hydroxide, evaporation, and drying in vacuo, a sample with no visible residue was obtained. Thus this two-step column chromatography sequence preferentially isolates only those compounds that possess both cis-diol and amino moieties like DHAC.

Derivatization was employed both to enhance the UV detectability of DHAC and to modify its retention on a reverse phase column. Zemlicka and Holy reported that treatment of cytidine with N,N-dimethylformamide diethylacetal (DMF-DEA) in dimethylformamide under mild conditions led to the N^4-dimethylaminomethyl-

ene derivative in high yield (19). Furthermore, the λ_{max} of this derivative was shifted to higher wavelength with a concomitant increase in extinction coefficient. More recently, this reagent was used to form N-dimethylaminomethylene alkyl ester derivatives of amino acids for GC and GC/MS studies (20). The fact that DMF-DEA produced these derivatives in high yield on a microscale prompted investigation of its use for DHAC quantitation. DHAC could be converted easily to its dimethylaminomethylene derivative (DHAC-DMAM, Figure 4) simply by reaction with DMF-DEA in pyridine and dimethylformamide at room temperature for 30 min. Solvent and excess reagent were simply removed in vacuo. Reproducible derivatization required complete removal of all reagents, so brief heating was employed to assist in this. Some 2',3'-O-dimethylaminomethylene derivative is also formed during this reaction, but this material is hydrolyzed to the DHAC-DMAM derivative by treatment with pH 7.4 borate buffer for 70 min at 50°. Formation of DHAC-DMAM was quantitative on a milli- to micromolar scale while yields of 70% to 80% were obtained with 1-5 nanomoles (0.28 - 1.4 µg), respectively. Because of extended conjugation in the base, this derivative exhibited a shift in its λ_{max} to 264 and a three-fold increase in extention coefficient compared to underivatized DHAC. This enhanced UV detectability, combined with better retention on a reverse phase HPLC column, resulted in good chromatographic sensitivity and lack of endogenous interferences. DHAC-DMAM was also quite stable in aqueous solution, showing only 5%, 15% and 24% decomposition after storage at 5°C for 5, 17 and 32 days, respectively. Thus standards may be used for several days and sample analysis immediately following derivatization is not mandatory.

An ideal internal standard should compensate for variations in both the isolation and derivatization procedures, act as a carrier for low levels of analyte, and have similar but not identical chromatographic properties. The choice of the proper inter-

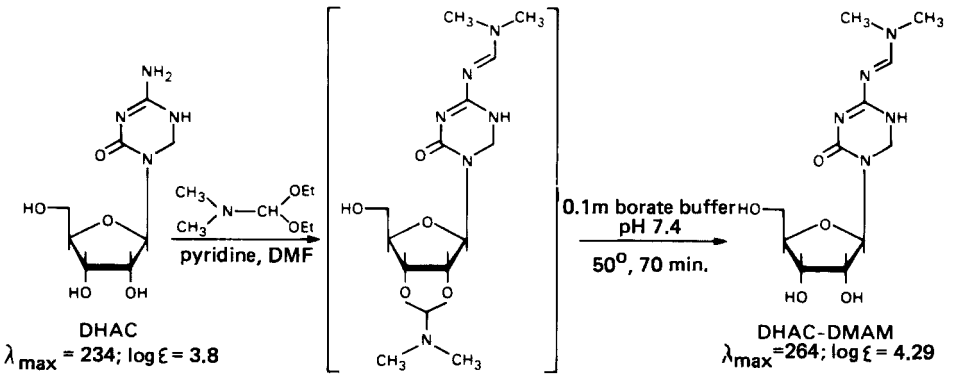


FIGURE 4. Chromogenic Derivatization of DHAC. Complete reaction conditions are described in Methods.

nal standard was especially critical for this assay because of the constraints both the isolation and derivatization procedures place on such a compound. The internal standard must bind both the phenylboronate gel and cation exchange resin, have a primary amino group to form the DMAM derivative, and possess chromatographic properties similar to those of DHAC-DMAM. Many commercially available ribosides with an exocyclic amino group in the aglycon were tested, but none proved completely satisfactory. Synthesis of 5'-chloro-5'-deoxy-5,6-dihydro-5-azacytidine (5'-Cl-DHAC, Figure 1) finally provided an internal standard that met all of the above criteria. This compound could be reproducibly isolated from plasma and its DMAM derivative formed in good yield. Furthermore, this derivative was retained on a reverse phase column so that it eluted shortly after DHAC-DMAM (Figure 3) and could likewise be monitored at 264 nm because it had exactly the same chromophore.

A column containing a high capacity octadecylsilane stationary phase that was fully capped, 5 μm Spherisorb ODS-2, was found to give the best separation under isocratic conditions. This

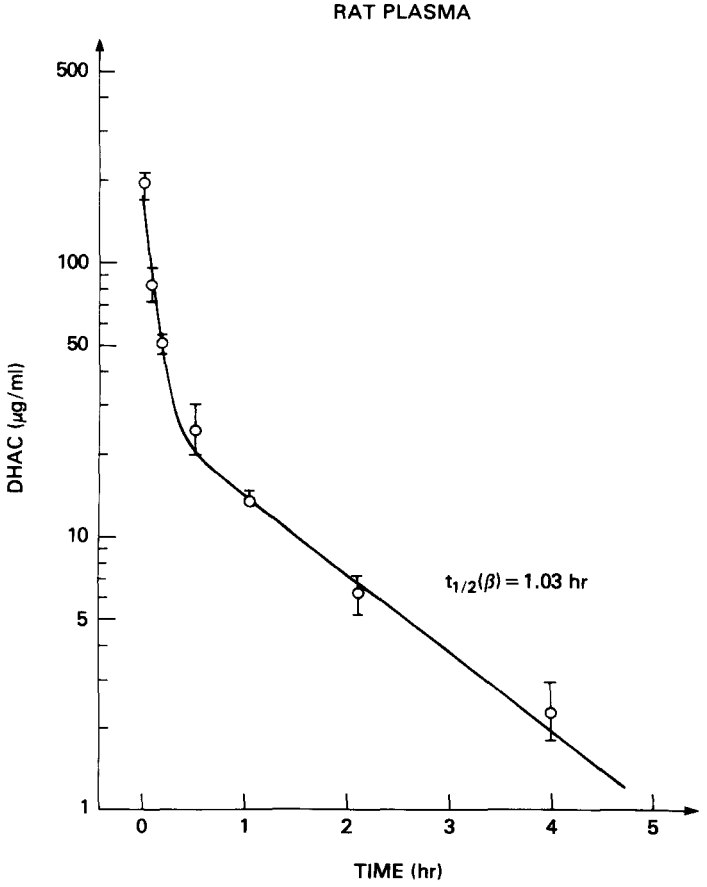


FIGURE 5. Plasma Pharmacokinetics of a Single 50 mg/kg Dose of DHAC in Male Sprague-Dawley Rats. Each point represents the mean DHAC concentration in 3 or more animals. The brackets about each point signify the range of measured DHAC concentrations.

column has also been reported to provide the best chromatographic separations of the more common deoxy- and ribonucleosides and their bases while using a pH 3.5 phosphate buffer mobile phase (21). For this analysis a mobile phase of 0.050 M formic acid in 20% acetonitrile/water gave the best chromatography and resulted

in k's of 2.6 and 4.8 for the DMAM derivatives of DHAC and 5'-Cl-DHAC, respectively.

This assay was applied to determine the single dose plasma pharmacokinetics of DHAC in rats receiving a dose (50 mg/kg) of the drug that would produce plasma concentrations equivalent to those anticipated in humans. A typical chromatogram is shown in Figure 3. The DMAM derivatives of both DHAC and the 5'-Cl-DHAC are clearly defined with no obvious interferences. For maximum sensitivity, the assay requires at least 1.0 ml of biological fluid. Such a sample size presents no problem for humans, but for 260-300 g rats where the total blood volume is only a few milliliters, serial sampling of the type necessary for pharmacokinetics would severely perturb the system. Therefore, rats of similar age, sex and weight were sacrificed at prescribed times after administration of DHAC and only a single sample was obtained from each animal. The data from these composite samples was then used to define the plasma pharmacokinetics.

DHAC exhibits biphasic behavior in the rat (Figure 5). Its plasma disappearance curve is described by a two-compartment open model where $t_{\frac{1}{2}}(\alpha)$ = 4.2 min and $t_{\frac{1}{2}}(\beta)$ = 61 min. Each point in Figure 5 represents multiple animals and the error bar represents the range of concentrations for these different animals. No problem was encountered in measuring DHAC levels 4 h after drug administration since plasma concentrations were well above the limit of sensitivity of the assay.

CONCLUSION

The data presented above show that this HPLC assay is suitable for measuring DHAC concentrations in human patients. This method possesses sufficient sensitivity and specificity to determine DHAC pharmacokinetics, even when the drug is given as a 24-hr continuous infusion. This is the case for our Phase I clinical trial where the initial dose of DHAC will be 1000 mg/m^2/day (42

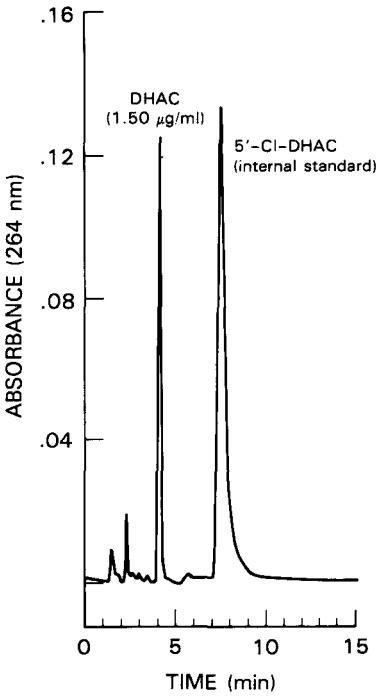


Figure 6. Representative HPLC Analysis of DHAC in Human Plasma. Chromatography conditions are as described in Methods. Normal human plasma was spiked with DHAC and incubated for 3 hr at 37°. A 0.5 ml aliquot of this plasma was diluted to 1.0 ml with distilled water and processed as indicated in Methods.

mg/m^2/hr). Based on extrapolation from single dose pharmacokinetics in the rat, steady state levels of only a few μg/ml DHAC are expected in humans at this entry level dose. Post-infusion plasma levels which will be used to determine pharmacokinetics will, of course, be much lower. Figure 6, which shows the analysis of DHAC in spiked human plasma after incubation at 37° to determine drug stability, indicates that there are no obvious interferences from endogenous plasma components and that adequate sensitivity does indeed exist. Determination of DHAC disposition

and pharmacokinetics in human patients participating in a Phase I clinical trial is currently in progress using this assay. Thus, a sample preparation method which combines the specificity of affinity and ion exchange chromatography for isolation from the biological matrix with the enhanced sensitivity of chromogenic derivatization allows the reverse phase HPLC determination of DHAC in plasma and urine.

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SEMI-PREPARATIVE HPLC SEPARATIONS OF E AND Z ISOMERS OF NEW AROMATIC RETINOIDS

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ABSTRACT

Semi-preparative scale HPLC separations of E and Z isomers of new aromatic retinoids with $\alpha \simeq 1$ are reported. Clean separations with quantitative yields were achieved in less than 1 hour by normal phase HPLC using an analytical instrument.

INTRODUCTION

Synthetic retinoids are extremely effective drugs in various types of keratinization disorders (2). In addition, they exert antiinflammatory effects and seem to possess immunomodulatory properties such as lymphocytes and macrophages (2). The application of HPLC to the separation of retinoids continues to expand at a rapid rate (3,4). Much work has been done in developing methods for the analysis and separation of retinoids in μg and ng quantities (4). There are instances (5) that E isomers were crystallized from retinoid mixtures leaving up to 42% of E and Z mixtures in mother liquors. The corresponding Z isomers were not isolated although Z isomers of some retinoids show significant biological activity (6) and 13-cis retinoic

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acid is less toxic than all-trans retinoic acid (2). Crystals obtained after repeated crystallizations of an E and Z retinoid mixture from different solvents were still reported to be a mixture (7). It has also been reported that synthetic retinoids, an E and Z mixture (5a and 6a in reference 7) could not be separated by HPLC using different solvent systems.

During our work on the synthesis of new aromatic retinoids for testing as potential anti-cancer agents, we were faced with the problem of separating the following retinoids in significant quantities: a) methyl (Z)- and methyl (E)-p-[2-(5,6,7,8-tetrahydro-8,8-dimethyl-2-naphthyl)propenyl]benzoates (1 and 2), b) (1E,3Z)- and (1E,3E)-m-[4-(5,6,7,8-tetrahydro-8,8-dimethyl-2-naphthyl)-1,3-pentadienyl]phenols (3 and 4), c) (Z)- and (E)-3-methyl-5-[2-(5,6,7,8-tetrahydro-8,8-dimethyl-2-naphthyl)-propenyl]phenols (5 and 6), and d) methyl (2E,4E,6Z)- and methyl (2E,4E,6E)-3-methyl-7-(5,6,7,8-tetrahydro-8,8-dimethyl-2-naphthyl)-2,4,6-octatrienoates (7 and 8). We have achieved excellent HPLC separations of the retinoid isomers having $\alpha \simeq 1$ (TLC analysis) in a semi-preparative scale. Structures and α (selectivity factor) values of the retinoid isomers are shown in Figure 1.

MATERIALS AND METHODS

HPLC separations were carried out on a Waters Associates ALC 201 liquid chromatograph equipped with a differential refractometer R401 using a Whatman Partisil M9 10/50 column with a chart speed of 1/2" per min. Fractions were characterized by ^{1}H NMR at 300 MHz using a Bruker WM 300 spectrometer and ^{13}C NMR at 22.5 MHz using a Jeol FX 90Q spectrometer. TLC was performed on pre-coated Kieselgel 60 plates (Merck). The synthesis and anti-tumor activity of the new aromatic retinoids will be published elsewhere (8).

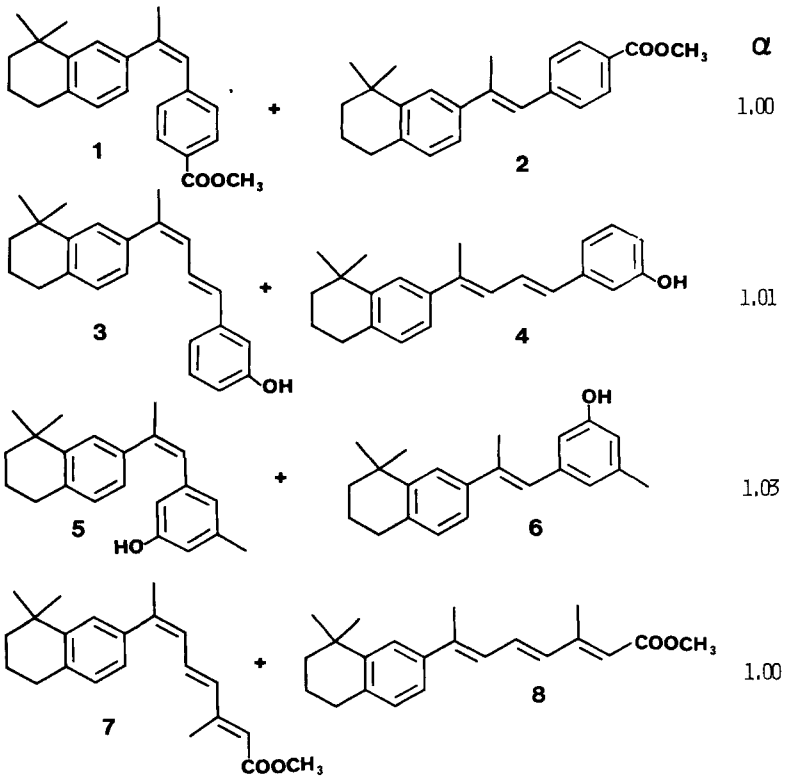

FIGURE 1. Structures and α values of Z and E isomers of new aromatic retinoids.

RESULTS AND DISCUSSION

The mixture of 1 and 2 (ca. 3:2 by NMR analysis) showed a single spot on silica gel plates; Rf 0.22 (solvent system: 4% EtOAc/hexane); selectivity factor α =1.00 (TLC analysis). Figure 2a shows the HPLC separation of retinoids 1 and 2 with the solvent system 4% EtOAc/hexane at a flow rate of 1.54 ml/min. Injection of 38 mg of the mixture of 1 and 2 afforded 21 mg of 1 and 14 mg of 2 (92.1% yield) in 43 min.

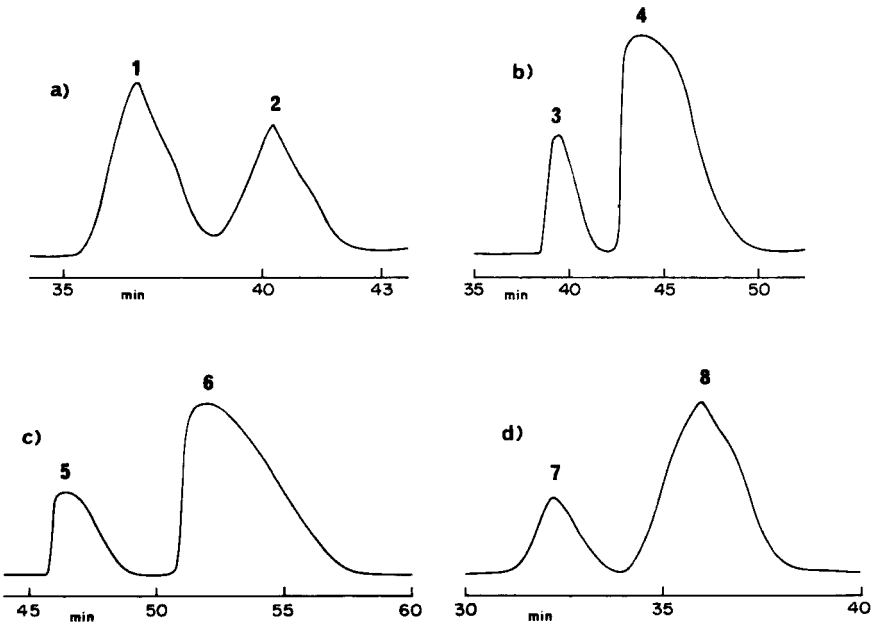

FIGURE 2. Separations of Z and E isomers of retinoids a) 1 and 2; b) 3 and 4; c) 5 and 6; d) 7 and 8.

The mixture of 3 and 4 (ca. 1:4 by NMR analysis) showed almost a single spot on silica gel plates; Rf 0.21 (solvent system: 15% EtOAc/hexane); $\alpha \simeq 1.01$. Figure 2b shows the HPLC separation of retinoids 3 and 4 with the solvent system 15% EtOAc/hexane at a flow rate of 1.2 ml/min. Injection of 64 mg of the mixture of 3 and 4 furnished 13 mg of 3 and 49 mg of 4 (96.9% yield) in 50 min.

The mixture of 5 and 6 (ca. 15:85 by NMR analysis) showed very closely overlapping spots on silica gel plates; Rf 0.205 and 0.21 (solvent system: 12% EtOAc/hexane); $\alpha \simeq 1.03$. Figure 2c shows the HPLC separation of retinoids 5 and 6 with the solvent system 12% EtOAc/hexane at a flow rate of 1.2 ml/min. Injection of 53 mg of the mixture of 5 and 6 gave 8mg of 5 and 43 mg of 6 (96.2% yield) in 1 hour.

The mixture of 7 and 8 (ca. 35:65 by NMR analysis) showed a single spot on silica gel plates; Rf 0.25 (solvent system: 5% EtOAc/hexane); $\alpha \simeq 1.00$. Figure 2d shows the HPLC separation of retinoids 7 and 8 with the solvent system 5% EtOAc/hexane at a flow rate of 1.44 ml/min. Injection of 40 mg of the mixture of 7 and 8 furnished 13 mg of 7 and 24 mg of 8 (92.5% yield) in 40 min.

Excellent separations with yields of 92% were achieved in a) and d) where $\alpha \simeq 1.00$. In b) and c) where $\alpha \simeq 1.01$ and 1.03, 96-97% yields were obtained with clean separations. In all the four cases, Z isomers were eluted first followed by the corresponding E isomers.

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AN ISOCRATIC HPLC METHOD FOR THE DETERMINATION OF CEPHALOSPORINS IN PLASMA

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ABSTRACT

An isocratic reversed-phase liquid chromatographic method for the determination of eight cephalosporins in human plasma using UV detection at 254 nm is described. Plasma proteins were precipitated using acetonitrile prior to injection of a 10 µl aliquot onto an octadecylsilane column. The mobile phases consisted of 6-11% acetonitrile in sodium dihydrogen phosphate (0.01M). The minimum detectable limit for each drug was less than 1 µg/ml of plasma. Possible interference from other drugs which might be administered concurrently is discussed. The reproducibility and precision of the method for cephalosporin assay are shown from the analysis of plasma containing 5-500 µg/ml of plasma. The chromatographic behavior of the eight cephalosporins was examined by varying mobile phase conditions.

INTRODUCTION

Cephalosporins are a family of antibiotics commonly used for the treatment of infections caused by gram-positive cocci and gram-negative bacilli. Toxicities from cephalosporin therapy have been documented in cases of renal impairment (1,2) and during combination therapy with aminoglycosides (3). Monitoring of serum

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concentrations of the cephalosporins ensures adequate drug levels for treatment of infections while avoiding potentially toxic concentrations (4).

Quantitative analyses for cephalosporins in human serum have routinely been done by microbiological assay methods which are subject to interference from other antibiotics commonly used in combination therapy. These assay interferences can be avoided by the use of high performance liquid chromatography (HPLC). A number of recent publications have described HPLC methods for assaying cephalosporins in plasma or serum (5-23). Two review articles discussing the methods of analysis of antibiotics including cephalosporins have recently appeared (24-25). Many of these published methods suffer certain limitations including 1) the use of a lengthy extraction procedure, 2) the lack of an internal standard, 3) the need for ion-pairing reagents, 4) the use of protein precipitation reagents which can cause co-precipitation or degradation of the drugs or are toxic, and 5) the inclusion of limited information on the retention times of other drugs.

The present study was undertaken to meet the need for a single, simple HPLC method to monitor several commonly used cephalosporins without interference from other antibiotics. The drugs studied (cefamandole, cefazolin, cefonicid, cefoperazone, cefotaxime, cefoxitin, cephalothin and cephapirin) represent the three generations of cephalosporins and includes a recently

released product. The method is simple and rapid, requiring only precipitation of proteins with acetonitrile and injection of an aliquot of the supernatant into the chromatograph. The chromatographic behavior of these eight cephalosporins was studied using an octadecylsilane column with combinations of phosphate or acetate buffer and acetonitrile or methanol as mobile phases. The method is applicable to the direct determination of plasma levels in the presence of one or more of several drugs which might be prescribed concurrently. The applicability of the method has been demonstrated by the analysis of serum or plasma from patients receiving cephalosporins.

MATERIALS AND METHODS

Instrumentation

A Hewlett-Packard Model 1084B liquid chromatograph with a variable wavelength UV detector and autoinjector was equipped with an Ultrasphere-ODS (Beckman Instruments) column, 150 mm long and 4.6 mm i.d. The degassed mobile phase was pumped through the column at 2.0 ml/min using isocratic conditions. The column compartment was maintained at 45°C, and the detector was set at a wavelength of 254 nm.

Chemicals and Reagents

Reagent grade sodium dihydrogen phosphate, sodium acetate and acetic acid were used. HPLC grade methanol and acetonitrile were obtained from Fisher Scientific Co. Cefamandole nafate (Mandol^{R}, Eli Lilly and Co.), cefazolin sodium (Ancef^{R}, Smith Kline and

French Labs), cefotaxime sodium (Claforan^{R}, Hoechst-Roussel Pharmaceuticals, Inc.), cefoxitin sodium (Mefoxin^{R}, Merck, Sharp and Dohme), cephalothin sodium (Keflin^{R}, Eli Lilly and Co.) and sodium cephapirin (Bristol Laboratories) were obtained commercially. Cefonicid sodium (Smith Kline and French Labs) and cefoperazone sodium (Pfizer Pharmaceuticals) were obtained courtesy the manufacturers.

Drug Solutions

The powdered drugs were reconstituted to an equivalent of 100 mg/ml water, and aliquots of these solutions were stored frozen for up to one month. Working dilutions of 0.25, 1.0 and 10.0 mg/ml water were prepared daily from the frozen aliquots for each drug.

Internal Standard Solution

A solution of 1.25 mg cephapirin/ml water was prepared and then diluted with acetonitrile to a final concentration of 50 µg/ml.

Mobile Phases

Sodium dihydrogen phosphate, 0.01M, was prepared in deionized distilled water. The pH was not adjusted. Sodium acetate, 0.01M, was prepared in deionized distilled water, and the pH was adjusted to 4.0 with acetic acid. The mobile phases were mixtures of a buffer with methanol or acetonitrile.

Sample Preparation

To 0.5 ml of plasma in a 10x75 mm pyrex tube were added an aliquot (2.5 to 50 µl) of a drug working solution and 1.0 ml of

acetonitrile containing the internal standard. The tubes were vortexed for 10 seconds and centrifuged for 10 minutes at 1500xg. An aliquot of the supernatant was transferred to a polypropylene microvial (P. Weidmann & Co., Romanshorn, Switzerland) before injection of 10 µl into the chromatograph.

Quantitation

A standard curve was constructed for each drug utilizing three replicates simulating concentrations of drugs from 5 to 500 µg/ml of plasma. The mobile phases used were phosphate buffer containing the following percentages of acetonitrile: cefonicid, 6%; cefazolin, cefotaxime and cefoxitin, 7.5%; and cefamandole, cefoperazone and cephalothin, 11%. The chromatograms were recorded at a chart speed of 5 mm/min. The peak heights were measured and the ratios (drug/internal standard) were calculated and plotted versus concentration expressed as micrograms per milliliter of plasma.

Patient Samples

Plasma or serum samples from patients receiving cephalosporin therapy were analyzed in duplicate using the same procedure. The amount of drug in patient samples was calculated by comparison with a standard curve prepared daily.

Interferences

The possible interference of normal plasma constituents was tested by the analysis of blank plasma samples. The interference of other drugs was tested by direct injection of aqueous or methanolic drug solutions.

Recovery

The recovery of each cephalosporin from spiked plasma was compared with that from water.

RESULTS AND DISCUSSION

Deproteinization of plasma samples with acetonitrile containing internal standard is a simple and rapid means of preparing the samples for HPLC. Monitoring the effluent at 254 nm afforded good sensitivity since cephalosporins exhibit appreciable absorbance at this wavelength. Slightly increased sensitivity might be obtained by using the maximum absorbance wavelength (λmax) for each drug (Table 1).

The chromatographic conditions used were chosen after comparison of two buffers and two organic modifiers as the mobile phases on an octadecylsilane reversed-phase column. The buffers tested were sodium acetate (0.01M, pH4) and sodium dihydrogen phosphate (0.01M). The pH of the phosphate solution was not adjusted, but was found to be 4.7. Methanol and acetonitrile were also compared for use in the mobile phase. The chromatographic behavior of the eight cephalosporins was examined and showed that both the magnitude and relative order of the capacity factors of the compounds tested are effected dramatically by the change from acetate to phosphate buffer, by the change from methanol to acetonitrile, and by even a small change in percent of organic modifier (Tables 2a and 2b). The use of phosphate buffer and acetonitrile both contributed to improved resolution of the

TABLE 1. MAXIMUM ABSORBANCE WAVELENGTHS

DRUG	λ_{max}*, nm
Cefamandole	265
Cefazolin	276
Cefonicid	270
Cefoperazone	232
Cefotaxime	238
Cefoxitin	239
Cephalothin	240
Cephapirin	264

* Ultraviolet spectrum of each compound scanned by stop-flow method during chromatography. The mobile phase consisted of 0.01M NaH_2PO_4 and acetonitrile.

cephalosporins, but complete separation of all eight compounds simultaneously was not obtained even with the use of gradient analysis. The cephalosporins would normally not be used concurrently with each other for therapy so patient samples would not be expected to have interference among the cephalosporins. All chromatograms were therefore run isocratically using phosphate buffer and with the percent acetonitrile chosen to provide convenient analysis time for a single drug and internal standard (K between 2 and 10). Elution with only 6% acetonitrile in phosphate buffer was used to resolve cefonicid from the normal plasma constituents. The other cephalosporins were eluted at higher percent acetonitrile to reduce analysis time. Maintaining

TABLE 2a. VARIATION OF CAPACITY FACTOR (K) WITH MOBILE PHASE CHANGE

COMPOUND	NaH_2PO_4, 0.01M					
	+5% CH_3CN	+10% CH_3CN	+15% CH_3CN	+10% CH_3OH	+15% CH_3OH	+20% CH_3OH
Cefonicid	4.08	1.08	0.57	3.32	1.25	0.65
Cefoxitin	12.97	4.23	1.67	11.8	5.32	2.78
Cefotaxime	15.85	3.03	0.98	15.83	5.58	2.40
Cefazolin	16.42	3.17	1.02	18.42	5.78	2.42
Cephapirin	17.63	3.73	1.25	>25	10.75	4.93
Cefamandole	>25	12.42	3.25	>25	20.57	6.15
Cefoperazone	>25	14.42	2.70	>25	22.57	8.02
Cephalothin	>25	18.25	4.85	>25	>25	13.72

TABLE 2b. VARIATION OF CAPACITY FACTOR (K) WITH MOBILE PHASE CHANGE (continued)

COMPOUND	CH_3COONa, 0.01M, pH4					
	+5% CH_3CN	+10% CH_3CN	+15% CH_3CN	+10% CH_3OH	+15% CH_3OH	+20% CH_3OH
Cefonicid	3.63	1.44	1.00	3.45	1.30	0.68
Cefoxitin	10.38	3.74	1.92	11.80	5.55	2.87
Cefotaxime	11.88	2.78	1.41	17.50	6.10	2.57
Cefazolin	11.55	2.89	1.39	16.63	5.82	2.40
Cephapirin	5.96	1.88	1.15	7.43	4.20	2.02
Cefamandole	>25	9.06	3.18	>25	19.37	7.67
Cefoperazone	>25	9.88	2.75	>25	19.90	5.73
Cephalothin	>25	13.07	4.64	>25	>25	13.42

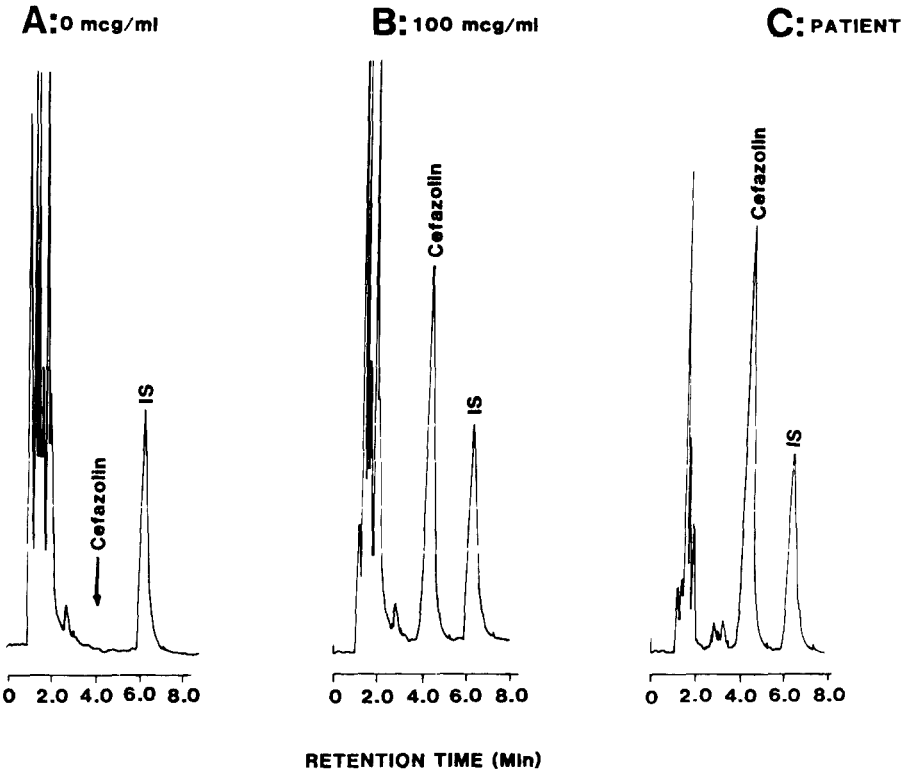

Figure I. Typical Chromatograms of Plasma Extracts

the column at 45°C also contributed to resolution of the compounds. No interference from normal plasma constituents was observed (Figure 1a) and several drugs which might be prescribed concurrently with cephalosporins were chromatographed. Their capacity factors are listed in Table 3.

The use of acetonitrile for precipitation of the serum and plasma proteins has the advantages of having low toxicity, of being the organic modifier in the mobile phase and of resulting in

TABLE 3. CAPACITY FACTORS (K) OF DRUGS WHICH MIGHT BE PRESCRIBED CONCURRENTLY WITH THE CEPHALOSPORINS

COMPOUND	0.01M NaH_2PO_4		
	+5% CH_3CN	+10% CH_3CN	+15% CH_3CN
Moxalactam	0.87 & 1.05 (doublet)	0.35	0.11
Carbenicillin	1.37	0.62	0.18
Ticarcillin	2.92	0.36	0.16
Acetaminophen	4.18	0.77	0.93
Salicylic Acid	4.42	2.37	1.37
Theophylline	6.92	1.15	0.88
Caffeine	9.20	4.15	1.78
Pennicillin G	>25	>25	5.59
Sulfamethoxazole	>25	>25	5.95
Chloramphenicol	>25	>25	11.88
Piperacillin	>25	>25	12.66
Amikacin	>25	>25	>25
Chlortetracycline	>25	>25	>25
Clindamycin	>25	>25	>25
Erythromycin	>25	>25	>25
Gentamicin	>25	>25	>25
Nafcillin	>25	>25	>25
Tetracycline	>25	>25	>25
Tobramycin	>25	>25	>25

TABLE 4. PRECISION OF CEPHALOSPORIN ASSAY

Amount Added ug/ml	Amount Found, ug/ml*			
	Cefamandole	Cefazolin	Cefonicid	Cefoperazone
5	6.6±1.5	5.4± 1.4	6.7±0.3	5.9± 0.7
25	25.5±0.3	24.0± 1.3	27.4±0.8	25.2± 1.0
50	51.7±0.6	51.7± 0.6	52.7±2.2	50.9± 2.1
75	75.0±1.9	74.4± 2.3	75.6±1.2	74.9± 5.1
100	98.9±2.3	101.5± 3.2	97.1±1.7	101.7± 2.2
200	197.5±4.0	206.2± 3.8	207.4±6.3	207.2± 4.6
300	303.6±6.9	293.0± 5.3	302.8±1.7	291.7± 7.8
400	403.8±6.0	397.6±13.1	404.9±3.6	392.0± 4.7
500	495.8±11.5	505.6± 7.4	495.7±8.9	506.3±11.0
Correlation Coefficient	0.9994	0.9987	0.9989	0.9989
Total N	27	27	27	27
R^2	0.9987	0.9974	0.9978	0.9977
y-Intercept	0.0018	0.0208	0.0331	0.0059

* Mean ± Std. dev.

(continued)

high recoveries for most of the cephalosporins. The percentage of recoveries were: cefamandole 96.3%, cefazolin 89.1%, cefonicid 74.7%, cefoperazone 83.3%, cefotaxime 99.1%, cefoxitin 92.4%, and cephalothin 97.3%. The lower recovery of some of the drugs is probably due to co-precipitation of the drugs with protein.

TABLE 4. PRECISION OF CEPHALOSPORIN ASSAY (continued)

Amount Added ug/ml	Amount Found, ug/ml*		
	Cefotaxime	Cefoxitin	Cephalothin
5	4.3± 0.2	5.2± 0.4	5.6± 0.7
25	22.9± 3.1	26.5± 0.2	27.9± 2.0
50	50.7± 1.8	51.7± 0.9	50.4± 3.1
75	77.2± 7.3	74.2± 2.3	76.0± 3.4
100	102.6± 1.2	99.4± 2.3	98.3± 5.7
200	203.8± 4.8	207.3±4.5	210.4± 8.2
300	298.9± 8.2	303.1±6.4	310.6±11.7
400	399.8± 6.5	400.1±3.9	404.9±11.6
500	496.3±10.7	496.2±10.2	491.7± 9.5
Correlation Coefficient	0.9995	0.9990	0.9995
Total N	27	27	27
R^2	0.9990	0.9980	0.9989
y-Intercept	-0.0115	0.0225	0.0144

* Mean ± Std. dev.

The ratios of the peak heights of the drugs to the peak height of the internal standard were calculated. Statistical analysis of the data by linear regression indicated linearity and reproducibility in the range of 5 to 500 μg/ml plasma (Table 4). This range includes the therapeutic range. The minimum detectable limit for the compounds by this method is less than 1 μg/ml plasma

(less than 3 ng/10 µl injection). The injection volume was maintained at 10 µl because larger volumes resulted in peak broadening.

The method has been applied to the analysis of patient samples in our laboratory (Figure 1c). Major advantages of this method for the analysis of cephalosporins in patient samples are its precision, simplicity, sensitivity and rapidity. All the drugs are determined from small volumes (0.5 ml or less) of serum or plasma with minimal sample preparation. The use of an isocratic mobile phase, an internal standard and UV detection at 254 nm contribute to its simplicity. The applicability of the method to cephalosporins from all three generations including a recently released product makes it feasible for routine patient monitoring. In addition, the high sensitivity of quantitation indicates its applicability for pharmacokinetic studies. The method is especially well suited to analysis of samples from patients receiving a combination of antibiotics which would preclude the use of microbiological assay methods.

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ION INTERACTION CHROMATOGRAPHY OF CLAVULANIC ACID ON A POLY(STYRENE-DIVINYLBENZENE) ADSORBENT IN THE PRESENCE OF TETRABUTYLAMMONIUM SALTS

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ABSTRACT

The effect of tetrabutylammonium, inorganic coanions, and inert electrolytes, on the clavulanic anion retention on PRP-1 column, formed with 10 μm spherical uniform particles from a polystyrene-divinylbenzene with high pore volume and large surface area, is studied. The results obtained are applied to the chromatographic separation of clavulanic acid from β-lactamic antibiotics and to the determination of clavulanate anion in fermentation broths.

INTRODUCTION

Clavulanic acid (CA) is Z-(2R,5R)-3(2-hydroxyethylidene) -7-oxo-4-oxa-1-azabicylo(3,2,0)-heptane-2-carboxylic acid (1). It is a potent inhibitor of β-lactamases (2) which is used mixed with β-lactamase sensitive penicillins to protect them against hydrolysis by the enzyme.

Iodometric and hydroxamic methods frequently used for lactamic antibiotic titration are not appropriate for clavulanic acid quantitative analysis (3).

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Some recent works describing the clavulanic acid spectrophotometric titration by reaction with imidazole (4) have been issued, as well as the determination by HPLC of the derivative obtained (5).

In the present work we have studied the effect of tetrabutylammonium (TBA) salts, inorganic coanions, mixed solvents, and addition of inert electrolytes, on the clavulanic anion retention on PRP-1 column. The PRP-1 column is prepared from polystyrene-divinylbenzene resin in the form of 10 μm spherical uniform particles with high pore volume and large surface area.

MATERIALS AND METHODS

Reagents

Amoxicillin, Cephalexin, and Sodium Ampicillin, were gifts by Antibióticos, S.A. (Madrid, Spain), and they were used as supplied.

Sodium clavulanate was extracted from a pharmaceutical preparation ("Augmentin", Beecham Research Labs., England). Tablets, finely pulverized, were resuspended in cool water and then filtered. The filtrate was extracted at pH 2.5 with n-butanol. The organic phase was extracted again with water, adjusting the mixture to pH 7. The aqueous phase was lyophilized. All these operations were carried out at low temperature (5º C) and as rapidly as possible to avoid the clavulanic acid decomposition. The nature of the product obtained was checked on IR, UV and nmr.

The chemicals were of the highest commercial grade available and were used without further purification. Tetrabutylam-

monium chloride, acetate, formate, propionate, and perchlorate were prepared by passing the tetrabutylammonium bromide solution through Amberlite resin IRA-400 of the forms of chloride, acetate, formate, propionate, and perchlorate, respectively.

Instruments

All the chromatograms were obtained using a modular chromatograph equipped with a Model 6000 A pump, a U6K injector, a Model 441 UV (220 nm) detector, and a strip-chart recorder. All of them manufactured by Waters Ass. A 4.1 mm i.d. x 150 mm, 10 μm PRP-1 (polystyrene-divinylbenzene copolymer) column supplied in a prepacked form by Hamilton Co. was also used.

Chromatographic procedure

The PRP-1 column was conditioned by passing a mobile phase containing tetrabutylammonium salt solution for 1 or 2 hours at 1 ml/min flow rate. When a tetrabutylammonium salt is to be removed the column is first washed by passing al least 20 column volumes of a 50% water-acetonitryle solution through the column and then charged in the desired quaternary ammonium anionic form.

Mixed solvents in the mobile phase are expressed as percent by volume.

Basic mobile phases were prepared from phosphate salts and sodium hydroxide. Ionic strength was controlled when necessary by adding known amounts of inorganic electrolyte.

Sodium clavulanate and amoxicillin were dissolved in the corresponding mobile phases used, injecting 10 μl of these solutions in most cases. The void volume, V_o, was determined from retention volumes found for several samples not retained

on PRP-1 column at the given eluting condition. Depending on these conditions, V_o was about 1.1 ml. All the tests were performed at room temperature and at 1 ml/min flow rate.

RESULTS AND DISCUSSION

The main mobile phase parameters influencing the analyte retention are the nature of the quaternary ammonium salt and its concentration, the nature of the coanion, pH, type and concentration of mixed solvents, and ionic strength. Though these parameters are generally related, the adjustment of the three first ones are usually most critic. In our case, the working pH has always been between 6 and 7 due to the instability of the clavulanic acid at pH different from neutrality (6).

Effect of R_4N^+ concentration

In order to carry out the present study, the retention times of clavulanate and amoxicillin on a PRP-1 column were measured using mobile phases formed by 0.001 M phosphate buffer at pH 6 and acetonitryle (9-1 v/v) containing tetrabutylammonium bromide at different concentrations. The results obtained are given in Fig. 1.

In a system formed by a nonpolar phase of polystyrene-divinylbenzene copolymer, and mobile phases formed by solvents in which water predominates and which contains R_4N^+ cations and their corresponding coanions, the procedure that determines the chromatographic separation is probably based on a series of equilibria in which anion exchanges predominate. ISKANDARINI and PIETRZYK (7) have deduced Equation 1 from the study of major equilibria that contribute to an organic analyte

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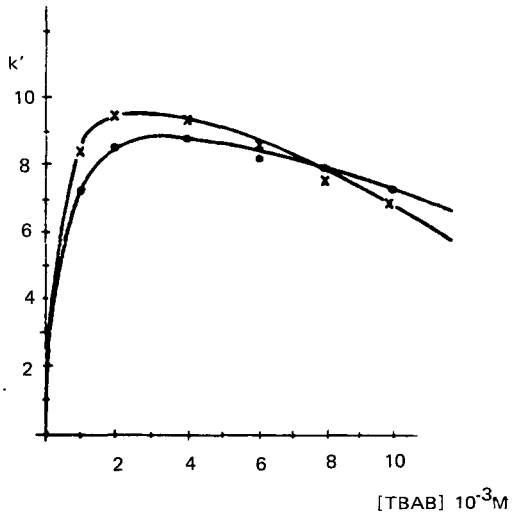

Figure 1. Effect of tetrabutylammonium bromide concentration on the capacity factor of sodium clavulanate (•) and amoxicillin (x) on PRP-1 column. The mobile phase conditions are 1:9 $CH_3CN:H_2O$ 1.0 mM phosphate buffer pH 6 and variable TBAB concentrations.

anion retention, under conditions similar to those experienced by us. Such equation expresses the capacity factors in function of a series of parameters.

$$\frac{1}{k'} = \frac{1}{qK_oK_{(R_4NXA)_s}} \cdot \frac{1}{(R_4N^+)_m} + \frac{K_{(R_4NCA)_s}}{qK_oK_{(R_4NXA)_s}} \cdot \left[C^-\right]_m +$$

$$+ \frac{1}{qK_o}\left[1 + \frac{K_{R_4NX} \cdot K_{(R_4NCA)_s}}{K_{(R_4NXA)_s}}\right]\left[X^-\right]_m \qquad \text{(Eq. 1)}$$

where: k' is the capacity factor for a determined analyte.

$[R_4N^+]_m$ $[C^-]_m$ and $[X^-]_m$ are the concentrations for the mobile phase of quaternary ammonium salt, of coanion and of analyte anion, respectively. "q" is the ratio of stationary phase volume to mobile phase volume. K_o is the sorption capacity for the stationary phase and a measure of the number of sites that can be occupied in the retention process.

$K_{(R_4NXA)_s}$ and $K_{(R_4NCA)_s}$ are the retention equilibrium constants of R_4NX and R_4NC on the stationary phase. X represents the analytic anion and C the R_4N^+ coanion.

K_{R_4NX} is the equilibrium constant for the anion exchange:

$$(R_4NCA)_s + [X^-]_m \rightleftharpoons (R_4NXA)_s + [C^-]_m$$

We have experimentally found (Fig. 1) that on increasing R_4N^+ concentration the values of the capacity factors of the analyte anion also increase, reaching an optimum value from which they begin to increase. Equation 1 describes this phenomenon at least qualitatively. In fact, on increasing R_4N^+, its coanion C^- concentration also increases at a same ratio. The coanion exerts a competitive action with the analyte anion adsorption, expressed in Equation 1 by the second number to be added. On increasing the coanion concentration, the second number to be added predominates on the first one in Equation 1; this justifies the presence of a maximum in Fig. 1

Effect of R_4N^+ coanion

Few works have studied the effect of the nature of anions in solution or accompanying the R_4N^+ salt on the retention of the organic anionic analytes.

Table 1 shows k´ values determined by amoxicillin and clavulanic acid retention on PRP-1 column in the presence of tetrabu-

TABLE 1

Effect of Tetrabutylammonium Coanions on Amoxicillin and Sodium Clavulanate Retention on PRP-1 Column.

	Capacity factors k′							
	$Butyr^-$	$Prop^-$	$Acet^-$	$Form^-$	ClO_4^-	$SO_4^=$	Cl^-	Br^-
Clavulanate	3.5	5.1	12.4	6.4	0.54	3.6	4.7	5.7
Amoxicillin	2.7	7.0	34.8	14.9	0.81	3.4	4.8	3.6

tylammonium and different types of coanions, at an ionic strength of 0.01 completed with the sodium salt of the corresponding coanion, always at pH between 6 and 7. At this pH, both clavulanic acid and amoxicillin are in the anion form.

As it can be seen in Table 1, the nature of coanions exerts a remarkable effect on the clavulanate and amoxicillin anion retention. However, this effect is not the same on amoxicillin as on clavulanic acid. The order of the effect of different coanions, from maximum to minimum retention for amoxicillin is as follows:

$$Ac^- > Form^- > Prop^- > Cl^- > Br^- > SO_4^= > Butyr^- > ClO_4^-$$

while the order for clavulanate is:

$$Ac^- > Form^- > Br^- > Prop^- > Cl^- > SO_4^= > Butyr^- > ClO_4^-$$

Given the composition of the mobile phase used, it is probable that the phenomenon which rules the clavulanate ion retention will be an anion exchange. However, we must stand out that the experimental verification of Eq. 1 does not prove that the process is necessarily an anion exchange, since Equation 1 can also be derived where only ion pairs formation between R_4N^+ and the different anion is considered. In the latter case, the

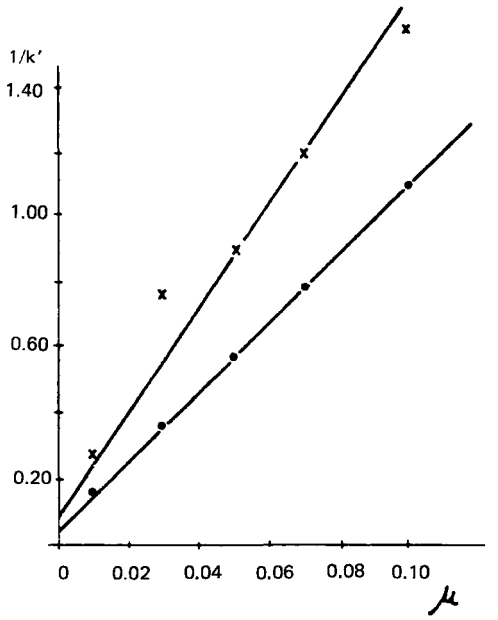

Figure 2. Effect of ionic strength on amoxicillin (x) and clavulanate ion (•) retention. Column: PRP-1. Mobile phase: (1-9 v/v.) acetonitryle 1.0 mM phosphate buffer pH 6, 3.0 mM TBAB and variable ionic strength (NaBr).

equilibrium constant in Equation 1 would be the ion pairs constants. We think ion pairs formation will probably play a more important role for mobile phases having a high organic solvent/water ratio, which does not occur in our case.

Effect of ionic strength

The effect of ionic strength in mobile phase, on clavulanic acid and amoxicillin retention on PRP-1 column, with a mobile phase formed by 0.001 M phosphate buffer, pH 6-acetonitryle (9-1 v/v) which is at the same time 0.03 M in tetrabutylammonium bromide at an ionic strength between 0.01 and 0.1 set with NaBr,

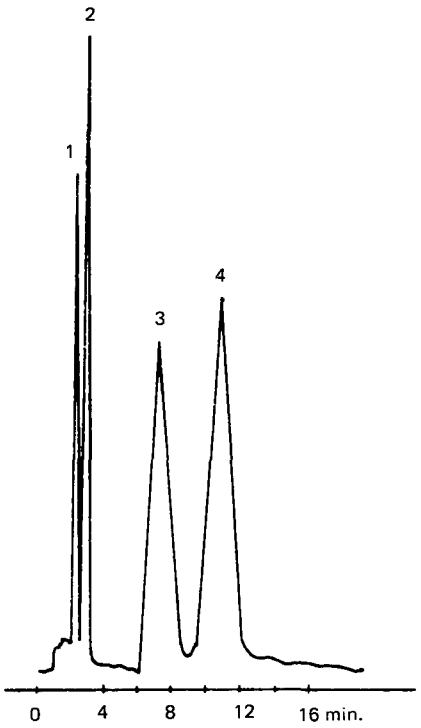

Figure 3. Column, 150 x 4.1 mm i.d., 10 μm PRP-1. The mobile phase conditions are 1-9 acetonitryle-1.0-mM phosphate buffer pH 6, 3.0 mM TBAB, μ= 0.05 (NaBr). Flow rate, 1.0 ml/min U.V. detector at 230 nm. 1 Amoxicillin, 2 Sodium clavulanate, 3 Cephalexin, 4 Sodium Ampicillin.

is shown in Fig. 2. As the ionic strength increases, $1/k'$ also increases, resulting in a linear ratio as can be predicted from Equation 1.

All the experimental results seem to suggest that clavulanic acid and amoxicillin retention on PRP-1 column, in the presence of tetrabutylammonium salt, is the results of a complex set of equilibrium, taking place within a double layer at

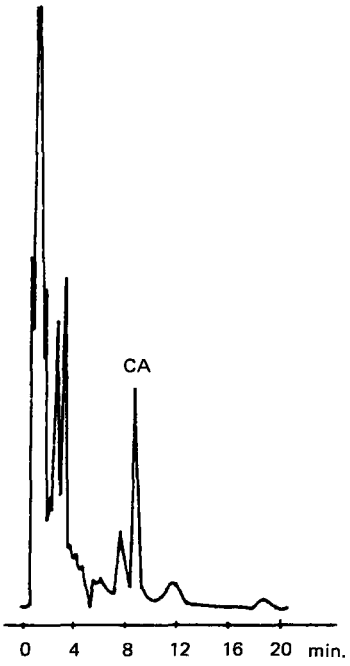

Figure 4. Column, 150 x 4.1 mm i.d., 10 μm PRP-1. The mobile phase conditions are 1-9 acetonitryle-1.0-mM phosphate buffer pH 6, 3.0 mM TBAB. Flow rate, 1.0 ml/min. Sample: Streptomyces clavuligerus ATCC 27064 fermentation broth.

the stationary phase surface. The coanion and/or anion of the buffer would also contribute on the second layer and would be a part of a competitive equilibrium.

Applications

The above shown may be applied to the chromatographic separation of clavulanic acid in different mixtures. Thus, for example, Fig. 3 illustrates the separation of a mixture of clavulanic acid, amoxicillin, cephalexin, and ampicillin using a PRP-1 column and a mobile phase formed by (1-9 v/v) aceto-

nitryle-10 mM phosphate, pH 6, in the presence of 3.0 mM TBAB and μ = 0.05 (NaBr).

Fig. 4 shows an example of clavulanic acid separation in a Streptomyces clavuligerus ATCC 27064 fermentation broth, using a mobile phase (1-9 v/v) acetonitryle-1.0 mM phosphate solution, pH 6, containing 3.0 mM TBAB.

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AN HPLC METHOD FOR MEASURING 5-FLUOROURACIL IN PLASMA

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ABSTRACT

5-Fluorouracil in plasma was determined by extraction with methyl isobutyl ketone, evaporation of the ketone, and reverse phase high performance liquid chromatography of the evaporation residue. With UV detection at 280 nm the lower limit of detection is 10.0 ng/ml and interfering peaks eliminated. The method is highly reproducible.

INTRODUCTION

5-Fluorouracil (5-FU) is a frequently used chemotherapeutic agent in many cancer treatment protocols. Despite frequent use and twenty years of opportunity to study this drug, the optimal dose and method of administration are uncertain. Part of this uncertainty is due to its rapid clearance ($T_{\frac{1}{2}}$ is 5-15 minutes) from the body which decreases the time cancer cells are exposed to the drug. Blood level measurements and therefore pharmacokinetic studies have been difficult to obtain and are highly variable since very small amounts of drug are present. Recent use of a continuous intravascular infusion of this drug has necessitated the development of a sensitive and accurate method for

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analyzing 5-FU in nanogram per milliliter concentrations in plasma samples. The method described here has a lower limit of detection of 10 ng/ml and is reproducible and accurate. No interfering peaks in the chromatogram have been detected.

MATERIALS AND METHODS

Instrumentation

A Waters Associates (Milford, Mass.) HPLC system was used consisting of a model WISP 710B autoinjector, a model 6000M solvent delivery system, a model 440 wavelength detector set at 280 nm and .005 aufs, a model 720 data module, a column heater set at 39°C, and a Waters model 730 system controller.

The column (Alltech) was 25 cm reverse phase C_{18}, 10 μ particle size. A 3 cm pellicular C_{18} guard column was also used. The mobile phase was .05M NaH_2PO_4 buffer to which 3.2 g/l of tetrabutylammonium hydroxide (Aldrich) was added for a pH of 6.2. The column and guard column were protected from microrganisms and fine particles with a 2 micron inline filter. Flow rate was 1 ml/min (600 psi av.) for the first 10 minutes then increased to 3 ml/min (1200 psi av.) for the next 25 minutes. The retention time for 5-FU averaged 5.40 minutes, $k' = 0.80$. Column life averaged 120 injections.

Calibrations

Calibration curves were prepared by diluting a stock solution of 5.0 μg/ml 5-FU (Aldrich Chemical Co., Milwaukee, Wisconsin) from 12 ng/ml to 100 ng/ml in water. The stock solution was also added to pooled plasma to achieve final concentrations of 25 ng/ml and carried through the entire procedure to produce another linear calibration curve with intercepts at or near zero. Linear water and pooled plasma calibration curves were obtained for each set of patient plasma samples analyzed. The ratio of the slope of the plasma calibration curve to the slope of the water calibration curve gave the efficiency of the extraction.

Procedure

Patient blood samples were drawn from a peripheral vein into a 7 ml Vacutainer tube (Becton-Dickinson) containing sodium heparin. This was centrifuged at 3500 RPM for 5 minutes, and the plasma was removed and frozen until the analysis was performed.

Methyl isobutyl ketone (5 ml) and 6N HCl (2 drops) were added to 1 ml plasma, vortexed for 30 seconds and centrifuged at 3500 RPM for 5 minutes. One further extraction of the plasma with methyl isobutyl ketone (5 ml) was performed and the organic phase combined and evaporated at 40-45°C under a stream of nitrogen. The residue was dissolved in 200 µl of mobile phase, centrifuged at 3500 RPM for 5 minutes and the supernatant transferred to 1.5 ml polypropylene microfuge tubes which were centrifuged at 12,000 RPM for one hour at 5°C. 25 µl of this supernatant was injected into the HPLC apparatus.

RESULTS

A typical chromatogram of plasma alone and with 95 ng/ml of 5-FU added is shown in Figure 1. No interfering peaks have been identified in a large number of patients with varying disease processes while most were receiving multiple other drugs.

The relationship of peak height to amount of 5-FU injected in water and extracted from plasma is shown in Figure 2. The relationship is linear over the range 6×10^{-10} gm/ml to 2×10^{-5} gm/ml with typical correlation coefficients of 0.999 for 5-FU in water and 0.997 for 5-FU in plasma. The extraction efficiency of the procedure averaged 49.3% $\pm$4.8 (SD) over a period of several weeks. Five separate extractions of the same plasma sample on one day gave an average value 70.3 $\pm$ 2.45 (SD) ng/ml 5-FU for a coefficient of variation of 3.5%. Ninety-six injections of the same water standard gave an average peak height of 551.9 $\pm$ 16.6 mm for a coeffieient of variation of 3.0%.

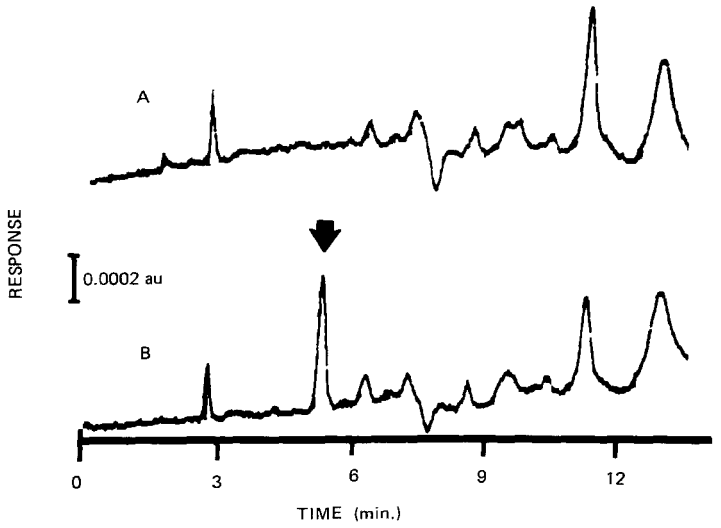

Figure 1A. A typical chromatogram of human plasma from time of injection to 14 min at 0.005 aufs.
B. The same plasma sample to which 95 ng/ml 5-FU (arrow) had been added. Retention time is 5.37 min.

DISCUSSION

Plasma 5-fluorouracil has been determined by gas (1,2,3,4,5) and liquid chromatography. (6,7) The liquid chromatography methods have required a separate clean-up procedure to attain a sensitivity of 100 ng/ml (7) or require a variable wavelength detector.(6) Our method achieves a lower limit of detection of 10 ng/ml while using the commonly available 280 nm detector.

The methyl isobutyl ketone extraction removes interfering peaks from the chromatogram and concentrates the sample for higher sensitivity. The evaporation step caused a loss of less than 10% of the 5-FU from the original sample, but 40% at the 5-FU was not extracted from the plasma. This loss however, has been consistent between samples and between days, and is measured for each batch of plasma samples processed and is not considered a major

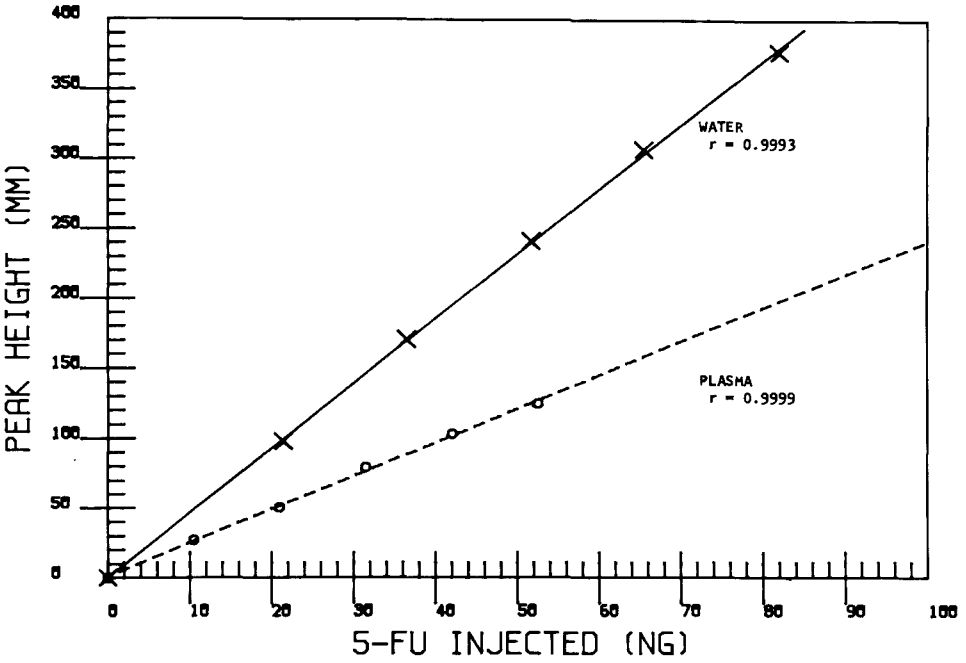

Figure 2. 5-FU was added to water and injected directly into the HPLC. Similar amounts of 5-FU were added to plasma which was then extracted and injected into the HPLC. "r" is the correlation coefficient for the linear regression equations.

disadvantage. Adherence of 5-FU to glass has been reported (1), but this was not detected in a series of experiments conducted by us.

The mobile phase chosen in the HPLC step contains tetra-n-butyl-ammonium ion. Without this ion, resolution of the 5-FU peak from nearby peaks was poor.

When a 254 nm detector was used, 5-FU absorbance was higher than at 280 nm, but resolution from a nearby peak was difficult. At 280 nm the source of this nearby peak had little or no absorbance and, consequently, the interference disappeared.

The method does not employ an internal standard. When 5-bromouracil was used as a standard its retention frequently coincided with one or more

plasma peaks. Therefore, a separate set of plasma standards was used giving a new calibration curve for each series of patient plasma samples analyzed.

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DETERMINATION OF LIPOPHILIC CHARACTER OF A SERIES OF DERMORPHIN-RELATED OLIGOPEPTIDES BY MEANS OF REVERSED-PHASE HPLC

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In recent times there has been a growing interest in the determination of chromatographic parameters of lipophilicity with regard to their use in the study of quantitative structure-activity relationship (1, 2). Very good correlations had been shown between the chromatographic parameters and the log P or π values as a measure of the partition coefficient between octanol and water (2). The reversed phase TLC R_m values in two different chromatographic systems and the reversed-phase HPLC log k' values of a series of dermorphin-related oligopeptides have been previously determined (3, 4). The purpose of the present work was to study the relationship between log k' values on one hand and R_m or $\Sigma\pi$ values on the other one in view of QSAR studies. In fact the discovery of enkephalin and endorphins with high affinities for opioid receptors added new dimensions to the study of structure-activity relationship of opioid agonists (5, 6, 7, 8).

MATERIALS and METHODS

The test compounds are reported in Table 1 (9, 10). The HOPL reversed-phase chromatography was performed on a Waters 6000 A chromatograph using a μ Bondapak C_{18} column (300 x 3.9 mm I.D.) (Waters), packed with Silica Gel (particle size 10 μm) with a C_{18} chemically bonded non-polar stationary phase. A UV detector (Waters Model 480) at 214 μm and Hamilton 802 chromatographic syringes (25 μl) were also used. the dermorphin derivatives were separated using methanol-water mixtures as the mobile phase at a flow-rate of 1 ml/min. The methanol concentration ranged from to 80%. Samples were dissolved in methanol (1 mg/ml) and applied to the column in 5 μl volumes. The solutions were first filtered to reduce contamination. The experiments were performed at room temperature (20-22 °C). The retention times were expressed as log capacity factor (k') were $k' = \frac{Tx - To}{To}$

The R_m values had been previously determined by means of a reversed-phase TLC technique (4). A stationary non-polar phase was obtained by impregnating a Silica Gel g layer with silicone oil DC 200 (350 cS) from Applied Science Laboratories. The polar mobile phase

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saturated with silicone oil was an aqueous buffer (sodium acetate-veronal buffer 1/7 M at pH 7.0) alone or mixed with various quantities of methanol or acetone.
The $\Sigma\pi$ values have been calculated from the data of Hansch et al. (11) and Fauchére et al. (12).

RESULTS and DISCUSSION

The linear relationship between the TLC R_m values and the composition of the mobile phase has been previously described as a condition which allows to calculate on extrapolated R_m value at 0% of organic solvent in the mobile phase (13, 14, 15). The extrapolated R_m values should be an expression of the partitioning of compounds between silicone oil and water, i.e. in a standard system where all the compounds could be compared. The linear relationship has been described in liquid-liquid partition chromatography (16) and in reversed-phase HPLC (17). In particular it was pointed out that the extrapolated hydrophobic parameters are practically independent of the organic solvent in the mobile phase (17, 4). The plots of Fig. 1 show a linear relationship between the log k' values and the methanol concentration in the mobile phase. The equations describing such linear relationship yielded the log k' values at 0% reported in Table 1 are also reported the R_m values from the two TLC systems previously described (4), as well as the $\Sigma\pi$ values. The $R_{m\ MeOH}$ and $R_{m\ Me_2CO}$ values are the intercepts of the equations describing the linear relationship between the R_m values and the concentration of methanol and acetone respectively in the mobile phase (4). A very good correlation is shown by both eq. 1 and 2 between the R_m values from the two TLC HPLC systems and the log k' values from the HPLC system.

	n	r	s	
$\log k' = 0.553 + 1.117\ R_{m\ MeOH}$	23	0.922	0.464	(1)
($F=119.18$; $P<0.005$)				
$\log k' = 0.456 + 1.238\ R_{m\ Me_2CO}$	23	0.921	0.466	(2)
($F=118.00$; $P<0.005$)				

In both equations the slopes are rather close to 1 and therefore the TLC and HPLC systems are similar in measuring the lipophilic character of the present series of compounds. It can be pointed out that the log k' values range from 0.88 to 5.24 with a difference of 4.36 which is much larger than that shown by the R_m values in both TLC systems.
In fact while the R_m values in the methanol system range from 0.94 to 4.06 with a difference of 3.12, in the acetone system they range from 0.92 to 4.19 with a difference of 3.27. This should be an advantage of the HPLC data over the R_m values in QSAR studies.
In Table 1 are also reported the $\Sigma\pi$ values as calculated from the data of Hansch (11) and Fauchére (12). In a previous paper a highly significant relationship has been shown between the R_m and the $\Sigma\pi$ values (4). As regards the present data eq. 3 shows a rather lower correlation coefficient between log k' and $\Sigma\pi$ values.

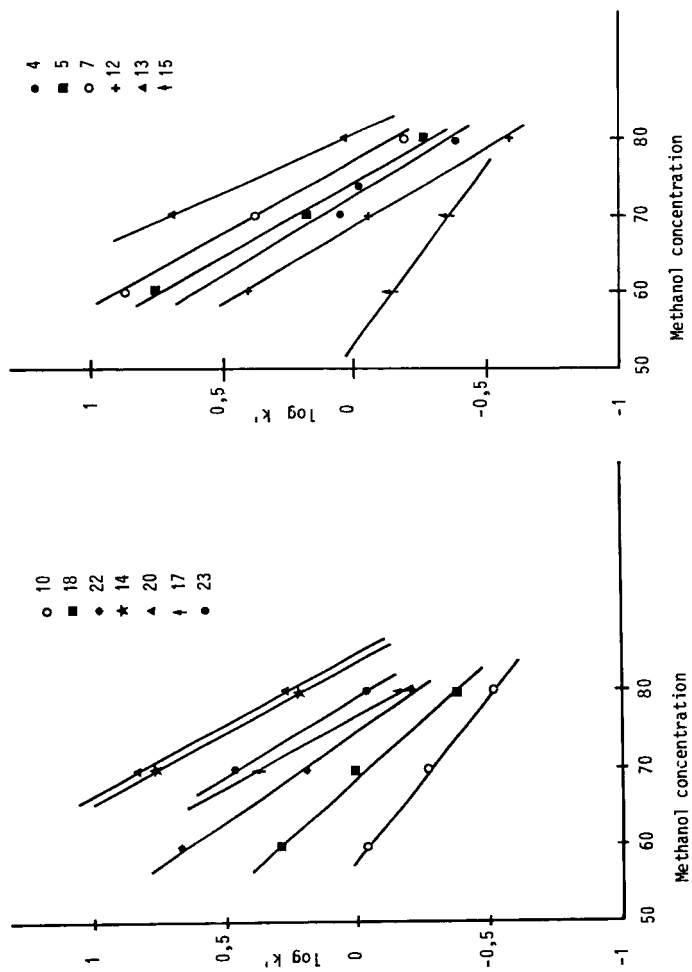
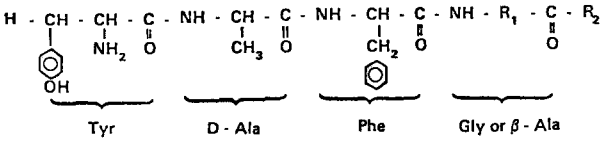
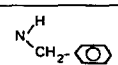
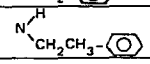
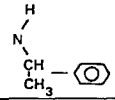
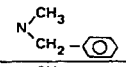
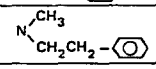
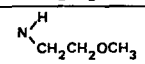
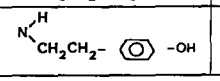


Fig. 1. Relationship between log k' values and methanol concentration in the mobile phase.

Table 1 - log k' R_m and $\Sigma\pi$ values of dermorphin-related oligopeptides



Cpd. No.	R_1	R_2	Log k'	R_m Methanol	R_m Acetone	$\Sigma\pi$
1	CH_2		2.35	1.36	1.28	-1.23
2	CH_2		2.86	1.41	1.44	0.08
3	CH_2		2.69	1.95	1.94	1.18
4	CH_2		3.56	2.24	2.17	0.78
5	CH_2		3.87	3.09	2.56	1.34
6	CH_2		3.48	3.07	2.75	1.34
7	CH_2		4.03	2.93	2.69	1.34
8	CH_2		3.57	2.99	2.73	1.90
9	CH_2		3.20	2.30	2.23	0.91
10	CH_2		1.40	1.42	1.34	-0.59
11	CH_2		1.77	1.56	1.41	0.06
12	CH_2		3.43	1.93	2.17	0.67

(continued)

Table 1 (continued)

Cpd. No.	R_1	R_2	log k'	R_m Methanol	R_m Acetone	$\Sigma\pi$
13	CH_2		5.24	3.73	3.62	2.14
14	CH_2		4.61	4.01	4.19	2.70
15	CH_2	OH	1.18	0.94	0.92	-0.67
16	CH_2	O-CH_2CH_3	2.26	1.45	1.50	0.38
17	CH_2		4.30	3.13	2.59	1.66
18	$(CH_2)_2$		2.35	1.36	1.31	-0.95
19	$(CH_2)_2$		3.99	3.14	2.62	1.62
20	$(CH_2)_2$		4.82	4.06	3.54	2.42
21	$(CH_2)_2$	OH	0.88	1.03	0.96	-0.39
22	$(CH_2)_2$	O-CH_2CH_3	3.30	1.91	1.87	0.66
23	$(CH_2)_2$		4.04	3.09	2.74	1.94

	n	r	s	
$\log k' = 2.407 + 0.924\ \Sigma\pi$	23	0.871	0.390	(3)

($F=65.86$; $P<0.005$)

However the slope is close to 1 which should indicate that the HPLC system is very similar to the π system in measuring the lipophilic character of the present series of compounds. The range of the log k' values is still wider than that of the $\Sigma\pi$ values which range from -1.23 to 2.70 with a difference of 3.93.

On the other hand it has been pointed out that the range of the $\Sigma\pi$ values is wider than that of the R_m values in both TLC systems (4). In particular it was pointed out that this was mainly due to the $\Sigma\pi$ values for compounds 1 and 18 which are characterized by an amino group. In the π system the amino group seemed to be more hydrophilic than in the TLC systems. A similar result seems to arise from the present HPLC data. In fact cpds 1 and 18 are among those showing the largest deviations from the linear regression described by eq. 3, in the π system being more hydrophilic than in the HPLC one. When eq. 4 was calculated without cpds 1 and 18 a higher correlation coefficient was obtained.

	n	r	s	
$\log k' = 2.095 + 1.140\ \Sigma\pi$	21	0.920	0.433	(4)

($F=104.21$; $P<0.005$)

The slope still close to 1 confirms the similarity of the two systems in measuring the lipophilic character.

In conclusion the log k' values are very well correlated on one hand with the TLC R_m values and on the other one with the calculated $\Sigma\pi$ values. This should show their reliability as an expression of the lipophilic character of drugs in QSAR studies. An advantage of the HPLC procedure at least for the present series of dermorphin-related oligopeptides might be represented by the wider range of the log k' values which should allow better correlation studies with biological data.

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LC CALENDAR

1984

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/R801-106, 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 eStreet, 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.

FEBRUARY 25 - MARCH 1: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, New Orleans, LA. Contact: Paul E. Bauer, 1985 Pittsburgh Conference, 437 Donald Rd., Dept. FP, Pittsburgh, PA, USA.

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., or Prof. S. Ebel, Institute for Pharmacies, A. M. Hubland, D-8700 Wuerzburg, West Germany.

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 1-6: 6th International Symposium on Bioaffinity Chromatography & Related Techniques, Prague, Czechoslovakia. Contact: Dr. J. Turkova, Institute of Organic Chemistry & Biochemistry CSAV, Flemingovo n. 2, CS-166 10 Prague 6, Czechoslovakia.

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

SEPTEMBER 21-26: XVIth International Symposium on Chromatography, Paris, France. Contact: G.A.M.S., 88,Boulevard Malesherbes, F-75008 Paris, France.

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.

The Journal of Liquid Chromatography will publish announcements of interest to liquid chromatographers in every issue of the Journal. To be listed in the LC Calendar, we will need to know: Name of the meeting or symposium, sponsoring organization, when and where it will be held, and whom to contact for additional details. You are invited to send announcements to Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P. O. Box 1440-SMS, Fairfield, CT, 06430, USA.

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LC NEWS

AUTOMATED PREPARATIVE-SCALE HPLC INSTRUMENT utilizes axial compression column technology to produce high efficiency columns with all rigid stationary phases. The column is prepared just before using by axially compressing a slurry of the packing to produce a uniform chromatographic nbed in which deleterious wall effects and diffusional band spreading are all but eliminated. User-friendly automation features include sample injection, fraction collection, recycle, column conditioning, safety alarms, step gradients, etc. Elf Aquitaine Development Corp., JLC/84/7, P. O. Box 1678-Murray Hill Station, New York, NY, 10157, USA.

HPLC INJECTOR STATION for the Laboratory Automation System combines HPLC sample introduction with automated sample preparation procedures. Samples are automatically prepared by the system and then directly introduced to the HPLC via either syringe injection or aspiration from a sample tube. Zymark, Corp., JLC/84/7, Zymark Center, Hopkinton, MA, 01748, USA.

pH GRADIENT CONTROLLER/PROGRAMMER uses a discontinuous, non-linear approach for feedback control, which is ideally suited to pH control, both batch and continuous. Varying of plug-in range resistor (potentiometer) permits stepwise or continuous programming of pH. Luft Instruments, Inc., JLC/84/7, Old Winter Street, Lincoln, MA, 01773, USA.

IMPROVED HPLC SEPARATIONS with ion pair reagents for ionic solutes without the use of ion exchange columns. Ion pair reagents modify the mobile phase to effectively attenuate or enhance solute retention, improve peak symmetry and control selectivity. Both cationic and anionic species are available, as well as perfluoroalkanoic acids and triethylamine. Pierce Chemical Co., JLC/84/7, P. O. Box 117, Rockford, IL, 61105, USA.

TLC STICK is a solid, elongated block of silica gel designed for use in a wide variety of separations. It can be spotted with a maximum of 300mg or 20ml of sample...1,000 to 10,000 times more sample than other related methods. Recovery is achieved by slicing the desi"ed band from the stick and extracting with an appropriate solvent. Alltech Associates, Inc., Applied Science Labs, JLC/84/7, 2051 Waukegan Rd., Waukegan, IL, 60031, USA.

HPLC COLUMN BLOCK HEATERS are available for precise control of column temperature to improve sample loading, baseline stability, separation efficiency, and resolution. Phenomenex, Inc., JLC/84/7, 426 Via Corta, Bldg. 305, Palos Verdes Estates, CA, 90274, USA.

MAXIMIZING HPLC PRECISION BY SKILLFUL INJECTION is a report that can help both the beginner and the experienced chromatographer. Some very practical questions are addressed: "How much sample must be wasted to fill a sample loop?"; "When you are judging the amount of sample being loaded by reading a syringe, how much sample can you inject without impairing accuracy?" Ask for Tech Note No. 5. Rheodyne, Inc., JLC/84/7, P. O. Box 996, Cotati, CA, 94928, USA.

COMPUTING INTEGRATOR for re-integrating, re-plotting, recalculating features 32K RAM peak storage. It offers 6 external event controls, autosampler interface, choice of thermal and impact printer plotters, IEEE interface, 100 data slices per second programmable sampling rate. Applied Chromatography Systems (USA), Inc., JLC/84/7, Suite 125, 315 S. Allen Street, State College, PA, 16801, USA.

MINIATURE INERT VALVES, fittings and controllers include 2, 3, 4, 6, and 8-port variations, large or small port, ON-OFF selection, distribution, sampling, and other flow directional control applications. Hamilton Co., JLC/84/7, P. O. Box 10030, Reno, NV, 89520, USA.

HPLC DETECTOR RESOLVES EVEN HIDDEN COMPOUNDS by monitoring at two different user-selectable wavelengths. Thus, sample comparisons can be made on the basis of absorbance, absorbance ratios, sums and differences within a single sample cell. The detector uses a deuterium lamp, a single high pressure flow cell (up to 4,000 psi, variable angle concave grating, two photodiode pairs, and measures between 190 and 750 nm. Micromeritics Instrument Corp., JLC/84/7, 5680 Goshen Springs Road, Norcross, GA, 30093, USA.

ACETYLCHOLINE ANALYZER utilizes a rapid reverse phase separation of acetylcholine and choline, and a post-column enzymatic reactor to achieve detection limits of 2 pmol or better. Detection is based upon the electrochemical oxidation of hydrogen peroxide realeased in the enzymatic reaction using a platinum electrode. Bioanalytical Systems, Inc., JLC/84/7, 2701 Kent Avenue, West Lafayette, IN, 47906, USA.

PORTABLE PIPETTING AID is rechargeable and can give over two hours of continuous operation with one charge. It will accept all standard glass and plastic pipettes, including Pasteur, serological, and transfer types. Sarstedt, Inc., JLC/84/7, P. O. Box 4090, Princeton, NJ, 08540, USA.

THE FILTER BOOK is a handy reference tool for filtration users. It contains informatoon on how to select the right filter for any

labortory filtration application. Gelman Sciences, Inc., JLC/84/7, 600 S. Wagner Road, Ann Arbor, MI, 48106, USA.

VARIABLE WAVELENGTH CHROMATOGRAPHY DETECTOR has a master-blazed holographic grating which virtually eliminates stray light and provides high accuracy in absorbance measurements, with an 8 microliter cell. Pye Unican Ltd., JLC/84/7, York Street, Cambridge CB1 2PX, England.

GPC/HPLC FLOWMETER measures liquid flow rates from 0.1 to 100 cc/min with accuracies up to 0.1%. The instrument uses a thermal pulse time of flight technique that is independent of changes in the properties of the solvent or ambient temperature. Two glass-encapsulated thermistors protrude into the liquid stream about 1" apart. A heat pulse, generated by the upstream thermistor, moves downstream where it is detected by the second thermistor. The time required for the pulse to reach the downstream thermister is a function of the liquid flow rate. Molytek, Inc., JLC/84/7, 2419 Smallman Street, Pittsburgh, PA, 15222, USA.

POLYACRYLAMIDE RIGID GEL FOR SEC/GPC may be operated at high pressures and undergoes minimum swelling and shrinkage when transferred between polar eluents. Applications include separations of polysaccharides, polyphenols, and synthetic aqueous polymers. Polymer Laboratories, JLC/84/7, Essex Road, Church Stretton, Shropshire, SY6 6AX, UK.

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F. D. Pierce and H. R. Brown
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2. In general, the chapter title and the abstract, as well as the tables and references, are typed single-spaced. All other text discussion should be typed 1½-line spaced, if available, or double-spaced. Prestige elite characters (12 per inch) are recommended, if available.

3. It is essential to use black typewriter ribbon (carbon film is preferred) in good condition so that a clean, clear impression of the letters is obtained. Erasure marks, smudges, creases, etc., may result in return of the manuscript to the authors for retyping.

4. Tables should be typed as part of the text but in such a way as to separate them from the text by a three-line space at both top and bottom of each table. Tables should be inserted in the text as close to the point of reference as possible, but authors must make sure that one table does not run over to the next page, that is, no table may exceed one page. The word TABLE (capitalized and followed by an Arabic number) should precede the table and be centered on the page. The table title should have the first letters of all main words in capitals. Titles should be typed single-spaced. Use the full width of the type page for the table title.

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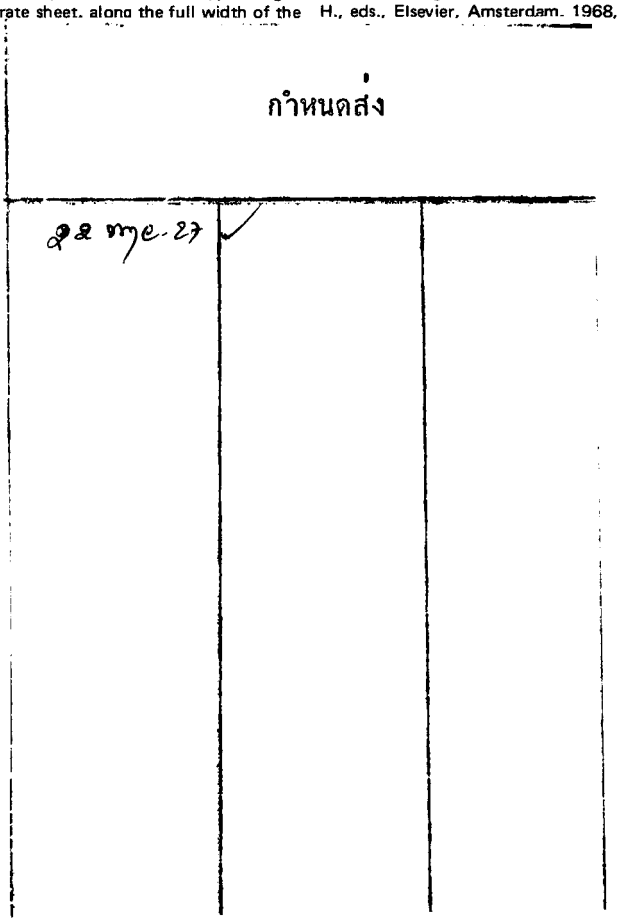

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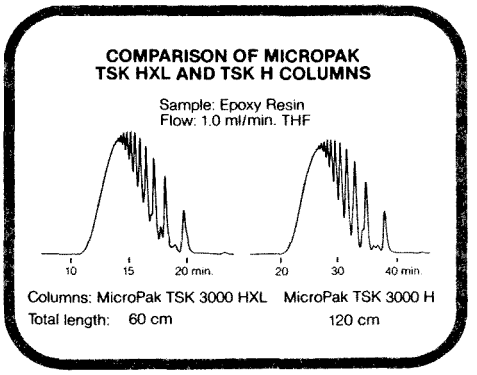

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SPECIFICATIONS	
Column Dimensions	250mm x 7.8mm
Packing Material	10um Styrene DVB
End Fittings	Parker-Hannefin (comp. screw)
Porosities	40Å-10^7Å, MXL, MXM, MXH, Linear
Efficiency (p/m)	>30,000
Max. Flow Rate	3.0ml/min
Temp. Range	ambient-140°C
Warranty	**120 days** (unconditonal)

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ULTRAGEL 10^4Å	20,000 - 300,000	545.	445.
ULTRAGEL 10^5Å	100,000 - 1,000,000	545.	445.
ULTRAGEL 10^6Å	500,000 - 10,000,000	545.	445.
ULTRAGEL 10^7Å	1,000,000 - >20,000,000	645.	545.
ULTRAGEL MXL	0 - 40,000	595.	495.
ULTRAGEL MXM	5,000 - 500,000	595.	495.
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