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PHYSICAL SCIENCES DATA 2

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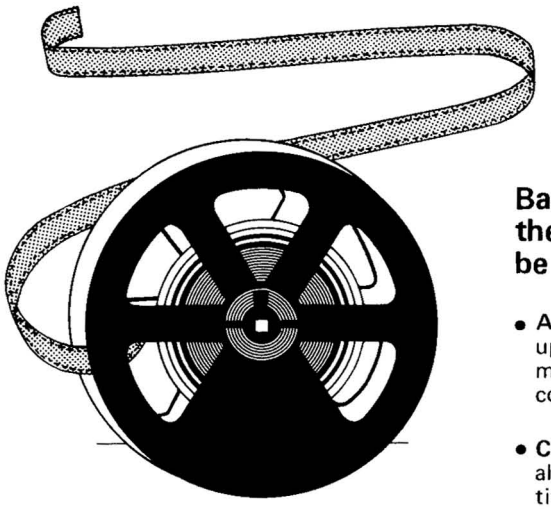

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LIQUID CRYSTALS

I. SYNTHESIS AND APPLICATION AS STATIONARY PHASES IN GAS–LIQUID CHROMATOGRAPHY*

K. P. NAIKWADI, D. G. PANSE, B. V. BAPAT and B. B. GHATGE*
National Chemical Laboratory, Poona-8 (India)

(First received January 25th, 1980; revised manuscript received March 3rd, 1980)

SUMMARY

2-R_1-4′-R-4-(4-*n*-alkoxybenzoyloxy)azobenzenes, where R = *n*-butyl, methyl and methoxy, R_1 = H and methyl and *n*-alkoxy = methoxy, ethoxy and *n*-butoxy were synthesized. They have long liquid crystalline "nematic" ranges and were used as stationary phases for the separation of positional isomers of di- and trisubstituted benzene. It was observed that lateral substitution (R_1) on the middle ring has a profound influence on the relative retentions of these compounds. The separation of mixtures of free phenolic isomers such as *m*- and *p*-cresols was difficult, whereas the separation of α- and β-naphthols could be easily achieved. Similarly, the separation of mixtures of free bases such as toluidines or picolines was difficult, but napthylamines were separated with great ease. Also, the complete separation of all positional isomers of monochlorobiphenyl was achieved by using these liquid crystalline substrates.

INTRODUCTION

The first application of liquid crystals as stationary phases in gas–liquid chromatography (GLC) was described in 1963[1], and subsequent papers have been reviewed by Kelker and Von Schivizhoffen[2] and Schroeder[3]. Recently several reports on the separation of the alkylnaphthalenes[4], polycyclic aromatic hydrocarbons and their derivatives[5–8], phenol ethers[9], disubstituted benzenes[10,11] and high-boiling hydrocarbons[12] using liquid crystalline stationary phases have appeared. The synthesis and use as stationary phases of nematic compounds with $-\overset{\overset{O}{\|}}{C}-O-$, $-CH=N-$ and $-\overset{\overset{O}{\uparrow}}{N}=N-$ linkages have been described[3]. Recently, liquid crystals with $-\overset{\overset{O}{\|}}{C}-O-$ and $-\overset{\overset{O}{\uparrow}}{N}=N-$ linkages[12] have been investigated for their substrate behaviour.

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However, the use of liquid crystals with lateral substitution (R^1) as stationary phases in GLC has not previously been studied. Therefore, it was decided to explore the possibility of applying such laterally substituted liquid crystalline stationary phases in GLC. This paper dexcribes the synthesis of such laterally substituted liquid crystals and a study of the effect of lateral substitution on selectivity when they are used as stationary phases in GLC.

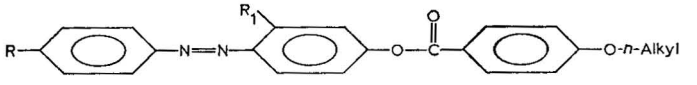
EXPERIMENTAL

Materials

Liquid crystalline compounds I–V were synthesized by known methods[13,14]. Compounds III, IV and V were synthesized by using *m*-cresol instead of phenol with *p*-substituted phenyldiazonium chloride. All of the compounds were purified to give constant transition temperatures. The structures and the transition temperatures of the compounds studied as stationary phases are given in Table I.

TABLE I

STRUCTURES AND TRANSITION TEMPERATURES OF THE COMPOUNDS STUDIED



Compound No.	*R*	R_1	*Alkyl*	*Transition temperatures (°C)**		*Nematic range (°C)*
				C–N	*N–I*	
I	OCH_3	H	$n\text{-}C_4H_9$	116	280	164
II	$n\text{-}C_4H_9$	H	$n\text{-}C_4H_9$	94	234	140
III	OCH_3	CH_3	C_2H_5	125	244	119
IV	CH_3	CH_3	C_2H_5	125	220	95
V	OCH_3	CH_3	CH_3	160	253	93

* C–N = crystal to nematic liquid crystal; N–I = nematic liquid crystal to amorphous isotropic liquid.

The solid support used was 80–120-mesh Celite. The Celite was coated with the liquid crystalline compounds by using benzene or ethanol as solvent followed by gradual elimination of the solvent by evaporation on hot water-bath. This coated Celite was dried in an oven at 80°C for 1 h and packed in aluminium column of 4.0 mm I.D. The column parameters are given in Table II.

Solutes

Individual positional isomer samples were GC pure; only metamethylanisole showed a detectable anisole impurity.

The oven temperatures and flow-rates of the carrier gas (hydrogen) are given in Table III.

Apparatus

An AIMIL dual-column chromatograph equipped with a thermal conductivity detector with hydrogen as the carrier gas was used to obtain retention

TABLE II

COLUMN PARAMETERS

Column No.	*Stationary phase*	*Length of column (m)*	*Total wt. of packing (g)*	*Amount of stationary phase used (wt.-%)*
1	I	2.00	10.220	10
2	II	2.00	9.700	10
3	III	1.74	7.900	10
4	IV	1.77	8.820	10
5	V	1.77	8.293	10
6	II	1.76	7.260	3
7	III	1.78	8.389	3

TABLE III

COLUMN TEMPERATURES AND CARRIER GAS FLOW-RATES

Parameter	*Column No.*												
	1			*2*		*3*		*4*		*5*		*6*	
Operating temperature (°C)	117	130	163	95	129	130	142	126	138	161	178	96	166
Flow-rate of hydrogen (ml/min)	30	29.7	23.1	37.5	30.7	27.2	26.6	42.8	36.3	32.8	25.5	40.0	37.0

times. The chromatograms shown in Figs. 1 and 2 were obtained on a Hewlett-Packard 700 chromatograph.

Procedure

Individual samples were injected with a 10-μl syringe using the smallest detectable sample volume. All columns were conditioned at 200°C for 6 h. The injector and detector temperatures were 150°C and 225°C, respectively, and retention times were measured from air peak maxima to sample peak maxima. The flow-rate of the carrier gas was measured using a soap-film flow meter.

RESULTS AND DISCUSSION

Table IV lists the measured retention times at various temperatures for a number of di- and tri-substituted benzene isomers. Table V gives relative retentions calculated from Table IV.

When the retention times at two different temperatures are observed on any column, it is found that with an increase in temperature, that is sufficiently higher than the crystal to nematic transition temperature, the selectivity of the stationary phase decreases.

Changes in the oven temperature have a profound influence on the nature of the stationary phase. These changes affect the nature of the column packing and the flow-rate of the carrier gas (see Table III).

TABLE IV

RETENTION TIMES OF SUBSTITUTED BENZENE ISOMERS IN COLUMNS WITH LIQUID CRYSTALLINE STATIONARY PHASES

Compound	*Column Nos. and operating temperatures (°C)*										
	1		*2*		*3*		*4*		*5*		*6*
	117	*130*	*95*	*129*	*130*	*142*	*126*	*138*	*161*	*178*	*96*
m-$CH_3C_6H_4CH_3$	1.48	1.12	2.34	1.37	0.73	—	0.97	—	—	—	0.78
p-$CH_3C_6H_4CH_3$	1.68	1.25	2.61	1.50	0.85	—	1.10	—	—	—	0.87
m-$ClC_6H_4CH_3$	3.25	2.38	5.01	2.83	1.67	1.36	2.12	1.70	0.75	—	1.82
p-$ClC_6H_4CH_3$	3.95	2.86	6.07	3.34	2.05	1.63	2.56	2.06	0.90	—	2.09
m-ClC_6H_4Cl	5.25	3.61	8.02	4.37	2.60	2.11	3.30	2.63	1.15	0.97	2.72
p-ClC_6H_4Cl	6.47	4.47	10.47	5.25	3.35	2.65	4.32	3.45	1.40	1.15	3.57
m-$BrC_6H_4CH_3$	6.20	4.41	10.50	5.28	3.02	2.39	3.88	3.08	1.31	1.12	3.42
p-$BrC_6H_4CH_3$	7.71	5.31	12.93	6.18	3.82	2.95	4.83	3.78	1.56	1.30	4.18
m-$CH_3C_6H_4OCH_3$	—	—	—	—	2.73	—	3.35	2.62	1.15	—	3.07
p-$CH_3C_6H_4OCH_3$	—	—	—	—	3.40	—	4.17	3.13	1.35	—	3.62
1,2,3-$Cl_3C_6H_3$	—	—	—	—	—	—	—	—	3.25	2.62	1.50**
1,2,4-$Cl_3C_6H_3$	—	—	—	—	—	—	—	—	4.20	3.31	1.83**
m-$ClC_6H_4NH_2$	7.20*	—	—	—	—	—	—	—	7.97	6.00	—
p-$ClC_6H_4NH_2$	8.37*	—	—	—	—	—	—	—	9.20	6.88	—

* Operating temperature 163°C.

** Operating temperature 166°C.

TABLE V

RELATIVE RETENTIONS (α) OF *p*-DISUBSTITUTED BENZENES (*m*-ISOMER = 1.000)

Compound	*Column Nos. and column temperatures (°C)*											*Maximum α-value in literature*
	1		*2*		*3*		*4*		*5*		*6*	
	117	*130*	*95*	*129*	*130*	*142*	*126*	*138*	*161*	*178*	*96*	
p-$CH_3C_6H_4CH_3$	1.135	1.116	1.115	1.09	1.169	—	1.128	—	—	—	1.115	1.16 (ref. 3)
p-$ClC_6H_4CH_3$	1.215	1.20	1.21	1.18	1.227	1.199	1.208	1.206	1.20	—	1.148	—
p-ClC_6H_4Cl	1.232	1.238	1.30	1.20	1.288	1.256	1.309	1.311	1.21	1.186	1.312	1.26 (ref. 3)
p-$BrC_6H_4CH_3$	1.244	1.204	1.21	1.17	1.265	1.234	1.245	1.227	1.200	1.161	1.223	—
p-$CH_3C_6H_4OCH_3$	—	—	—	—	1.246	—	1.245	1.195	1.174	—	1.180	—
1,2,4-$Cl_3C_6H_3$	—	—	—	—	—	—	—	—	1.292	1.263	1.22	—
p-$ClC_6H_4NH_2$	1.162	—	—	—	—	—	—	—	1.154	1.147	—	—

When the relative retention (α) for any sample on column 1 is compared with that on column 2, it is found that column 1 is more selective than column 2. This is in accordance with previous results[2,3] that compounds with a longer nematic range have a higher selectivity. The same trend of selectivity based on the nematic range is observed for most of the samples on columns 3–5.

Compounds III and IV have lateral substitution (R_1), whereas compounds I and II have not, which affects the relative retentions. Compounds I, II, III and IV have nematic ranges of 164°C, 140°C, 119°C and 95°C, respectively. Now, based on previous results, compound I and II are supposed to show a higher selectivity. However, compounds III and IV, with narrow nematic ranges, show a higher selectivity than compounds I and II for most of the samples (Table V). This observation leads us to conclude that a certain lateral substitution in a typical liquid crystalline compound may decrease the nematic range but may give a higher selectivity. These results encouraged us to synthesize liquid crystalline compounds with other lateral substitutions, the results of which will be published elsewhere.

The relative retentions (α) observed on our columns and those observed on other liquid crystals that were assumed to be more selective are given in last row of Table V. The compounds we have studied show higher α-values. Of the five stationary phases studied, column 3 (stationary phase III) gave the most promising results.

Changes in the percentage of stationary phase seem to have a negligible effect on relative retention; this can be seen for columns 2 and 6.

In order to avoid long retention times and broadening of the peaks, higher boiling compounds were separated on columns 6 and 7 with a low percentage of stationary phases. Retention times and relative retentions observed on column 6 are given in Table VI.

TABLE VI

RESULTS FOR HIGHER BOILING SAMPLES ON 3 WT-% STATIONARY PHASE II (COLUMN 6)

Compound	*Retention time at 166°C (min)*	*Relative retention (α)* *	*Literature value of α*
2-Chlorobiphenyl	6.55		
3-Chlorobiphenyl	10.92		
4-Chlorobiphenyl	15.33	1.404	1.27 (ref. 15)
α-Naphthylamine	16.47		
β-Naphthylamine	21.02	1.277	
α-Naphthol	16.42		
β-Naphthol	21.50	1.309	1.30 (ref. 16)

* Relative to 3- or α-isomer = 1.00.

Separation of monochlorobiphenyls

The separation of chlorinated biphenyls is important owing to their toxicological properties. A good separation of monochlorobiphenyl isomers is achieved on the liquid crystalline stationary phases (Fig. 1). Stationary phase II (column 6) gives higher relative retentions (α) for monochlorobiphenyl isomers than those in the literature[15,17,18].

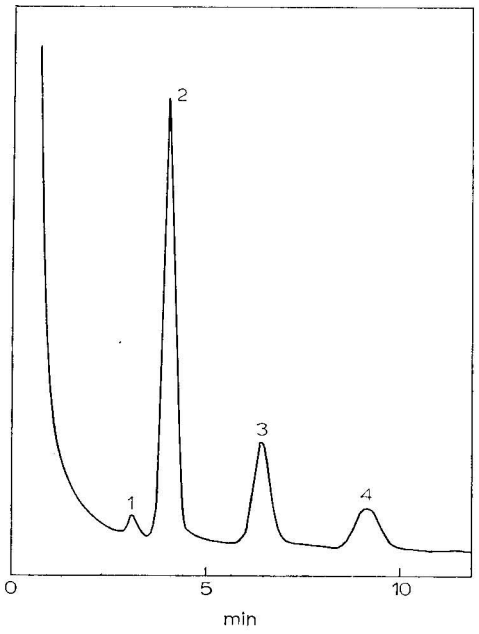


Fig. 1. Gas chromatogram of a mixture of monochlorobiphenyls on column 7. Oven temperature, 190°C; injector temperature, 240°C; detector temperature, 290°C; flame-ionization detector; nitrogen flow-rate, 40 ml/min. Peaks: 1 = biphenyl; 2 = 2-chlorobiphenyl; 3 = 3-chlorobiphenyl; 4 = 4-chlorobiphenyl.

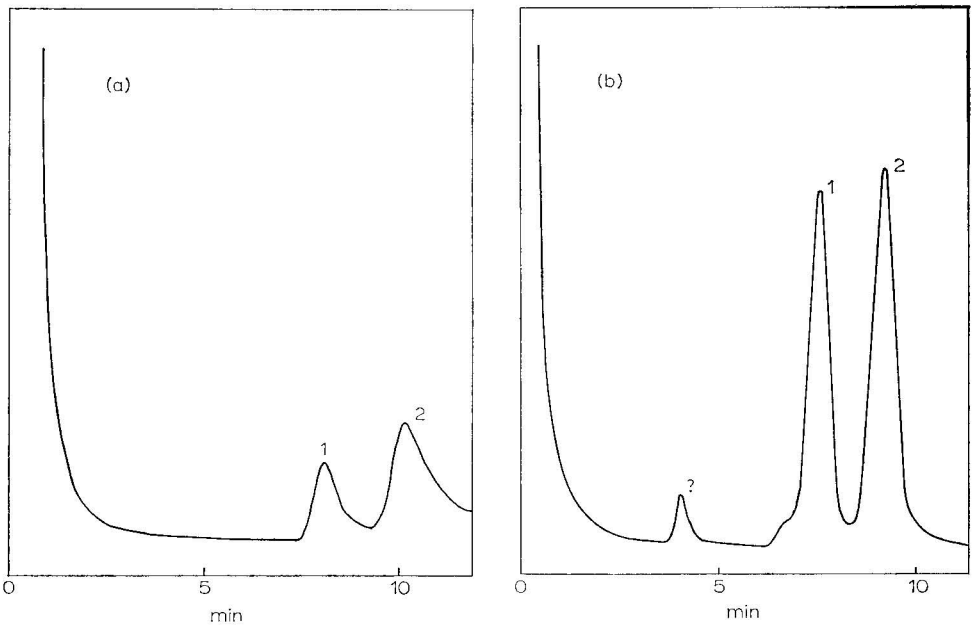


Fig. 2. (a) Gas chromatogram of naphthols on column 7. Oven temperature, 195°C; other conditions as in Fig. 1. Peaks: 1 = α-naphthol; 2 = β-naphthol. (b) Gas chromatogram of naphthylamines on column 7. Conditions as in Fig. 2a. Peaks: 1 = α-naphthylamine; 2 = β-naphthylamine.

Complete separations of α-naphthol from β-naphthol and of α-naphthylamine from β-naphthylamine were achieved (Fig. 2a and b). The relative retentions (α) are comparable to previous results[12,16].

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N-DOCOSANOYL-L-VALINE-2-(2-METHYL)-*n*-HEPTADECYLAMIDE AS A STATIONARY PHASE FOR THE RESOLUTION OF OPTICAL ISOMERS IN GAS–LIQUID CHROMATOGRAPHY

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(Received March 5th, 1980)

SUMMARY

The synthesis and chromatographic properties of N-docosanoyl-L-valine-2-(2-methyl)-*n*-heptadecylamide are described. It has been employed as a stationary phase at column temperatures up to 200°C and it shows excellent stereoselectivity for various classes of compounds, including, in particular, N-trifluoroacetyl (N-TFA) esters of α- and γ-amino acids, aromatic N-TFA amines, N-TFA-O-acyl amino alcohols and N-TFA-α-methylvaline isopropyl ester. The influence of lengthening the chains of R′ and R‴ on the properties of the diamides R′CONHCH(R″)CONHR‴ is discussed.

INTRODUCTION

It is well established that diamides of the type R′CONHCH(R″)CONHR‴, when used as stationary phases in gas chromatography (GC), show high stereoselectivity for enantiomeric amides derived from amino acids, amines, amino alcohols[1–3], α-hydroxy acids[4] and esters of aromatic diols[4]. Studies in our laboratory were concerned with the influence on chromatographic properties and chiral recognition of modifications of R′ and R‴[2,3] and, more recently, of R″[5]. Other workers have examined the use of diamide phases where R′ is a polymeric matrix[4,6]. At present these various phases are being employed routinely for the GC analysis of enantiomeric mixtures, particularly of α-amino acids, using capillary and packed columns[7–10]. The increasing application of these solvents lends special interest to the pursuit of the above systematic investigations, which might elucidate the mechanism of chiral recognition observed and solve remaining analytical problems. Also, the further development of simple preparative procedures for these phases and control of the optical purity during synthesis is important.

In this paper we report the synthesis and properties of a diamide phase derived from valine (R″ = *i*-Pr), with R′ = n-$C_{21}H_{43}$ and R‴ = $-\overset{\displaystyle CH_3}{\underset{\displaystyle CH_3}{\overset{|}{\underset{|}{C}}}}-(CH_2)_{14}-CH_3$.

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EXPERIMENTAL

Materials

The N-trifluoroacetyl (N-TFA) isopropyl esters of the amino acids and the TFA derivatives of the other compounds resolved were prepared by esterification with 1.25 *N* hydrochloric acid–isopropanol in a sealed tube at 105–110°C for 4 h, followed by trifluoroacetylation, as described previously[11].

Synthesis of the stationary phase

*N-Carbobenzoxy-*L*-valine-2-(2-methyl)-n-heptadecylamide (1).* To a stirred solution of N-carbobenzoxy-L-valine-N-hydroxysuccinimide ester[3] (0.001 mole) in dry chloroform (60 ml) kept at −5 to −10°C, 2-methyl-2-heptadecylammonium chloride[3] (0.001 mole) and 2 equivalents of N-methylmorpholine in chloroform (40 ml) were added dropwise. Reaction was continued for 48 h and the mixture was then left to reach room temperature. The crude product was washed successively with water, 2% hydrochloric acid, water and 5% sodium hydrogen carbonate solution. After drying over magnesium sulphate, the solvent was removed rapidly.

L-*Valine-2-(2-methyl)-n-heptadecylamide (2).* The above crude product was dissolved in 50 ml of absolute methanol and reduced with hydrogen in the presence of 10% palladium–charcoal for 4 h. Filtration of the catalyst and evaporation of the solvent left the expected compound, which was identified by nuclear magnetic resonance (NMR) spectroscopy.

*N-Docosanoyl-*L*-valine-2-(2-methyl)-n-heptadecylamide (3).* The diamide was obtained by coupling compound 2 (0.001 mole) with the docosanoate of N-hydroxysuccinimide (0.001 mole) in dry chloroform in the presence of 1 equivalent of N-methylmorpholine at −5 to −10°C for 48 h. The reaction mixture was treated with active charcoal, filtered and then washed as for compound 1. After evaporation of the solvent, the crude product was coated on 30–60-mesh Chromosorb W AW and placed on top of a column containing silica gel deactivated with 6% of water. The fraction eluting with 10% ethyl acetate in *n*-hexane was the desired compound, as demonstrated by NMR spectroscopy (m.p. 54–57°C; yield 46%). Elemental analysis: found, C 78.10, H, 13.18%; calculated for $C_{45}H_{90}N_2O_2$, C 78.19, H 13.13%; $[\alpha]_D^{25}$ −15.2 (*c* 1.85 in chloroform). The optical purity was 95%, as determined by GC of the N-TFA-valine isopropyl ester, obtained after hydrolysis of the phase at 110°C for 4 h with a 1:1 mixture of 6 and 1.25 *N* hydrochloric acid in isopropanol*, followed by trifluoroacetylation[11].

Chromatographic conditions

A stainless-steel capillary column (50 m × 0.5 mm I.D.) was mounted in a Varian Series 2700 gas chromatograph, provided with a stream splitter and a flame-ionization detector (FID). The temperatures were: injector 240°C and detector 240°C; the column temperatures used are indicated in the tables. The carrier gas (helium) flow-rate was 3 ml/min. The resolution factors ($r_{II/I}$, see tables) were not corrected for the optical purity of the stationary phase[2]. Thermogravimetric analysis was performed with a Perkin-Elmer TG-S-1 instrument provided with a thermo-

* It was shown that under these conditions the diamides are not racemized[12].

balance. The amount of the sample was 2.8 mg; between 50 and 300°C the temperature was raised at a rate of 8°/min. Loss of weight was observed to start above 200°C.

RESULTS AND DISCUSSION

As mentioned in the Introduction, the use of diamide stationary phases in the enantiomeric analysis of amino acids has become routine in many laboratories. There remain, however, some aspects of the method that could still be improved, for instance the resolution of aspartic acid and of proline, the degree of peak overlap of some protein amino acids and the ease of synthesis of the stationary phases and their thermal stability.

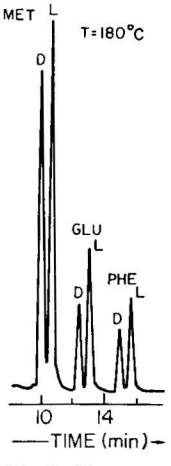
The separation problems can be solved, at least in part, by the use of highly efficient capillary columns. However, it would be advantageous to develop stationary phases or combinations of them and/or to employ solute derivatives that would permit the complete enantiomeric analysis of mixtures of protein amino acids with less peak overlap, and preferably also on packed columns[3].

As to thermal stability, incorporation of the diamide moiety into a polymeric matrix increases its thermal stability[4,6]. It has been shown[3], however, that modification of the substituents R′ and R‴ equally permits bleeding of "low-molecular-weight" diamides to be reduced. Thus, N-docosanoyl-L-valine-*tert.*-butylamide (4) and N-lauroyl-L-valine-2-(2-methyl)-*n*-heptadecylamide (5) could be used at column temperatures of 180–190°C[3].

The synthesis of stationary phase 3 has been carried out as part of a study on the influence of the chain lengthening of R′, R″ and R‴ on the thermal stability and chromatographic behaviour of the diamides[3].

Thermal stability

Thermogravimetric analysis showed that bleeding should start only at about 200°C. As can be seen in Figs. 1 and 2, a stable baseline was obtained when operating



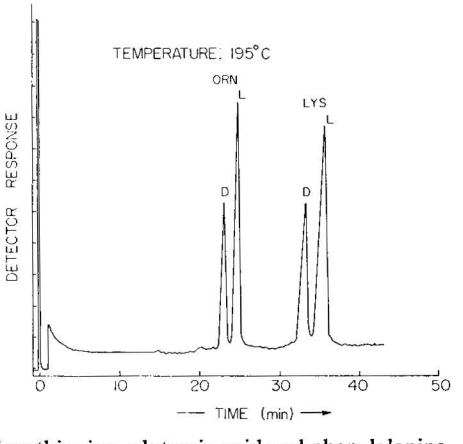


Fig. 1. Chromatogram of N-TFA-isopropyl esters of methionine, glutamic acid and phenylalanine.

Fig. 2. Chromatogram of N-TFA-isopropyl esters of ornithine and lysine.

at 180 and 195°C with the instrument set at $4 \cdot 10^{-11}$ full-scale deflection. The stationary phase was also used for the resolution of some less volatile protein amino acids at 200°C (Table I) and, in general, performed well when exposed for long periods to the above relatively high temperatures. It should be emphasized that efficient purification of 3 and other similar stationary phases, including a final clean-up by chromatography on silica gel, is considered essential for good thermal performance[§].

Chromatographic properties

Data on the resolution of amino acids, amino alcohols and amines are given in Tables I, II and III, respectively.

TABLE I

RESOLUTION OF N-TFA-AMINO ACID ISOPROPYL ESTERS

For chromatographic conditions, see Experimental.

Protein α-amino acids	*α-Amino acids*			*T (°C)*
	Enantiomer	r^*	$r_{L/D}$**	
ALA	D-	4.76	1.155	120
	L-	5.50		
THRE	D-	6.74	1.098	120
	L-	7.40		
VAL	D-	7.70	1.104	120
	L-	8.50		
GLY	—	9.12	—	120
allo-ILE	D-	11.78	1.108	120
	L-	13.06		
ILE	D-	13.06	1.105	120
	L-	14.44		
SER	D-	12.30	1.100	120
	L-	13.56		
LEU	D-	16.08	1.230	120
	L-	19.80		
PRO	D-	19.60	1.025	120
	L-	20.08		
ASP	D-	37.12	1.042	120
	L-	38.68		
CYS	D-	18.98	1.178	140
	L-	22.36		
MET	D-	10.54	1.066	180
	L-	11.24		
GLU	D-	12.90	1.060	180
	L-	13.68		
PHE	D-	16.07	1.049	180
	L-	16.85		
ORN	D-	20.08	1.072	200
	L-	21.54		
LYS	D-	28.60	1.064	200
	L-	30.40		
TRP	D-	62.52	1.041	200
	L-	65.08		

[§] Some workers[4] have claimed that N-docosanoyl-L-valine-*tert.*-butylamide (4), when coated on glass capillaries, could not be used above 140°C. However, to make a meaningful statement on the thermal behaviour of these stationary phases one has to ascertain their purity.

TABLE I *(continued)*

Non-protein α-amino acids	*Enantiomer*	r^*	$r_{L/D}$**	*T (°C)*
α-Aminobutyric	D-	6.34	1.148	120
	L-	7.28		
α-Aminopentanoic	D-	11.70	1.174	120
	L-	13.74		
α-Aminohexanoic	D-	6.48	1.126	150
	L-	7.30		
α-Aminoheptanoic	D-	10.75	1.102	150
	L-	11.85		
α-Aminooctanoic	D-	12.30	1.081	160
	L-	13.30		
tert.-Leucine	D-	8.06	1.052	120
	L-	8.48		
Phenylglycine	D-	19.90	1.043	150
	L-	20.76		
γ-Amino acids	*Enantiomer*	r^*	$r_{D/L}$**	*T (°C)*
γ-Aminovaleric	L-	31.80	1.046	120
	D-	33.26		
γ-Amino-δ-methyl-	L-	61.78	1.076	120
hexanoic	D-	66.50		
γ-Amino-ε-methyl-	L-	110.50	1.070	120
heptanoic	D-	118.20		
α-Methyl-α-amino acids	*Enantiomer*	r^*	$r_{II/I}$**	*T (°C)*
α-Methylvaline	I	10.88	1.048	110
	II	11.40		
α-Methylnorvaline	I	9.26	1.000	110
	II	9.26		
α-Methylleucine	I	12.36	1.000	110
	II	12.36		
α-Methylnor-	I	16.04	1.000	110
leucine	I	16.04		

* Corrected retention time (minutes).

** $r_{L/D}$ = resolution factor = ratio of the corrected retention time of the L- over that of the D-enantiomer, calculated with r values expressed to the second decimal place.

α-Amino acids. Comparison with data for homologous low-molecular-weight diamides, *e.g.*, N-lauroyl-L-valine-2-(2-methyl)-*n*-heptadecylamide (5)[3], shows lower resolution factors [Ala: $r_{L/D} = 1.166$ on 5 (130°C), 1.155 on 3 (120°C). Glu (180°C): $r_{L/D} = 1.069$ on 5, 1.060 on 3. Met (180°C): $r_{L/D} = 1.080$ on 5, 1.066 on 3]. This can be readily interpreted as being due to the lengthening of the chains of R′ and R‴, which "dilutes" the effect of the central diamide group responsible for chiral recognition. However, the r values are higher throughout than for the polymeric chiral phases and are still amply sufficient for resolution on packed columns of 14 of the 16 optically active protein amino acids examined, and of aspartic acid and proline on capillaries. Using readily available starting materials, it is possible to synthesize higher homologues of 3, which might further increase the permissible operating temperature.

TABLE II

RESOLUTION FACTORS OF N-TFA-O-ACYL DERIVATIVES OF AMINOALKANOLS

For chromatographic conditions, see Experimental.

Aminoalkanol	*Enantiomer*	*Acyl group*								
		Propionyl			*Isobutyryl*			*Pivaloyl*		
		r^*	$r_{(D/L)}$**	T (°C)***	r^*	$r_{(D/L)}$**	T (°C)***	r^*	$r_{(D/L)}$**	T (°C)***
2-Aminopropan-1-ol	L-	14.58	1.040	120	9.40	1.051	140	12.00	1.075	140
	D-	15.16			9.88			12.90		
2-Aminobutan-1-ol	L-	25.00	1.076	120	14.56	1.089	140	15.86	1.112	140
	D-	26.90			15.86			17.64		
2-Aminopentan-1-ol	L-	42.28	1.089	120	10.94	1.075	160	11.48	1.097	160
	D-	46.00			11.76			12.60		
2-Aminohexan-1-ol	L-	69.88	1.087	120	16.86	1.071	160	18.14	1.096	160
	D-	76.00			18.06			19.88		
2-Aminoheptan-1-ol	L-	51.88	1.071	140	19.86	1.062	170	20.88	1.082	170
	D-	55.58			21.10			22.60		
2-Aminooctan-1-ol	L-	88.80	1.071	140	31.98	1.067	170	33.14	1.083	170
	D-	95.10			34.14			35.90		
2-Amino-3-methyl-	L-	28.44	1.094	120	16.90	1.105	140	18.66	1.125	140
butan-1-ol	D-	31.12			18.68			21.00		
2-Amino-4-methyl-	L-	24.10	1.057	140	29.84	1.085	140	30.04	1.130	140
pentan-1-ol	D-	25.48			32.38			33.94		

* See Table I.

** See Table I

*** Temperature at which good peak resolution and relatively short retention were observed.

TABLE III

RESOLUTION OF CHIRAL N-TFA-AMINES

For chromatographic conditions, see Experimental.

2-Amino-n-alkanes ($R\text{-}CH\text{-}CH_3$ with NH_2)	*Enantiomer*	r^*	$r_{D/L}$**	R_S***	T (°C)
2-Aminoheptane	L-	11.72	Shoulder		110
	D-	12.06			
2-Aminooctane	L-	23.82	1.021	~0.2	110
	D-	24.32			
2-Aminononane	L-	42.86	1.026	~0.6	110
	D-	44.00			
2-Aminodecane	L-	88.10	1.028	~0.5	110
	D-	90.56			
3-Aminocyclenes	*Enantiomer*	r^*	$r_{II/I}$**	R_S***	T (°C)
3-Aminocyclohexene	I	7.96	1.000		120
	II	7.96			
	I	35.50	1.020	~0.3	80
	II	36.20			
	I	70.10	1.021	~0.4	65
	II	71.60			
Aromatic N-TFA-amines	*Enantiomer*	r^*	$r_{S/R}$**	T (°C)	
α-Phenylethylamine	*R*	16.68	1.035	130	
	S	17.26			
α-(1-Naphthyl)ethylamine	*R*	42.80	1.033	180	
	S	44.20			

* See Table I.

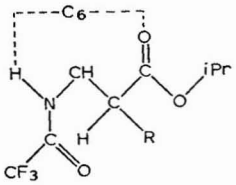
** See Table I.

*** R_S defined by the expression $2d/(w_1 + w_2)$, where d is the distance between the peak maxima and w_1 and w_2 are the half widths (at the baseline) of the first and second peak, respectively.

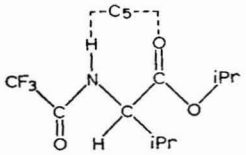
Increasing the chain length, *e.g.*, of R′ in 3, shifts the relative retentions of some amino acids, *e.g.*, Phe, as compared with Glu, is more retained on 3 than on 5 (at 180°C). Another result of chain lengthening is a decrease in the melting point, *e.g.*, 54–57° *vs.* 85°C for 4. Low-melting stationary phases permit the use of a wider temperature programming range and advantage to be taken of the higher resolution factors at the lower temperatures [*e.g.*, $r_{L/D}$ at 80°C (120°C): Ala, 1.306 (1.155); Pro, 1.040 (1.025); Leu, 1.470 (1.230)]. These effects could be combined to solve problems of peak overlap with protein amino acids on packed and, in special instances, on capillary columns. Two or more thermally stable homologous diamides, coupled with different temperature programming profiles, could be employed consecutively for this purpose. Alternative or additional possibilities for reducing peak overlap, such as coupling in series of a second column coated with an achiral phase or the injection of derivatives other than N-TFA isopropyl esters, have been discussed earlier[3].

β- and γ-amino acids. The β-amino acids examined (β-aminobutyric, β-amino-γ-methylpentanoic and β-amino-δ-methylhexanoic acid) showed either no resolu-

tion or, in the best instances, only a hint of a shoulder. As has been reported recently[13], the β-amino acids, which can form hydrogen bonds via a "C_6" conformation:



have considerably lower resolution factors than the corresponding α-amino acids, which can hydrogen bond via a "C_5" ring:



The above-mentioned diluting effect of chain lenghtening further decreases chiral recognition. It should also be recalled that there is a strong influence for this class of compounds of the nature of the R''' substituent on stereoselectivity, as manifested by the reversal of the order of emergence on N-lauroyl-L-valine-*tert.*-butylamide as compared with N-lauroyl-L-valine-6-undecylamide[13].

γ-Amino acids. Data on the γ-amino acids examined are given in Table I. They all show reversal of the order of emergence with respect to α-amino acids, as has also been noted on other diamide phases. This chromatographic behaviour has been tentatively correlated with the predominant formation of solvent–solute hydrogen-bonded association[2,14,15] of the "C_5–C_7" type, with "non-parallel" orientation of the alkyl groups at the respective asymmetric carbons for the less strongly retained L-enantiomer (Fig. 3). Frank *et al.*[4] have also used this type of structure to explain the stereoselectivity observed with their Chirasil-val phase. It is of interest that in the example given[4], where the solute was pentafluoropropionyl-L-lactic acid cyclohexylamide and the association was of the "C_5–C_7" type, the enantiomer with the higher retention ($r_{L/D} > 1$) showed, as expected, a parallel orientation of the pertinent alkyl groups.

α,α-Dialkyl-α-amino acids. N-TFA derivatives of this type of amino acid have been separated into their enantiomers on carbonylbis-(N-L-valine isopropyl ester)[16]. On "low-molecular-weight" diamides, however, resolution has been observed only in exceptional instances, *e.g.*, for α-methylvaline on 3 (Fig. 4); ($r_{II/I} = 1.085$ at 80°C). Isovaline, α-methylnorvaline, α-methylnorleucine and α-methylleucine gave only one peak. It seems that for this class of compounds the polymeric diamides have an advantage, as isovaline is reported to have been resolved on Chirasil-val[9].

2-Amino-1-alcohols. It has been already reported that aliphatic 2-aminoalkan-1-ols in the form of their N-TFA-O-acyl esters can be readily resolved[13]. The same is true for stationary phase 3, as can be seen in Table II and Fig. 5. The order of

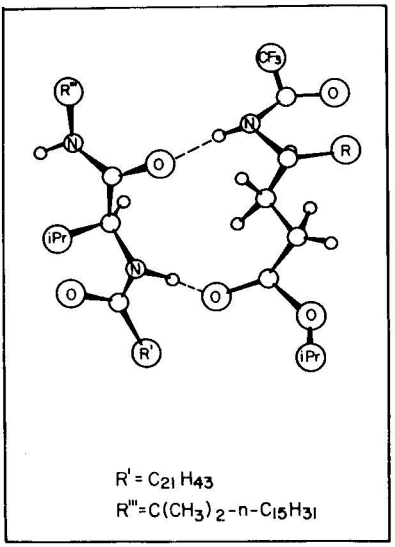


Fig. 3. Scheme of the hydrogen-bonded association for γ-amino acid derivatives with "non-parallel" orientation of the alkyl substituents at the asymmetric carbons for the less strongly retained L-enantiomer.

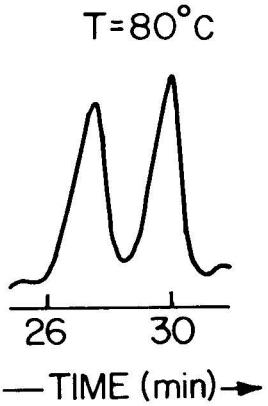


Fig. 4. Resolution of N-TFA-α-methylvaline isopropyl ester.

emergence is the reverse of that for the α-amino acids, *i.e.*, the D-enantiomers are more strongly retained on the L-phase. This behaviour, which is similar to that of the above γ-amino acid derivatives, has been discussed elsewhere[13].

Amines. N-Acyl derivatives of amines, which lack the additional carbonyl group present in amino acids and amino alcohols, are resolved by a different mechanism than the latter. It has been established that their resolution is, in general, best achieved with stationary phases such as carbonylbis-(N-L-valine isopropyl ester)[16] and N-lauroyl-S-α-(1-naphthyl)ethylamine[17]. In agreement with this, the

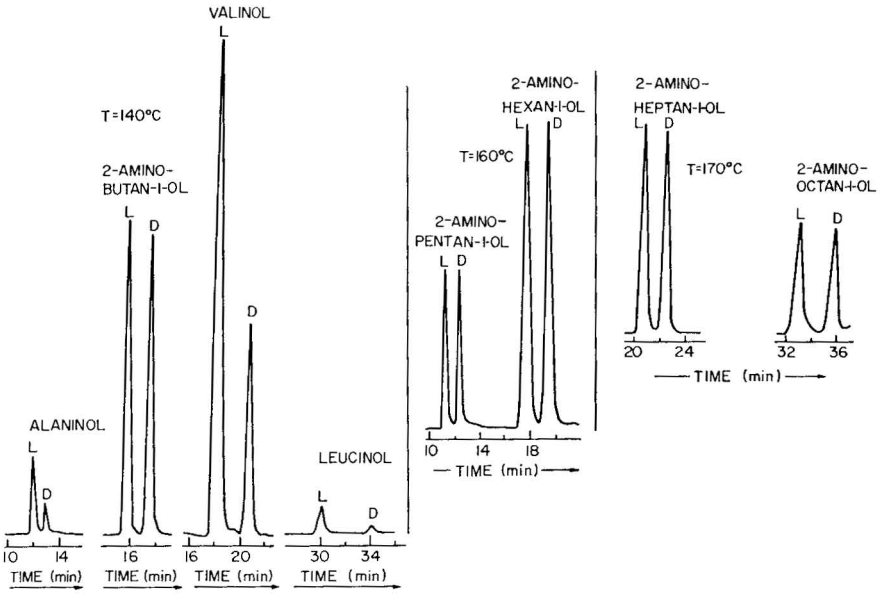


Fig. 5. Chromatogram of N-TFA-2-amino-alkane-1-ol pivaloyl esters.

separation of enantiomeric N-TFA aliphatic amines (Table III) on 3 is inefficient. The aromatic amines, on the other hand, although having far smaller r values than on N-lauroyl-S-α-(1-naphthyl)ethylamine, show good peak resolution (Table III, Fig. 6). It is also of interest that 3-aminocyclohexene could be relatively well resolved on 3 at 65°C, whereas conditions for the resolution of seven- and eight-membered homologues could not be found. On stationary phases showing typical amine stereoselectivity[16,17], the cyclohexene derivative is the one most difficult to separate.

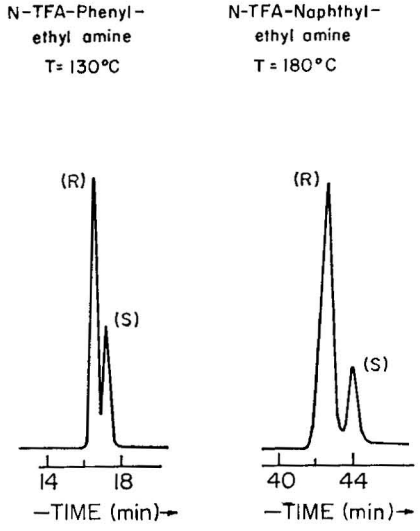


Fig. 6. Chromatogram of aromatic N-TFA amines.

CONCLUSIONS

Chain lengthening of the R substituents of the diamides R'CONHCH(R")-CONHR"', as in N-docosanoyl-L-valine 2-(2(methyl)-*n*-heptadecylamide (3) led to a stationary phase with considerable thermal stability and high stereoselectivity for a wide range of compounds. In particular, N-TFA-amino acid esters are resolved with relatively large resolution factors. The results indicate that further pursuit of systematic studies on the influence of the size and nature of the substituents should lead to optimization of analytical procedures and reveal many more significant details of the properties of these phases and the mechanism of their stereoselective action.

The conditions employed for the synthesis of 3 led to a product of high optical purity.

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R_M VALUES FROM REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY AS PARAMETERS OF LIPOPHILICITY IN QUANTITATIVE STRUCTURE–ACTIVITY RELATIONSHIPS IN FOUR SERIES OF ARYLALIPHATIC ACIDS

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SUMMARY

Series of β-aryl-*n*-butyric (I), arylacetic (II), α-methyl-β-arylpropionic (III) and cinnamic (IV) acids were subjected to reversed-phase thin-layer chromatography on silica gel impregnated with silicone oil. In the series of acids I, II, and IV, characterized by a broad range of lipophilicity of the aromatic substituents, a non-linear course of the relationship between R_M values and π parameters was found. The influence was studied of these non-linear relationships on the usability of R_M values for the characterization of lipophilicity in quantitative structure–activity relationships. A change from a linear form of activity–π dependence to a parabolic form of activity–R_M dependence was found solely in instances in which the statistical significance of the linear dependence was characterized by a high correlation coefficient, approaching 0.99. In such exceptional instances, requiring the highly accurate determination of the biological activity concerned, a fallacious quadratic activity–lipophilicity relationship may thus appear. In most of the regression equations studied, however, the use of R_M values instead of π parameters did not influence the activity–lipophilicity relationships.

With the aid of values obtained from partition chromatography, the lipophilicity of some disubstituted derivatives of acids I–IV was characterized, in which the addiditivy of the π parameters could be expected to fail as a result of the action of the *ortho*-effect of their substituents. For this purpose either the R_M values were used directly, or π parameters calculated from the relationship between π and R_M values were used.

INTRODUCTION

Recently we investigated[1] the lipophilicities of β-aryl-*n*-butyric and arylacetic acids with the aid of partition thin-layer chromatography (TLC). We used silica gel impregnated with silicone oil, which is frequently used[2–5] for the evaluation of lipophilicity in biological correlations. Applying the method proposed by Hulshoff and Perrin[6,7], we found that in the chromatographic separation of both series of acids an adsorption mechanism participated, in addition to the partition mechanism. The

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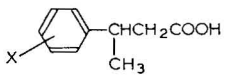
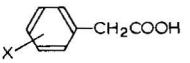
dichotomy of the mechanism manifests itself in deviations from linearity in the correlations between the tabulated π parameters and the experimental R_M values. Within a broader range of lipophilicity, the non-linear correlation between the π and R_M values can be substituted[1] either by a quadratic dependence or by a pair of straight lines with different regions of lipophilicity of the substituents.



I



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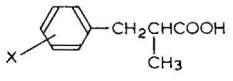
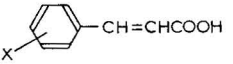


III



IV

In this study we applied the R_M values to correlations of certain biological activities in series of β-aryl-*n*-butyric (I), arylacetic (II), α-methyl-β-arylpropionic (III) and cinnamic (IV) acids. We aimed to establish the way in which the non-linear correlation between π and R_M modified the dependences of biological activities on R_M values. We studied first the inhibition of the heat denaturation of serum albumin and the activation of fibrinolysis, phenomena which depend solely on the lipophilicity, as ascertained in previous studies[8–12]. We also performed, for the series of acids I, regression analyses of erythrocyte membrane stabilization against hypotonic haemolysis and of the anti-inflammatory activity in the test for inhibition of kaolin-induced oedema. These processes are dependent on both the lipophilicity and the polar effects of aromatic substituents[13].

EXPERIMENTAL

Chromatography

The stationary phase was prepared by shaking 25 g of silica gel GF_{254} for 90 sec with a mixture of x% (v/v) of silicone oil with 6 ml of acetone and diluting with dioxane to 50 ml. The glass plates (10 × 20 cm) were covered with a 0.25-mm layer of a slurry of the support using standard equipment. The volatile components of the impregnating solutions were evaporated within 16 h at 20°C.

Solutions (1%) of acids I–IV in methanol were prepared, and 5-μl samples were applied to the plate 3 cm from the lower edge. After evaporating the methanol at 20°C, ascending, one-dimensional TLC was carried out using 50% acetone containing a citrate buffer (pH 3.4) as the mobile phase. A chromatographic chamber was equilibrated for 16 h with the mobile phase at 20°C. After migration for 15 cm the plates were removed and, after evaporating the remaining mobile phase, the acids were detected under UV light (λ = 254 nm). Each chromatogram contained six compounds, two acids serving as reference samples. In the individual chromatograms the R_F values of the standards did not differ by more than 0.02.

Sample preparation

β-Aryl-*n*-butyric acids (I) were prepared by the method proposed by Asano *et al.*[14] and described in detail elsewhere[8,15]. Arylacetic acids (II) were prepared by

the Wilgerodt reaction; alkoxy derivatives were obtained[16] by alkylation of the methyl esters of the corresponding 4-hydroxyarylacetic acids and subsequent hydrolysis. α-Methyl-β-arylpropionic acids (III) were obtained[10] either by hydrogenation of substituted α-methylcinnamic acids or by hydrolysis and decarboxylation of the corresponding α-benzyl-α-methylmalonates. Cinnamic acids (IV) were prepared by the Wittig reaction of substituted benzaldehydes with ethoxycarbonylmethylene phosphorane[17].

Biochemical testing

Inhibition of the heat denaturation of bovine serum albumin was determined according to Mizushima[18] as described in ref. 8. The efficiency was expressed by the molar concentration, C^I, causing 50% inhibition.

Activation of fibrinolysis was measured by the "hanging clot" method[19]. The efficiency was expressed by the minimum molar concentration, C^F, that dissolved the coagulum after incubation for 24 h at 37°C.

Stabilization of erythrocyte membranes against hypotonic haemolysis was determined by the method proposed by Kalbhen *et al.*[20], modified by using whole rat blood[21]. The activity was expressed as the concentration, C^{St}, in moles per litre, bringing about 50% inhibition of haemolysis.

Kaolin oedema inhibition was measured according to Hillebrecht[22] as described in detail in ref. 23. The effects of the compounds tested were expressed in terms of the percentage inhibition of inflammation, and the activity index, I^K, was calculated as the ratio of the effects of the compound tested and of the standard; the standard was 3-chloro-4-benzyloxyphenylacetic acid[23].

Calculations

In the regression analysis, the π parameters derived[24] for arylacetic (in the series I–III) and benzoic (in the series IV) acids were used. The π parameters for alkoxy and for higher alkyl groups were calculated from the value for the methoxy group, or the methyl group, and from the following increments[25]: $\Delta\pi = 0.5$ for aliphatic CH_2, 0.41 for cyclic CH_2, -0.2 for branching and -0.3 for a double bond. For the calculation of $\Sigma\pi$ for disubstituted derivatives, the difference between the lipophilicity[26] of remaining aromatic parts, $C_6H_4<$ and $C_6H_3<$, was taken into consideration, so that the value 0.23, corresponding[27] to 0.5 log P of hydrogen, was subtracted from the sum of both substituents.

The coefficients in the regression equations were calculated from experimental results by multiple regression analysis using the least-squares method on a Hewlett-Packard 9820 computer. The statistical significances of the regression equations were tested by the standard deviation, s, the coefficient of multiple correlation, r, and the Fischer–Snedecor criterion, F. Individual parameters were evaluated statistically by Student's t-test at the minimal significance level $\alpha = 0.001$; the exceptions are noted in the text.

RESULTS AND DISCUSSION

β-Aryl-n-butyric acids

In the series of acids Ia–In the R_M values were determined with two concen-

TABLE I

CHROMATOGRAPHIC BEHAVIOUR AND BIOLOGICAL ACTIVITIES OF β-ARYL-*n*-BUTYRIC ACIDS (I)

No.	*X*	π^*	R_F**		R_M**		*Log (1/C^I)****	*Log (1/C^F)****	*Log (1/CSt)*§	*Log (I^K)*§
			A	*B*	*A*	*B*				
Ia	H	0	0.65	0.58	−0.27	−0.14	3.046	0.922	3.088	0.51
Ib	4-CH_3O	0.01	0.655	0.61	−0.28	−0.19	3.097	1.046	3.079	0.48
Ic	4-CH_3	0.45	0.60	0.525	−0.18	−0.04	3.444	1.097	3.127	0.47
Id	4-Cl	0.70	0.59	0.495	−0.16	0.01	3.495	1.398	3.288	0.27
Ie	4-*iso*-C_3H_7O	0.81	0.58	0.45	−0.14	0.09	3.514	1.398	— §§	— §§
If	4-C_2H_5	0.90	0.56	0.45	−0.10	0.09	3.770	1.456	3.191	−0.39
Ig	4-Br	0.90	0.57	0.46	−0.12	0.07	3.602	1.523	3.258	−0.24
Ih	3-Br	0.91	0.565	0.46	−0.11	0.07	3.569	1.523	3.352	−0.24
Ii	4-*iso*-C_3H_7	1.40	0.52	0.39	−0.03	0.19	3.863	1.699	3.281	−0.28
Ij	4-*iso*-C_4H_9	1.90	0.435	0.30	0.11	0.37	4.125	2.155	3.414	−0.16
Ik	4-*iso*-C_5H_{11}	2.25	0.335	0.225	0.30	0.54	4.377	2.222	3.430	−0.15
Il	4-*n*-C_5H_{11}	2.45	0.32	0.20	0.33	0.60	4.398	— §§§	3.513	−0.04
Im	4-*n*-$C_6H_{13}O$	2.51	0.30	0.195	0.37	0.62	4.280	— §§§	— §§§	— §§
In	4-2′-ethylhexyl	3.90	0.14	0.06	0.80	1.19	— §§§	— §§§	3.895	−0.25
Io	3-Br-4-*iso*-C_3H_7	2.08 (1.70†)	0.435	0.305	0.11	0.36	4.046	2.097	3.470	−0.13
Ip	3-Br-4-*iso*-C_4H_9	2.58 (2.20†)	0.355	0.23	0.26	0.52	4.301	2.301	3.650	0.04
Ir	3-Br-4-*n*-C_5H_{11}	3.28 (2.77†)	0.275	0.16	0.42	0.73	4.456	— §§§	— §§	— §§
Is	4-$C_6H_5CH_2$	2.34 (1.96†)	0.40	0.295	0.18	0.38	— §§	— §§	3.421	−0.56

* π parameters taken from refs. 24 and 25.

** Chromatography was performed on silica gel containing (A) 3.5% and (B) 7.5% of silicone oil.

*** Activities taken from refs. 8 and 15.

§ Activities taken from ref. 13.

§§ Not established.

§§§ Insoluble under the conditions of the test.

† Average values calculated from R_M values using eqns. 1 and 2; these values were used in correlation analysis of biological activities (eqns. 3, 6, 9 and 12).

trations of silicone oil in the silica gel (see Table I). The correlations between the π parameters and the R_M values are expressed by eqn. 1 for the 3.5% concentration and eqn. 2 for the 7.5% concentration.

	n	r	s	F	
$\pi = 4.030\, R_{M(3.5)} - 1.138\, [R_{M(3.5)}]^2 + 1.316$	14	0.994	0.131	460	(1)
$\pi = 3.481\, R_{M(7.5)} - 0.625\, [R_{M(7.5)}]^2 + 0.622$	14	0.998	0.082	1168	(2)

From these equations, with experimental R_M values inserted, the $\Sigma\pi$ values of substituents were calculated for the acids Io–Ir. The values obtained are lower than would correspond to the tabulated figures (see Table I), probably owing to a hydrophobic interaction between the *ortho*-substituents. For the acids Ia–Ir, regression analysis of the results of the test for the inhibition of heat denaturation of serum albumin led to eqn. 3. If in this series of acids the lipophilicity is characterized by the experimental R_M values, then the dependence of the inhibitory activity on lipophilicity is expressed by eqns. 4 and 5.

	n	r	s	F	
$\log(1/C^{\mathrm{I}}) = 0.504\,\pi + 3.148$	16	0.986	0.080	493	(3)
$\log(1/C^{\mathrm{I}}) = 2.243\, R_{M(3.5)} - 2.575\, [R_{M(3.5)}]^2 + 3.905$	16	0.993	0.060	446	(4)
$\log(1/C^{\mathrm{I}}) = 2.118\, R_{M(7.5)} - 1.050\, [R_{M(7.5)}]^2 + 3.468$	16	0.988	0.076	275	(5)

In eqns. 4 and 5, the $R_M{}^2$ terms are statistically significant at the $\alpha = 0.005$ level. This apparent parabolic activity–lipophilicity relationship evidently results from the quadratic relationships in eqns. 1 and 2, respectively.

For fibrinolysis activation, in this series of acids we calculated the regression eqn. 6. Owing to insolubility of the more strongly lipophilic derivatives Il, m, n and r under the conditions of the test, the lipophilicity range is narrower in this instance. Consequently, when the R_M values are used, a linear relationship between the activation of fibrinolysis and lipophilicity remains preserved, as is evident from eqns. 7 and 8. Additional insertion of $R_M{}^2$ terms into these equations lowers their statistical significance.

	n	r	s	F	
$\log(1/C^{\mathrm{F}}) = 0.608\,\pi + 0.942$	13	0.987	0.077	428	(6)
$\log(1/C^{\mathrm{F}}) = 2.395\, R_{M(3.5)} + 1.715$	13	0.966	0.124	156	(7)
$\log(1/C^{\mathrm{F}}) = 1.932\, R_{M(7.5)} + 1.314$	13	0.978	0.100	245	(8)

For the stabilization of erythrocyte membranes against hypotonic haemolysis, the dependence on the lipophilicity and polar effects of substituents was calculated, expressed by eqn. 9. In this equation the partial correlation coefficients have the value $r(\pi) = 0.951$ and $r(\sigma) = 0.124$. By using the R_M values for the characterization of lipophilicity, the linear dependence on lipophilicity did not change, as is evident from eqns. 10 and 11.

	n	r	s	F	
$\log(1/C^{\mathrm{St}}) = 0.206\,\pi + 0.246\,\sigma + 3.068$	15	0.981	0.045	158	(9)
$\log(1/C^{\mathrm{St}}) = 0.757\, R_{M(3.5)} + 0.290\,\sigma + 3.322$	15	0.988	0.037	246	(10)
$\log(1/C^{\mathrm{St}}) = 0.608\, R_{M(7.5)} + 0.261\,\sigma + 3.201$	15	0.988	0.037	241	(11)

We assume that the statistical significance of R_M^2 values in the regression equations are influenced by the statistical significance of the lipophilicity–activity linear relationship.

An analogous result was obtained in assessments of the dependence of anti-inflammatory activity on the lipophilicity parameters π and R_M. In this series of acids I we found[13] that kaolin oedema inhibition had a quadratic dependence on lipophilicity and a linear dependence on the polar constants σ of aromatic substituents (eqn. 12), with partial correlation coefficients $r(\pi) = 0.71$, $r(\pi^2) = 0.46$ and $r(\sigma) = 0.31$. In this instance also the original quadratic dependence of activity on lipophilicity underwent no change when the R_M values were used for characterizing the lipophilicity (see eqns. 13 and 14).

	n	r	s	F	
$\log I^K = 0.302\pi - 0.056\pi^2 + 0.175\sigma - 0.515$	15	0.952	0.052	35.5	(12)
$\log I^K = 0.718\, R_{M(3.5)} - 0.873\,[R_{M(3.5)}]^2 + 0.209\sigma - 0.223$	15	0.967	0.044	52.1	(13)
$\log I^K = 0.764\, R_{M(7.5)} - 0.534\,[R_{M(7.5)}]^2 + 0.182\sigma - 0.356$	15	0.957	0.049	40.4	(14)

Arylacetic acids

In the series of acids IIa–IIs the relationships between the π parameters and the R_M values, determined on silica gel impregnated with 3% silicone oil, are expressed by eqns. 15 and 16.

	n	r	s	F	
$\pi = 3.571\, R_M + 2.236$	18	0.995	0.119	1824	(15)
$\pi = 3.513\, R_M - 0.787\, R_M^2 + 2.322$	18	0.998	0.076	2254	(16)

In eqn. 16 the quadratic term is statistically significant at the $\alpha = 0.001$ level. On insertion of the experimental R_M value into eqn. 16, $\Sigma\pi$ for the substituents of derivative IIt was calculated (see Table II); the decrease in lipophilicity against the assumed sum of the tabulated π values is evidently a consequence of the *ortho*-effect of the two alkoxy groups.

In the series of arylacetic acids the dependence of the inhibition of the heat denaturation of serum albumin on lipophilicity is expressed by a linear correlation (eqn. 17). When characterizing the lipophilicity by R_M values, we obtain eqn. 18, in which the quadratic term is statistically significant at the $\alpha = 0.005$ level.

	n	r	s	F	
$\log (1/C^1) = 0.590\, \pi + 2.615$	16	0.988	0.083	585	(17)
$\log (1/C^1) = 1.759\, R_M - 1.406\, R_M^2 + 4.003$	16	0.991	0.077	337	(18)

This result corresponds to an analogous dependence expressed by eqn. 3 or 4 for the series of acids I. For fibrinolysis activation a linear dependence on lipophilicity was found, expressed by eqn. 19. When lipophilicity was characterized by R_M values, the linear correlation remained preserved (eqn. 20), probably again as a

TABLE II

CHROMATOGRAPHIC BEHAVIOUR AND BIOLOGICAL ACTIVITIES OF ARYLACETIC ACIDS (II)

No.	*X*	π^*	R_F**	R_M	*Log* $(1/C^I)$***	*Log* $(1/C^F)$***
IIa	H	0	0.795	−0.59	—§	—§§
IIb	4-CH_3O	0.01	0.795	−0.59	2.523	—§§
IIc	4-Cl	0.70	0.725	−0.42	3.000	—§§
IId	4-*iso*-C_3H_7O	0.81	0.705	−0.38	3.056	1.000
IIe	4-C_2H_5	0.90	0.70	−0.37	3.056	1.222
IIf	3-Cl-4-CH_2=$CHCH_2O$	1.16	0.675	−0.32	3.377	1.398
IIg	3-Cl-4-*iso*-C_3H_7O	1.26	0.64	−0.25	3.511	1.398
IIh	4-*iso*-C_3H_7	1.40	0.645	−0.26	3.488	1.398
IIi	4-*tert.*-C_4H_9	1.68	0.59	−0.16	3.678	1.699
IIj	4-*iso*-C_4H_9	1.90	0.57	−0.12	3.724	1.699
IIk	4-cyclo-C_6H_{11}	2.46	0.50	0	4.125	2.222
IIl	4-cyclo-$C_6H_{11}CH_2O$	2.47	0.46	0.07	4.046	2.301
IIm	3-Cl-4-cyclo-$C_6H_{11}O$	2.51	0.49	0.02	4.131	2.222
IIn	4-*n*-$C_6H_{13}O$	2.51	0.46	0.07	4.034	2.222
IIo	3-Cl-4-cyclo-$C_6H_{11}CH_2O$	2.92	0.40	0.18	4.347	2.398
IIp	3-Cl-4-*n*-$C_6H_{13}O$	2.96	0.40	0.18	4.201	2.398
IIr	4-2′-ethylhexyl	3.90	0.265	0.47	—§§§	—§§§
IIs	3-Cl-4-*n*-$C_8H_{17}O$	3.96	0.215	0.56	—§§§	—§§
IIt	3-CH_3O-4-cyclo-$C_6H_{11}CH_2O$	2.28 (1.91†)	0.56	−0.10	3.801	2.000

* π parameters taken from refs. 24 and 25.

** Chromatography was performed on silica gel containing 2.5% of silicone oil.

*** Activities taken from ref. 16.

§ Not established.

§§ Inactive.

§§§ Insoluble under the conditions of the test.

† Average value calculated from R_M value using eqns. 15 and 16; this value was used in correlation analysis of biological activities (eqns. 17 and 19).

consequence of the lower statistical significance of the linear dependence of fibrinolysis activation on lipophilicity.

	n	*r*	*s*	*F*	
$\log (1/C^F) = 0.645\,\pi + 0.590$	14	0.982	0.094	328	(19)
$\log (1/C^F) = 2.419\,R_M + 2.076$	14	0.977	0.107	248	(20)

α-Methyl-β-arylpropionic acids

In this series of acids (IIIa–IIIm), the linear correlation between the tabulated π parameters and R_M values is expressed by eqn. 21.

	n	*r*	*s*	*F*	
$\pi = 2.827\,R_M + 0.625$	13	0.993	0.084	754	(21)

Owing to a narrower range of lipophilicity of the aromatic substituents, $(0 < \pi < 1.9)$, the parabolic π *versus* R_M relationship is statistically less significant. $\Sigma\pi$ values were calculated for the 3,4-disubstituted derivatives IIIn–IIIp (see Table III) by inserting experimental R_M values into eqn. 21.

TABLE III

CHROMATOGRAPHIC BEHAVIOUR AND BIOLOGICAL ACTIVITIES OF CYCLOHEXYLAMMONIUM SALTS OF α-METHYL-β-ARYLPROPIONIC ACIDS (III)

No.	*X*	π*	R_F**	R_M	*Log* $(1/C^I)$***	*Log* $(1/C^F)$***
IIIa	H	0	0.60	−0.18	3.192	0.921
IIIb	4-CH_3O	0.01	0.61	−0.19	3.169	0.921
IIIc	3-CH_3O	0.04	0.615	−0.20	3.169	—§
IIId	3-Cl-4-CH_3O	0.46	0.535	−0.06	3.327	—§§
IIIe	3-Cl	0.68	0.49	0.02	3.498	1.222
IIIf	4-Cl	0.70	0.50	0	3.524	1.222
IIIg	4-*iso*-C_3H_7O	0.81	0.47	0.05	3.607	1.347
IIIh	4-Br	0.90	0.48	0.03	3.620	1.398
IIIi	3-Cl-4-*iso*-C_3H_7O	1.26	0.38	0.21	3.896	—§§§
IIIj	4-*iso*-C_3H_7	1.40	0.345	0.28	3.982	1.824
IIIk	4-*tert.*-C_4H_9	1.68	0.28	0.41	4.071	2.000
IIIl	3-Cl-4-*iso*-C_4H_9O	1.76	0.28	0.41	4.201	—§§§
IIIm	4-*iso*-C_4H_9	1.90	0.26	0.45	4.292	2.222
IIIn	3-CH_3O-4-*iso*-C_3H_7O	0.62 (0.41†)	0.535	−0.06	3.327	1.097
IIIo	3-CH_3O-4-*n*-$C_6H_{13}O$	2.32 (1.80†)	0.275	0.42	4.171	2.097
IIIp	3-Br-4-*iso*-C_3H_7	2.08 (1.73†)	0.29	0.39	4.071	2.000

* π parameters taken from refs. 24 and 25.
** Chromatography was performed on silica gel containing 7.5% of silicone oil.
*** Activities taken from ref. 10.
§ Inactive.
§§ Not established.
§§§ Insoluble under the conditions of the test.
† These values, calculated from R_M values using eqn. 21, were used in correlation analysis of biological activities (eqns. 22 and 23).

In the series of acids III the dependences of the inhibition of denaturation of serum albumin and of fibrinolysis activation on lipophilicity are expressed by eqns. 22 and 23.

	n	*r*	*s*	*F*	
$\log(1/C^I) = 0.569\,\pi + 3.135$	16	0.993	0.047	1069	(22)
$\log(1/C^F) = 0.679\,\pi + 0.830$	12	0.990	0.070	493	(23)

In line with eqn. 21, in this instance substitution of π parameters by experimental R_M values led to the linear correlations in eqns. 24 and 25, equivalent to the preceding equations.

	n	*r*	*s*	*F*	
$\log(1/C^I) = 1.634\,R_M + 3.483$	16	0.989	0.061	621	(24)
$\log(1/C^F) = 1.960\,R_M + 1.247$	12	0.992	0.063	623	(25)

Cinnamic acids

In the series of acids IVa–IVl the correlations between the π parameters and the R_M values are expressed by the quadratic dependence in eqn. 26.

	n	*r*	*s*	*F*	
$\pi = 3.660\,R_M - 1.232\,R_M^2 + 1.130$	12	0.997	0.077	724	(26)

from which the values of $\Sigma\pi$ were calculated for derivatives IVm and IVn (see Table IV). Owing to the poor solubility of cinnamic acids under the conditions of both tests, it was possible to assess only the inhibition of denaturation of serum albumin in

TABLE IV

CHROMATOGRAPHIC BEHAVIOUR AND BIOLOGICAL ACTIVITY OF CINNAMIC ACIDS (IV)

No.	*X*	π*	R_F**	R_M	*Log* $(1/C^I)$***
IVa	H	0	0.655	−0.28	3.252
IVb	4-CH_2=$CHCH_2O$	0.78	0.69	−0.35	3.495
IVc	3-Cl	0.83	0.55	−0.09	3.509
IVd	4-Cl	0.87	0.53	−0.05	3.569
IVe	4-*iso*-C_3H_7O	0.88	0.54	−0.07	3.479
IVf	3-Cl-4-CH_2=$CHCH_2O$	1.38	0.47	0.05	3.757
IVg	4-*iso*-C_3H_7	1.40	0.44	0.10	3.801
IVh	3-Cl-4-*iso*-C_3H_7O	1.58	0.44	0.10	3.792
IVi	4-*iso*-C_4H_9	1.90	0.385	0.20	4.149
IVj	4-cyclo-$C_6H_{11}O$	2.18	0.315	0.34	—§
IVk	4-*n*-$C_6H_{13}O$	2.58	0.24	0.51	—§
IVl	3-Cl-4-*n*-$C_6H_{13}O$	3.18	0.16	0.72	—§
IVm	3-CH_3-O-4-*iso*-C_3H_7O	0.79 (0.52§§)	0.595	−0.17	3.306
IVn	3-CH_3O-4-*n*-$C_6H_{13}O$	2.49 (2.26§§)	0.295	0.38	4.279

* π parameters taken from refs. 24 and 25.

** Chromatography was performed on silica gel containing 7.5% of silicone oil.

*** Activities taken from ref. 12.

§ Insoluble under the conditions of the test.

§§ These values, calculated from R_M values using eqn. 26, were used in correlation analysis of the inhibition of serum albumin denaturation (eqn. 27).

eleven derivatives with lipophilicity not exceeding $\Sigma\pi = 2.2$ (acid IVn). For these compounds, the linear dependence on the lipophilicity was calculated, characterized either by the π parameters (eqn. 27) or by R_M values (eqn. 28).

	n	r	s	F	
$\log (1/C^I) = 0.487\,\pi + 3.123$	11	0.975	0.076	172	(27)
$\log (1/C^I) = 1.734\,R_M + 3.657$	11	0.981	0.066	235	(28)

CONCLUSION

For the four series of acids investigated, the results of regression analysis of various biological activities showed that the use of R_M values, obtained by reversed-phase TLC, for the characterization of lipophilicity was not markedly influenced by deviations from linearity of the π *versus* R_M correlations. Of nine examples of relationships between biological activity and π parameters studied, the application of R_M values for the characterization of lipophilicity led to a significant change in the activity–lipophilicity relationships solely in eqns. 4, 5 and 18. In these instances a linear relationship between the activity and π parameters is substituted by the fallacious parabolic dependence on lipophilicity expressed by R_M values. These pitfalls in the use of R_M values can probably be expected only in instances when the statistical

significance of the above linear relationship is characterized by a correlation coefficient approaching 0.99. Such exceptional instances also require a highly accurate determination of the biological activity concerned.

In several examples of compounds with combinations of substituents in which the additivity of π parameters could be expected to fail (acids Io–Is, IIt, IIIn–IIIp, IVm and IVn), it was possible to characterize their lipophilicity by quantities obtained from partition chromatography. For this purpose either the R_M values were used directly, or π parameters calculated from the relationship between π and R_M values were used.

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REVERSED-PHASE AND SOAP THIN-LAYER CHROMATOGRAPHY OF PHENOLS

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SUMMARY

The chromatographic characteristics of 60 phenols on layers of silanized silica gel alone and impregnated with anionic and cationic detergents have been investigated using elution with water–alcohol mixtures at different pH. The validity of the relationships between the R_F values, the pH of the eluent and the pK_a of the phenols has been verified on thin layers of silanized silica gel alone and impregnated with 4% DBS. Many interesting separations of polyhydroxybenzenes and dichloro-, trichloro-, dinitro- and alkylphenols have been carried out.

INTRODUCTION

Reversed-phase chromatography on silanized silica gel and soap thin-layer chromatography (TLC) have been used with good results in recent years in the study of basic organic compounds[1–6]. In this study we have applied such techniques to the separation of acidic organic compounds. The compounds considered included alkylphenols, halogenated phenols, nitrophenols and polyhydroxybenzenes, many of which we have already studied on anion and cation exchangers with cellulose, paraffinic and polystyrene matrices[7–9]. It is possible, therefore, to compare the results achieved on layers of these exchangers and those of silanized silica gel alone and impregnated with detergents.

EXPERIMENTAL

The test compounds (Supelco, Bellefonte, PA, U.S.A.) were dissolved in 95% ethanol. The concentration of the solutions were 1–2 mg/ml and 1-μl volumes were deposited on the layer. With halogenated phenols, more concentrated solutions (6–10 mg/ml) were employed. Fresh solutions were used for those phenols which easily decompose (pyrogallol, gallic acid and pyrocatechic acid).

The phenols were detected by the Boute reaction[10], exposing the wet layers successively to nitrogen dioxide and ammonia vapours. The solution of 2,6-dibromophenol is violet coloured. A violet spot, due to impurities in the commercial product,

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is visible at the application point before the exposure of the layer to nitrogen dioxide vapour.

The layers (thickness 300 μm) were prepared with a Chemetron automatic apparatus by mixing 20 g of silanized silica gel 60 HF (C_2) (Merck, Darmstadt, G.F.R.) in 50 ml of 95% ethanol with a known concentration of detergent. The detergents used were triethanolamine dodecylbenzenesulphonate (DBS) and N-dodecylpyridinium chloride (N-DPC).

All the work was carried out at 25°C. The migration distance was 11 cm unless otherwise stated.

RESULTS AND DISCUSSION

Table I gives the R_F values of 60 phenols on layers of silanized silica gel alone (columns A) and impregnated with 4% DBS (columns B), eluting with aqueous–organic solutions containing the same percentage of methanol (30%) but with different pH values (apparent pH between 5 and 11.3).

The DBS concentration refers to the alcoholic solution in which the silanized silica gel was suspended when the layers were prepared.

Layers of silanized silica gel alone

On these layers, the chromatographic behaviour of the phenols depends on their acid–base characteristics, as the R_F values increase with the increase in the apparent pH of the eluent owing to the progressive deprotonation of the phenolic OH group.

On the basis of the pK_a values reported in Table I, the R_F values obtained with the solution at pH 5 refer to the non-dissociated form of most phenols, and those with the solution at pH 11.3 refer to their deprotonated form.

The influence of the substituent groups on the chromatographic behaviour of the compounds can be seen from the behaviour of the phenol and of the eluent at the lowest pH value, so that the deprotonation effect can be excluded in the retention mechanism.

The introduction into the ring of CH_3, CH_2CH_3, NO_2, Br and Cl groups involves an increase in the retention by the layer, whereas the opposite behavior is observed on introduction of an OH group. This last occurrence is similar to that observed on the same layer with the introduction of an OH group into the aromatic ring of catecholamines[1] and of an NH_2 group in the case of primary aromatic amines[3]. The behaviour of polyhydroxybenzenes, in contrast, is completely different from that observed on Dowex 50-X4 (Na^+) thin layers, where such compounds are less retained than phenol[8].

The influence of the ionic strength on the chromatographic behaviour of phenols is shown from the data in columns 4A and 4B in Table I; on changing the ionic strength of the eluent from 0.1 to 0.01 a considerable increase in the R_F values is observed. Such behaviour is similar to that obtained on Dowex 50-X4 (Na^+) layers[8].

In alkaline media, gallic and pyrocatechic acids, phloroglucinol, pyrogallol and the three esters of gallic acid are oxidized in air and become visible as brown spots (elongated in some instances) before the exposure of the layers to nitrogen

TABLE I

R_F VALUES OF PHENOLS ON THIN LAYERS OF SILANIZED SILICA GEL (A) ALONE AND (B) IMPREGNATED WITH 4% DBS SOLUTION

Eluents: (1) 0.1 *M* CH_3COOH + 0.1 *M* CH_3COONa in 30% CH_3OH (pH 5.00); (2) 0.1 *M* KH_2PO_4 in 30% CH_3OH (pH 7.00); (3) 0.1 *M* NH_3 + 0.1 *M* NH_4Cl in 30% CH_3OH (pH 9.02); (4) 1 *M* NH_3 + 0.1 *M* NaCl in 30% CH_3OH (pH 11.30); (5) 1 *M* NH_3 in 30% CH_3OH (pH 11.30).

Phenol	*1*		*2*		*3*		*4*		*5*		$pK_a(25°C)$*
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	
Phenol	0.36	0.35	0.36	0.35	0.37	0.35	0.51	0.47	0.73	0.62	10.02
m-Cresol	0.21	0.19	0.22	0.19	0.24	0.20	0.38	0.31	0.53	0.40	10.09
p-Cresol	0.21	0.19	0.22	0.19	0.23	0.19	0.35	0.29	0.48	0.36	10.27
o-Cresol	0.22	0.20	0.22	0.20	0.23	0.20	0.35	0.29	0.48	0.35	10.32
3,5-Dimethylphenol	0.13	0.12	0.13	0.12	0.13	0.12	0.24	0.21	0.33	0.25	10.19
3,4-Dimethylphenol	0.14	0.12	0.14	0.12	0.14	0.12	0.22	0.19	0.29	0.24	10.36
2,5-Dimethylphenol	0.13	0.11	0.13	0.11	0.13	0.11	0.22	0.19	0.26	0.21	10.41
2,3-Dimethylphenol	0.13	0.11	0.13	0.11	0.13	0.11	0.22	0.19	0.26	0.20	10.54
2,4-Dimethylphenol	0.12	0.11	0.12	0.11	0.13	0.11	0.20	0.18	0.26	0.19	10.60
2,6-Dimethylphenol	0.13	0.12	0.13	0.12	0.13	0.12	0.20	0.18	0.25	0.20	10.63
2,3,5-Trimethylphenol	0.07	0.06	0.07	0.06	0.07	0.06	0.13	0.10	0.14	0.12	—
2,4,6-Trimethylphenol	0.07	0.06	0.07	0.06	0.07	0.06	0.13	0.10	0.13	0.10	10.88
2,3,6-Trimethylphenol	0.07	0.06	0.07	0.06	0.07	0.06	0.13	0.10	0.14	0.11	—
m-Ethylphenol	0.11	0.11	0.11	0.11	0.12	0.11	0.25	0.21	0.32	0.28	9.9**
p-Ethylphenol	0.12	0.11	0.12	0.11	0.12	0.11	0.23	0.20	0.28	0.24	10.0**
o-Ethylphenol	0.11	0.10	0.11	0.10	0.11	0.10	0.20	0.18	0.25	0.21	10.2**
o-Chlorophenol	0.20	0.19	0.21	0.19	0.32	0.24	0.69	0.68	0.93	0.89	8.48
m-Chlorophenol	0.16	0.12	0.16	0.12	0.20	0.14	0.50	0.42	0.82	0.64	9.02
p-Chlorophenol	0.16	0.12	0.16	0.12	0.20	0.14	0.42	0.33	0.67	0.50	9.38
2,6-Dichlorophenol	0.12	0.10	0.16	0.13	0.56	0.55	0.72	0.73	0.95	0.88	6.79, 6.79***
2,5-Dichlorophenol	0.09	0.06	0.11	0.06	0.34	0.28	0.62	0.62	0.93	0.85	7.50, 7.35***
2,3-Dichlorophenol	0.08	0.06	0.09	0.06	0.28	0.22	0.59	0.58	0.93	0.85	— , 7.45***
2,4-Dichlorophenol	0.08	0.05	0.08	0.05	0.22	0.16	0.55	0.52	0.81	0.78	7.89, 7.75***
3,5-Dichlorophenol	0.07	0.04	0.07	0.04	0.14	0.09	0.43	0.42	0.79	0.64	8.18, 7.93***
3,4-Dichlorophenol	0.07	0.04	0.07	0.04	0.12	0.07	0.39	0.35	0.76	0.57	— , 8.39***
2,3,6-Trichlorophenol	0.05	0.05	0.13	0.12	e.s.	0.45	e.s.	0.49	0.83	0.82	6.12
2,4,6-Trichlorophenol	0.04	0.04	0.09	0.08	0.37	0.37	0.49	0.47	0.81	0.73	6.42
2,3,5-Trichlorophenol	0.03	0.03	0.05	0.04	0.26	0.25	0.43	0.43	0.76	0.69	7.23
2,4,5-Trichlorophenol	0.03	0.03	0.05	0.04	0.25	0.22	0.43	0.43	0.78	0.70	7.33
2,3,4-Trichlorophenol	0.03	0.03	0.05	0.03	0.23	0.18	0.43	0.43	0.77	0.68	7.59
3,4,5-Trichlorophenol	0.02	0.02	0.03	0.02	0.12	0.09	0.33	0.34	0.64	0.52	7.74
2,3,5,6-Tetrachlorophenol	0.02	0.02	0.12	0.12	0.24	0.25	0.33	0.33	0.64	0.50	5.44
2,3,4,5-Tetrachlorophenol	0.01	0.01	0.02	0.02	0.14	0.14	0.29	0.30	0.61	0.46	6.96
Pentachlorophenol	0.01	0.01	0.09	0.09	0.14	0.13	0.24	0.21	0.46	0.33	5.26
o-Bromophenol	0.18	0.15	0.18	0.15	0.26	0.20	0.68	0.59	0.93	0.88	8.44
m-Bromophenol	0.13	0.10	0.13	0.10	0.19	0.12	0.49	0.38	0.78	0.62	9.03
p-Bromophenol	0.13	0.10	0.13	0.10	0.18	0.11	0.42	0.30	0.65	0.50	9.36
2,6-Dibromophenol	0.08	0.07	0.11	0.09	0.49	0.46	0.64	0.64	0.93	0.89	6.6
2,4-Dibromophenol	0.05	0.03	0.06	0.04	0.11	0.10	0.51	0.43	0.85	0.75	7.8
p-Nitrophenol	0.26	0.23	0.31	0.26	0.67	0.66	0.83	0.89	0.96	0.92	7.15
o-Nitrophenol	0.20	0.17	0.23	0.19	0.62	0.61	0.79	0.83	0.96	0.92	7.23
m-Nitrophenol	0.25	0.22	0.25	0.22	0.36	0.29	0.69	0.68	0.96	0.92	8.40
2,6-Dinitrophenol	0.53	0.68	0.54	0.76	0.66	0.77	0.71	0.78	0.96	0.92	3.71
2,4-Dinitrophenol	0.48	0.58	0.51	0.74	0.63	0.77	0.71	0.78	0.96	0.92	4.09

(Continued on p. 342)

TABLE I *(continued)*

Phenol	*1*		*2*		*3*		*4*		*5*		*pK*$_a$*(25°C)*
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	
2,5-Dinitrophenol	0.32	0.31	0.49	0.65	0.63	0.73	0.70	0.77	0.96	0.92	5.22
3,4-Dinitrophenol	0.21	0.18	0.40	0.51	0.58	0.68	0.70	0.78	0.96	0.92	5.43
2-Chloro-5-methylphenol	0.11	0.09	0.11	0.09	0.13	0.12	0.55	0.45	0.89	0.77	—
4-Chloro-3-methylphenol	0.08	0.06	0.08	0.06	0.09	0.06	0.28	0.20	0.51	0.30	—
4-Chloro-2-methylphenol	0.08	0.06	0.08	0.06	0.09	0.06	0.25	0.16	0.44	0.24	—
Gallic acid	0.92	0.94	0.91	0.94	e.s.	e.s.	e.s.	e.s.	e.s.	e.s.	4.41
Pyrocatechic acid	e.s.	0.93	0.85	0.95	e.s.	e.s.	e.s.	0.95	e.s.	0.95	—
Phloroglucinol	0.76	0.73	0.74	0.72	e.s.	e.s.	0.95	0.95	0.95	0.95	8.45
Pyrogallol	0.74	0.68	0.74	0.67	e.s.	e.s.	e.s.	e.s.	e.s.	e.s.	9.01
Resorcinol	0.58	0.58	0.56	0.55	0.56	0.55	0.74	0.70	0.95	0.87	9.81
Hydroquinone	0.67	0.66	0.66	0.66	0.67	0.68	e.s.	e.s.	e.s.	e.s.	10.35
Orcinol	0.47	0.48	0.46	0.46	0.47	0.46	0.62	0.57	0.89	0.80	—
BHA[§]	0.02	0.02	0.02	0.02	0.02	0.02	0.04	0.03	0.05	0.03	—
n-Propyl gallate	0.19	0.24	0.21	0.23	e.s.	e.s.	e.s.	e.s.	e.s.	e.s.	—
Octyl gallate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	—
Dodecyl gallate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	—

* Refs. 11, 12 and 13.
** pK_a values at 28°C.
*** pK_a values at 29°C (ref. 14).
§ 2, (3)-*tert.*-Butyl-4-hydroxyanisole (mixed isomers).

dioxide vapour. Hydroquinone is oxidized only in strongly alkaline media because, on eluting with the solution at pH 9.02, it does not give rise to the brown spot.

Layers of silanized silica gel impregnated with 4% DBS

In the presence of DBS, as shown in Table I, a decrease in the R_F values for most compounds is observed, with the exception of the phenols with marked acidic characteristics, such as dinitrophenols and gallic and pyrocatechic acids, for which higher R_F values than those on the layers without detergent are observed.

The affinity sequence of the phenols on the two layers is, however, similar over the whole pH range explored, except for the above-mentioned compounds and some polyhydroxybenzenes. Such behaviour can be used from an analytical standpoint on layers impregnated with detergent for the separation of pyrogallol from phloroglucinol and of dinitrophenols.

A characteristic of these layers is the extraordinary compactness of the spots, so that the separation of compounds that differ only by 0.05 in their R_F values can be carried out. This is important in view of the shorter elution time on the impregnated layer (about 45 min) than on silanized silica gel alone (about 55 min).

On changing the ionic strength of the eluent from 0.1 to 0.01, an increase in the R_F values is observed on layers impregnated with 4% DBS (see columns 4B and 5B in Table I), even if such an increase is generally less marked than on layers without detergent.

Another peculiar characteristic of the impregnated layer is the possibility of using eluents with containing less than 30% of methanol, so that both the retention and the resolving power of the layers can be further increased[5].

Table II gives the R_F values of phenol and alkylphenols, eluting with solutions

TABLE II

R_F VALUES OF PHENOLS ON LAYERS OF SILANIZED SILICA GEL IMPREGNATED WITH 4% DBS SOLUTION

Eluents: ammonia solutions in 20% methanol.

Phenol	*Ammonia concentration (M)*				
	1	*4*	*6*	*8*	*10*
Phenol	0.60	0.73	0.78	0.79	0.80
m-Cresol	0.37	0.57	0.63	0.63	0.63
p-Cresol	0.31	0.48	0.54	0.57	0.57
o-Cresol	0.32	0.48	0.53	0.55	0.56
3,5-Dimethylphenol	0.21	0.37	0.42	0.44	0.46
3,4-Dimethylphenol	0.19	0.32	0.38	0.41	0.42
2,5-Dimethylphenol	0.17	0.29	0.35	0.37	0.38
2,3-Dimethylphenol	0.15	0.26	0.32	0.34	0.34
2,4-Dimethylphenol	0.14	0.24	0.30	0.31	0.32
2,6-Dimethylphenol	0.16	0.27	0.32	0.35	0.36
2,3,5-Trimethylphenol	0.07	0.14	0.17	0.20	0.22
2,3,6-Trimethylphenol	0.06	0.10	0.16	0.19	0.21
2,4,6-Trimethylphenol	0.06	0.09	0.14	0.17	0.19
2,3,5,6-Tetramethylphenol	0.03	0.05	0.11	0.14	0.15
m-Ethylphenol	0.21	0.38	0.46	0.49	0.51
p-Ethylphenol	0.19	0.34	0.38	0.43	0.44
o-Ethylphenol	0.16	0.27	0.31	0.35	0.36

with a constant methanol concentration (20%) and increasing ammonia concentrations (from 1 to 10 *M*). Under these elution conditions the behaviour of the alkylphenols in the deprotonated form is pointed out; 2,3,5,6-tetramethylphenol, whose detection is possible only in strongly alkaline medium, has also been studied.

From the data in Table II it should be noted that, as the ammonia concentration in the eluent is increased, an increase in the R_F values is observed for all phenols. This increase is sharp at ammonia concentrations up to 6 *M* and becomes negligible at higher ammonia concentrations. The differences in the R_F values of the isomers, which are not very marked at ammonia concentrations below 4 *M*, reach their highest values at concentrations above 6 *M*.

It should be noted that, relative to phenol, the R_F values of the alkylphenols gradually decrease as the number of methyl groups in the ring increases. Therefore, from the R_F values of an alkylphenol, the number of methyl groups in the molecule can be obtained.

The affinity sequence of the isomers of cresols, ethylphenols and dimethylphenols is opposite to that of their pK_a values.

Layers of silanized silica gel impregnated with N-DPC

On these layers the retention of phenols is higher than that on silanized silica gel alone, and is similar to that observed on layers impregnated with DBS, at least for those phenols which are prevalently in the non-dissociated form.

The phenols in the deprotonated form are strongly retained owing to an anion-exchange process with the functional group of N-DPC in addition to the liquid–liquid partition process. As the apparent pH of the eluent is increased, a

decrease in the R_F values is observed, which is different to that found on layers of silanized silica gel alone and impregnated with DBS. Such behaviour causes a levelling of the R_F values of the phenols and therefore the different acid–base characteristics of the compounds cannot be used for their separation.

The best results can be achieved with an increase in the methanol concentration in the eluent (from 30 to 50%) and a decrease in the amount of N-DPC on the layer (from 4 to 1%). For example, on layers impregnated with 1% N-DPC and with 0.1 *M* ammonia solution in 50% methanol as eluent, a great difference between the R_F values of phenol (0.49) and cresols (0.34–0.38) with respect to those of polyhalogenated phenols (0.09–0.18) is observed.

As the concentration of methanol in the eluent is further increased (to 80%) a general increase in the R_F values is obtained, but this does not result in a better resolving power because many compounds give rise to elongated spots.

Retention mechanism

The parameters that determine the retention of phenols on layers of silanized silica gel alone and impregnated with DBS are the same that affect retention on cation exchangers[8]. We considered it useful, therefore, to verify also on silanized silica gel layers the validity of the relationship

$$\frac{1}{R_F} - 1 = \left(\frac{1}{R_{F_{ac}}} - 1\right) \frac{[H^+]}{K_a + [H^+]} + \left(\frac{1}{R_{F_{alk}}} - 1\right) \frac{K_a}{K_a + [H^+]} \qquad (1)$$

where $R_{F_{ac}}$ and $R_{F_{alk}}$ are the R_F values of the protonated and deprotonated form of the phenols obtained on eluting with acidic and alkaline solutions, respectively. Applying eqn. 1 to some phenols chosen on the basis of their pK_a values, the curves in Fig. 1, which refer to layers of silanized silica gel, were obtained.

The theoretical curves were constructed according to the pK_a values relative to solutions containing 30% methanol and drawn by adding 0.58 to the corresponding

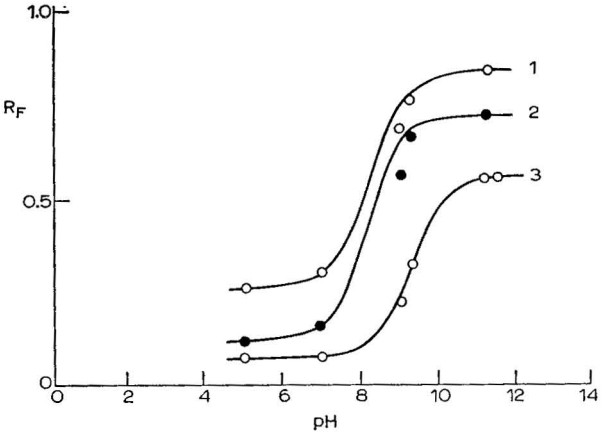


Fig. 1. R_F values *versus* the apparent pH of the eluent for phenols on silanized silica gel thin layers. (1) *p*-Nitrophenol (pK_a = 7.73); (2) 2,6-dichlorophenol (pK_a = 7.37); (3) 2,4-dichlorophenol (pK_a = 8.47). The pK_a values refer to aqueous—organic solutions containing 30% methanol.

pK_a in aqueous solution[15]. The experimental points are those reported at the different apparent pH values of the eluent in Table III; they are in good agreement with the theoretical curves and support the validity of eqn. 1 even on these layers. The experimental points at pH 9.02 are lower than the theoretical values on curves 1 and 2, owing to an apparent pH value on the layer that is smaller than that of the eluent for the formation of a pH gradient on the layer similar to that observed on cation exchangers[8].

TABLE III

R_F VALUES OF PHENOLS ON SILANIZED SILICA GEL THIN LAYERS OBTAINED WITH ELUENTS AT DIFFERENT pH VALUES

Eluents: 0.1 *M* CH_3COOH + 1 *M* CH_3COONa in 30% CH_3OH (pH 5.00); 0.1 *M* KH_2PO_4 in 30% CH_3OH (pH 7.00); 0.1 *M* NH_3 + 0.1 *M* NH_4Cl in 30% CH_3OH (pH 9.02); 0.2 *M* NH_3 + 0.1 *M* NH_4Cl in 30% CH_3OH (pH 9.30); 1 *M* NH_3 + 0.1 *M* NaCl in 30% CH_3OH (pH 11.30); 2 *M* NH_3 + 0.1 *M* NaCl in 30% CH_3OH (pH 11.60).

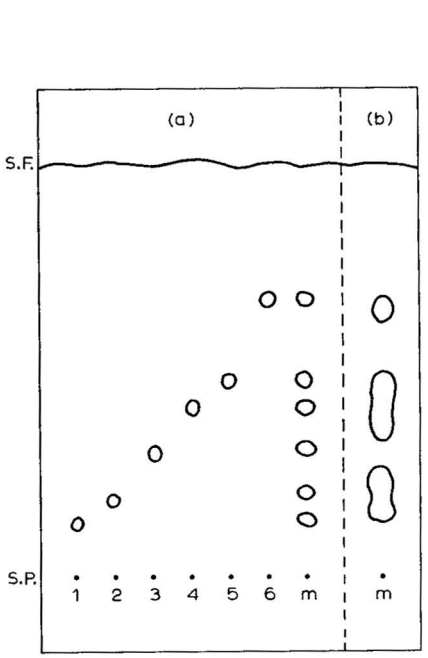
p-Nitrophenol		*2,6-Dichlorophenol*		*2,4-Dichlorophenol*	
R_F	*pH*	R_F	*pH*	R_F	*pH*
0.26	5.00	0.12	5.00	0.08	5.00
0.31	7.00	0.16	7.00	0.08	7.00
0.67	9.02	0.56	9.02	0.22	9.02
0.75	9.30	0.66	9.30	0.32	9.30
0.83	11.30	0.72	11.30	0.55	11.30
				0.56	11.60

Analytical applications

Among the separations that can be effected on the basis of the R_F values obtained with the different eluents, we carried out separations of the three chloro- and bromophenols and of the two tetrachlorophenols, on layers of silanized silica gel alone and impregnated with 4% DBS. In comparison with reversed-phase chromatography, however, soap TLC permits the separation of a larger number of phenols, as smaller differences in their R_F values are necessary owing to the compactness of the spots.

For example, the separation of the three methylchlorophenols cannot be performed on layers of silanized silica gel alone, although the difference between the R_F values of 4-chloro-2-methylphenol and 4-chloro-3-methylphenol is greater than that observed on impregnated layers (see columns 5A and 5B in Table I), where such a separation can be effected by eluting with ammonia solution at pH 11.30, both alone and in the presence of 0.1 *M* sodium chloride. It should be noted that the difference in the R_F values of the two above-mentioned isomers is only 0.04 with the eluent of higher ionic strength.

Fig. 2a shows the separation of the six dichlorophenols on layers of silanized silica gel impregnated with 4% DBS, eluting with 0.2 *M* ammonia and 0.1 *M* ammonium chloride in 30% methanol (apparent pH 9.30); such a separation cannot be achieved on layers without detergent (Fig. 2b). With 0.1 *M* ammonia and 0.1 *M* ammonium chloride in 30% methanol as the eluent (apparent pH 9.02) and with



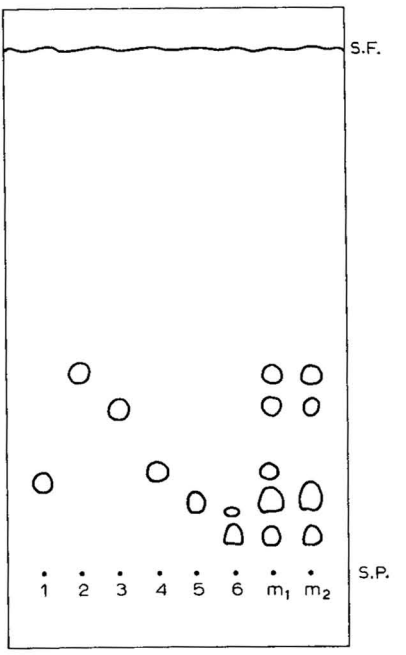


Fig. 2. Thin-layer chromatogram of dichlorophenols on (a) silanized silica gel impregnated with 4% DBS solution and (b) silanized silica gel alone. Eluent: 0.2 *M* ammonia + 0.1 *M* ammonium chloride in 30% methanol (pH = 9.30). (1) 3,4-Dichlorophenol; (2) 3,5-dichlorophenol; (3) 2,4-dichlorophenol; (4) 2,3-dichlorophenol; (5) 2,5-dichlorophenol; (6) 2,6-dichlorophenol; (m) mixture. S.P. = start point; S.F. = solvent front.

Fig. 3. Thin-layer chromatogram of trichlorophenols on silanized silica gel impregnated with 4% DBS solution. Migration distance: 14 cm. Eluent: 0.1 *M* ammonia + 0.1 *M* ammonium chloride in 30% methanol (pH = 9.02). (1) 2,4,5-Trichlorophenol; (2) 2,3,6-trichlorophenol; (3) 2,4,6-trichlorophenol; (4) 2,3,5-trichlorophenol; (5) 2,3,4-trichlorophenol; (6) 3,4,5-trichlorophenol; (m_1) mixture of 1, 2, 3, 4, 5 and 6; (m_2) mixture of 1, 2, 3, 5 and 6. S.P. = start point; S.F. = solvent front.

a migration distance of 14 cm, the separation shown in Fig. 3 was obtained on layers impregnated with 4% DBS; this separation, however, does not concern all six trichlorophenols which are present in mixture m_1 because 2,4,5-trichlorophenol and 2,3,4-trichlorophenol can not be separated, as indicated in the chromatogram relative to mixture m_2. With 3,4,5-trichlorophenol, two spots are observed owing to the presence of by-products (probably 2,3,4-trichlorophenol).

Fig. 4 shows the separation of the four dinitrophenols and of a large number of polyhydroxybenzenes on impregnated layers using 0.1 *M* acetate buffer in 30% methanol as eluent and with a long migration distance (15 cm) so that phloroglucinol can be separated from pyrogallol. On layers of silanized silica gel alone the separation of the four dinitrophenols is difficult owing to the small difference in the R_F values of 2,6-dinitrophenol and 2,4-dinitrophenol.

Finally, numerous separations of mixtures of phenol and alkylphenols can be effected on layers impregnated with 4% DBS. For example, separations of phenol, *m*-cresol, *p*-cresol, 3,5-dimethylphenol, 2,3-dimethylphenol, 2,3,5-trimethylphenol

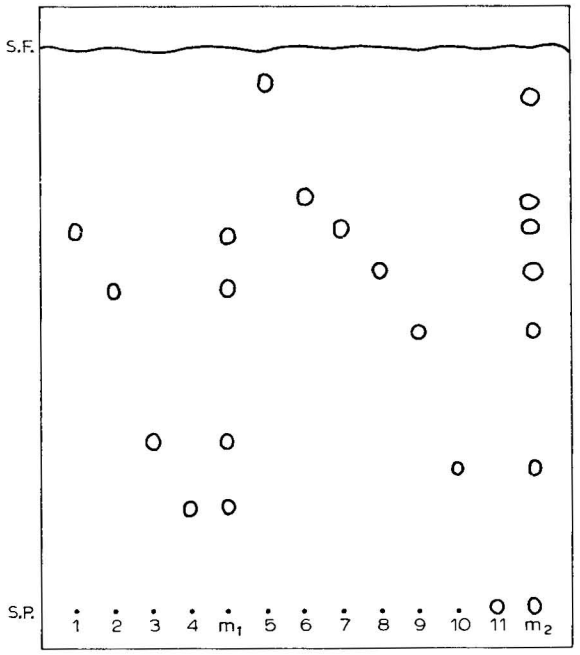


Fig. 4. Thin-layer chromatogram of dinitrophenols and polyhydroxybenzenes on silanized silica gel impregnated with 4% DBS solution. Migration distance: 15 cm. Eluent: 0.1 *M* acetic acid + 0.1 *M* sodium acetate in 30% methanol (pH = 5.00). (1) 2,6-Dinitrophenol; (2) 2,4-dinitrophenol; (3) 2,5-dinitrophenol; (4) 3,4-dinitrophenol; (m_1) mixture of dinitrophenols; (5) gallic acid; (6) phloroglucinol; (7) pyrogallol; (8) resorcinol; (9) orcinol; (10) *n*-propyl gallate; (11) octyl gallate; (m_2) mixture of polyhydroxybenzenes. S.P. = start point; S.F. = solvent front.

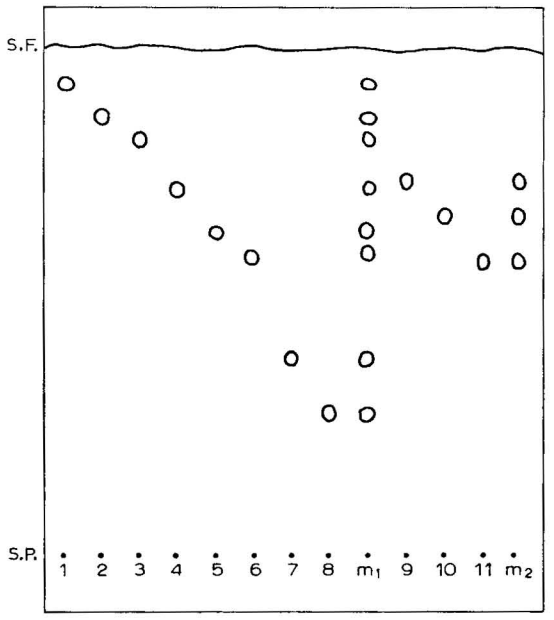


Fig. 5. Thin-layer chromatogram of phenol and of some mono- and polyalkyl derivatives on silanized silica gel impregnated with 4% DBS solution. Migration distance: 13.5 cm. Two successive developments with 10 *M* ammonia in 20% methanol. (1) Phenol; (2) *m*-cresol; (3) *p*-cresol; (4) 3,5-dimethylphenol; (5) 2,5-dimethylphenol; (6) 2,3-dimethylphenol; (7) 2,3,5-trimethylphenol; (8) 2,3,5,6-tetramethylphenol; (m_1) mixture; (9) *m*-ethylphenol; (10) *p*-ethylphenol; (11) *o*-ethylphenol; (m_2) mixture of ethylphenols. S.P. = start point; S.F. = solvent front.

and 2,3,5,6-tetramethylphenol and of the three ethylphenols have been effected by eluting with solutions with a constant methanol concentration (20%) and different ammonia concentrations (6, 8 and 10 *M*). With two successive developments in the same eluent (10 *M* ammonia in 20% methanol) the separation of a large number of alkylphenols was achieved (see Fig. 5).

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DIRECT DERIVATIZATION AND GAS CHROMATOGRAPHIC DETERMINATION OF BARBITURATES IN AUTOPSY LIVER TISSUES

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SUMMARY

A new gas chromatographic procedure is presented for the determination of barbiturates in autopsy tissues (liver and blood). The barbiturates are separated from the interference of co-extracted, free fatty acids in an acid-catalyzed methylation of the fatty acids and quantified as the N,N-dimethyl derivatives following reaction with dimethyl sulphate. Derivatization and recovery are shown to be simple, efficient procedures leading to substantially higher analytical results.

INTRODUCTION

The extraction of small amounts of drugs from biological materials is usually accompanied by troublesome lipids unless, as in the case of urine or saliva, the sample is essentially lipid-free. The amount and nature of the lipids present is determined by the type of material constituting the sample. Thus, the extraction of liver tissues by common organic solvents leads to the isolation[1] of large amounts of free fatty acids in addition to much smaller amounts of lipids of varying polarity, such as triglycerides, phospholipids, cholesteryl esters and cholesterol. As a consequence, the recovery from liver tissues and blood of *acidic* drugs or other toxic substances, as distinct from *basic* compounds, is always complicated by the co-extracted fatty acids since their behaviour will be very similar in any separation process based on chromatography or partitioning between two immiscible solvents under controlled pH. When considering the estimation of an acidic compound present in tissues, the solvent chosen ought to result in the complete removal of the compound regardless of the amount of co-extracted material. Thereafter, the procedure is required to cope with the removal of lipids (or, indeed, other co-extractives) in a manner which does not lead to the simultaneous loss of the acidic compound. Although obvious, these two requirements are, in practice, difficult to meet fully and existing methodologies have either ignored them or accepted the analytical results as, at best, semi-quantitative (for a review, see ref. 2).

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In this paper, we present a new procedure for the estimation of barbiturates isolated from autopsy liver tissues after extraction with methanol. Interference from free fatty acids, the major component of the co-extractives, is eliminated by an acid-catalyzed esterification (using methanol–hydrogen chloride) and the unaffected free barbituric acids are recovered with the aid of dilute alkali. The acids are finally alkylated with dimethyl sulphate prior to gas chromatography (GC) on a non-polar (SE-30) column.

EXPERIMENTAL

Synthesis of barbituric acid derivatives

The N,N-dimethyl derivatives of amobarbital, pentobarbital and phenobarbital were prepared[3] by reacting the free acid (1 g) with dimethyl sulphate in a mildly alkaline medium. Crude derivatives were isolated by extraction of the solvent-free residues with hexane (2 × 40 ml) and conveniently purified by passing the extract (dried with anhydrous sodium sulphate) through a short column of alumina (10 × 2.5 cm) with further development of the column using 1% methanol (v/v) in hexane. Fractions (11 × 10 ml) showing a single compound when examined by GC were combined, the solvent removed and the product dried under vacuum, over phosphorus(V) oxide. Identity and purity were confirmed by microanalysis, infrared and mass spectroscopy.

Reagents and standard solutions

All solvents and reagents were chemically pure, commercially available materials. Apart from the solvents which were re-distilled from an all-glass apparatus, the reagents were used without further treatment.

Potassium carbonate. Saturated aqueous solution.

Sodium hydroxide. 0.1 *M* in water.

Methanol–hydrogen chloride. Prepared by slowly saturating anhydrous methanol (700 ml, 10 min) with dry hydrogen chloride (Matheson, Coleman & Bell, East Rutherford, NJ, U.S.A.) and diluting to 1 l. The acid concentration was determined by titrating aliquots (10 ml) with standardized alkali (1.00 *M*) using phenolphthalein indicator. It was usually 1.5–2.0 *M* and suitable for use without further dilution.

Solutions of barbituric acids. Prepared by dissolving an accurately weighed amount of amobarbital, pentobarbital or phenobarbital (about 0.030 g) in methanol and diluting to 100 ml. A ten-fold dilution of the solution gave a convenient working concentration.

Solutions of N,N-dimethyl derivatives of barbituric acids. Prepared by dissolving the appropriate and accurately weighed, pure dimethyl derivative (about 0.025 g) in hexane and diluting to 100 ml. A working solution was obtained by making a ten-fold dilution of each stock solution.

Solution of internal standard. 0.1% (w/v) *n*-octadecane in CS_2.

Gas chromatography

A Becker Model 417 gas chromatograph was used. It was fitted with a coiled borosilicate column (5 ft. × 1/4 in. O.D.) packed with 10% (w/w) SE-30 on Chromosorb W AW DMCS (80–100 mesh). General operating conditions were:

carrier gas nitrogen flow, 45 ml/min; injection port temperature, 210°C; column, 180°C; flame-ionization detector, 210°C.

A solution (1–2 μl) of the barbiturate derivative (from extract or standard solution) contained in a known volume of the internal standard solution was injected on the column. Concentrations of the barbituric acid were calculated from a calibration plot of peak height ratio (derivative/*n*-octadecane) *versus* concentrations of the corresponding dimethyl derivative. The dilution of the solution to be chromatographed was adjusted to correspond to the concentration range 0.030–0.140 mg derivative per ml of the calibration plot (see captions to Table IV). However, a calibration plot was prepared for each batch of five or six determinations.

Conversion studies

(i) Amobarbital (30 μg) was added to a test-tube (10 × 1.8 cm O.D.) and the solvent carefully removed under vacuum at 35°C). Aqueous potassium carbonate (0.3 ml), dimethyl sulphate (0.07 g) and methanol (0.2 ml) were added. The tube was then lightly stoppered and placed in boiling water until the reaction had ceased (*ca.* 10 min). The contents of the tube were diluted with water (2 ml), extracted with hexane (2 ml) and the extract carefully reduced to dryness. This residue was dissolved in 2.0 ml solution containing the internal standard, then analyzed by GC, as above.

(ii) A variation of (i) differed only in that benzene was used as the extracting solvent.

Efficiency of the conversion reaction was determined separately for pentobarbital (32.4 μg) and phenobarbital (33.5 μg) using benzene only in the extraction step. Conditions for GC remained the same.

Recovery studies

Analyses were carried out with an homogeneous matrix obtained from a freeze-dried, drug-free tissue (5 g). This was exhaustively extracted by refluxing in boiling methanol (160 ml) for 1.5 h. The cooled mixture was filtered under vacuum, then the extract was divided into five equal portions and thereafter handled as a set of five replicates. To each was added a known amount of amobarbital (30 μg) and the solvent removed under vacuum (at 35°C). The dry residue was then heated in a water-bath at 85–90°C with the methanol–hydrogen chloride reagent (50 ml) in a tightly stoppered test-tube. When cool, the contents of the tube were reduced to approximately 20 ml, diluted with water (50 ml) and extracted with chloroform (2 × 20 ml). The combined organic phase was reduced in volume (*ca.* 20 ml) and extracted with sodium hydroxide solution (2 × 10 ml). The aqueous phase was immediately acidified with hydrochloric acid (10 *M*, 0.5 ml) then re-extracted with chloroform (2 × 10 ml). Solvent was completely removed from the extract and the residue treated as in (ii), above.

Tissue analyses

Although similar procedurally, some details are repeated because of differences in the final scale adopted for the actual analyses.

Fresh tissues (Method B). A sample (5 g) of the comminuted tissue was boiled in methanol (50 ml, 30 min) under reflux. The cold methanolic extract* was decanted

* A single extraction in methanol is referred to later as Method A.

through a small, coarse filter-paper and the residue subjected to two similar extractions. The combined extracts were reduced in volume to *ca.* 20 ml in a rotary evaporator, 50 ml 0.2 *M* hydrochloric acid were added and the solution extracted with chloroform (3 × 20 ml). The combined extracts were again reduced to dryness under vacuum (at 35°C) and the *dry* residue reacted with methanol–hydrogen chloride reagent (30 ml) in a tightly stoppered test-tube at 85°C (in a water-bath) for 30 min. The cooled contents of the tube were reduced to about 10 ml, diluted with water (20 ml) and extracted with chloroform (2 × 10 ml). The extracts were combined, extracted with sodium hydroxide solution (2 × 10 ml) and the total alkaline extract immediately acidified with hydrochloric acid (10 *M*) and re-extracted with chloroform (2 × 10 ml) to recover the barbituric acid. This extract was reduced to dryness and, with the aid of a minimal amount of chloroform, quantitatively transferred to a methylation tube (10 × 1.8 cm O.D.). Solvent was carefully removed and methylation completed (≈ 5 min) by heating the stoppered tube at 85°C after adding dimethyl sulphate (0.07 g), potassium carbonate solution (0.3 ml) and methanol (0.3 ml). When cold, the product was diluted with water (2 ml) and extracted with benzene (2 × 2 ml). The organic phase was taken to dryness and the residue dissolved in an accurately measured volume of the internal standard solution. Aliquots (1–2 μl) were injected onto the column and the concentration of barbituric acid derivative determined from the calibration plot. The concentration of the barbituric acid in the tissue was obtained from:

$$\text{Barbituric acid (mg/kg sample)} = (\text{concentration of derivative in extract, } \mu\text{g}/\mu\text{l}) \times V \times \frac{1000}{W} \times F$$

where W = mass (g) of tissue taken, V = final volume (ml) of internal standard solution and F = (molecular weight of the barbituric acid)/(molecular weight of corresponding N,N-dimethyl derivative).

Freeze-dried tissues. The same procedure was employed for freeze-dried tissues, however, a smaller sample (1 g) was used and the dry methanol-soluble residue methylated directly, thereby eliminating the acid–chloroform extraction step.

Blood. The same procedure was employed as in *Fresh tissues* except that the acidified blood (2 ml) was extracted directly with chloroform (3 × 10 ml) and the combined extracts reduced to a dry residue for reaction with methanol–hydrogen chloride (20 ml).

RESULTS AND DISCUSSION

This procedure for the analysis of barbiturates was developed primarily because of two problems in the application of existing spectrophotometric methods to the toxicological analysis of liver tissues and, to a lesser extent, blood. In the first of these, the inevitable precipitation of fatty acids (isolated by centrifugation and confirmed by GC) at low pH precludes measurement of the absorption* of radiation at 240 nm and, despite effective methylation of the fatty acids (as outlined in the Experimental section) and the removal of the barbiturate from the resulting substrate,

* Unsaturated fatty acids have insignificant absorption at 240 nm.

the yellow-brown colour and high absorbance values of the extracts (and aqueous residues) persist. The second problem is related to the, as yet, unidentified chromogens originating from other matrix components[4], and is also partially dependent on the analytical procedure employed.

The selective esterification of free fatty acids in the presence of the barbiturates is the basis for the separation of these two classes of compounds. The mechanism probably involves protonation of the pyrimidine nucleus of the barbiturate, thereby effectively blocking any alkylation, with the simultaneous, unhindered esterification of carboxylic acids of lipid origin by the oxonium ion derived from the alcohol (here, $CH_3\overset{+}{O}H_2$). At the same time, any other carboxylic acids present in the original extract would also be expected to undergo esterification, and remain (or be lost) with the fatty acid esters when separated from the barbiturate in the prevailing acidic environment at the completion of the esterification reaction. Identification of these carboxylic acids would be dependent only upon an effective separation from the fatty acid esters of similar GC retention times. Again, the acid-catalyzed esterification ought to be equally effective in separating fatty acids from other acidic drugs possessing a cyclic amide structure provided that the compounds are not acid-sensitive.

Efficiency of the barbiturate derivatization

Based on the use of the pure derivative, the combined efficiency of the conversion of amobarbital into the N,N-dimethyl derivative and its extraction by hexane and benzene is compared in Table I. The data show significantly better results when extraction of the derivative is carried out with benzene, although the reproducibility in both cases is comparable. The efficiency attainable with benzene

TABLE I

COMPARISON OF CONVERSION EFFICIENCIES FOR AMOBARBITAL USING HEXANE AND BENZENE SOLVENTS

Values obtained for 30 μg amobarbital.

Conversion efficiency (%)	
Hexane extraction	*Benzene extraction*
81.6	95.7
77.6	87.7
76.2	95.2
77.6	94.1
74.9	88.2
79.8	94.1
79.8	90.9
84.3	
79.8	
86.1	
81.6	
Mean 79.9	92.3
n 11	7
S.D. 3.36	3.33

is confirmed by the data for amobarbital, pentobarbital and phenobarbital shown in Table II where, in each case, the two-step process of conversion and extraction has an efficiency exceeding 95%, with standard deviations ranging between 4.0 and 6.0%.

TABLE II

CONVERSION EFFICIENCIES FOR AMOBARBITAL, PENTOBARBITAL AND PHENOBARBITAL

Values obtained for ≈30 μg of free acid with benzene as solvent in the extraction of the derivatized drug.

Conversion efficiencies (%)		
*Amobarbital**	*Pentobarbital*	*Phenobarbital*
100.0	99.5	91.6
93.5	100.6	87.4
95.0	90.5	91.6
90.0	100.6	94.8
92.0	102.2	95.9
95.0	93.4	98.6
97.5	102.2	100.1
91.8	96.7	104.9
92.8	94.5	104.9
94.0		
100.5		
98.5		
103.6		
Mean 95.7	97.8	96.6
n 13	9	9
S.D. 4.02	4.21	6.05

* Overall amobarbital results from Tables I and II ($n = 20$): mean 94.5; S.D. 4.1; R.S.D. 4.3%. Results are for samples processed in five batches.

Recovery of barbiturate from a tissue matrix

Table III shows the recovery of amobarbital added to methanol-soluble tissue components which, with the described procedure, averages greater than 90% with a standard deviation of *ca.* 2.7%. The lowest recovery (see Set 3) was obtained when the solutions were left to stand overnight before completing the analysis, and can reasonably be attributed[5,6] to adsorption of the barbiturate onto the surfaces of the glass containers.

Analysis of fresh tissues

Results for the analysis of amobarbital in liver tissues and one blood specimen are shown in Table IV. The simpler procedure involving only a single extraction in methanol (Method A) yielded results which, when compared with those obtained following the triple extraction of Method B, can only be regarded as semi-quantitative. Nevertheless, in most cases, even Method A yielded higher values than the official results for the tissue analyses. Apart from the more effective recovery of soluble material by the triple extraction procedure, explanation[4] for the improved barbiturate recovery lies in the progressive dehydration of the tissue by each

TABLE III

RECOVERY OF AMOBARBITAL FROM TISSUE EXTRACTS AFTER METHYLATION AND ALKYLATION REACTIONS

Values for the recovery of 30 μg of free acid after extraction, derivatization and isolation of the dimethyl derivative. Overall mean 90.6; S.D. 2.7; R.S.D. 3.0%.

Recovery (%)			
	Set 1	*Set 2*	*Set 3*
	93.50	91.9	89.1
	90.8	93.2	88.6
	88.1	94.7	90.2
	90.8	90.2	86.5
	93.5	91.9	85.5
Mean	91.3	92.4	88.0
n	5	5	5
S.D.	2.26	1.68	1.93

TABLE IV

DATA FOR ANALYSES OF "FRESH" LIVER TISSUES

Age of samples at time of analysis (official results, mg/kg); 1, 18 months (12.7); 2, 5 years (32.0); 3 18 months (18.0); 4 3 months (20.0); 5, 3 months (83.0); 6, 3 months (20.0).

Method	*Sample no.*	*Final volume* (ml)*	*Sample mass (g)*	*Amobarbital concentration*	
				*mg/kg***	*Mean*
A	1	2.0	4.9	21.0	
			4.5	18.9	20.2
			4.7	20.7	
A	2	4.0	4.9	61.0	61.5
			5.1	61.9	
A	3	2.0	5.0	20.8	
			4.3	24.5	22.5
			4.6	22.3	
A	4	2.0	4.8	41.8	
			6.0	49.4	42.7
			3.6	36.8	
A	5	10.0	4.6	71.4	
			4.7	89.2	80.6
			4.0	81.2	
—	6***	2.0		25.8	
				24.9	24.6
				23.1	
B	1	4.0	5.8	20.3	
			5.0	20.9	22.3
			8.2	25.6	
B	2	4.0	5.1	80.0	
			4.6	78.7	80.1
			5.5	81.5	
B	4	4.0	5.9	60.8	57.8
			5.1	54.7	
B	5	10.0	8.4	110.9	
			4.3	119.5	120.2
			5.0	130.2	

* Adjusted to correspond to calibration plot.

** Values are means from duplicate determinations.

*** Blood specimen; results in mg/l.

successive volume of methanol employed for the extraction. Indeed, comparison of the result for a freeze-dried tissue (84.7 mg/kg for sample 2 of Table IV) suggests that after three extractions the recovery of the barbiturate from fresh tissue is still incomplete, and that solvent extraction of fresh tissue is less efficient and slower than for a freeze-dried form of the same tissue containing less than 10% moisture. The variation observed for sample 5 (and, to a lesser extent, sample 4) with both methods implies that, because of the small sample required for analysis, great care is needed when preparing the entire specimen prior to sampling. Although obvious precautions such as thorough mixing of the thawed tissues were exercised, close attention to the actual comminution of the specimen (especially fibrous tissues in it) may be essential. It is difficult, furthermore, to assess the effect upon the data of Table IV of chemical changes which accompany lengthy storage of tissues and lead to the release of additional amounts of the drug. In fact, there is little information available for comparison, and the conflict which exists may even implicate the analytical methods employed. For example, the release of protein-bound barbiturate[7,8] and loss of water from decomposing tissue[7] were considered to account for increasing concentrations found in tissues held at room temperatures for several months. However, in another study[9], under similar conditions, a marked fall in the concentration occurred, yet, when stored at 4°C, little loss of the drug was observed. Additional support for the data of Table IV as an improvement in methodology (rather than as evidence for the release of protein-bound barbiturate) is provided by the analytical results for fresh tissues (see Table V) obtained using three alternative extraction[10] procedures. Significantly, all values fall below that of the freeze-dried tissue for reasons which probably involve inadequacies in methodology (including, incomplete extraction of samples and losses due to adsorption on precipitates). With the exception of ketones, for reasons given below, correct use of polar, water-miscible solvents, such as

TABLE V

RESULTS OF AMOBARBITAL ANALYSES FOLLOWING EXTRACTION OF FRESH TISSUES BY DIFFERENT METHODS

For details of the extraction see ref. 10. After treatment, residue from each sample (5 g) was rinsed two or three times with the appropriate solvent (water or chloroform). Regardless of the method of extraction, estimation was completed as in *Tissue analyses*. Value obtained for the corresponding freeze-dried tissue, 86.0 mg/kg. Official result, 32 mg/kg.

Method of extraction	*Sample mass (g)*	*Amobarbital concentration*	
		*mg/kg**	*Mean*
Stas–Otto Extraction**	6.5	61.3	62.2
	5.5	63.1	
Tungstate protein precipitation***	5.3	52.9	51.9
	5.1	50.9	
Direct chloroform extraction§	6.8	47.1	47.4
	5.6	47.7	

* Final volume 2.0 ml. Concentration range for calibration plot 0.061–0.244 mg dimethyl derivative per ml.

** Two extractions with ethanol (50 ml; 2 h then 1 h).

*** Using 30 ml of specified reagent solutions.

§ Extraction in refluxing solvent (50 ml, 1.5 h).

methanol and ethanol, greatly improves the likelihood of complete drug recovery from fresh tissues and, more effectively than other water-miscible solvents (or water-immiscible solvents which function by partitioning rather than extraction), facilitates the denaturing and insolubilizing of proteins. Of course, emphasis on the removal of proteins[10,11] seems an irrelevance in comparison with the more difficult problems posed by lipids and other co-extractives of a more polar and reactive nature or, still related to the recovery problem, the potential losses introduced by the use of metal salts as protein precipitants[11–13]. Thus, in our view, extraction in boiling (or re-cycling) solvent is successful for removing free or loosely-bound drug. Presumably, enzymatic degradation of protein[11,14] can liberate, in addition, any occluded or more strongly protein-bound drug, whereas acid hydrolysis or specific enzymes are needed to free the drug covalently-bound to the protein.

Typical chromatograms of tissue extracts are reproduced in Figs. 1 and 2. In Fig. 1a, the chromatogram of an alkaline extract of methanol-soluble compounds

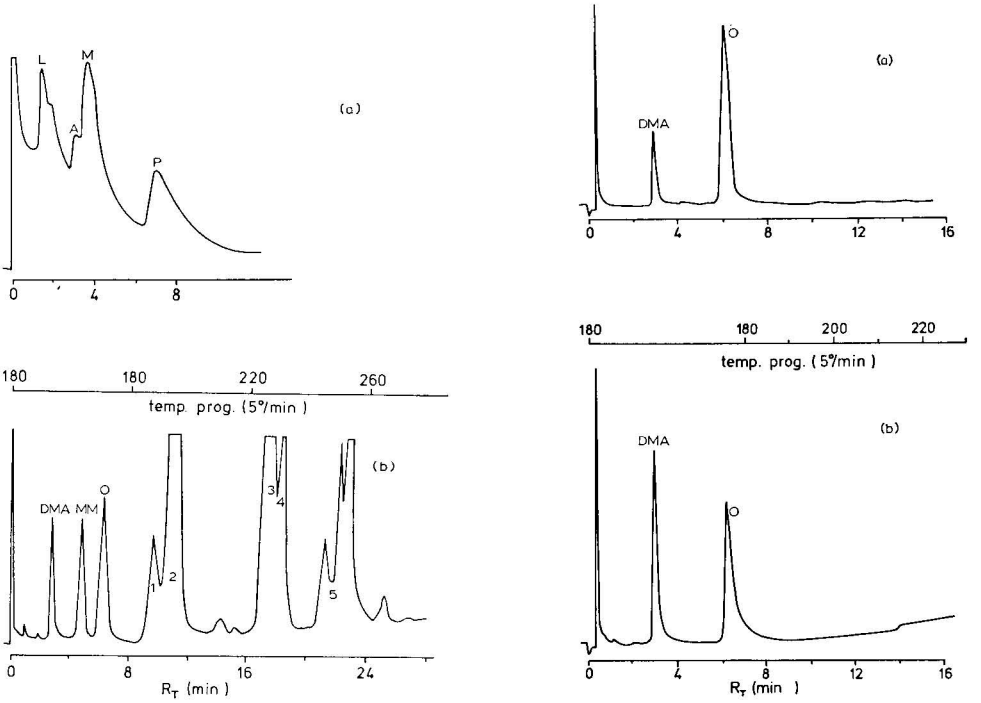

Fig. 1. Chromatograms of tissue extracts. (a), Without derivatization and under isothermal conditions at 190°C. Amobarbital (A) appears as a small peak ahead of the main source of interference, myristic acid (M). Broad peaks due to lauric (L) and palmitic (P) acids are also shown on an elevated baseline. (b), After alkylation with dimethyl sulphate. In the isothermal portion, dimethyl amobarbital (DMA) is clearly separated from methyl myristate (MM) and the internal standard, octadecane (O). Other fatty acid esters elute as the temperature rises: 1 = methyl palmitoleate; 2 = methyl palmitate; 3 = methyl oleate; 4 = methyl stearate; and 5 = higher fatty acid esters.

Fig. 2. Chromatograms of typical extracts obtained after methylation and alkylation steps in the new procedure. The complete absence of fatty acid esters is shown in the isothermal run (a) and confirmed in (b) under temperature programmed conditions (after elution of octadecane) for the same sample. See Fig. 1 for abbreviations.

shows the presence of amobarbital as a small shoulder on the larger myristic acid peak. Poor resolution of the peaks due to lauric and myristic acids, together with an elevated baseline, precludes useful identification and estimation of barbiturates. As shown in the isothermal region of Fig. 1b, conversion of fatty acids and the barbituric acid to the corresponding methyl derivatives facilitates a complete separation of the N,N-dimethylamobarbital from the laurate (under the solvent peak) and myristate esters. For these experimental conditions, the most suitable internal standard was *n*-octadecane. However, this applies only in the absence of the metabolite 3'-hydroxyamobarbital whose retention time on this column is close to that of the internal standard. The efficacy of the new procedure in removing interfering lipids is demonstrated by typical chromatograms of lipid-free extracts (see Fig. 2). There is no evidence in either case of traces of fatty acid esters in the isothermal (Fig. 2a) or temperature-programmed runs (Fig. 2b) so that greater flexibility in the choice of an internal standard is possible and the presence of other acidic drugs, particularly those with longer retention times, is more easily observed.

Finally, as referred to earlier, despite the removal of UV-absorbing endogenous[15] carboxylic acids with the long-chain fatty acids, there is no overall improvement in the composition of the remaining extract which simplifies the subsequent measurement of barbiturates by spectrophotometry. Advantage in the methylation procedure is gained only when the analysis is completed by GC. As will be discussed elsewhere, the absorption of UV-radiation (as well as the formation of intense colour) is related to complex and on-going reactions involving compounds present in all tissues. Perhaps of greater interest is the fact that this reaction (the Maillard reaction) acts as a source of highly reactive intermediates, especially carbonyl compounds[4], which may be responsible for the loss of, or failure to detect, certain drugs other than barbiturates because of the scavenging action of the numerous intermediates.

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METABOLIC PROFILES OF PENAEID SHRIMP: DIETARY LIPIDS AND OVARIAN MATURATION*

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SUMMARY

The major impediment to the culture of penaeid shrimp in captivity in the United States has been an inability to obtain ovarian maturation and spawning. Lipid profiles of tissues (gonads, hepatopancreas, and tail muscle) of *Penaeus setiferus* caught at sea have shown that cholesterol is the dominant sterol and that polyunsaturated fatty acids known to be essential in man comprise a significant portion of the fatty acid fraction. A proprietary marine ration contains cholesterol, but is devoid of polyunsaturated fatty acids. Ovarian maturation and spawning were obtained when the shrimp diet was supplemented with an annelid rich in lipids containing these compounds. The biochemical significance of these findings is discussed.

INTRODUCTION

Wild stocks of shrimp are being depleted, their natural habitat is threatened by pollution, and increasing energy costs are adversely affecting the economics of shrimping. These factors alone are sufficient to stimulate an interest in the breeding of shrimp in captivity. Of greater long-term significance, however, is that the achievement of this objective would open the door to selective breeding to obtain animals with superior characteristics. Previous attempts to obtain spawning of

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penaeid shrimp in captivity in the United States have been less than successful: ovarian maturation has not been obtained except in isolated instances[1,2].

The study of metabolic profiles has provided insight to many human biochemical disorders[3]. Notable among these are the discovery of new inborn errors of amino acid metabolism[4–6], the diagnosis of diabetes mellitus by analysis of urinary volatiles[7], and the investigation of rheumatoid arthritis[8] and breast lesions[9]. We have used a similar approach to the investigation of the maturation of female penaeid shrimp.

The classes of compounds which we selected for study were steroids and fatty acids[10]. Shrimp are unable to biosynthesize steroids[11], which are used by these animals as molting hormones and membrane components. The essential fatty acids also feature as membrane components and, in some animals, are precursors of prostaglandins which are important in reproduction[12].

The species of shrimp studied was the white shrimp *Penaeus setiferus*, native to the Gulf of Mexico. Steroid and fatty acid profiles for the gonads, hepatopancreas (digestive gland), and tail muscle of male and female specimens were determined, and compared with the corresponding dietary profiles.

EXPERIMENTAL

Animals

P. setiferus were obtained from the Gulf of Mexico off the Texas coast. All collections were made during the natural spawning season, which runs from May to September. The stages of gonadal development were estimated using gonadal indices[13]. Appropriate tissues were removed as soon as possible after capture and were frozen until analyzed.

Materials

All solvents were Mallinckrodt Nanograde (Mallinckrodt, St. Louis, MO, U.S.A.) except for anhydrous diethyl ether (U.S.P. grade) and hydrochloric acid and sodium hydroxide (reagent grade). Samples were exposed only to Teflon, aluminium, or glass rinsed with chloroform.

Extraction

The samples were homogenized in 20 ml of water and were saponified with 4 ml of 4 *M* sodium hydroxide by heating at 110°C for 2 h. Basic and neutral components (including sterols) were extracted with 2 × 15 ml of diethyl ether (extract A). After acidification with 2 ml of concentrated hydrochloric acid, acidic components (including fatty acids) were extracted with 2 × 15 ml of diethyl ether (extract B). Each extract (A and B) was fractionated by chromatography on separate 20 × 1 cm glass columns containing activated silica gel (80–200 mesh). In each case, a 40-ml benzene eluate was discarded and the components of interest were eluted with 40 ml of ethyl acetate. Thus, a sterol and a fatty acid fraction were obtained.

Analysis

Sterols were analyzed as trimethylsilyl (TMS) ethers, prepared using bis-(trimethylsilyl)acetamide (Supelco, Bellefonte, PA, U.S.A.) and fatty acids as methyl

esters, prepared using dimethylformamide dimethylacetal (Pierce, Rockford, IL, U.S.A.). Gas chromatography was performed using Perkin-Elmer 3920B instruments equipped with flame ionization detectors and 2 m × 2 mm I.D. silanized glass columns containing 3% OV-1 on Gas-Chrom Q (100–120 mesh), programmed from 100 to 270°C (fatty acid methyl esters) or 200 to 270°C (sterol TMS ethers). The injection port and interface temperatures were, respectively, 250 and 280°C. Some analyses were performed using a similar column with SP-2330 as the stationary phase or a 10-m glass capillary column coated with OV-101. Combined gas chromatography–mass spectrometry (GC–MS) was performed using a Hewlett-Packard 5992A instrument equipped with a 2 m × 2 mm I.D. silanized glass column containing 3% OV-1 on Gas-Chrom Q, with appropriate temperature programs and an electron energy of 70 eV.

RESULTS AND DISCUSSION

Shrimp sterols

The relative concentrations of sterols in the three major tissues (gonad, hepatopancreas, tail muscle) of both sexes of *P. setiferus* are given in Table I. Cholesterol was always the predominant sterol, and 22-dehydrocholesterol was found in most samples. Desmosterol was also found in some samples. 24-Methylcholesterol and sitosterol were encountered only in the hepatopancreas. The identities of all of these compounds were verified by GC–MS.

TABLE I

RELATIVE CONCENTRATIONS OF STEROLS* IN TISSUES OF *P. SETIFERUS* FROM THE GULF OF MEXICO

*Specimen***	*Tissue****	*n*	*Cholesterol*	*Δ²²*	*Δ²⁴*	*24-Me*	*24-Et*
Male	G	6	98.7 ± 1.9§	0.7 ± 0.8			
	H	6	93.9 ± 7.4	2.7 ± 3.0	4.2 ± 1.4	2.5 ± 1.6	0.6 ± 1.3
	T	6	82.2 ± 40.3	0.7 ± 0.9	0.2 ± 0.6		
Female	G	2	100 ± 0.0				
(stage 1)	H	1	96.2	3.8			
	T	2	100 ± 0.0				
Female	G	4	99.0 ± 0.8	1.0 ± 0.8			
(stage 2)	H	4	84.0 ± 11.3	5.5 ± 3.8	5.1 ± 3.7	3.3 ± 2.6	2.2 ± 1.7
	T	4	99.5 ± 1.0	0.3 ± 0.5	0.3 ± 0.5		
Female	G	2	98.1 ± 2.7	1.0 ± 1.3			
(stage 3)	H	3	89.8 ± 9.0	4.6 ± 1.8	3.1 ± 3.2	1.6 ± 2.8	0.9 ± 1.6
	T	3	100 ± 0.0				
Female	G	8	92.5 ± 15.2	0.7 ± 0.8	0.3 ± 0.8		
(stage 5)	H	8	82.5 ± 4.7	6.4 ± 1.9	5.2 ± 1.2	4.0 ± 1.2	2.0 ± 1.1
	T	8	100 ± 0.0				

* Δ^{22} = 22-dehydrocholesterol; Δ^{24} = desmosterol; 24-Me = 24-methylcholesterol; 24-Et = sitosterol.

** For females, stage of development of ovaries is indicated: stage 1 is immature, stage 5 is about to spawn. No stage 4 animals were collected.

*** G = gonad; H = hepatopancreas; T = tail muscle.

§ Mean ± standard deviation.

TABLE II

RELATIVE CONCENTRATIONS OF SELECTED FATTY ACIDS IN TISSUES OF *P. SETIFERUS* FROM THE GULF OF MEXICO

*Specimen**	*Tissue***	*n*	*16:0*	*16:1*	*18:0*	*18:1*	*20:4*	*20:5*	*22:6*
Male	G	4	13.5 ± 4.6***	4.1 ± 0.6	10.1 ± 3.7	9.9 ± 1.3	9.5 ± 2.9	17.3 ± 2.5	17.5 ± 0.9
	H	5	20.7 ± 3.2	6.3 ± 0.7	9.9 ± 2.1	8.0 ± 1.0	4.7 ± 1.5	7.3 ± 1.4	5.1 ± 0.8
	T	5	15.7 ± 1.8	5.9 ± 0.8	12.7 ± 3.7	11.2 ± 1.8	6.9 ± 1.6	13.9 ± 5.4	12.8 ± 3.4
Female	G	1	19.1	7.6	8.3	19.8	8.3	10.1	6.9
(stage 1)	H	2	24.8 ± 1.1	7.5 ± 1.3	8.5 ± 0.1	9.6 ± 0.8	4.6 ± 0.6	8.0 ± 2.3	5.6 ± 0.6
	T	2	17.6 ± 1.4	8.3 ± 1.8	11.0 ± 0.8	12.8 ± 0.2	6.6 ± 0.9	14.6 ± 3.5	11.5 ± 1.2
Female	G	2	21.8 ± 3.3	11.0 ± 1.1	8.0 ± 1.1	16.1 ± 0.4	4.3 ± 0.6	9.4 ± 2.1	6.8 ± 2.1
(stage 2)	H	2	21.8 ± 2.1	10.9 ± 0.2	8.4 ± 0.3	10.9 ± 1.6	5.0 ± 0.1	7.7 ± 1.1	5.0 ± 0.6
	T	2	17.7 ± 1.9	8.5 ± 0.6	11.2 ± 1.2	12.0 ± 0.1	6.2 ± 1.8	13.1 ± 2.7	10.7 ± 2.5
Female	G	2	20.2 ± 2.8	10.9 ± 0.6	7.8 ± 0.4	15.2 ± 0.8	4.3 ± 0.7	12.8 ± 2.1	8.7 ± 1.6
(stage 3)	H	3	22.1 ± 2.3	10.7 ± 0.8	8.3 ± 0.3	9.4 ± 1.5	4.4 ± 0.4	8.6 ± 2.2	4.8 ± 0.1
	T	3	18.5 ± 1.1	8.7 ± 0.3	11.9 ± 0.9	11.8 ± 0.3	5.1 ± 1.2	16.2 ± 4.5	11.4 ± 3.3
Female	G	6	21.2 ± 2.8	10.5 ± 1.4	8.8 ± 2.3	15.2 ± 0.9	4.1 ± 1.0	9.9 ± 2.5	7.0 ± 2.0
(stage 5)	H	6	21.4 ± 1.2	9.6 ± 2.1	7.9 ± 0.6	9.8 ± 1.0	4.4 ± 0.5	8.8 ± 2.2	5.4 ± 1.1
	T	6	17.8 ± 1.4	7.9 ± 0.7	10.6 ± 1.9	12.3 ± 1.5	6.1 ± 1.4	14.1 ± 2.2	11.7 ± 1.7

* For females, stage of development of ovaries is indicated: stage 1 is immature, stage 5 is about to spawn. No stage 4 animals were collected.

** G = gonad; H = hepatopancreas; T = tail muscle.

*** Mean ± standard deviation.

Cholesterol has been known in marine invertebrates since the turn of the century[14], and is a common shrimp sterol[15]. It is derived from the diets of species which are unable to synthesize it[16–18].

22-Dehydrocholesterols (22-*cis* and 22-*trans*) are also widely distributed marine sterols[19–21]. The origin of this compound is not clear. In mammals, the formation of (22*R*)-22-hydroxycholesterol is the first step in the conversion of cholesterol into C_{21}, C_{19}, and C_{18} steroids[22]. Such steroids in crustaceans apparently do not share the significance of their counterparts in mammals[23]. It is tempting to speculate that (22*R*)-22-hydroxycholesterol is dehydrated to 22-dehydrocholesterols in some marine organisms and, like cholesterol, is transferred through the marine food web.

Desmosterol was first reported in a crustacean and has been found since in other crustaceans[24]. These animals can convert dietary desmosterol into cholesterol[25,26].

It is of interest that we found 24-methylcholesterol and sitosterol in the hepatopancreas but not in the gonads or tail muscle. Both of these phytosterols can be converted into cholesterol by crustaceans[15,27–31]. Our data indicate that the metabolism of these dietary sterols takes place in the hepatopancreas or, perhaps, some tissue other than the gonads and tail muscle.

Shrimp fatty acids

Analytical data for fatty acids in all three tissues of *P. setiferus* examined are given in Table II. Their identities were verified by GC–MS. Most samples contained the full range of saturated and unsaturated fatty acids from C_{14} to C_{22}. Compounds included in Table II are the major fatty acids: palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), arachidonic (20:4), 5,8,11,14,17-eicosapentaenoic (20:5), and 4,7,10,13,16,19-docosahexaenoic (22:6) acids. Their relative concentrations are expressed as percentages of the total fatty acid content.

The saturated and monounsaturated fatty acids are presumably most important as membrane components. Those which are likely to be most significant to reproduction are certain of the polyunsaturated fatty acids. The most prominent of the latter compounds in many samples are arachidonic acid, 5,8,11,14,17-eicosapentaenoic acid, and 4,7,10,13,16,19-docosahexaenoic acid.

The C_{20} acids are precursors of prostaglandins in many animals[12]. While there are no reports on endogenous prostaglandins in crustaceans, experiments performed *in vitro* have resulted in the conversion of the 20:3 acid into prostaglandin E_1 in low yield by lobster stomach and gill homogenates[32]. Since prostaglandin concentrations are particularly high in human seminal fluid[33–35] and since these compounds have been implicated in the stimulation of uterine contractions during labour[12], it has been suggested[10] that a role of the C_{20} acids in the reproduction of shrimp is mediated by prostaglandins.

Prostaglandins and prostaglandin analogues have been produced *in vitro* using sheep vesicular gland homogenates only from C_{19}, C_{20}, and C_{21} substrates[36,37]. There have been no reports of the formation of prostaglandin analogues from the 22:6 acid or other C_{22} acids. A correlation has been noted, however, between the sex ratio of zooplankton and the content of heneicosahexaene in algal feed at the nauplius stage[38], and it has been suggested[10] that the 22:6 acid is the active compound, while the hydrocarbon is merely a decarboxylation product.

There is strong circumstantial evidence to suggest that polyunsaturated fatty acids are involved in some capacity in the reproductive process, and attention to this possibility should be given in designing feedstocks for shrimp mariculture.

Dietary lipids

The fatty acid content of many crustaceans reflects that of their diets[39–41]. Of particular interest is the observation that the lipid content of the coconut crab is very similar to that of coconuts[42]. Thus, the foregoing discussion has shown that attention should be given to the sterol and fatty acid content of the diets of penaeid shrimp if they are to be expected to spawn in captivity.

The fatty acid composition of a commercially available marine chow is given in Table III. Cholesterol is the only sterol in this formulation. While the sterol content appears to be appropriate, there is clearly a deficiency of polyunsaturated fatty acids. If this feedstock is to be used for maturation, it should be supplemented with additives containing sufficient essential fatty acids.

TABLE III

RELATIVE CONCENTRATIONS OF SELECTED FATTY ACIDS IN COMMERCIALLY AVAILABLE MARINE CHOW AND THE BLOODWORM *G. DIBRANCHIATA*

Specimen	*16:0*	*16:1*	*18:0*	*18:1*	*20:4 + 5**	*22:6*
Marine chow	21.6	3.3	7.6	27.1	3.5	1.7
Bloodworm (live)	12.9	4.5	6.6	10.7	21.5	10.0
Bloodworm (frozen)	19.4	2.8	4.3	11.1	26.3	10.7

* 20:4 and 20:5 not resolved on OV-1.

Among the feedstock supplements which we have investigated is the bloodworm *Glycera dibranchiata*. The fatty acid profile (Table III) of this annelid appears to be appropriate for use with shrimp.

Feeding trials

P. setiferus were maintained in captivity with a diet supplemented with *G. dibranchiata*. The fatty acid profiles of these animals after three months on this diet are given in Table IV. These profiles are very similar to those for the wild shrimp in Table II. Ovarian development and spawning were obtained 3–4 weeks after the experiment was initiated: 63 egg collections yielded a total of 4.3 million eggs[43]. Fertilization did not take place since the male shrimp had a *Vibrio* infection. More recently, however, this protocol has resulted in the production of viable larvae of *P. stylirostris*[44].

Even though the fatty acid profiles of the wild and captive shrimp were similar, there were significant ($P < 0.05$) differences between the total lipid contents of the two groups of animals, as shown in Table V. The hepatopancreas lipid content of the captive shrimp was 2.0–4.4 times that of the wild shrimp, the gonad lipid content of the captive shrimp was 1.2–2.0 times that of the wild shrimp, while the tail muscle lipid content of the captive shrimp was only 1.1–1.4 times that of the wild shrimp. The higher concentrations of the lipids in the captive shrimp indicate

TABLE IV

RELATIVE CONCENTRATIONS OF SELECTED FATTY ACIDS IN TISSUES OF *P. SETIFERUS* GROWN IN THE LABORATORY

*Specimen**	*Tissue***	*n*	*16:0*	*16:1*	*18:0*	*18:1*	*20:4*	*20:5*	*22:6*
Female	G	0	—	—	—	—	—	—	—
(stage 1)	H	1	21.0	4.4	5.5	7.4	2.1	12.5	17.2
	T	1	14.7	3.9	10.1	10.0	10.2	17.9	18.2
Female	G	3	19.6 ± 1.0***	5.1 ± 0.9	6.5 ± 0.0	15.9 ± 1.5	3.8 ± 0.8	16.8 ± 0.7	18.8 ± 0.8
(stage 2)	H	3	25.6 ± 2.5	5.8 ± 1.4	5.1 ± 0.7	9.5 ± 0.6	2.0 ± 0.6	10.4 ± 0.9	13.2 ± 2.2
	T	3	19.0 ± 1.0	4.9 ± 0.3	11.0 ± 0.5	13.1 ± 1.1	4.8 ± 0.3	16.5 ± 0.7	17.6 ± 0.9
Female	G	2	20.4 ± 0.5	6.8 ± 0.0	5.8 ± 0.2	14.8 ± 0.9	3.0 ± 0.1	15.5 ± 0.2	19.6 ± 0.1
(stage 3)	H	2	29.4 ± 1.8	8.1 ± 0.1	3.7 ± 0.4	8.3 ± 0.2	1.6 ± 0.1	8.9 ± 0.8	10.8 ± 2.3
	T	2	19.9 ± 1.4	6.2 ± 0.4	10.2 ± 0.4	12.6 ± 1.2	4.4 ± 0.1	16.7 ± 1.3	17.8 ± 0.8
Female	G	2	21.0 ± 1.0	6.9 ± 1.3	5.7 ± 0.0	16.1 ± 1.6	2.8 ± 0.3	13.5 ± 0.7	18.6 ± 1.8
(stage 4)	H	2	26.5 ± 2.3	8.5 ± 1.2	2.9 ± 0.1	9.3 ± 0.8	1.7 ± 0.0	11.2 ± 1.3	14.0 ± 0.4
	T	2	17.9 ± 1.1	5.6 ± 0.2	9.7 ± 0.1	13.6 ± 0.3	4.3 ± 0.6	15.0 ± 0.9	17.0 ± 1.8
Female	G	2	23.0 ± 0.8	7.1 ± 1.8	7.0 ± 0.1	14.7 ± 0.4	2.5 ± 0.1	12.6 ± 0.4	18.5 ± 1.4
(stage 5)	H	1	26.0	4.7	5.7	8.9	2.1	9.1	13.0
	T	2	18.5 ± 2.1	4.8 ± 1.1	10.8 ± 0.8	12.7 ± 0.2	4.5 ± 0.4	18.1 ± 1.3	19.6 ± 2.0

* Stage of development of ovaries is indicated: stage 1 is immature, stage 5 is about to spawn.

** G = gonad; H = hepatopancreas; T = tail muscle.

*** Mean ± standard deviation.

TABLE V

TOTAL LIPID CONTENT (%) IN TISSUES OF *P. SETIFERUS* FROM THE GULF OF MEXICO AND GROWN IN THE LABORATORY

*Specimen**	*Tissue***	*Gulf shrimp*		*Captive shrimp*	
		n	*% lipid*	*n*	*% lipid*
Male	G	5	2.9 ± 0.7***	0	—
	H	5	24.1 ± 20.4	0	—
	T	5	4.0 ± 0.4	0	—
Female (stage 1)	G	2	6.0 ± 1.8	0	—
	H	2	22.6 ± 12.7	1	44.9
	T	2	4.2 ± 0.1	1	4.6
Female (stage 2)	G	2	7.7 ± 9.1	3	15.4 ± 1.9
	H	2	13.6 ± 0.1	4	32.9 ± 20.6
	T	2	3.5 ± 0.1	4	4.8 ± 0.4
Female (stage 3)	G	2	13.6 ± 0.3	5	19.4 ± 2.3
	H	3	15.1 ± 2.1	5	66.8 ± 9.8
	T	2	3.6 ± 0.2	5	5.1 ± 0.3
Female (stage 4)	G	0	—	3	20.1 ± 0.9
	H	0	—	3	51.4 ± 19.7
	T	0	—	3	4.6 ± 0.3
Female (stage 5)	G	6	16.1 ± 1.1	3	19.5 ± 1.1
	H	4	11.7 ± 5.9	3	23.6 ± 6.8
	T	6	3.8 ± 0.2	3	4.6 ± 0.2

*, **, *** Footnotes as in Table IV.

that the artificial diet of the captive shrimp, while adequate for inducing maturation and spawning, is not optimum. Further refinement of the artificial diet is required to reduce the accumulation of lipids in animals which are fed with it.

CONCLUSIONS

Fatty acid and sterol profiles were obtained for penaeid shrimp caught at sea. These profiles were duplicated in captive animals, and spawning resulted.

There have been many reports of seasonal changes in the lipid content and composition of crustaceans: *Astacus astacus*[45], *Astacus leptodactylus*[46], *Orconectes rusticus*[47], *Palaemon carcinus*[48], and *Panulirus polyphagus*[48]. These changes probably reflect differences in diet rather than changes in temperature. Minor variations in lipid content throughout the molt cycle, however, have been observed[49,50].

Our success in using *G. dibranchiata* as a feedstock additive is probably also related to the diet of the bloodworm. The essential fatty acids probably derive ultimately from phytoplankton, and other routes through the food web to shrimp could be envisioned.

Finally, it is not impossible that a factor other than dietary lipid content was responsible for the promotion of ovarian development. Now that spawning is possible in captivity on a reproducible basis, experiments can be performed to resolve some of these issues.

ACKNOWLEDGEMENTS

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FIELD DESORPTION MASS SPECTROMETRY OF HYPOTHALAMIC OLIGOPEPTIDES

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SUMMARY

A high-performance liquid chromatographic system employing a chemically bonded alkyl phase and a tetraalkylammonium phosphate buffer has been evaluated for separation of synthetic mixtures of hypothalamic oligopeptides. Separation of two peptides in the mixture having very similar adjusted retention times has been achieved. Sensitivity down to 5 ng is attained. Peptides range in size from three to 31 amino acids. Field desorption mass spectra illustrate novel structural elucidation methods for individual peptides and signal a quantification method.

INTRODUCTION

This paper describes a high-performance liquid chromatographic (HPLC) method to separate mixtures of biological oligopeptides in a fast and facile manner, optimizing sensitivity, speed and resolution of the separation to provide a purified peptide fraction for quantification and structural elucidation studies. Towards this end, the triethylamine phosphate (TEAP)–acetonitrile system is used with a μBondapak C_{18} HPLC column. Synthetic mixtures of hypothalamic oligopeptides containing from three to 31 amino acids are employed. A field desorption (FD) spectrum of an underivatized hexapeptide illustrates the utility of the novel ionization method in peptide chemistry to provide molecular ions of underivatized oligopeptides and for quantification.

Reversed-phase (RP) HPLC using chemically bonded alkyl stationary phases has become an important and powerful tool in peptide separation chemistry. Long chain alkyl silanes are chemically bonded to silanol groups located on the silicon surface of the packing material. An end-capping reaction follows where trimethylsilyl groups are chemically bonded to most of the remaining unreacted silanol groups. Various authors reviewed HPLC column technology and provided a theoretical basis for separations afforded by RP columns[1–3]. HPLC retention indices related to compound lipophilicity were estimated[4]. Horváth and co-workers[5–7] show separation on RP columns is a function of increasing hydrophobicity of the peptide–paired-ion–solvent hydration complex.

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The number of ionic equilibria in which an oligopeptide can participate[8] requires paired-ion chromatography. A buffer system provides ions to pair with charges on a peptide at a pH value sufficiently low to suppress ionization of carboxyl groups. Ion-pair HPLC is extensively reviewed by Tomlinson *et al.*[9]. For purposes of peptide chemistry, an extensively studied, elaborated and used buffer system is the TEAP buffer system. Hancock and co-workers[10–18] studied this system and applied it to various separations. Rivier *et al.*[19] used the TEAP system for separation of peptides and Rivier[20] studied use of trialkylammonium phosphate buffers in RP-HPLC for high resolution and high recovery of peptides. A radioactive peptide was quantitatively recovered from a column. Resolution, flow-rate, temperature, buffer composition, reproducibility and sensitivity were studied.

Other authors expanded the role of HPLC in separation of peptides and proteins including neurohypophyseal proteins[21], polypeptide antibiotics[22], bacitracin[23] polypeptides and proteins[24,25], neuroendocrine peptides[26], amino acids, peptides and derivatives[27], peptide diastereoisomers[28,29] and analysis of the purity of commercial peptides[30]. Other studies include fluorogenic detection of oligopeptides using *o*-phthalaldehyde, fluorescamine and ninhydrin[31], with an aim towards increasing sensitivity of HPLC detection using fluorometric detectors.

MATERIALS AND METHODS

Apparatus

The Waters (Milford, MA, U.S.A.) HPLC system employed consisted of two 6000A solvent delivery systems, U6K injector, R401 detector, 660 solvent programmer, 450 variable-wavelength UV detector and M420 fluorescence detector. A μBondapak C_{18} column (30 $\times$ 0.4 cm) (Registry number 068733) was employed. All experiments were performed at ambient temperature. The 0.5-μm filters were purchased from Waters Assoc. A Precision Sampling pressure lock syringe, Series B110, 10 μl (Supelco, Bellefont, PA, U.S.A.) was employed.

Peptides, chemicals, buffer

Triethylamine (Lot no. 03059.24) was purchased from Pierce (Rockford, IL, U.S.A.), phosphoric acid (Lot no. 790884) from Fisher Scientific (Pittsburgh, PA, U.S.A.), acetonitrile from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.); thyrotropic releasing hormone (TRH), met-enkephalin, bradykinin, angiotensin, eledoisin-related peptide, Tyr^{11} and Tyr^{1} SS and β-endorphin from Bachem (Torrance, CA, U.S.A.), substance P and somatostatin (SS) from Sigma (St. Louis, MO, U.S.A.). Water was obtained from a laboratory distillation apparatus. Ionic strength of the TEAP buffer was 76 m*M*. TEAP buffer was prepared by titrating a 0.25 *N* phosphoric acid solution to pH 3.2 with distilled triethylamine. Buffers and solvents were filtered through 0.5-μm Millipore filters to remove solid particles and degas solutions to avoid bubble formation upon decompression after the column.

Field desorption mass spectrometry

FD spectra were obtained on a Varian (Bremen, G.F.R.) 731 mass spectrometer. Initial experiments were done at the National Bureau of Standards (Gaithersburg, MD, U.S.A.) and later experiments on a Varian 731 in our laboratory. A

5-μl volume of a solution containing 1 μg/μl of the underivatized oligopeptide was applied to the benzonitrile-activated FD emitter wire with assistance from a micromanipulator–microscope–syringe system. This volume of solution corresponds to 6.65 nmol peptide (Fig. 2) and sufficed for several FD mass spectral magnetic scans recorded with an oscillographic recorder. Source temperature: 70°C. Extraction lens voltage: 3 kV. Accelerating voltage, 8 kV. Resolution: 1000.

RESULTS

Results obtained in this study include an HPLC chromatogram illustrating separation of the synthetic oligopeptide mixture, a FD mass spectrum of an underivatized hexapeptide, a table collecting chromatographic and statistical parameters for an HPLC reproducibility study and a list of integrals of Rekker's hydrophobic fragmental constants for constituent amino acids for ten hypothalamic oligopeptides.

Fig. 1 contains the HPLC chromatogram illustrating separation of a synthetic mixture of ten hypothalamic peptides. A 2-μl volume of a solution containing 1 μg of each peptide per μl TEAP was injected onto the column. Size of the peptides range from a tripeptide TRH to β-endorphin which contains 31 amino acids. Separation of this complex mixture of peptides occurred within 78 min. Peak shapes are sharp, symmetrical and narrow. Excellent resolution occurs between Tyr^{11} somatostatin and substance P, these peptides having t_A values 45.40 and 46.02, respectively. A linear flow program (Waters curve 6) from 2% to 70% solution B in 78 min was employed. Solution A was TEAP, pH 3.2; solution B was TEAP–acetonitrile (40:60 v/v).

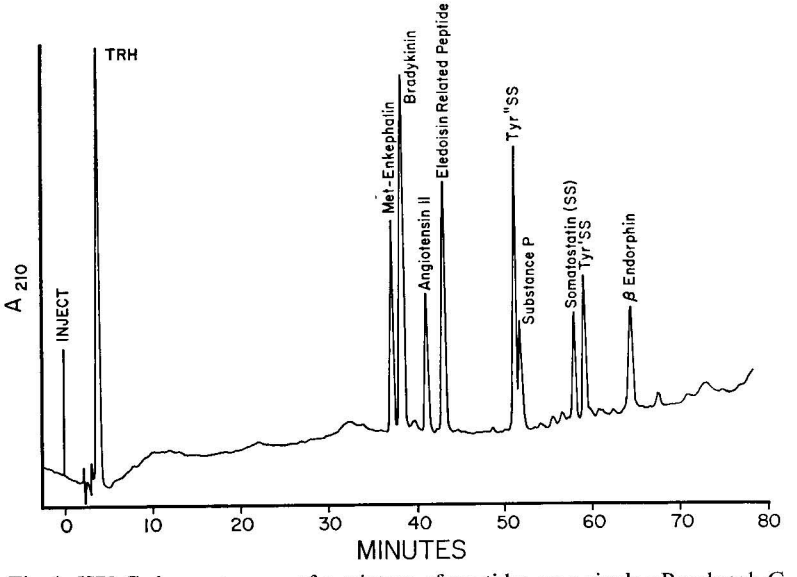

Fig. 1. HPLC chromatogram of a mixture of peptides on a single μBondapak C_{18} column (30 × 0.4 cm). Back pressure: 2000 p.s.i. Flow-rate: 1.5 ml/min. Linear flow program: 2% to 70% B in 78 min. Solutions: A, triethylamine–phosphoric acid (TEAP), pH 3.2; B, TEAP–acetonitrile (40:60). A 2-μl volume of a solution of 1 μg of each peptide per μl TEAP is injected. 0.1 a.u.f.s., 210 nm; chart speed 0.2 in./min.

TABLE I

ADJUSTED RETENTION TIMES FOR BIOLOGIC PEPTIDES

Data obtained on a μBondapak C_{18} column (30 × 0.4 cm). Back pressure: 2000 p.s.i. Flow-rate: 1.5 ml/min. Linear flow program: 3% to 60% B in 65 min. Chromatogram was run from 2% to 70% in 78 min. Solutions: A, triethylamine–phosphoric acid (TEAP), pH 3.2; B, TEAP–acetonitrile (40:60). A 2-μl volume of a solution of 1 μg of each peptide per μl of TEAP is injected. 0.1 a.u.f.s., 210 nm, $\mu = 0.08$ *M*. $\bar{t}_A$ = Mean, σ = standard deviation, V = coefficient of variation = $100\sigma/\bar{t}_A$. Adjusted retention (t_A) = $t_R - t_0$.

Peptide	*Chromatogram*							$\bar{t}_A$	σ	V
	1	*2*	*3*	*4*	*5*	*6*	*7*			
TRH	1.06	1.63	1.73	0.77	0.61	0.89	1.57	1.18	0.46	38.98
Met-enkephalin	—	32.91	31.85	32.32	31.85	32.40	33.21	32.42	0.55	1.70
Bradykinin	32.91	33.78	32.44	33.05	32.83	33.05	34.25	33.19	0.62	1.86
Angiotensin II	35.76	36.36	35.31	33.05	35.49	36.16	36.93	35.58	1.24	3.49
Eledoisin-related peptide	37.32	38.21	36.97	37.56	37.26	37.85	38.97	37.73	0.68	1.80
Tyr^{11} SS	—	—	44.64	45.22	44.88	45.85	46.41	45.40	0.73	1.61
Substance P	44.98	46.89	45.27	45.94	45.53	46.56	46.97	46.02	0.80	1.74
Somatostatin (SS)	50.53	52.54	50.98	51.59	51.16	52.48	52.73	51.72	0.87	1.68
Tyr^{1} SS	—	—	—	—	52.26	53.60	53.88	53.25	0.87	1.63
β-Endorphin	56.32	57.56	56.85	57.58	57.06	58.80	58.89	57.58	0.97	1.68

Sensitivity of the UV detector for the data reported was 0.1 absorbance units full scale (a.u.f.s.). The wavelength selected for this study (210 nm) reflects experience of other workers[20,25] to optimize sensitivity towards oligopeptides rather than proteins where in the former case, the probability increases that an aromatic residue is not present.

Retention time (t_0) for a completely unretained compound was determined in two ways. First, the position of the center for the first baseline disturbance[32–34] in the chromatogram in Fig. 1 was measured as 2.05 min. Multiple injections ($n = 8$) yielded 2.01 $\pm$ 0.08 min for t_0. In the second method, t_0 is calculated from column geometry as $t_0 = Ld/1.5\ F$ where d is the inside column diameter (0.39 cm), F the solvent flow-rate (1.5 ml/min) and $L = 30$ cm. A t_0 value of 2.03 min is calculated.

Adjusted retention time (t_A) is defined as observed retention time (t_R) minus t_0[34]. Multiple injections of this mixture of oligopeptides yielded data assembled in Table I: adjusted retention times for individual injections; average of the multiple injections ($\bar{t}_A$); σ (standard deviation) and V (coefficient of variation) defined as $100\sigma/t_A$. Standard deviations range from 0.46 to 1.24 and yield coefficients of variation (excluding the first eluting TRH peak) between 1.6 and 3.5%. This reproducible system provides a potential method of identification from t_A values alone.

Table II collects adjusted retention time (t_A) values *versus* Rekker integrals[35] of the five most hydrophobic values (RI_5) on one hand, and *versus* all residues (RI) on the other hand.

Fig. 2 contains the low resolution FD mass spectrum of a synthetic model hexapeptide Lys-Glu-Thr-Tyr-Ser-Lys, molecular weight 754 at an emitter heating current (e.h.c.) of 21 mA. The only ions recorded in this mass spectrum occur at m/z 755, 756 and 757 and represent $(M + H)^+$, $(M + 2H)^{+\cdot}$ and $(M + 3H)^+$, respectively.

DISCUSSION

Interactions between hydrophobic side chains of oligopeptides and chemically bonded C_{18} phases reduce molecular surface area exposed to an aqueous solvent and provide the basis for strong bonding between a peptide and the reverse phase. The high surface tension of water contributes to enhancing retention of a peptide to hydrophobic stationary phases. As the percentage of the organic modifier increases during gradient elution, intermolecular peptide–phase interactions decrease and increasingly hydrophobic oligopeptides elute from the column. TEAP plays a rôle in this process by decreasing hydrophilicity of polar peptides by ion-pairing and consequently reducing overall charge of the peptide–ion-pair complex[36]. This model for paired-ion chromatography elution of oligopeptides on RP-HPLC is supported by the correlation observed in Table II between each oligopeptide's integral of Rekker's hydrophobic fragmental constants (Rekker integral, RI) of each constituent amino acid[25,35] and t_R in the RP-HPLC system. Empirical improvement in this correlation was observed by O'Hare *et al.*[25], where they considered only the five most hydrophobic residues Trp, Phe, Leu, Ile and Tyr. In the RI column, Table II, two pairs of peptides (met-enkephalin, 32.42 *vs.* bradykinin, 33.19; and Tyr^{11} SS, 45.40 *vs.* substance P, 46.02) are reversed and do not obey the correlation. In the RI_5 column, one pair (bradykinin and angiotensin) is reversed.

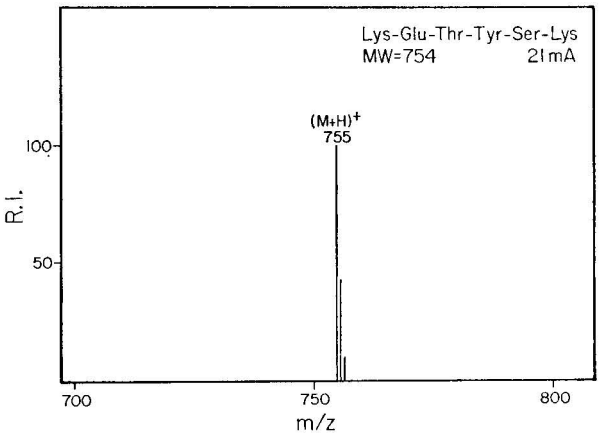

Fig. 2. FD mass spectrum of underivatized hexapeptide, Lys-Glu-Thr-Tyr-Ser-Lys.

TABLE II

ADJUSTED RETENTION TIMES *vs.* REKKER INTEGRALS FOR FIVE MOST HYDROPHOBIC VALUES (RI_5) AND ALL VALUES (RI)

Pyrollidone carboxylic acid (PCA) estimate = − 0.06.

Peptide	t_A	RI_5	*RI*
TRH	1.18	0	0.72
Met-enkephalin	32.42	3.94	5.02
Bradykinin	33.19	4.48	4.47
Angiotensin II	35.58	3.94	7.05
Eledoisin-related peptide	37.73	6.22	8.72
Tyr^{11} SS	45.40	6.25	10.15
Substance P	46.02	6.47	9.01
Somatostatin (SS)	51.72	6.79	10.69
Tyr^{1} SS	53.25	9.10	12.39
β-Endorphin	57.58	14.14	12.80
		$r^2 = 0.76$	$r^2 = 0.86$
		$y = 0.20x - 1.78$	$y = 0.22x - 0.78$

The RI_5 data yield a coefficient of determination value (r^2) of 0.76 while the RI method yields a value of 0.86. This present study statistically favors calculating the sum of all Rekker constants as opposed to the sum of only the five most hydrophobic residues. Nonetheless, it is apparent in either case reversals are noted and therefore it is likely that other factors such as conformation, charge, etc., play a role in the RP-HPLC in addition to hydrophobicity. It is interesting to note in passing that the concept of relating RP-HPLC elution with increasing hydrophobicity closely parallels the situation of correlating gas charomatographic (GC) elution with integrals of retention index increments of amino acid residues in oligopeptide-derived O-trimethylsilylated perfluorinated dideuteroalkyl polyamino alcohols[37].

The HPLC system employed in this study for resolution of a synthetic mixture of hypothalamic oligopeptides has several important parameters. Speed of separation for such a complex mixture is increased: within 78 min the mixture of peptides

ranging from three to 31 amino acids is resolved. Speed of separation is important for a rapid, sensitive and highly resolving chromatographic system to provide metabolic profiles for biologic extracts of hypothalamic oligopeptides. Gas chromatograms are common for drug and steroid metabolic profiles in various body fluids.

Sensitivity of UV detection is sufficient for many peptide studies and options currently under study exist to further increase sensitivity. Somatostatin is one hypothalamic oligopeptide currently being investigated in many laboratories[38] and a rapid, unequivocal identification and quantification method is required. At 210 nm, 5 ng (pmol) of SS is detected at the appropriate retention time. In a single rat hypothalamus *ca.* 40 ng SS are present[39]. Thus, sufficient sensitivity exists for detection and quantification of somatostatin in one rat hypothalamus by means of UV detection. Another method under development which promises increased sensitivity includes fluorescence detection[25]. Structural assignment and quantification of oligopeptides may be afforded by FD mass spectrometry (MS) operated in the selected ion monitoring (SIM) mode using appropriate stable isotope-labeled internal standards[40].

In addition to speed and sensitivity of peptide separations, in many cases the most important parameter is resolution of peptides. The TEAP buffer used with the C_{18} column has provided continued excellent resolution of synthetic mixtures of peptides, a necessary preliminary study to looking at extracts of hypothalamic tissue. For example, t_R values of Tyr[11] somatostatin and substance P are quite close and further chromatographic refinements (such as temperature, buffer, pH, flow-rate, column) in this separation may further resolve this pair. The high resolution of this RP-HPLC system is necessary for biologic extracts and will follow either gel or Sep-pak chromatography separations of proteins from peptides in biologic extracts[41].

Reproducibility of retention times (t_R) for multiple injections of peptide mixtures becomes an important parameter for peptide identification for chromatographic systems that do not have a follow-up step such as MS for structural elucidation of individual HPLC peaks. Reproducibility of t_R values was studied in a series of multiple injections of mixtures of oligopeptides. Data in Table I collect t_R values (individual and average), standard deviation (σ) and coefficient of variation (V). Retention times (t_R) are reproducible with an average coefficient of variation of 1.91%. By injecting known amounts of standards first, followed by injection of an unknown mixture of the same compounds, it should be possible to identify and quantify individual components in a mixture of oligopeptides. For cases involving mixtures of unknown compounds, a structural elucidation method is required following HPLC separation.

MS offers an attractive option to elucidate, identify and quantify an oligopeptide in a purified HPLC fraction. FD-MS in particular provides a fast and facile method for unambiguous identification because it is possible to produce ions only in the molecular ion region. On the other hand, for unknown underivatized oligopeptides, sequence-determining fragment ions may be produced by either increasing e.h.c. or by following FD by collision activation (CA)[42,43].

The type of data one obtains with FD-MS is illustrated by the FD spectrum of an underivatized hexapeptide Lys-Glu-Thr-Tyr-Ser-Lys given in Fig. 2. In contrast to the plethora of peaks commonly found in electron impact (EI) or chemical ionization (CI) mass spectra of derivatized oligopeptides[44], the spectrum in Fig. 1 is extra-

ordinarily clean. Unambiguous identification of sequence ions by either CA or by increasing e.h.c. is corroborated by accurate mass measurement (not performed) of the protonated molecular ion, $C_{33}H_{54}N_8O_{12}$ 755.3939. Similar results are obtained with enkephalins and other oligopeptides[45].

It is clear that, in order to employ MS techniques, one must avoid a non-volatile buffer salt such as TEAP. Appropriate alternate volatile buffers which can be removed by lyophilization are TEA–formate, TEA–acetate or ammonium acetate[29]. Alternatively, individually resolved RP-HPLC peptide fractions can be desalted by employing Waters Sep-pak cartridges after HPLC and before MS analysis[41].

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PREPARATIVE SEPARATION OF THE PYRROLIZIDINE ALKALOIDS, INTERMEDINE AND LYCOPSAMINE, AS THEIR BORATE COMPLEXES

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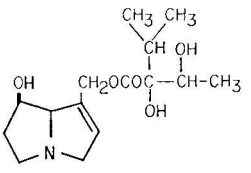
SUMMARY

Intermedine and lycopsamine are diastereoisomers containing vicinal glycol groups of different configuration. The difference in the degree to which they complex with borate is the basis of two procedures for their separation from mixtures on a preparative scale. In the first, the mixture dissolved in chloroform is passed through a column of glass powder moistened with a solution of borax. Intermedine elutes first with chloroform and is cleanly separated from lycopsamine, the more strongly complexing of the pair.

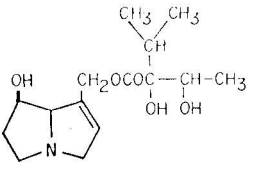
In the other procedure, in which lycopsamine elutes first, a mixture is dissolved in 0.1 *M* borax and the solution passed through a cation-exchange resin (Bio-Rad AG 50W-X2) impregnated with 0.1 *M* borax.

INTRODUCTION

The diastereoisomeric alkaloids, intermedine (1) and lycopsamine (2), occur together in a number of genera of the Boraginaceae, *e.g. Amsinckia*[1] and *Echium*[2] and in the genus *Parsonsia*[3] of the Apocynaceae. They are of interest both for their toxicity and for their conversion into pheromones by certain species of insects[4]. The physical properties of these two alkaloids are closely similar and their complete separation from mixtures by column, thin-layer or paper chromatographic methods has not yet been achieved, even on an analytical scale. Resolution satisfactory for analytical purposes is possible by gas chromatography of the mixed alkaloids as their



(1)



(2)

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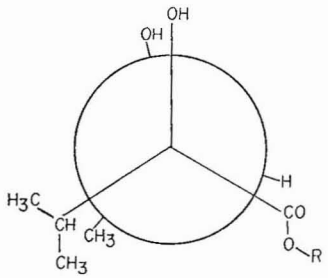
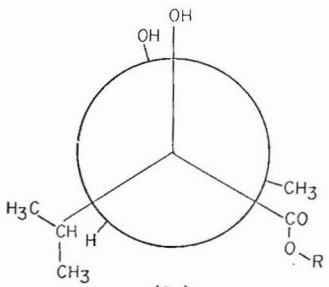
trimethylsilyl[5] or alkylboronate[3] derivatives. When first described from *Amsinckia* species, each alkaloid was isolated in small amounts by counter-current distribution of extracts, but the preparation of the pure alkaloids on a larger scale has remained one of the most difficult separation tasks in the pyrrolizidine alkaloid field.

In an earlier study of the paper electrophoresis of pyrrolizidine alkaloids[6] it was shown that these compounds migrate as cations in non-complexing electrolytes, but that, in sodium borate buffer, compounds containing complexable pairs of hydroxyl groups form anionic complexes to an extent determined by their stereochemistry, and the cationic migration is thereby reduced or changed into anionic migration. In a sodium carbonate electrolyte (pH 9.2), intermedine and lycopsamine were found to have identical cationic mobilities (1.54 cm/h·kV of total potential applied) but, in borate buffer of the same pH, they migrated toward the anode with mobilities of 0.29 and 2.35 cm/h·kV, respectively, and this large difference allowed rapid separation of the isomers, albeit on an analytical scale.



(1a)



(2a)

The greater anionic mobility of lycopsamine is a result of its forming the more stable borate complex, the reason for which is indicated by comparison of the Newman projection formulae (1a) and (2a). Formula (1a) represents a projection along the C_2–C_3 bond of the trachelanthate moiety of intermedine. It is seen that with the hydroxyl groups eclipsed (a condition ideal for the formation of a 5-membered cyclic borate complex) a destabilising non-bonded interaction is set up by the eclipsing of the C_2-isopropyl and C_3-methyl groups. The corresponding projection formula (2a) for the viridiflorate moiety of lycopsamine shows how eclipsing of its *erythro* hydroxyls leads to a conformationally more stable arrangement in which the C_2-isopropyl group interacts only with an eclipsed C_3-H.

The difference in degree to which intermedine and lycopsamine complex with borate has now been used in two different procedures for their separation on a preparative scale.

EXPERIMENTAL

Materials

The mixture of intermedine (approx. 30%) and lycopsamine (approx. 70%) contained a small amount of echimidine and was the product of an earlier investigation of the alkaloids of *Amsinckia* species[1]. The long-stored mixture was cleaned up by dissolving it in dilute sulphuric acid, filtering the solution from a black precipitate,

washing the filtrate with diethyl ether and with chloroform, adding ammonium hydroxide to pH 9.5, saturating the basic solution with sodium chloride and extracting it with at least five lots of chloroform.

Borate partition column. Powdered soda glass (passing 150 mesh; 48 g) was moistened with a 5% aqueous solution of disodium tetraborate (borax) (8 ml) and packed in light petroleum in a 55 × 1.1 cm column with the aid of a perforated plunger. The alkaloid mixture (47.7 mg) was dissolved in a small volume of chloroform which was absorbed into glass powder and evaporated under vacuum. The glass powder was then packed on top of the column. Chloroform was applied as eluent and fractions of approximately 5 ml collected and titrated with toluene-4-sulphonic acid (0.01 *M*) in chloroform using dimethyl yellow as indicator. The titres indicated two peaks (Fig. 1), comprised of fractions 13–24 and 51–80, which were combined appropriately for recovery of alkaloid. Each bulk fraction was evaporated and the residue taken up in dilute sulphuric acid. The solutions were made alkaline by adding ammonium hydroxide and then washed several times with light petroleum to remove dimethyl yellow, saturated with sodium chloride and extracted with five lots of chloroform. Fractions 13–24 gave 14 mg and fractions 51–80 gave 23.5 mg colourless crystalline alkaloid with the electrophoretic properties in borate buffer of pure intermedine and lycopsamine, respectively.

In a larger scale run, 1.7 g mixed alkaloid was applied to a 70 × 2.2 cm column packed with 340 g glass powder bearing 55 ml borate solution. Fractions of 40 ml were collected and examined by spotting on filter paper and spraying with manganese sulphate–potassium permanganate–sulphuric acid reagent[7]. Fractions were grouped appropriately and worked up as before. Fractions 5–15 gave 0.58 g intermedine and fractions 23–60 gave 0.83 g lycopsamine, both electrophoretically pure.

Intermedine. After recrystallisation from acetone, intermedine formed thick prisms, m.p. 141–142°, $[\alpha]_D^{20} + 9.8°$ (c, 1.49 in ethanol). ^{1}H NMR, δ(100 Mhz, $CDCl_3$): 0.93, d, 6H, $(CH_3)_2CH$; 1.20, d, 3H, CH_3CHOH; 4.80, AB nearly *s**, 2H, H9; 5.94, m, 1H, H2.

Lycopsamine. After recrystallisation from acetone, lycopsamine formed thick prisms, m.p.. 132–134°, $[\alpha]_D^{20} + 5.7°$ (c, 0.89 in ethanol). ^{1}H NMR, δ(100 Mhz, $CDCl_3$): 0.89, d, 3H, CH_3CH; 0.93, d, 3H, CH_3CH; 1.25, d, 3H, CH_3CHOH; 4.76, 4.81, ABq*, 2H, H9; 5.91, m, 1H, H2. The m.p. of a mixture of lycopsamine and intermedine was depressed to 119–125°.

Ion-exchange separation of borate complexes of intermedine and lycopsamine

A solution of mixed alkaloids (0.52 g) in 0.1 *M* di-sodium tetraborate (1 ml) was applied to the top of a 100 × 3 cm column of Bio-Rad AG 50W-X2 resin, (200–400 mesh; sodium form in 0.1 *M* borate). Elution was continued with 0.1 *M* borate at a flow-rate of 2 ml/min. Fractions of 10 ml were collected and examined for alkaloid by spotting on filter paper and spraying with manganese sulphate–potassium permanganate–sulphuric acid reagent. Visual assessment of the spots indicated that the alkaloids had eluted as in Fig. 2. Fractions were grouped appropriately and worked up by adding glucose (20 g/100 ml), potassium chloride (30 g/100 ml) and 10 *M* sodium hydroxide (3–4 ml/100 ml, until pH 11 was indicated by test papers).

* AB nearly *s*: AB quartet which has the appearance of a broadened singlet; ABq: AB quartet.

Immediate extraction with five lots of chloroform gave products as follows: fractions 1–30, 10 mg echimidine; fractions 31–70, 200 mg lycopsamine; fractions 71–110, 121 mg intermedine. Electrophoresis confirmed that the peaks shown in Fig. 2 were pure single components.

RESULTS AND DISCUSSION

The separation of intermedine and lycopsamine on the borate partition column depended on differences in the chloroform solubility of the free bases and their respective borate complexes. The free bases are easily soluble in chloroform but the salt-like complex anions may reasonably be expected to have little or no solubility in this solvent. The order of elution of the alkaloids was therefore determined by the proportion in which each existed as the free base in its equilibrium mixture and, as expected, intermedine appeared first and was cleanly separated from lycopsamine (Fig. 1). Each alkaloid crystallised readily from its pure fraction.

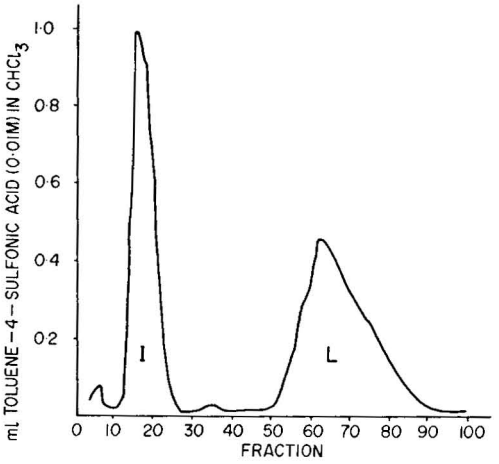


Fig. 1. Elution pattern of intermedine (I) and lycopsamine (L) from borate partition column.

In the other procedure, in which the alkaloid mixture was dissolved in 0.1 *M* sodium borate and the aqueous solution passed through a cation-exchange resin, it was expected that the negative charge induced on the alkaloid molecules by borate complexing would hinder binding to the resin and that the order of elution would, in this case, depend on the equilibrium proportion in which each alkaloid existed as its anionic complex. Lycopsamine, as the more strongly complexed of the pair of alkaloids, was indeed the first eluted, and complete separation from intermedine was again achieved (Fig. 2).

Difficulty was experienced in recovering lycopsamine in high yield from the borate solution, and, to a lesser extent, this was also true for intermedine. In the procedure adopted, glucose was added to decompose the alkaloid complexes by competing for borate, and the freed bases were salted out into chloroform by adding potassium chloride after making the solution strongly alkaline. In a trial experiment with lycopsamine, recovery was 80%.

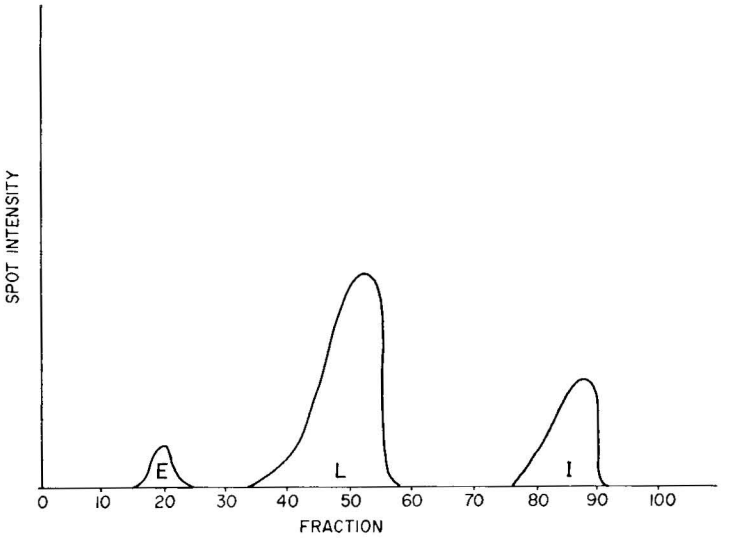


Fig. 2. Elution pattern of echimidine (E), lycopsamine (L) and intermedine (I) from Bio-Rad AG 50W-X2 ion-exchange column.

Both methods are suitable for separating gram quantities of the alkaloids and make intermedine and lycopsamine readily available in a pure state for the first time. The methods should be applicable to other mixtures of diastereoisomeric alkaloids which differ in being viridifloric and trachelanthic esters, for example, those of 7-angelylheliotridine which occur together in *Heliotropium supinum* L[8].

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AFFINITY CHROMATOGRAPHIC INTERACTIONS OF PROTEASES WITH LOW-MOLECULAR-WEIGHT SOYBEAN PROTEASE INHIBITORS

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SUMMARY

One of the five low-molecular-weight soybean protease inhibitors, protease inhibitor V (PI-V, Bowman–Birk inhibitor) with a molecular weight of 8000 daltons, is used for the affinity chromatographic purification of trypsin, chymotrypsin and kallikrein. The PI-V covalently bound to Sepharose 4B adsorbs bovine trypsin, chymotrypsin or porcine kallikrein at neutral pH. The adsorbed enzyme is eluted from the column with buffer containing a higher salt concentration and a lower pH. This low-molecular-weight PI-V affinity gel can separate trypsin and chymotrypsin from mixtures of these crude enzymes and can purify both enzymes to a homogeneous degree.

INTRODUCTION

Kunitz inhibitor, a soybean protease inhibitor with a molecular weight of 21,400 daltons, has been widely used to inhibit trypsin activity. Recently, this inhibitor, covalently linked to Sepharose 4B gel, has been employed to purify trypsin and chymotrypsin. It was used for affinity chromatography of trypsin by Mosolov and Lushnikova[1], Bartling and Barker[2], Porath and Sundberg[3], Liepnieks and Light[4,5], Sundberg and Porath[6], of chymotrypsin by Porath[7], of kallikrein by Fritz *et al.*[8] and of both trypsin and chymotrypsin by Amneus *et al.*[9]. This product is available also from commercial sources for purification of both trypsin and chymotrypsin[10].

We have described the isolation and purification of five low-molecular-weight (7000–8000 daltons) soybean protease inhibitors[11] and determined their usefulness as ligands for affinity chromatography of proteases[12]. Immunologic tests show that inhibitors I through IV are fully cross-reactive with each other but are

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distinct from inhibitor V (PI-V, Bowman–Birk inhibitor)*. Enzyme inhibition tests show that only inhibitor V inhibits both trypsin and chymotrypsin and is the most potent inhibitor for trypsin among them. For these reasons, PI-V may be useful as a ligand for the affinity purification of specific proteases. Since PI-V is only about a third the size of Kunitz inhibitor and can completely inhibit both crystalline bovine trypsin and chymotrypsin at an equimolar concentration, we believe that its use as a ligand would further increase the capacity of an affinity column for proteases. Furthermore, pepsin digestion of PI-V cleaves it into a tryptic and a chymotryptic inhibitor fragment, both of which can be separated by gel filtration chromatography[13]. Thus a specific affinity column for a single protease may be prepared. In the study reported here, we have observed the interaction of PI-V with trypsin, chymotrypsin and kallikrein by affinity chromatography and applied the procedure to the purification of these proteases from crude enzyme mixtures.

MATERIALS AND METHODS

Low-molecular-weight protease inhibitor PI-V was purified from the Tracy cultivar of soybean as previously described[11]. Crystalline bovine pancreatic trypsin (165 units/mg) and chymotrypsin (37 units/mg) were obtained from Worthington (Freehold, NJ, U.S.A.). TAME, BTEE, BAN, crude trypsin (10.4 units/mg in tryptic activity and 5.8 units/mg in chymotryptic activity) and porcine pancreatic kallikrein (2.5 units/mg based on BTEE activity) were purchased from Sigma (St. Louis, MO, U.S.A.). Sepharose 4B and Sephadex G-100 were Pharmacia (Uppsala, Sweden) products. All other chemicals and reagents used were of reagent grade.

Sepharose 4B gel was activated with cyanogen bromide, according to the method of Porath *et al.*[14]. Two and a half milligrams each of trypsin, chymotrypsin or PI-V were covalently conjugated to each millilitre of activated Sepharose 4B gel. About 100 ml of each type of gel were packed to a column (24 × 2.3 cm) for the chromatographic study. Each of the packed columns was washed with ten volumes of 1% ethanolamine, 0.1 *M* sodium borate buffer containing 1 *M* NaCl at pH 8.0 (buffer 2), 0.1 *M* sodium acetate buffer containing 1 *M* NaCl at pH 4.0 (buffer 3), buffer 3 adjusted to pH 2.0 with concentrated HCl (buffer 4) and finally each column was reequilibrated with pH 7.0, 0.05 *M* Tris–HCl buffer containing 1 m*M* $CaCl_2$ (buffer 1). All column elutions were carried out at 4°C in a cold room. Column flow-rates were adjusted to about 2 ml/min. Crystalline trypsin (50 mg), chymotrypsin (50 mg) or crude kallikrein (100 mg) was dissolved in 5 ml of buffer 1 for application to a PI-V–Sepharose 4B column (bed volume 100 ml). After the wash with three column volumes of buffer 1, the columns were eluted first with three volumes of buffer 2, then three volumes of buffer 3 and finally with three volumes of buffer 1 to re-equilibrate the column. For the separation of enzyme mixtures, 120 mg crude trypsin (containing 35% of chymotryptic activity) were applied to a PI-V–Sepharose 4B column (26 × 1.5 cm, bed volume 50 ml). Stepwise elution with buffers of different pH and salt concentrations was performed as described above.

* Abbreviations: TAME = *p*-tosyl arginyl methyl ester; BTEE = benzoyl tyrosyl ethyl ester; BAN = N-*tert.*-butyloxy-alanyl nitrophenyl ester; PI-V = Protease inhibitor V, Bowman–Birk inhibitor; SDS = sodium dodecyl sulfate.

The gradient elution procedure was accomplished with a linear gradient of three column volumes of pH 8.0 buffer (buffer 2) to three volumes of pH 4.0 buffer containing 0.5 *M* sodium acetate and 0.5 *M* calcium chloride. The affinity column could be regenerated with three volumes of buffer 4 and then equilibrated with ten volumes of buffer 1. The protein eluted from the column was monitored by measuring the absorbance of the eluate at 280 nm.

Tryptic activity was measured by the rate of hydrolysis of TAME as monitored by the increase in absorbance at 247 nm. Chymotryptic activity was measured by the rate of hydrolysis of BTEE as monitored by the changes in absorbance at 256 nm[15]. Elastase-like activity was measured by the rate of hydrolysis of BAN as monitored by the changes in absorbance at 347.5 nm[16]. Kallikrein activity was monitored by both BTEE and BAN assays and expressed as the change in absorbance per mg of enzyme in 10 min (*A* unit). Tryptic or chymotryptic activity was expressed as micromoles of TAME or BTEE hydrolyzed per minute.

Daily urine samples from six normal adults were pooled and concentrated from about 10 l to 40 ml with an Amican hollow fiber concentrator equipped with a cartridge for a molecular weight cutoff at 10,000 daltons. The concentrated sample was dialysed against buffer 1 and the enzymes purified by passage through the PI-V–Sepharose 4B column.

Fractions with kallikrein activity, eluted after the application of buffer 3, were pooled and concentrated in a Diaflo concentrator. Further purification was achieved by chromatography on a Sephadex G-100 column (157 × 0.9 cm). The fractions containing a single symmetric protein peak after gel filtration were pooled, dialysed and lyophilized for characterization by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate according to the method of Weber and Osborn[17].

RESULTS

The elution profile of 50 mg crystalline bovine trypsin on a PI-V–Sepharose 4B column is shown in Fig. 1a; the profile of 50 mg crystalline bovine chymotrypsin is depicted in Fig. 1b. In both Fig. 1a and 1b, the peak eluted by buffer 1 (peak I) was inactive. The peak eluted by buffer 2 (peak II) had less than 10% of the original specific activity. The protein peak eluted with pH 4.0 buffer (peak III) was very active. The fraction represented by the trailing end of peak III could not be completely eluted until the column was re-equilibrated with buffer 1 containing 1 m*M* of $CaCl_2$. More than 90% of the protein applied to the column was recovered in the eluate. Residual uneluted enzymes, if any, were removed from the column by washing with buffer 4 and then equilibrating with pH 7.0 buffer. The elution profile of 50 mg each of the PI-V on a trypsin–Sepharose 4B column (left) and on a chymotrypsin–Sepharose 4B column (right) is depicted in Fig. 2. PI-V was completely eluted by sequentially applying buffers 1, 2 and 3, including a final elution with buffer 4. Less than 20% of PI-V applied to the column was eluted with buffers 1 and 2. The main peak of PI-V was eluted with buffer 3. The elution profiles of PI-V on the trypsin column and chymotrypsin column were quite similar, suggesting that either enzyme can be used as a ligand for affinity purification of PI-V.

The elution profile of 100 mg of porcine pancreatic kallikrein (2.5 BTEE units/A_{280} protein unit) chromatographed on a PI-V Sepharose column is shown in Fig. 3.

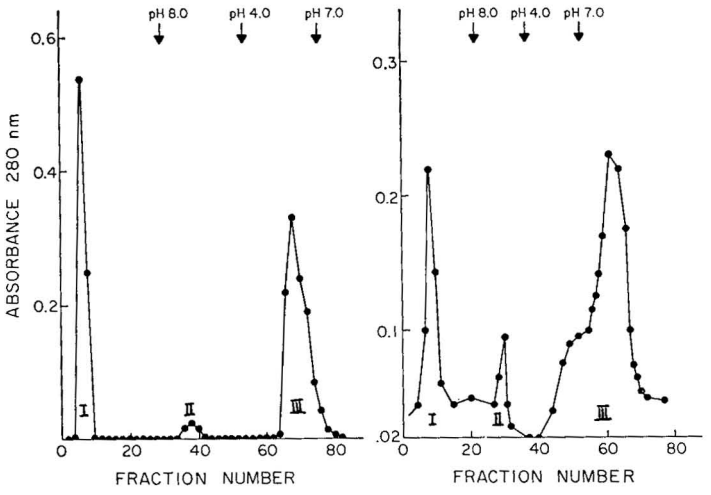

Fig. 1. Affinity chromatography of 50 mg of purified bovine trypsin on a PI-V–Sepharose 4B column (1a, left) and 50 mg of bovine chymotrypsin (1b, right) on the same column (100 ml). The arrow indicates the fraction at which buffer change occurred. Protein concentrations in the fractions were monitored by absorbance at 280 nm. Each fraction volume equals 10 ml.

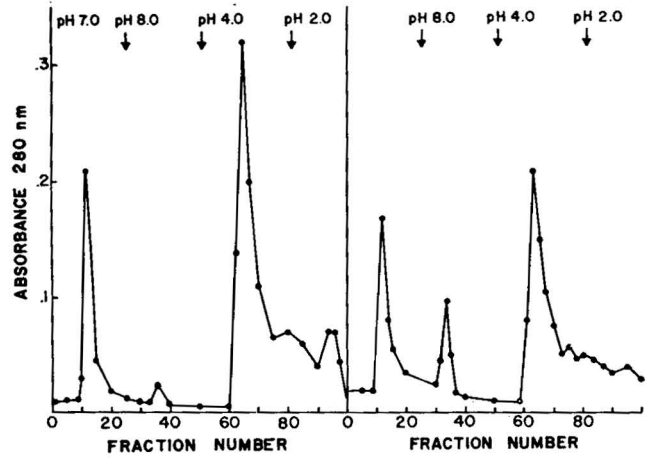

Fig. 2. Chromatography of 50 mg of purified PI-V each on a trypsin–Sepharose 4B column (left) and on a chymotrypsin–Sepharose 4B column (right) (both 100 ml). A pH 2.0 buffer was added to the elution profile before re-equilibration to ensure complete removal of PI-V from the column. Protein concentrations in the fractions were monitored by absorbance at 280 nm. Each fraction contains 10 ml.

The unadsorbed peak contained 50% of the protein applied but, as compared to the original sample, had less than 1% of the enzyme activity toward BTEE and BAN. The enzyme eluted with buffer 2 represented approximately 1% of the protein applied to the column. Compared to the original unfractionated sample, the main peak of activity eluted after the application of buffer 3 possessed a ten to twenty-fold

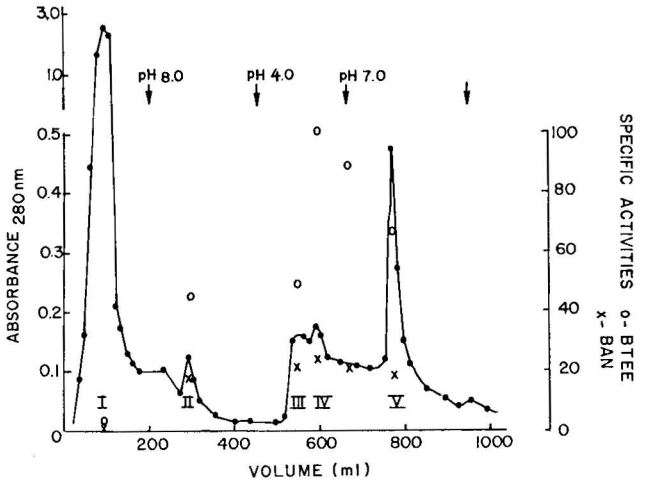

Fig. 3. Chromatography of 100 mg of purified porcine pancreatic kallikrein on a PI-V–Sepharose 4B column (100 ml). Specific activity toward BTEE (open circle) is expressed as change in absorbance at 256 nm in 10 min per A_{280} units of protein added. Specific activity toward BAN (×) is expressed as change in absorbance at 347.5 nm in 10 min per A_{280} units of protein added.

higher specific activity based on the BTEE assay. In contrast, the enzyme activity toward BAN increased only two-fold in peaks III, IV and V.

When crude human urine concentrate containing kallikrein was chromatographed on a PI-V–Sepharose 4B column (Fig. 4), the unadsorbed dark brownish fractions (peak I) contained no detectable enzyme activity toward either BTEE, BAN or TAME. The fractions eluted with buffer 2 showed low activity toward BTEE

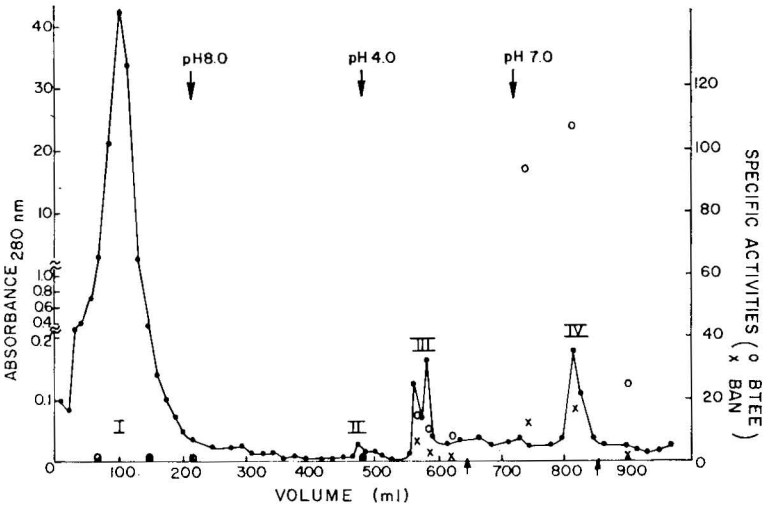

Fig. 4. Chromatography of concentrated daily urine from six normal adults on a PI-V–Sepharose 4B column (100 ml). Ten-ml fractions were collected. Fractions eluted from 630 ml to 850 ml were pooled, concentrated and chromatographed on a Sephadex G-100 column. Specific activity is expressed as in the legend of Fig. 3.

(1.2 units); the activity toward BAN was too low for measurement. The fractions eluted after the application of buffer 3 (peak III) exhibited appreciable kallikrein activity even though the protein concentration in these fractions, as measured by absorbance at 280 nm, was less than 0.2 *A*. When the column was re-equilibrated with buffer 1 (peak IV), an active peak toward BTEE (106 units) and BAN (17 units) was recovered. The activity ratio toward these two substrates was constant and was similar to that of a porcine pancreatic kallikrein repurified by the same procedure (Fig. 3).

The elution profile of 120 mg crude pancreatic trypsin (containing 35% chymotrypsin) on a PI-V–Sepharose 4B column (bed volume 50 ml) is shown in Fig. 5. Using a stepwise elution profile, the chymotrypsin was eluted with buffer 2 while the trypsin was retained on the column until the application of buffer 3. The two enzymes, therefore, were well separated. Chymotrypsin and trypsin activities were increased twelve-fold and sixteen-fold respectively, as shown in Fig. 5, left. Both enzymes were eluted as multiple peaks through the gradient when a second run with a pH and salt gradient elution was conducted. The major peak of both enzymes emerged after the application of buffer 3 (pH 4.0) at the completion of gradient elution. A seven-fold purification of chymotrypsin and twelve-fold purification of trypsin were observed even though the two enzymes were not separated.

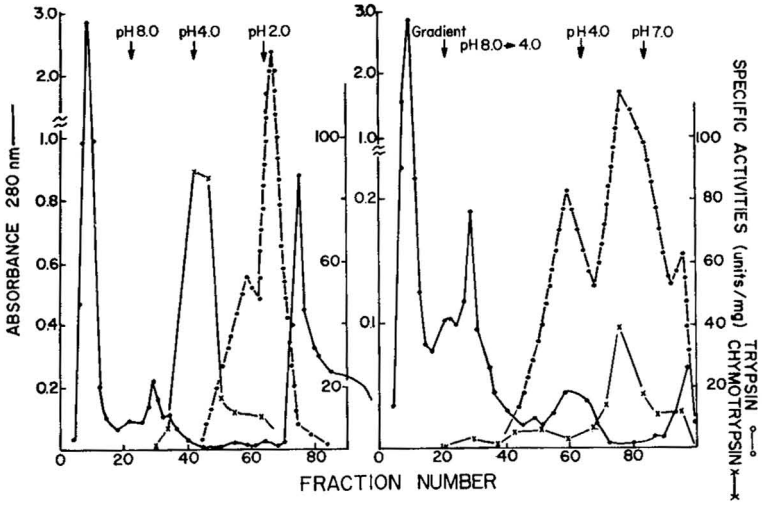

Fig. 5. Chromatography of 120 mg crude pancreatic trypsin (containing 35% chymotrypsin) on a PI-V–Sepharose 4B column (50 ml) by stepwise elution (left) and gradient elution (right). Fraction volume equals 7 ml. Specific activity of trypsin is expressed as micromoles TAME hydrolysed per min per mg protein added. Chymotrypsin activity is expressed as micromoles BTEE hydrolysed per min per mg protein added.

DISCUSSION

PI-V covalently linked to Sepharose 4B gel retains its affinity for both trypsin and chymotrypsin. The fact that the specific activity of neither crystalline trypsin nor purified chymotrypsin showed any further increase after affinity chromatography

implies that the crystalline enzymes were of high purity. However, substantial purification of a crude mixture of trypsin and chymotrypsin was seen. Since trypsin cleaves the PI-V molecule at the Lys-Ser bond (residues 16, 17) and chymotrypsin cleaves at the Leu-Ser bond (residues 43, 44) at equivalent molar ratio in solution, it is conceivable that the immobilized PI-V molecules on Sepharose 4B gel could have been modified by the proteases during the chromatographic procedures. Nevertheless, the binding capacity of the column remained unchanged after each run.

Purification of urinary protease can be achieved by one-step chromatography on a PI-V–Sepharose 4B column. The activity of this protease is similar to that of kallikrein based on substrate specificity. The most purified kallikrein exhibits only low activity toward TAME. The affinity of PI-V toward kallikrein is comparable to that of aprotinin, a low-molecular-weight pancreatic trypsin inhibitor which has been used for the affinity chromatography of urinary kallikrein[18].

In the stepwise elution procedure for the purification of crude enzymes, the chymotryptic and tryptic activities are well separated and both enzymes are recovered. The elution pattern indicated that the interaction of chymotrypsin with PI-V is affected by the binding of trypsin even though these two materials bind to separate sites on the PI-V molecule. A calcium and pH gradient elution profile shows the incomplete separation and partial loss of chymotryptic activity which may be due to denaturation of chymotrypsin at high calcium concentrations. Such an event would be responsible for the low recovery of chymotryptic activity. The recovery of tryptic activity, however, is increased as compared to that of the stepwise elution procedure.

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Note

Separation and detection of 2-aminoanthracene and its metabolites by high-performance liquid chromatography

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Recently, the carcinogen 2-aminoanthracene (2-AA) has been recommended for use as a positive control in the Salmonella/microsome mutagenicity assay since it causes mutations in all tester strains[1]. The metabolism of 2-AA to mutagenic products is also enhanced in the presence of lithocholic acid and its derivatives[2] and other modifiers of drug metabolism[3]. For metabolism studies with this carcinogen it is important to have an analytical method capable of detecting the compound and its metabolites. However, these metabolic products, which heretofore have been unavailable, are unstable and no chromatographic method for their detection has been reported. By derivatization of 2-AA and its metabolites to stable products, we can now separate them by high-performance liquid chromatography (HPLC). The application of the technique to some biological studies was examined.

EXPERIMENTAL

Reference standards and reagents

2-AA was obtained from Aldrich (Milwaukee, WI, U.S.A.). 2-Acetylaminoanthracene, 2-acetylamino-9,10-anthraquinone, 2-nitroanthracene, 2-nitroanthraquinone, N-hydroxy-2-aminoanthracene, and the monoacetoxy derivatives of the 5-, 6-, and 8-hydroxy-2-acetylaminoanthracenes were synthesized in our laboratory[4]. Glass-distilled organic solvents (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) were used. All other reagents were of the highest purity commercially available.

Preparation of microsomes and 2-AA metabolism samples

Male Sprague-Dawley rats (up to 250 g) were obtained from the National Cancer Institute Animal Facility in Bethesda, MD. The animals were housed in plastic shoe-box cages, and given water and Purina chow *ad libitum*. Liver 9000 *g* supernatant fractions (S9) were prepared from animals pretreated (IP) with Aroclor 1254 (one 500 mg/kg dose dissolved in corn oil 5 days before sacrifice). Liver S9 fractions were prepared as described previously[2].

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2-AA and its metabolites were assayed in the presence of the same concentrations of S9 protein which were present when this compound was tested in the Ames assay[3], *i.e.* 1 μg of 2-AA per 0.5 mg of AC-S9 protein. The assay mixture also contained the following components in a total volume of 1 ml: 100 μmoles potassium phosphate, pH 7.4, 3 μmoles $MgCl_2$, 5 μmoles glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 400 μmoles NADPH, and varying amounts of liver S9 fraction. The reactions were stopped by the addition of 1 ml of acetone and stored on ice; 2 ml of ethyl acetate were added and the solutions vortexed vigorously for 30 sec. After centrifugation, the organic phase was removed, dried over sodium sulfate and then acetylated with acetic anhydride (100 μl per 2.5 ml for 15 min at room temperature). The solutions were evaporated to dryness under N_2 and stored at $-20°C$. When analyzed as methylurea derivatives, the reactions were stopped by extraction with 5 ml of methylene chloride. The extracts were treated with 0.2 ml of methyl isocyanate and warmed at 37°C for 15 min prior to evaporation to dryness under N_2. The dried extracts were stored at $-20°C$.

Preparation of methylurea standards

Approximately 1 mg each of 2-AA and N-hydroxy-2-AA were dissolved in 3 ml of methylene chloride. Methyl isocyanate (0.3 ml) was added and after standing at room temperature for 2 h the solutions were evaporated to dryness under N_2. The residue was dissolved in a minimal volume of methanol and applied to a 0.25-mm silica gel TLC plate (20 × 20 cm, Analtech Rediplate). They were developed with isopropanol–hexane (5:95, v/v). After the plates were developed, the bands were visualized under long-wavelength UV light. The methylurea derivative of 2-AA migrated as a bright blue band. The band was scraped off the plate and eluted with acetone. The N-hydroxy-2-AA derivative separated into two major bands which appeared blue under the UV light. One of them had the same R_F as the 2-AA derivative. The other was more polar as might be expected of the methylurea derivative of N-hydroxy-2-AA. The N-hydroxy-2-AA was contaminated with, at least, 25% 2-AA. Infrared analysis of the isolated fraction indicated the presence of methylurea derivatives.

High-performance liquid chromatography

A modular HPLC system was used for this study. The unit consisted of a Laboratory Data Control (Riviera Beach, FL, U.S.A.) Constametric I and II pumps, a Gradient Master and Mixer; a Chromatronix dual-channel UV absorbance detector (Spectra-Physics, Santa Clara, CA, U.S.A.); a Rheodyne Model 7120 syringe-loading sample injector (Rheodyne, Berkeley, CA, U.S.A.); and a Fisher Recordall Series 5000 recorder (Fisher Scientific, Silver Spring, MD, U.S.A.) operated at 0.2 in/min chart speed and a 10 mV setting.

Separations were made on a 25 cm × 4.6 mm I.D. 10 μ-Partisil column (Whatman, Clifton, NJ, U.S.A.) operated at ambient temperature and a flow-rate of 1 ml/min of mobile phase 1–10% isopropanol in hexane with a linear gradient of 15 min followed by 45 min under the final conditions. An isocratic mobile phase of ethanol–heptane (20:80 v/v), was used for the separation of methylurea derivatives. A precolumn (4 cm × 2.1 mm I.D.) packed with Waters Assoc. (Milford, MA, U.S.A.) pellicular Corasil (37–50 μm) was used to ensure the stability of the analytical column.

Peak areas and retention times were determined with a Hewlett-Packard 3352A laboratory data system (Avondale, PA, U.S.A.). The residue obtained from metabolism samples was dissolved in 100 μl mobile phase (10% isopropanol in hexane) prior to HPLC analysis.

RESULTS AND DISCUSSION

A typical HPLC separation of 2-AA and its metabolites on a μPartisil column using a 15 min linear gradient from 1–10% propan-2-ol in hexane is shown in Fig. 1. The approximate amounts are given in the figure caption. Retention times, peak areas and peak shapes were highly reproducible under the conditions used. When an isocratic mobile phase of 10% isopropanol in hexane was used, baseline separation of all but the nitro derivatives was achieved. Table I shows the retention times of the compounds when resolved either by gradient or isocratic mobile phase conditions. Improved resolution of nitro-derivatives was obtained under gradient conditions. N-hydroxy-2-AA did not elute under these mobile phase conditions, presumably because it was strongly adsorbed to the silica gel column.

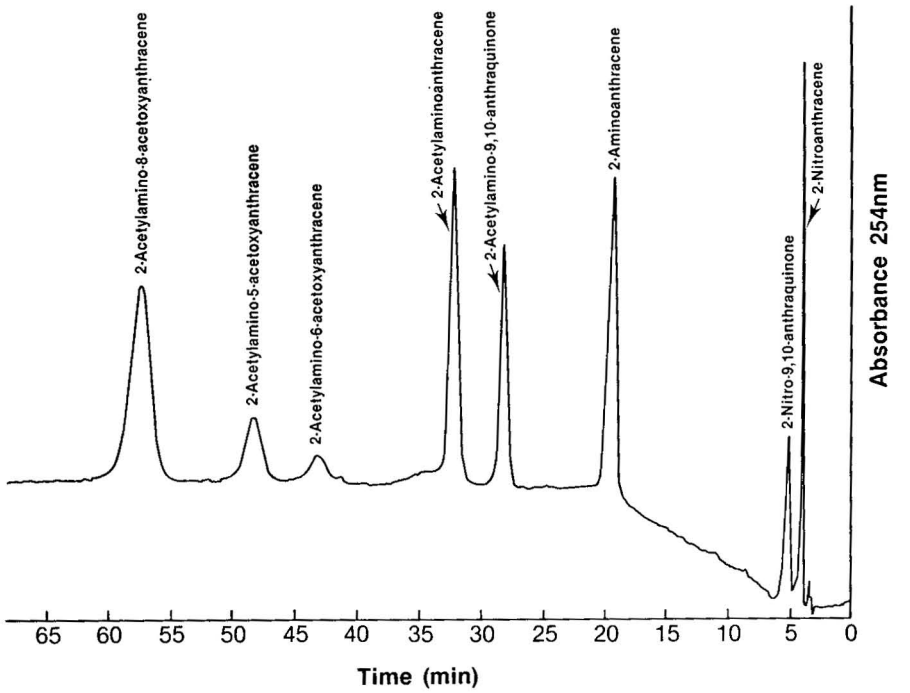

Fig. 1. HPLC profile of mixed standard. Approximate amounts are given in parentheses. 2-Nitroanthracene (45 ng), 2-nitro-9,10-anthraquinone (65 ng), 2-aminoanthracene (155 ng), 2-acetylamino-9,10-anthraquinone (300 ng), 2-acetylaminoanthracene (100 ng), 2-acetylamino-6-acetoxyanthracene (110 ng), 2-acetylamino-5-acetoxyanthracene (300 ng), and 2-acetylamino-8-acetoxyanthracene (500 ng) were separated on a 10 μ-Partisil column using a linear gradient of 1–10% isopropanol in hexane for 15 min followed by 45 min under the final conditions. UV absorbance was monitored at 254 nm with full-scale sensitivity at 0.04. Flow-rate of 1 ml/min and chart speed of 0.2 in/min were used.

TABLE I

RETENTION TIMES OF 2-AA AND METABOLITES UNDER GRADIENT AND ISOCRATIC MOBILE PHASE CONDITIONS

Compound	*1–10% Isopropanol in hexane (gradient)*	*10% Isopropanol in hexane (isocratic)*
2-Nitroanthracene	4.0	3.9
2-Nitro-9,10-anthraquinone	5.1	4.3
2-Aminoanthracene	17.6	11.4
2-Acetylamino-9,10-anthraquinone	27.7	17.9
2-Acetylaminoanthracene	31.7	22.7
2-Acetylamino-6-acetoxyanthracene	42.4	35.8
2-Acetylamino-5-acetoxyanthracene	47.3	41.7
2-Acetylamino-8-acetoxyanthracene	56.4	52.3

Metabolites of 2-AA produced by incubation with liver S9 fractions from rats pretreated with Aroclor 1254 were derivatized for the HPLC analysis. The results obtained after incubation of 1 μg of 2-AA per ml for 0, 5, and 15 min with 10 μl (0.25 mg/ml) S9 fractions showed that no derivatives of 2-AA were present. However, the disappearance of 2-AA both as a function of S9 protein concentration and time is shown in Fig. 2.

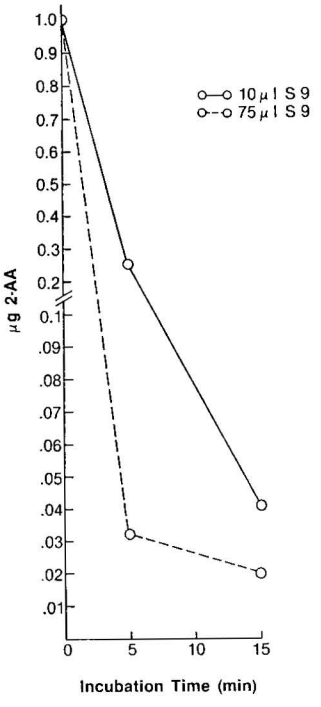

Fig. 2. Plot of disappearance of 2-AA as a function of time and protein concentration. 1 μg of 2-AA was incubated for 0, 5 and 15 min with 10 μl (○——○) and 75 μl (○- - -○) S-9 fractions.

To facilitate the elution of N-hydroxy-2-AA from the silica gel column, its methylurea (MU) derivative and also that of 2-AA were prepared and purified by TLC (see Experimental). With a stronger mobile phase of 20% isopropanol in hexane (isocratic) the N-hydroxy-2-AA-MU eluted at *ca.* 33 min as a broad peak, whereas 2-AA-MU eluted at 13 min as a sharp peak. When 20% ethanol in heptane (isocratic) was used as a mobile phase, sharper peaks of both N-OH-2-AA-MU and 2-AA-MU at *ca.* 15 and 7.4 min, respectively, eluted (Fig. 3).

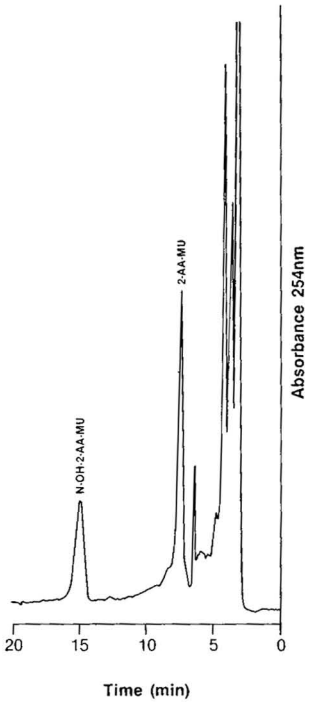

Fig. 3. HPLC profile of methylurea derivatives of 2-AA and N-hydroxy-2-AA. Conditions same as Fig. 1 with the exception of an isocratic mobile phase of ethanol–heptane (20:80 v/v).

The enthanol–heptane mobile phase was used to analyze extracts of incubations of 1 μg of 2-AA for 10 min with 0, 5, 10 and 25 μl (0, 0.12, 0.25 and 0.62 mg/ml) of S9-fraction. In Fig. 4 the increasing rate of disappearance of 2-AA with increasing amounts of S9 protein is shown. N-hydroxy-2-AA metabolite peaks, or other peaks which might correspond to phenolic metabolites, were not observed. Experiments to determine if the loss of N-hydroxy-2-AA results from non-specific binding to protein are continuing. Incubation of higher concentrations of 2-AA (10 μg/ml) also resulted in measurable losses of 2-AA, and no products eluted with expected metabolites even when 50% of the total extract was analyzed.

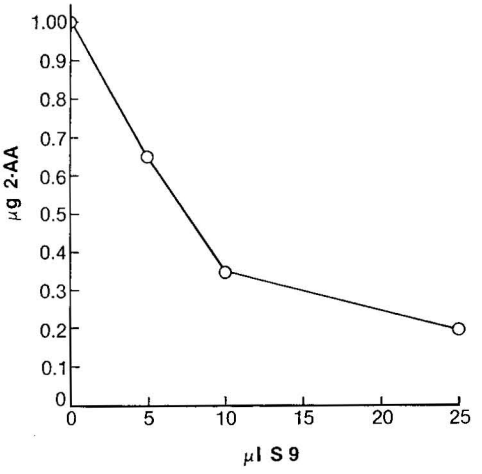

Fig. 4. Plot of disappearance of 2-AA as a function of protein concentration. 1 μg of 2-AA was incubated for 10 min with 0, 5, 10 and 25 μl S9 fractions.

CONCLUSIONS

A useful HPLC method for the separation and detection of 2-AA and its metabolites has been developed. The method was used to separate and quantitate the disappearance of 2-AA from *in vitro* incubations containing S9 fractions from rats. Even though total lipid extracts were used, the contaminating lipids did not interfere with the detection and elution of the metabolites in either mobile phase system. Although no N-hydroxy-2-AA and phenolic metabolites were detected, the sensitivity of the method could be substantially increased by using radiolabeled substrate. Hence the methods reported here have the potential for use in studies involving *in vivo* and *in vitro* studies of 2-AA metabolism.

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Note

Fingerprinting of heparins by low-amperage electrophoresis in barium acetate

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Heparin, a polysaccharide widely distributed in animal tissues and well known for its anticoagulant and antilipaemic properties, has a hybrid structure, the major features of which are known. However, it has become increasingly evident that heparin is not a single species, but consists of a family of polysaccharide chains having a number of structural features in common, but differing from each other in minor detail and in molecular weight. Although polydispersity is readily demonstrable by gel filtration, the elution profiles obtained are usually broad and show no real separation of distinct entities (for recent reviews, see ref. 1)*.

The concept of structural heterogeneity has been reinforced by a number of observations in the past few years. Most heparin preparations give two spots on agarose[3] or cellulose acetate[4] electrophoresis in barbital buffers, and are separated into two fractions, one non-mobile and the other mobile, in a barium acetate buffer[5].

A much more pronounced degree of heterogeneity has been reported in isoelectrofocusing experiments, which show up to 21 bands for heparins that give a maximum of two spots in conventional electrophoretic systems[6]. However, this behaviour does not necessarily imply that such a large number of sub-fractions are present, because it has been shown that the carrier ampholytes involved in complexing are themselves preferentially localized in well defined zones of the isoelectrofocusing slabs[7]. Thus, multiple banding seems to reflect, at least in part, "saturation" of these ampholyte zones by excess of heparin as it moves towards the anode. Single heparin bands transferred on to new slabs produced either 1–3[8] or up to 21[7] bands, depending apparently on the concentration of the polysaccharide in the band transferred, thus not providing real information on the number of sub-fractions.

In an attempt to provide a simple criterion for evaluating the heterogeneity of heparins, we have extended the original observation by Wessler[5] that a portion of heparin does not migrate on cellulose acetate strips during electrophoresis in a barium acetate buffer. We now report that by working at lower amperage and temperature for longer times, electropherograms are obtained showing well defined bands for heparin preparations that, otherwise, are pure by any generally accepted criteria.

* A heparin preparation from placenta gave, however, distinctly separated sub-fractions from Sephadex G-50[2].

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MATERIALS AND METHODS

Heparins (sodium salts) were commercial samples that appeared to be electrophoretically homogeneous in an acidic buffer (0.05 *M* potassium chloride in 0.1 *M* hydrochloric acid, adjusted to pH 1.1, 4°C)[9,10] that permits the detection of admixed hyaluronic acid, heparan sulphates and galactosaminoglycans. Contamination by more than 3–4% of hyaluronic acid or galactosaminoglycans was also ruled out by the ^{13}C nuclear magnetic resonance spectra. A preparation from pig mucosa was a reference standard from the University of Chicago (Drs. A. J. Cifonelli and M. B. Mathews). The anticoagulant activity of the preparations was between 145 and 170 USP units.

The electrophoreses were performed in barium acetate (0.1 *M*) adjusted to pH 5.8[11] with acetic acid, on Sepraphore III (Gelman, Milan, Italy) or Microphor (Elvi, Milan, Italy) cellulose acetate strips (160 × 25 mm), at a voltage corresponding to 0.4 mA/cm, at 4°C for 16 h. Before application of the sample, the strips were immersed for 30 min in 0.1 *M* barium acetate (pH 5.8) and the excess of buffer was eliminated by blotting with a paper towel. Heparins were applied as sodium salts (1–3 μl of 0.2% solutions in water or 0.1 *M* barium acetate, pH 5.8). After application of the spots (or bands), the strips were left for 5 min on the electrophoresis bridge before applying the appropriate voltage. After staining with 1% Alcian Blue (Bio-Rad Labs., Richmond, CA, U.S.A.) in a 1:1 (v/v) mixture of 0.05 *M* sodium acetate and 95% ethanol, the strips were washed with 5% acetic acid and air-dried.

The densitometric traces were recorded with a Zeiss KM3 spectrophotometer, equipped with a reflectance unit set at 380 nm (slit 3.5 × 1.5 mm). As the colorimetric response to Alcian Blue was expected to be different for different heparin subfractions, the relative areas of the electrophoretic bands were not assumed to be a direct measure of the relative concentration of the sub-fractions.

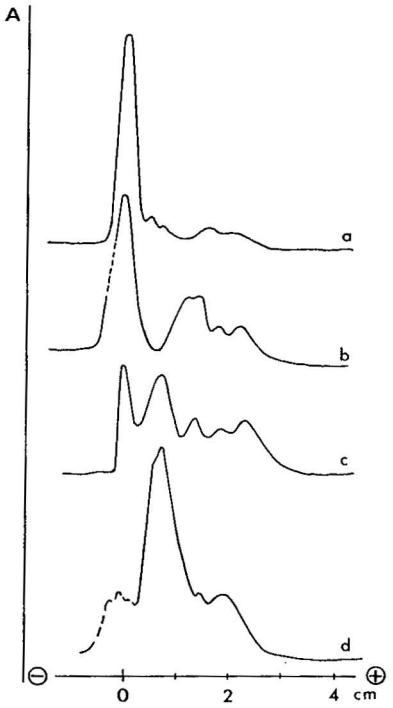
Strict adherence to the above electrophoretic and densitometric conditions is required for obtaining reproducible electropherograms. It should be noted in particular that the electrophoretic patterns vary somewhat for different brands of commercial cellulose acetate electrophoresis products, and that the relative intensity of the various heparin bands as measured by reflectometry is different when scanning is made by transmission densitometry. The latter effect appears attributable to the non-mobile fractions being in fact "precipitated" on the cellulose acetate strips, and thus preferentially concentrated on the surface of the strips.

RESULTS AND DISCUSSION

The electropherograms of four heparin samples from typical commercial sources (bovine lung and pig intestinal mucosa) are compared in Fig. 1. One of the two bovine lung preparations (a) consists largely of a single component, which hardly moves from the origin. The second bovine lung heparin (b) clearly shows at least three more, fastest moving, components. Fast-moving components are represented much more extensively in mucosal heparins (c) and (d). Except for the profile of the band near the origin (broken lines), such traces are reasonably reproducible, and substantially different from each other for more than 20 heparin preparations so far analysed. These electropherograms can thus be regarded as "fingerprints" of heparins

that otherwise show only minor differences in their chromatographic and bulk physico-chemical properties.

The electrophoretic mobility of heparins in barium acetate seems, at least in part, to be a function of molecular weight. In fact, different fractions obtained by gel filtration (Ultrogel AcA-44) gave different electrophoretic profiles by the present method, with a general trend towards lowest mobility by material of the highest molecular weight, and *vice versa*. As shown in Fig. 2, intermediate fractions gave prominent peaks in the intermediate zone. On the other hand, heparins with similar profiles on gel filtration gave different electrophoretic patterns in the present system.



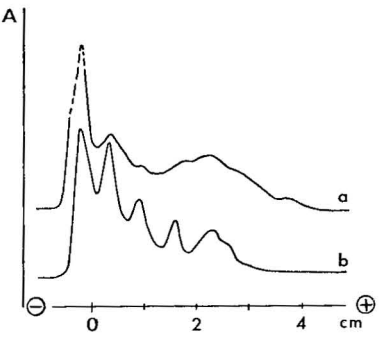


Fig. 1. Low-amperage electropherograms on cellulose acetate (0.1 *M* barium acetate, pH 5.8) of different heparin preparations: (a) from bovine lung (Upjohn, batch 070-ES); (b) from bovine lung (Upjohn, batch 746-DU); (c) from pig intestinal mucosa (Serva, batch 24590); (d) from pig intestinal mucosa (standard from the University of Chicago).

Fig. 2. Low-amperage electropherograms on cellulose acetate (0.1 *M* barium acetate, pH 5.8) of an unfractionated heparin [(a), from pig intestinal mucosa, Terhormon, batch 575-018] and an intermediate fraction obtained by gel filtration of the same heparin on Ultrogel AcA-44 (b).

Complexing with Ba^{2+} ions is likely to be the main factor influencing these separations, and it is concluded that the high-molecular-weight species complex Ba^{2+} ions stronger than do the low-molecular-weight species. This behaviour parallels that on isoelectrofocusing[6,8,12], where a trend is apparent in which high-molecular-weight fractions are more easily complexed by the carrier ampholytes than low-molecular-weight fractions.

The results show that "heparin" consists of a family of well differentiated species rather than a "continuum" of chains only barely distinguishable from each other. Although differences between these various species may prove to be relatively minor in terms of such criteria as overall composition, the number and/or arrangement of sites in them that determine their ability to complex with barium (and probably other divalent cations) must vary widely.

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Note

High-performance liquid chromatographic separation of unsaturated disaccharides derived from heparan sulfate and heparin

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An enzymatic method has been developed for the elucidation of the structures of heparan sulfate and heparin[1–4] which exhibit a more complex composition than most other glycosaminoglycans[5–7]. Heparinase digests heparin and "heparin-like" portions of heparan sulfate whereas heparitinase (heparanase) degrades the non-sulfate and low-sulfate portions of heparan sulfate. Both enzymes produce mainly disaccharides containing an α,β-unsaturated uronic acid on the nonreducing end. Five disaccharides have been isolated and characterized by Hovingh and Linker[8], which probably represent the disaccharide repeating units of heparan sulfate. The quantitation of the yield of disaccharides from different preparations of heparan sulfate and heparin was accomplished by small-scale enzymatic digestion of the polymers followed by paper chromatographic determination of the products. A similar paper chromatographic technique was used by Silva *et al.*[9] for the analysis of the disaccharide products formed from heparan sulfate.

We have developed a high-performance liquid chromatographic (HPLC) analytical method that is more rapid and sensitive than paper chromatographic procedure for quantifying these unsaturated disaccharides and is well suited to the investigation of the structures of heparan sulfate and heparin.

EXPERIMENTAL

Materials

Four unsaturated disaccharides from heparan sulfate and heparin were obtained as a much appreciated gift from Dr. Alfred Linker, University of Utah (Salt Lake City, UT, U.S.A.). These disaccharides are ΔDi–HS_b–I, a non-sulfated disaccharide from heparan sulfate, ΔDi–HS_b–II, a disaccharide from heparan sulfate containing one sulfate group on the acetylglucosamine unit, ΔDi–HS_b–III, a disaccharide from heparan sulfate containing N-sulfate glucosamine, and, ΔDi–He_a–I, a trisulfated disaccharide from heparin. The structures and preparation of these disaccharides have been reported previously[8,10]. The unsaturated disaccharides from chondroitin sulfates (ΔDi–OS, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi–6S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose; and ΔDi–4S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose) were purchased from

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Miles Labs. (Elkhart, IN, U.S.A.). The disulfated disaccharide, ΔDi–diS_B (2-acetamido-2-deoxy-3-O-(2-O-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose) was prepared and purified chromatographically from the chondroitinase ABC-digest of dermatan sulfate from pigskin (Miles Labs).

Methanol and acetonitrile (HPLC grade) and ammonium acetate were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). PIC-A reagent (tetrabutylammonium phosphate) for ion-pair chromatography was purchased from Waters Assoc. (Milford, MS, U.S.A.).

Apparatus

A modular HPLC system was used for the chromatographic studies which consisted of two Model 6000A solvent delivery systems, a Model 660 solvent programmer, a U6K universal injector, and a Model 440 two-channel absorbance detector (Waters Assoc.). The recorder used was a Houston Instrument (Austin, TX, U.S.A.) OmniScribe A5211-5 dual-pen recorder. Peak heights, peak areas and retention times were measured by an on-line Model Supergrator-1 integrator (Columbia Scientific Industries, Austin, TX, U.S.A.).

Prepacked HPLC columns, Partisil 10 PAC and Partisil 10 ODS, 10 μm, 25 cm $\times$ 4.6 mm I.D. (Whatman, Clifton, NJ, U.S.A.) were employed.

Procedure

In the first HPLC procedure, a column packed with a bonded cyano-amino-type polar material (Whatman Partisil 10 PAC) was used. The mobile phase was a ternary solvent system of acetonitrile, methanol and 0.5 *M* ammonium acetate (pH 6.5). This procedure was used for isocratic elution of non-sulfated, monosulfated and disulfated unsaturated disaccharides. The elution of the trisulfated disaccharide which is a more strongly retained compound in this system was achieved by increasing the aqueous ammonium acetate content in the mobile phase. However, more satisfactory results were achieved by employing a second column.

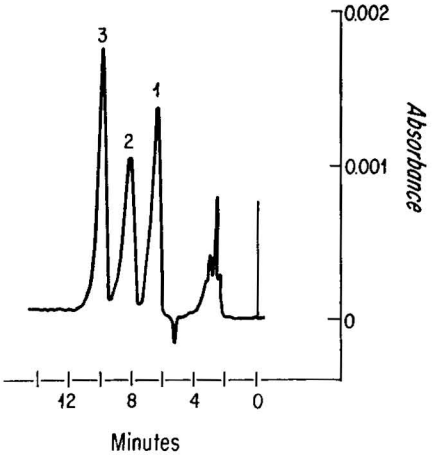
The second HPLC procedure was an ion-pair reversed-phase method which was designed specifically for the rapid separation and sensitive quantitation of disulfated and trisulfated disaccharides. A bonded C_{18} reversed-phase column was employed. The mobile phase consisted of 0.005 *M* PIC-A reagent mixed with methanol. The PIC-A reagent was prepared by mixing 15 ml (one bottle) of the pre-packaged reagent with 1 l of glass distilled water. The pH of the solution was 7.0. A solution of 10% methanol and 90% of this PIC-A reagent gave satisfactory results.

The flow-rate used for both procedures was 1.0 ml/min. All separations were performed at ambient temperatures. Details are given separately with each chromatogram.

RESULTS AND DISCUSSION

The results of HPLC separation and quantitation of the unsaturated disaccharides derived from chondroitin sulfates and dermatan sulfate have been reported previously[11–14]. An attempt was made to apply the same technique for the effective determination of the unsaturated disaccharides derived from enzymatic

degradation of heparan sulfate and heparin because of the similar structures of the two classes of disaccharides. Fig. 1 represents the isocratic separation of the three disaccharides, produced by digestion of heparan sulfate with heparitinase (ΔDi–HS_b–I, ΔDi–HS_b–II and ΔDi–HS_b–III), achieved by the Partisil 10 PAC column. The time required for this separation was 12 min. The composition of the mobile phase was systematically varied in order to select the optimal conditions for separation. The capacity ratio (k') of the disaccharides was determined as a function of the acetonitrile and methanol content of the mobile phase, keeping the content of aqueous ammonium acetate constant at 20% as shown in Fig. 2. The retention behavior of ΔDi–HS_b–I, ΔDi–HS_b–II and ΔDi–HS_b–III are essentially similar to that of ΔDi–OS, ΔDi–6S and ΔDi–4S, respectively. The k' values for the sulfated disaccharides first decrease with increasing acetonitrile content, pass through a minimum at about equal content of acetonitrile and methanol and then increase sharply. The k' values of the nonsulfated disaccharides increase slightly in the region in which k' values of sulfated disaccharides decrease and then increase sharply as did the sulfated disaccharides. The acetonitrile/methanol ratio in the mobile phase can be used for adjusting the relative retention of the three disaccharides. At a constant



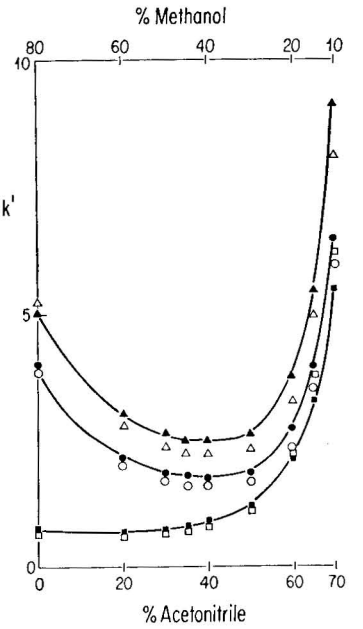


Fig. 1. HPLC of the three unsaturated disaccharides obtained from heparan sulfate: 1 = ΔDi–HS_b–I; 2 = ΔDi–HS_b–II; 3 = ΔDi–HS_b–III. Column, Whatman Partisil 10 PAC; mobile phase, acetonitrile–methanol–0.5 *M* ammonium acetate, pH 6.5 (60:20:20); flow-rate, 1.0 ml/min; pressure, 600 p.s.i.; UV detection at 254 nm, 0.005 a.u.f.s.

Fig. 2. Capacity ratio (k') of the unsaturated disaccharides as a function of the acetonitrile and methanol content of the mobile phase. ■ = ΔDi–HS_b–I; ● = ΔDi–HS_b–II; ▲ = ΔDi–HS_b–III; □ = ΔDi–OS; ○ = ΔDi–6S and △ = ΔDi–4S. Column, Whatman Partisil 10 PAC; mobile phase, acetonitrile–methanol–0.5 *M* ammonium acetate, pH 6.5 (X:Y:20), X, % acetonitrile, and Y, % methanol, X + Y = 80%; flow-rate, 1.0 ml/min.

ratio of the two organic solvents, an increase in the amount of aqueous ammonium acetate buffer resulted in the decrease of retention time of each disaccharide, which is probably due to the increase of the ionic strength and the increased solvation of the solutes as the solvent becomes more polar. When the pH of ammonium acetate was below 6.0, two peaks were observed for each disaccharide, especially for the nonsulfated one which suggests that anomeric forms were separated.

It was possible to separate the disulfated and trisulfated disaccharides derived from degradation of heparan sulfate and heparin with heparinase from non-sulfated and monosulfated disaccharides in one chromatogram by a concave gradient elution (curve No. 10, solvent programmer) with increasing ammonium acetate content from 20% to 45% within a 20-min period. The total chromatographic time was 30 min. However, a specific ion-pair reversed-phase HPLC method appears to be more rapid and sensitive for the determination of these disulfated and trisulfated disaccharides. Fig. 3 shows a separation of ΔDi–He_a–I from other disaccharides. The ΔDi–diS_B appeared at 5.5 min and was completely separated from ΔDi–He_a–I under these conditions. This peak probably locates the positions of disulfated disaccharides expected from heparan sulfate and heparin. The k' value of ΔDi–He_a–I decreases with increasing methanol content in the mobile phase.

Linear relationships between the amount of disaccharides injected (100 ng to 5 μg) and the peak area of disaccharides were observed in both HPLC procedures as shown in Fig. 4. Seven injections of a fixed amount of ΔDi–HS_b–III employing the

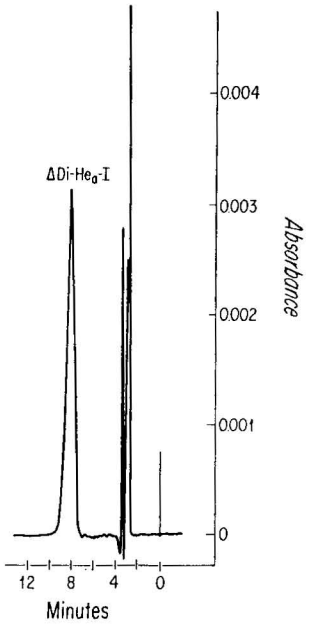


Fig. 3. Ion-pair chromatographic elution of the trisulfated disaccharide from a reversed-phase column. The peaks at about 3 min contain ΔDi–HS_b–I, ΔDi–HS_b–II, ΔDi–HS_b–III and solvent front. Column, Whatman Partisil 10 ODS; mobile phase, methanol–water (10:90) with PIC-A reagent; flow-rate: 1 ml/min; pressure, 800 p.s.i. The amount of ΔDi–He_a–I injected was 2.25 μg. UV detection at 254 nm, 0.005 a.u.f.s.

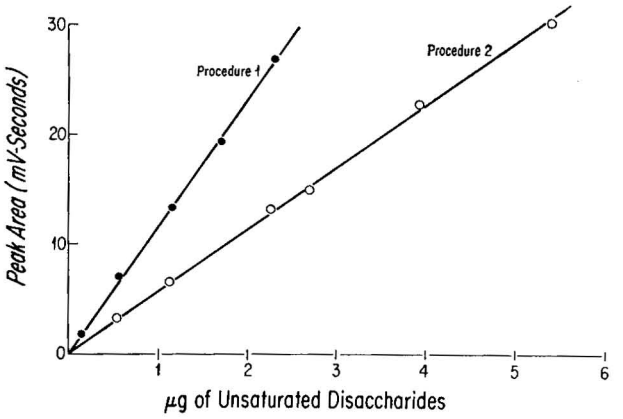


Fig. 4. Linearity of response plots for HPLC determination. ● = ΔDi–HS_b–III in Partisil 10 PAC column procedure; ○ = ΔDi–He_a–I in ion-pair reversed-phase (Partisil 10 ODS column) procedure.

Partisil 10 PAC column procedure and of ΔDi–He_a–I in the ion-pair chromatographic procedure gave relative standard deviations of 1.04% and 1.43% respectively. The detection limits were 20 ng (ΔDi–HS_b–III) employing the Partisil 10 PAC column and 40 ng (ΔDi–He_a–I) with the Partisil 10 ODS column with a signal-to-noise ratio of 3:1. These results demonstrate that the two HPLC procedures described here together provide a useful means for the quantification of unsaturated disaccharides which are produced from enzymatic degradation of heparan sulfate and heparin.

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Note

Chloranil, a sensitive detection reagent for pyrrolizidine alkaloids on thin-layer chromatograms

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For the detection of pyrrolizidine alkaloids on thin-layer chromatographic (TLC) plates, Mattocks[1] developed a reagent specific for hepatotoxic alkaloids with an unsaturated (3-pyrroline) ring in the basic moiety.

The alkaloids that are usually isolated as free bases after reduction of their corresponding N-oxides, which are often abundant in plant material, have to be converted into the N-oxides again after TLC separation of the free bases, by treatment with hydrogen peroxide, prior to further derivatization. The N-oxides are treated with acetic anhydride for their conversion into pyrroles, which give coloured compounds with Ehrlich reagent. Although the detection limit for retrorsine is low (0.25 μg), the complete detection method is lengthy and the conditions are critical. Another disadvantage of the method is that several hazardous chemicals are required. Because we experienced problems with the reagent, we discontinued investigations with this detection technique.

A second frequently used method for the detection of pyrrolizidine alkaloids involves the use of Dragendorff's reagent[2–7]. This reagent, however, is not only non-specific towards the different groups of alkaloids that can be detected, but also gives positive results with common plant sterols, triterpenes[8], α- and γ-pyrones and several other compounds[9], especially when used in combination with sodium nitrite as intensifier spray.

Chloranil is used as a spray for the detection of capsaicin and hydroquinone derivatives. It yields blue or red-brown spots with the former compounds after exposure to ammonia vapour[10]. A further use has been described for the detection of alkaloids that form charge-transfer complexes with π-acceptors such as chloranil[11].

In this paper we describe the use of chloranil as a sensitive and highly convenient spray reagent for the detection of pyrrolizidine alkaloids on TLC plates.

EXPERIMENTAL

Materials

Pyrrolizidine alkaloid extracts from *Echium vulgare*, *Omphalodes verna*, *Heliotropium europaeum*, *Symphytum officinale*, *Cynoglossum officinale*, *C. nervosum* and *Amsinckia intermedia* were derived according to Pedersen[5]. All reagents and

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solvents were of analytical-reagent grade. Lycopsamine, acetyllycopsamine (or diastereoisomers) and symphytine were purified from *Symphytum officinale* alkaloid extracts by means of ion-pair high-performance liquid chromatography on a Waters Prep LC/System 500 A under conditions described previously for TLC[12]

Detection reagents

Dragendorff's reagent in a modification according to Munier (not diluted) was used[13]. The intensifier spray was a 10% (w/v) solution of sodium nitrite[8].

Chloranil was used as a 1% (w/v) solution in toluene or methylene chloride. After spraying, the plates were heated at 105°C for 15 min. Sulphuric acid (2 *N*) was used as an intensifier spray.

Thin-layer chromatography

Ion-pair system. Silica gel thin-layer plates (pre-coated, Schleicher & Schüll, Dassel, G.F.R.) were impregnated with a 0.15 *M* solution of lithium chloride in methanol for 5 sec by dipping, followed by blotting and drying at 105°C. The plates were stored in a desiccator. Pyrrolizidine alkaloids were separated as ion pairs with chloroform–methanol (75:25) as eluent.

Straight-phase system. Pyrrolizidine alkaloids were separated on pre-coated silica gel TLC plates using chloroform–methanol–ammonia (85:14:1) as the eluent (see *Isolation and mass spectrometry of a main alkaloid from Omphalodes verna*).

Determination of responses to detection reagents

For determination of the responses to chloranil, amounts of 5 μg of several alkaloids in appropriate solvents were spotted on to silica gel thin-layer plates.

Minimal detectable amounts of lycopsamine, acetyllycopsamine (or diastereoisomers), symphytine, brucine and harmaline were determined by spotting decreasing amounts of the alkaloids on TLC plates.

The spots were revealed with Dragendorff and chloranil reagents followed by spraying with intensifier sprays.

Isolation and mass spectrometry of a main alkaloid from Omphalodes verna

Bands of an extract from *Omphalodes verna* in chloroform were applied to preparative TLC plates (20 × 20 cm, 1 mm silica gel layer; Merck, Darmstadt, G.F.R.) by means of a Camag Chromatocharger. After development of the plates with the straight-phase system, the borders of the plate were sprayed with Dragendorff reagent and the partially located main band was scraped off and powdered. The alkaloid was stripped from the silica gel by elution with methanol. Mass spectrometry of the alkaloid was performed on a Finnigan 3300 quadrupole mass spectrometer equipped with a 6110 data system with an electron energy of 70 eV and an ionizing current of 100 μA.

RESULTS AND DISCUSSION

Several alkaloids with different structural groups show a positive response to chloranil on TLC plates after spraying with a 1% (w/v) solution of chloranil in toluene. When methylene chloride was used as the solvent for chloranil, however, a considerable

decrease in the colour response occurred; toluene was therefore used as the solvent for chloranil throughout.

From Table I it can be seen that, depending on the circumstances during the detection procedure, several colours could appear even for a single compound. This phenomenon might be a possible aid in qualitative evaluations when screening for natural products.

TABLE I

COLOURS AND RESPONSES (RELATIVE TO BACKGROUND) OF SEVERAL ALKALOIDS (5 μg) DETECTED WITH CHLORANIL, IMMEDIATELY AFTER SPRAYING (A), FOLLOWED BY HEATING FOR 15 MIN AT 105°C (B) AND SUBSEQUENTLY SPRAYING WITH 2 *N* SULPHURIC ACID (C)

Order of increasing response: −, (±), ±, +, ++, +++, ++++.

Alkaloid	*A*		*B*		*C*	
	Colour	*Response*	*Colour*	*Response*	*Colour*	*Response*
Papaverine	Light brown	±	Grey	++	Grey	+++
Cinchonine	Brown	±	Brown	±	Brown	±
Theophylline	−	−	−	−	−	−
Emetine	Brown	+	Brown	+	Ochre	++
Scopolamine	−	−	−	−	−	−
Solanidine	Brown	+	Brown	±	Purple	±
Brucine	Blue	+	Blue	+	Purple	++
Caffeine	−	−	−	−	−	−
Narceine·HCl	Brown	±	White	+	Purple	+
Morphine	Red	+	Yellow	+	Red	++
Tropine	Purple	+	Purple	+	Brown	+
Quinine	Brown	±	Brown	±	Purple	±
Narcotine	−	−	Brown	(±)	Brown	(±)
Atropine	Brown	±	Purple	+	Red	+
Hyoscyamine	Brown	±	Purple	+	Purple	+
Strychnine	Brown	±	Purple	+	Purple	+
Codeine	Brown	±	Purple	+	Purple	+
Cephaline·HCl	Brown	±	Brown	+	Brown	++
Aconitine	Brown	±	Brown	±	Brown	±
Capsaicine	Brown	±	White	+	Brown	+
Nicotine	Purple/brown	++	Red/brown	+	Red	+
Berberine·HCl	Yellow	+	Yellow	++	Ochre	+++
Ephedrine	Yellow	±	Purple	+	Purple	
Piperidine	Blue	++	Grey	+	Grey	+
Harmaline	Green/black	+++	Black	++++	Black	++++
Symphytine	Green/brown	++	Brown	+++	Grey/brown	++++

* Yellow-coloured alkaloid.

Both harmaline and symphytine were highly sensitive to the reagent. From a comparison of the minimal detectable amounts of the pyrrolizidine alkaloids tested visible after spraying with chloranil and its intensifier and with Dragendorff's reagent and its intensifier, it could be concluded that a slightly improved detection limit could be achieved for acetyllycopsamine and symphytine by using chloranil (Table II). The use of sulphuric acid as the intensifier for chloranil did not give rise to a higher response with the alkaloids, but changed the background of the plate from purple

TABLE II

MINIMAL DETECTABLE AMOUNTS OF BRUCINE, LYCOPSAMINE, ACETYLLYCOPSAMINE AND SYMPHYTINE WITH DRAGENDORFF REAGENT AND CHLORANIL AFTER USE OF THEIR RESPECTIVE INTENSIFIER SPRAYS

Alkaloid	*Minimal detectable amount (μg)*	
	Dragendorff + nitrite	*Chloranil + acid*
Brucine	0.40	0.40
Harmaline	0.04	0.04
Lycopsamine	2.0	2.0
Acetyllycopsamine	0.20	0.15
Symphytine	0.20	0.15

to a very light brown, which led to an increase in the relative response due to enhanced contrast.

Other advantages of chloranil over Mattocks' and Dragendorff's reagents are that the method is very convenient because of the ease of preparation of the spray and that the spots remain visible on the TLC plates for more than a year. Further, chloranil is far more specific than Dragendorff's reagent.

The mass spectrum of the main alkaloid originating from *Omphalodes verna*, which was purified by means of preparative TLC, showed a base peak at *m/e* 124. The appearance of this peak together with a peak at *m/e* 83 (75%) indicates a monoester pyrrolizidine alkaloid with a (±)-trachelanthamidine or (±)-isoretronecanol nucleus[14,15]. These necines lack the 1,2-double bond in the basic moiety and could therefore be considered as non-hepatotoxic even when esterified at the C9 position[1]. After TLC separation of the original alkaloid extract derived from *Omphalodes verna*, a main and two minor spots appeared after spraying with Dragendorff's reagent. Chloranil, however, failed to give any coloured spots under all conditions tested.

Comparison of the alkaloid patterns obtained by TLC separation of extracts (ion-pair system) from *Echium vulgare*, *Heliotropium europaeum*, *Symphytum officinale*, *Amsinckia intermedia*, *Cynoglossum nervosum* and *C. officinale* and detection with Dragendorff's reagent and chloranil did not show any differences. These plants are known to contain pyrrolizidine alkaloids with necines having the double bond in the 1,2-position and with esterification of the primary hydroxyl group at C9[16]. Hence chloranil might be useful as a reagent for hepatotoxic pyrrolizidine alkaloids. This, together with the ease of preparation, the use of non-hazardous chemicals, the low detection limit and the stability of the spots, should be of great value in the detection of most pyrrolizidine alkaloids.

ACKNOWLEDGEMENTS

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Note

Specific detection of pyrrolizidine alkaloids on thin-layer chromatograms

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Pyrrolizidine alkaloids, occurring in various plant species, present a significant hepatotoxic hazard to grazing animals and may enter the human food chain via meat, milk products[1,2], grains, honey[3] and herbal teas.

Thin-layer chromatography (TLC) provides a rapid method for separation and monitoring of these alkaloids[4–6] in biological extracts but methods available for detection on the chromatographic plate are limited. The commonly used alkaloid-sensitive chromogenic spray reagents, such as Dragendorff's reagent and iodoplatinate, are non-specific, producing colors with most classes of naturally-occurring organic bases. The only specific pyrrolizidine alkaloid detection method developed to date is that of Mattocks[6] which is lengthy and somewhat inconvenient, involving spraying of TLC plates with three separate reagents and thorough drying between sprays. This method involves oxidation of the pyrrolizidine alkaloids to their N-oxides by hydrogen peroxide, treatment with acetic anhydride to convert the N-oxides to pyrroles and subsequent reaction with Ehrlich's reagent to give characteristic purple colours.

The method described herein also makes use of the pyrrole-specific Ehrlich's reagent but oxidation of the pyrrolizidine alkaloids to pyrroles is achieved rapidly and directly by treatment with *o*-chloranil, this reagent itself giving an intense, albeit transient, deep blue colour with the alkaloids.

EXPERIMENTAL*

o-Chloranil (tetrachloro-*o*-benzoquinone) was obtained from Aldrich (Milwaukee, WI, U.S.A.) and was dissolved in benzene to give a 1% solution. Ehrlich's reagent was prepared by dissolving *p*-dimethylaminobenzaldehyde (Eastman-Kodak, Rochester, NY, U.S.A.) (2.0 g) in absolute ethanol (100 ml) containing boron trifluoride etherate (2.0 ml)[7]. Acetic anhydride spray reagent was prepared as a 10% solution in benzene.

For TLC, solutions of pyrrolizidine alkaloids, or crude plant extracts, in

* Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

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chloroform were applied to silica gel 60 0.25-mm pre-coated plates, either glass- or aluminum-backed (E. Merck, Darmstadt, G.F.R.). The plates were developed with either chloroform–methanol–17% ammonium hydroxide (82.5:15.5:2) (solvent 1) or chloroform–acetone–ethanol–ammonium hydroxide (5:3:1:1)[6] (solvent 2), for a distance of approximately 15 cm.

The plates were dried, sprayed with *o*-chloranil solution (or acetic anhydride solution for N-oxides), dried on a steam-bath for *ca.* 1 min, resprayed with the Ehrlich's reagent and again heated on the steam-bath for 1 min.

RESULTS AND DISCUSSION

The R_F values in two different solvent systems and the levels of detection after TLC are shown in Table I for twelve pyrrolizidine alkaloids of the macrocyclic diester type, one necine base and three N-oxide derivatives.

TABLE I

THIN-LAYER CHROMATOGRAPHY OF PYRROLIZIDINE ALKALOIDS

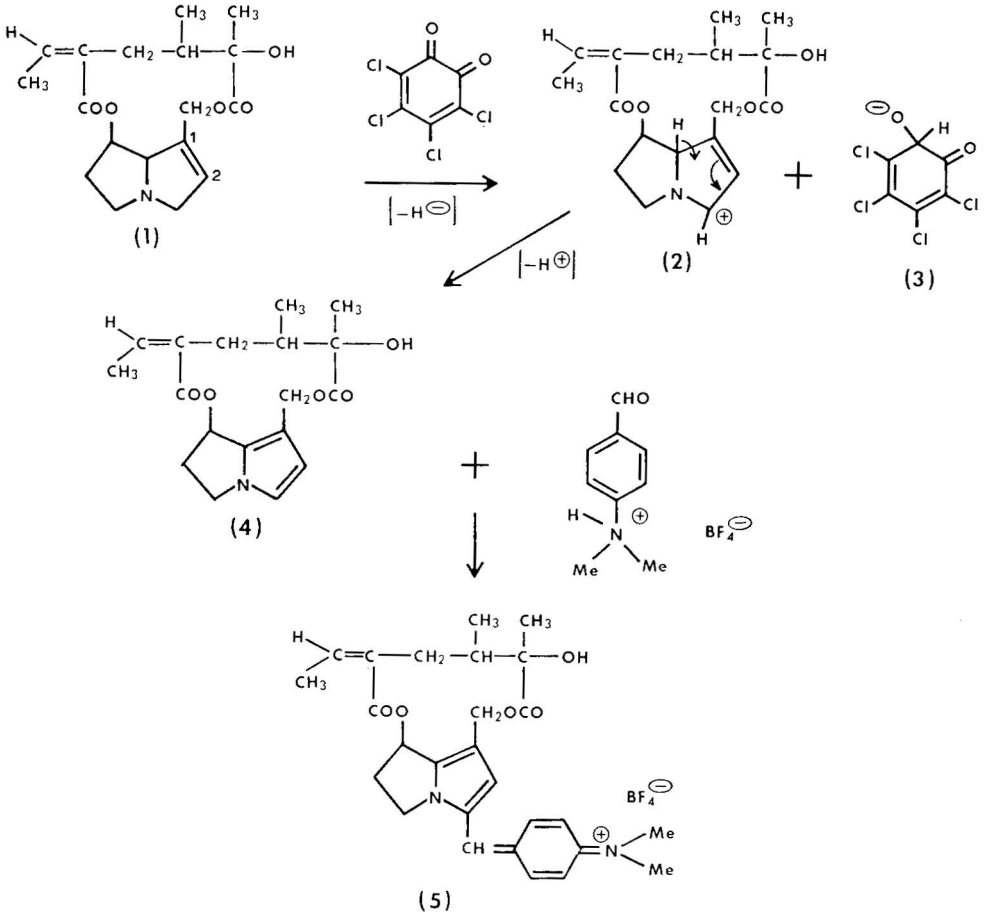
Solvents: 1 = chloroform–methanol–17% ammonium hydroxide (82.5:15.5:2); 2 = chloroform–acetone–ethanol–ammonium hydroxide (5:3:1:1).

Alkaloid	*Detection level (μg)*	R_F	
		Solvent 1	*Solvent 2*
Anacrotine	0.5	0.40	0.61
Integerrimine	2	0.62	0.82
Jacobine	1	0.37	0.79
Jacoline	0.5	0.29	0.52
Jaconine	2	0.61	0.79
Monocrotaline	1	0.39	0.63
Monocrotaline N-oxide	—	0.17	0.05
Platyphylline	25	0.46	0.78
Retronecine	<0.5	0.05	0.21
Retrorsine	2	0.35	0.54
Riddelliine	2	0.34	0.54
Riddelliine N-oxide	—	0.19	0.04
Senecionine	1	0.62	0.82
Senecionine N-oxide	—	0.38	0.15
Seneciphylline	2	0.61	0.82
Spectabiline	2	0.37	0.68

Previous TLC detection methods have involved oxidation of the alkaloids to their N-oxides which are then converted to the corresponding pyrroles. The latter react with Ehrlich's reagent (dimethylaminobenzaldehyde) under acidic conditions to give a characteristic deep purple color. Alkaloids lacking unsaturation in the 1,2-position fail to yield pyrroles and therefore do not react with Ehrlich's reagent. The sequence of three spray reagents (hydrogen peroxide, acetic anhydride and Ehrlich's reagent) required to convert the alkaloids to the pyrroles via their N-oxides, with subsequent detection, makes this method somewhat tedious and time-consuming. Direct oxidation to the pyrroles is obviously advantageous and can be achieved using

o-chloranil. Attempts to use dichlorodicyanobenzoquinone (DDQ) as an alternate oxidizing agent gave erratic results.

On spraying thin-layer chromatograms of pyrrolizidine alkaloids with a 1% solution of *o*-chloranil in benzene intense blue spots on a yellow background develop which fade quite rapidly at room temperature and within 1 min on heating the TLC plate on a steam-bath. On subsequent spraying with Ehrlich's reagent and further heating on a steam-bath the pyrrole-characteristic, stable purple spots develop. The blue color which forms on spraying with *o*-chloranil is probably due to formation of a charge-transfer complex (2 + 3), produced by abstraction of a hydride ion from a pyrrolizidine alkaloid, such as senecionine (1). Loss of a proton from the carbonium ion (2) would then yield the pyrrole (4) which reacts with Ehrlich's reagent to give the highly colored compound (5).



An alternative structure for the initial blue color could be a quinhydrone, formed by reduction of the *o*-chloranil. However, other readily oxidizable compounds which might be expected to lead to formation of the quinhydrone, such as quinols, catechols and various flavanoids, failed to give a blue color with *o*-chloranil. Deep blue aminovinylquinones have been reported to be formed on reaction of nitrogenous bases with chloranil but these are generally only produced with N-ethyl compounds[8]. Moreover, such quinones are relatively stable and reaction at the 2-position of the

pyrrolizidine ring to yield an aminovinylquinone would prevent oxidation to the pyrrole and subsequent reaction with Ehrlich's reagent. The formation of a charge-transfer complex therefore appears to account for the transient blue coloration most satisfactorily.

The detection level after TLC development for pyrrolizidine alkaloids having unsaturation at the 1,2-position ranged from 0.5 to 2 μg, compared to levels of 1 μg and 2 μg respectively for the pyrrolizidine non-specific Dragendorff's and iodoplatinate reagents. The necine base, retronecine, was detectable at levels well below 0.5 μg. Platyphylline, which lacks unsaturation at the 1,2-position and should therefore not be oxidizable to a pyrrole, gave a faint purple spot when applied to a TLC plate at a level of 25 μg and sprayed with *o*-chloranil followed by Ehrlich's reagent. The positive reaction at this high concentration was probably due to the presence of trace amounts of pyrrolizidine alkaloids having 1,2-unsaturation or to partial aerial oxidation of platyphylline to senecionine or its pyrrole.

The three N-oxides failed to react with *o*-chloranil and could therefore not be converted to the pyrroles by this method. However they were readily converted to the pyrroles by spraying with acetic anhydride[6,9] and subsequently detected by Ehrlich's reagent. Since many pyrrolizidine alkaloid-containing plants have a large proportion of the alkaloids in the form of their N-oxides[10], a crude extract of plant material can be analyzed for the presence of both parent alkaloids and N-oxides. Concurrent chromatography of a plant extract on opposite sides of a TLC plate, treatment of one side of the plate with *o*-chloranil spray reagent and the other side with acetic anhydride spray, followed by application of Ehrlich's reagent to the whole plate, provided detection of alkaloids and their N-oxides respectively. The presence of both pyrrolizidine alkaloids and N-oxides has been demonstrated in extracts from *Senecio longilobus*, *S. riddellii*, *S. vulgaris* and *Crotalaria spectabilis* using this procedure. In addition, pyrrolizidine alkaloids of the non-macrocyclic type were shown to be detectable by the *o*-chloranil–Ehrlich's reagent method by examination of extracts of *Amsinckia intermedia* and *Symphytum officinale*, which are known to contain mono- and di-ester pyrrolizidine alkaloids.

The solvent systems used provide useful complementary results for identification of pyrrolizidine alkaloids by TLC since R_F values are quite different in the two systems. In general the alkaloids move more slowly with increasing degrees of hydroxylation. In both systems retronecine and the N-oxides are relatively immobile, reflecting the high polarity of these compounds, enabling the alkaloids and their corresponding N-oxides to be readily distinguished by both R_F values and the different methods required for detection on the TLC plate.

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Note

Gas chromatographic determination of N-nitrosoamino acids

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N-Nitrosoamino acids, except for N-nitrososarcosine (NSAR), have been reported to be non-carcinogenic to experimental animals. However, these acids may be converted into carcinogenic N-nitrosoamines by decarboxylation during high-temperature cooking. N-Nitrosoproline (NPRO), for instance, was reported to be converted into carcinogenic N-nitrosopyrrolidine in a model system using conditions simulating the frying of bacon[1].

In the gas chromatographic determination of N-nitrosoamino acids, methods have been reported for the derivatization of the carboxyl groups by methylation[2,3] or silylation[4] to form volatile derivatives.

We have developed a method for the determination of trace amounts of N-nitrosoamino acids, including hydroxylated N-nitrosoamino acids such as N-nitrosohydroxyproline (NHPRO), based on the following principle. The carboxyl groups of NSAR, NPRO, NHPRO and N-nitrosopipecolic acid (NPIC) were esterified with diazomethane, and the nitroso groups further oxidized with peroxytrifluoroacetic acid (PTFA) to give N-nitroamino acid methyl esters. The hydroxyl group of NHPRO methylester was acylated with trifluoroacetic anhydride (TFA).

The derivatized N-nitrosoamino acids can be determined with high sensitivity using a gas chromatograph equipped with an electron capture detector (GC–ECD); in addition, these compounds can be confirmed by gas chromatography–mass spectrometry (GC–MS).

EXPERIMENTAL

Reagents

NSAR, NPRO, NHPRO and NPIC were synthesized by nitrosation of the corresponding amino acids according to the Lijinsky method[5]. Each N-nitrosoamino

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acid was dissolved in ethanol–ethyl acetate (1:4) to a level of 5 μg/ml and this solution was employed as the test solution for derivative formation.

Derivative formation

Esterification. To 0.1 ml of the test solution in a glass-stoppered test-tube was added 1 ml of diazomethane-saturated diethyl ether, the mixture was allowed to stand for 30 min at room temperature, and was then evaporated to dryness under a gentle stream of nitrogen.

Oxidation and acylation. To a test-tube containing an N-nitrosoamino acid methyl ester, 0.2 ml of PTFA reagent[6,7] [0.4 ml of 85–90% hydrogen peroxide and 2.5 ml of TFA were added to a 10-ml volumetric flask containing a few millilitres of dichloromethane; after gently shaking this mixture, CH_2Cl_2 was added to make the volume exactly 10 ml] and 1 ml of CH_2Cl_2 were added.

After brief shaking, the tube was heated under reflux for 2 h at 40°C. Following the oxidation reaction, the reaction mixture was evaporated to dryness under a gentle stream of nitrogen and 0.2 ml of TFA was added to the tube, which was then allowed to stand for 30 min at room temperature. The reaction mixture was then evaporated to dryness under a gentle stream of nitrogen. The resulting derivative was dissolved in 1 ml of CH_2Cl_2 and employed for GC–ECD analysis.

Operating conditions for GC–ECD and GC–MS

A Shimadzu GC-4BF gas chromatograph equipped with an ^{63}Ni electron-capture detector was used for the GC analysis of the derivatives of N-nitrosoamino acids; a glass column (2 m × 3 mm I.D.) packed with 3% DEGS–0.5% H_3PO_4 on Chromosorb W (60–80 mesh) pre-treated with HMDS was employed. The carrier gas was nitrogen at a flow-rate of 40 ml/min. The temperature of both the column and the detector was 170°C. A Shimadzu-LKB 9000 gas chromatograph–mass spectrometer was used for GC–MS analysis of the derivatives of test N-nitrosoamino acids. The glass column (2 m × 3 mm I.D.) was packed with the same materials employed for GC–ECD analysis. The carrier gas was helium at a flow-rate of 30 ml/min. The temperatures of the injection port and column oven were 240 and 180°C, respectively, and those of the separator and ion source were 250 and 260°C, respectively. The electron energy was 20–70 eV, the accelerating voltage was 3.0 kV and the trap current was 60 μA.

RESULTS AND DISCUSSION

There are two different ways of carrying out the derivatization of N-nitrosoamino acids to form N-nitroamino acid methyl esters. Firstly, after esterifying the carboxyl group of an N-nitrosoamino acid with diazomethane, the resulting N-nitrosoamino acid methyl ester can be oxidized with the PTFA reagent to form the N-nitroamino acid methyl ester. In the second method, the nitroso group of the N-nitrosoamino acid is oxidized with the PTFA reagent to give the N-nitroamino acid, followed by esterification with diazomethane to yield the N-nitroamino acid methyl ester. In the present study, we chose the former method. In a previous paper[3], we reported the rates of esterifications of N-nitrosoamino acids by diazomethane to be more than 95% for four test N-nitrosoamino acids.

The rates of oxidation of N-nitrosoamino acid methyl esters to form N-nitroamino acid methyl esters were determined in the following way. A 5-mg amount of each N-nitrosoamino acid was esterified with diazomethane and the resulting methyl ester was oxidized with PTFA reagent; then the unreacted N-nitrosoamino acid methyl ester was determined using a gas chromatograph equipped with an alkali flame-ionization detector. It was found that no appreciable amounts of the N-nitrosoamino acid methyl esters could be detected, indicating that the conversion of an N-nitrosoamino acid methyl ester to the corresponding N-nitroamino acid methyl ester may proceed almost to completion. It has been reported that the methyl ester of NHPRO exhibits a fairly low sensitivity to GC analysis, and this might be caused by non-specific adsorption of this compound, which has an OH group, on the packing materials[2,4,8]. We found that N-nitrohydroxyproline methyl ester showed a similar low sensitivity to GC analysis, probably for the same reason as the NHPRO methyl ester; the gas chromatogram of this compound is illustrated in Fig. 1. When the OH group of N-nitrohydroxyproline methyl ester was acylated with TFA reagent, the TFA derivative exhibited a fairly high sensitivity to GC analysis, as can be seen in Fig. 2.

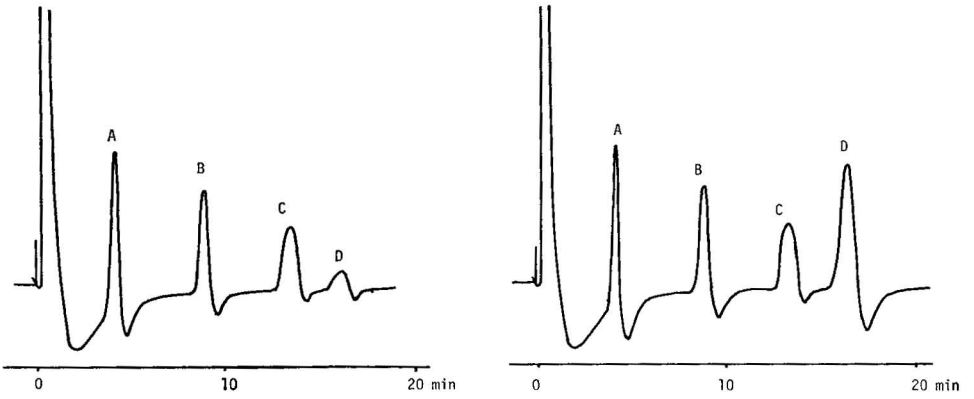

Fig. 1. Gas chromatogram of the esterified and oxidized derivatives of N-nitrosoamino acids before acylation with TFA. Sample size: 500 pg of each N-nitrosoamino acid. Peaks: A = NSAR; B = NPIC; C = NPRO; D = NHPRO.

Fig. 2. Gas chromatogram of the esterified and oxidized derivatives of N-nitrosoamino acids after acylation with TFA. Sample size and peaks as in Fig. 1.

Mass spectra of the four N-nitroamino acid methyl esters are shown in Fig. 3. Although no parent ions of the four derivatives could be detected, some abundant and characteristic fragments were observed. That is, fragments showing the loss of *m/e* 59 from the respective N-nitroamino acid methyl ester were *m/e* 89 for N-nitrosarcosine methyl ester, *m/e* 115 for N-nitroproline methyl ester, *m/e* 227 for N-nitrohydroxyproline methyl ester and *m/e* 129 for N-nitropipecolic acid methyl ester.

The detection limits of these N-nitroamino acid methyl esters by GC–ECD analysis were as low as 200 pg and, as shown in Fig. 4, linear relationships were observed in the range from 200 to 800 pg.

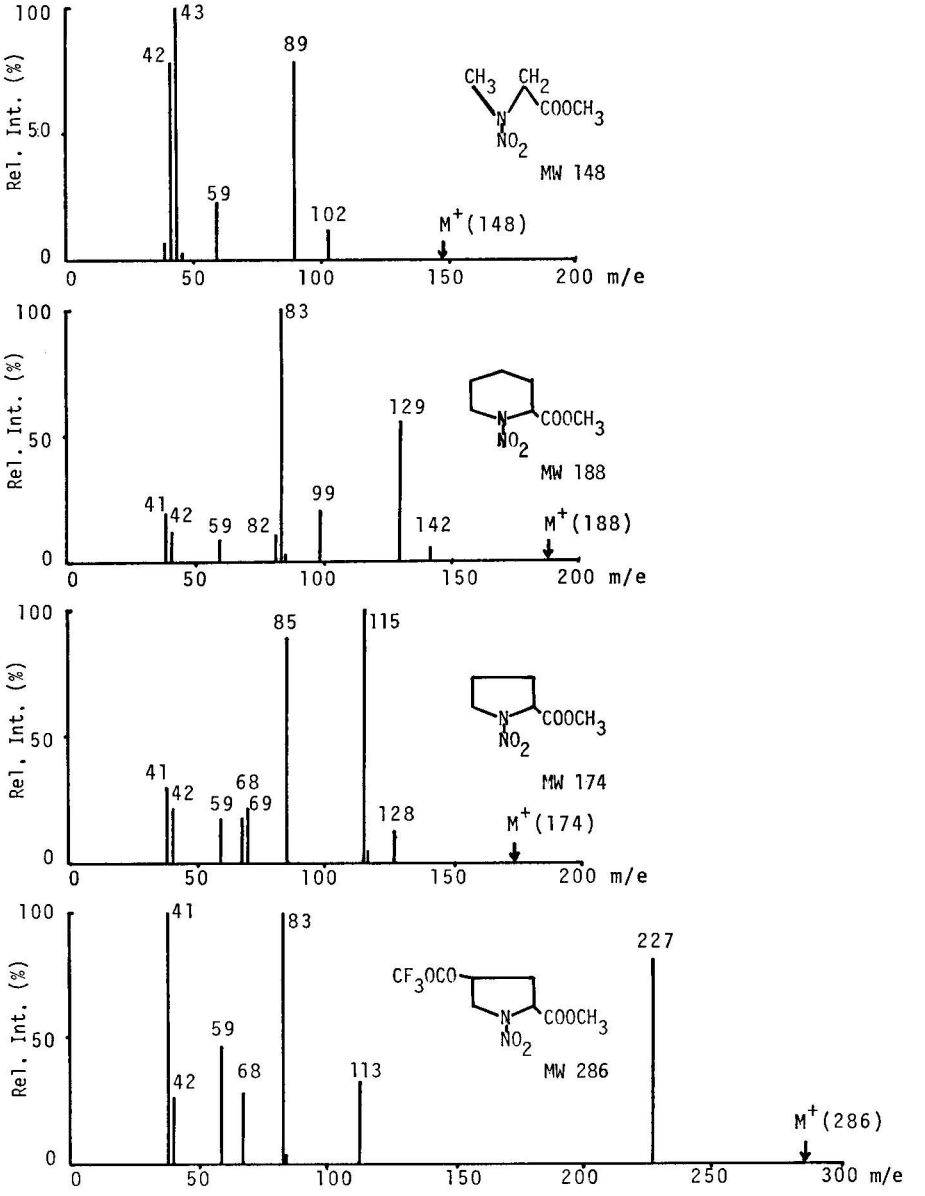

Fig. 3. Mass spectra of the esterified and oxidized derivatives of N-nitrosoamino acids after acylation with TFA.

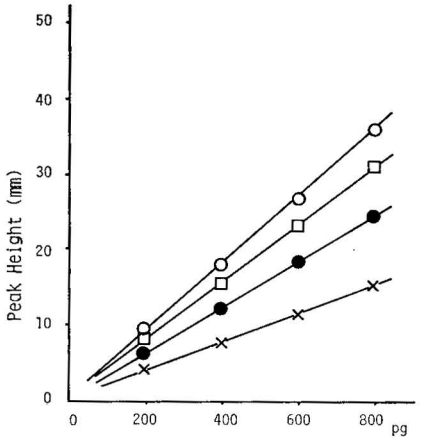

Fig. 4. Calibration graphs for the esterified and oxidized derivatives of N-nitrosoamino acids after acylation with TFA. ○, NSAR; □, NHPRO; ●, NPIC; ×, NPRO.

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Note

Determination of total monohydroxylated metabolites of diclofenac in urine by electron-capture gas–liquid chromatography

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The anti-inflammatory agent diclofenac sodium is extensively metabolised in man[1]. Stierlin and co-workers[2,3] have reported that up to 60% of the dose excreted appears in urine, less than 1% of which is eliminated unchanged. In man, phenolic metabolites are formed (Fig. 1) which are mainly conjugated to glucuronide and sulphate esters[3]. The purpose of this investigation was to establish a simple gas–liquid chromatographic method to quantitate the two major metabolites, 4-hydroxy- and 5′-hydroxy-diclofenac.

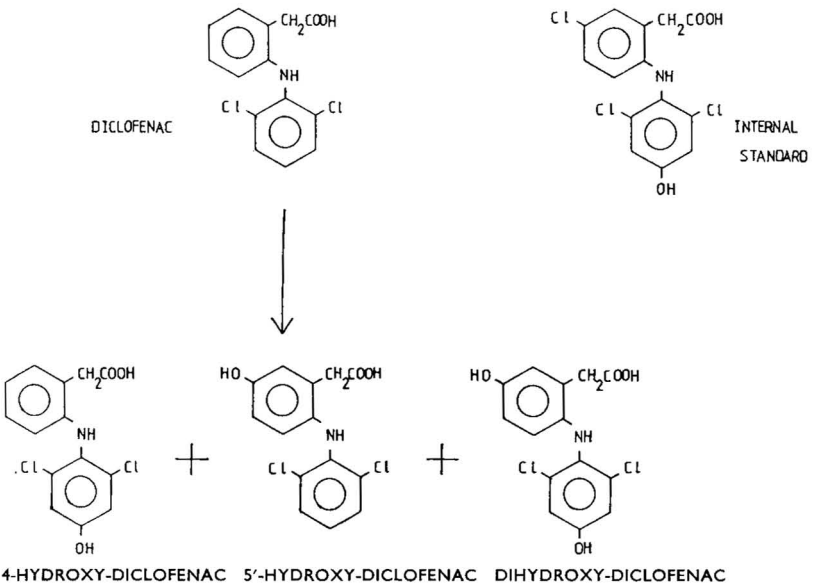

Fig. 1. Structure of diclofenac and its phenolic metabolites and of the internal standard used in their determination.

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MATERIALS AND METHODS

Diethyl ether and *n*-hexane, standard laboratory grade, were redistilled before use, and dichloromethane (Distol® grade; Fisons, Loughborough, Great Britain) was used without further purification. For extraction, ether and dichloromethane were mixed (3:1; v/v). Sodium acetate buffer (1.0 *M*, pH 5.0), tetrabutylammonium hydrogen sulphate (0.05 *M*) and sodium hydroxide (2.5 *M*) were used. Iodomethane (Sigma London, Poole, Great Britain) and ascorbic acid (Fisons) were used as supplied. All reagents were of analytical grade.

Standard solutions were prepared in distilled water as follows: 4-hydroxy-diclofenac (GP 47 766), 0.2 μg/ml; standard solution of 5′-hydroxy-diclofenac (GP 47 852), 0.2 μg/ml; and internal standard, CGP 7406, 0.8 μg/ml. Metabolites and internal standard were supplied by Ciba-Geigy (Basle, Switzerland). Metabolite solutions were prepared daily and internal standard solutions every two weeks.

Extraction of free metabolites from urine

A 1-ml volume of urine, diluted if necessary with 10 ml distilled water, and 0.5 ml of internal standard solution were acidified with 3 ml of sodium acetate buffer and 100 mg of ascorbic acid added as an anti-oxidant. A 3.5-ml volume of the extraction solvent was added and the mixture shaken mechanically for 20 min at 90 strokes/min, then centrifuged at 300 *g* for 10 min.

Methylation

The organic layer was removed and evaporated to dryness at 45°C by a gentle stream of nitrogen. The residue was dissolved in 2 ml sodium hydroxide to which 0.2 ml tetrabutyl ammonium hydrogen sulphate solution was added. To this, 0.05 ml of iodomethane in 3.5 ml dichloromethane was added. The mixture was shaken at room temperature for 20 min then centrifuged as before. After centrifugation, the dichloromethane layer was removed and evaporated to dryness. The residue was redissolved in 0.2 ml hexane and 3 μl injected into the gas-liquid chromatograph. The structure of the derivatives formed may be seen in Fig. 2.

Procedure for conjugates

Both diclofenac and its hydroxylated metabolites are excreted in urine in free and conjugated forms[3]. These conjugates may be hydrolysed by treatment with alkali and the total measured. A 400-mg amount of sodium hydroxide was added to 3 ml of urine which was then incubated at 75°C for 60 min. This hydrolysed urine was submitted to the extraction and derivatisation procedures described for the free metabolites.

Gas chromatography

Chromatography of the derivatives was performed on a Hewlett-Packard 5710A gas chromatograph equipped with an electron-capture detector (^{63}Ni, 15 mCi). A 2 m × 3 mm I.D. glass column was used packed with 3% OV-17 on Gas-Chrom Q, 80–100 mesh (Field, Richmond, Great Britain). The oven temperature was 245°C, the injection port 250°C and detector 300°C. The flow-rate of the carrier gas (argon–methane, 90:10) was maintained at 60 ml/min.

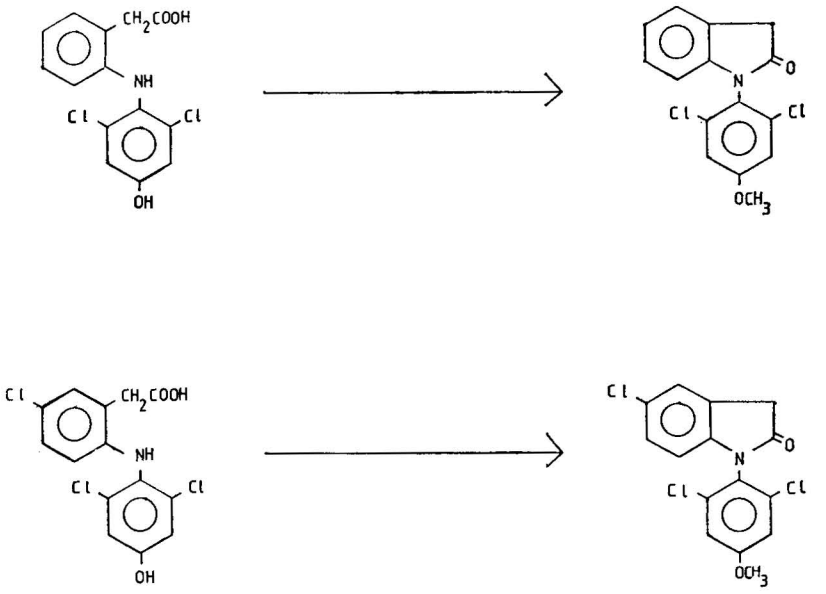

Fig. 2. Methylation of 4-hydroxy-diclofenac and the internal standard. 5′-Hydroxy-diclofenac undergoes a similar reaction.

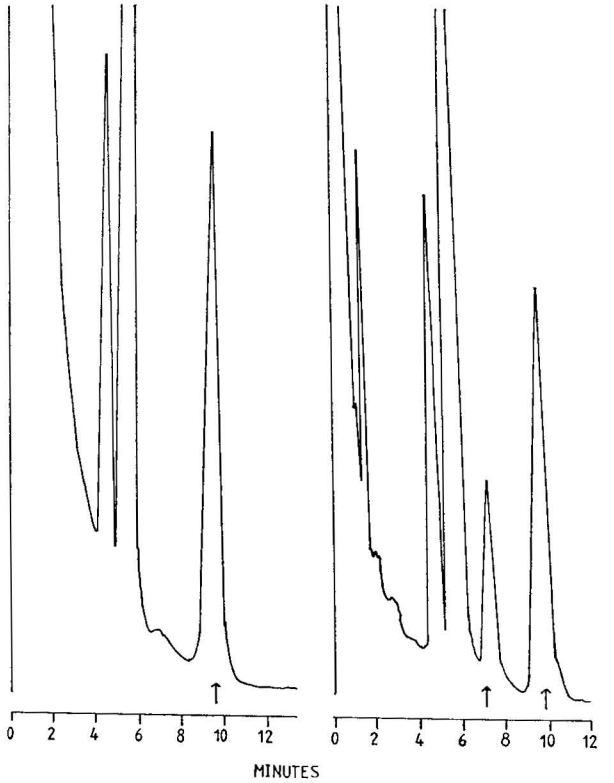

Fig. 3. Typical chromatograms obtained after injection of 400 ng/ml of internal standard only (left) and 200 ng/ml of metabolite with 400 ng/ml of internal standard (right).

RESULTS AND DISCUSSION

The two metabolites were not resolved and were measured as one peak. Quantitation was by measurement of peak height. Examples of typical chromatograms obtained can be seen in Fig. 3. Analysis of blank urine samples obtained from volunteers showed no substances interfering with the method of assay.

Known amounts of 4-hydroxy- and 5'-hydroxy-diclofenac were added to biological fluids and carried through the extraction and derivatization procedures. Peak heights of drug and internal standard were measured and calibration curves constructed. Calibration curves were linear within the range 0–500 ng/ml and metabolite levels could be determined down to 10 ng/ml.

The precision of the assay procedure was assessed from the coefficient of variation determined for five different concentrations of metabolite assayed on six independent occasions. The results may be seen in Table I. A coefficient of variation of 7% was obtained. The method has now been successfully used in a number of studies and no modifications have been necessary.

TABLE I

PRECISION OF THE ASSAY FOR TOTAL MONOHYDROXYLATED METABOLITES

Concentration (ng/ml)		*S.D.*	*C.V. (%)*
Actual	*Mean (n = 6)*		
100	98	5	5.1
200	205	11	5.3
300	295	13	4.3
400	405	26	6.4
500	498	18	3.7

NOTE ADDED IN PROOF

We have now established that the minor metabolite 3-hydroxy-diclofenac will also be measured by this method since its lactam derivative has the same retention time as the 4- and 5'-hydroxy derivatives under the conditions described.

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CHROM. 12,823

Note

High-performance liquid chromatographic separation and analysis of steroidal constituents of two solanaceous plants

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Various plants of the Solanaceae family are known to contain oxygenated steroids built on a C_{28} ergostane-type skeleton, such as withanolides[1], physalins[2], Nic derivatives[3–5] and the ixocarpalactones[6].

Withanolide E (Fig. 1, 1)[1] and its 4β-hydroxy derivative (2)[7,8] were found to be active against several tumour systems used in cancer chemotherapy screening, namely P338[1] and L1210[8] leukaemia, Lewis lung carcinoma and B-16 mouse melanoma[1].

In this paper, we report the application of an efficient, high-sensitivity high-performance liquid chromatographic (HPLC) method for the qualitative and quantitative analysis of two solanaceous plants: (a) *Physalis peruviana* L.[7,9] and (b) *Nicandra physaloides* (L.) Gaerth var. *albiflora*[10]. The former was found to contain steroidal lactones of the withanolide group with a 17α-oriented side-chain: withanolide E (1), 4β-hydroxywithanolide E (2), 2,3-dihydrowithanolide E (3)[7] and withanolide S (4)[9]. *Nicandra physaloides* (L.) Gaerth var. *albiflora* was found to contain two closely related steroids, namely nicalbin A (5) and nicalbin B (6). One of the interesting features of nicalbin A (5) is that the hemiacetalic hydroxyl group is axially oriented. Upon extraction with methanol or ethanol, the two epimeric C26-methyl acetals (7 and 8) and ethyl acetals (9 and 10), respectively, were obtained.

It should be emphasized that so far this is the only variety of this plant which does not contain Nic-1 (nicandrenone) (11), characterized by an expanded and aromatic ring D. This compound was a common constituent in all plants of *Nicandra physaloides* investigated by Begley *et al.*[3], Bates and Eckert[4] and our group[5].

EXPERIMENTAL

The HPLC apparatus was a Waters Assoc. Model 204 liquid chromatograph, equipped with a U6K injector, 6000A pump and Model 450 variable-wavelength detector. The column (stainless steel, 250 × 4.6 mm I.D.) was packed with LiChrosorb SI-100, 5 μm. The eluents were 2-propanol (Frutarom, Haifa, Israel), acetonitrile (spectrograde, BDH, Poole, Great Britain) and methylene chloride (BDH). The methylene chloride distilled and then passed through a 60 × 2 cm I.D. column, the bottom half of which was filled with silica gel 60

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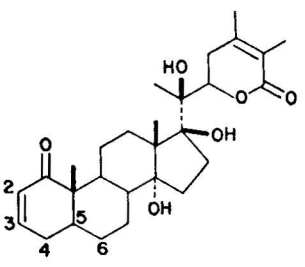
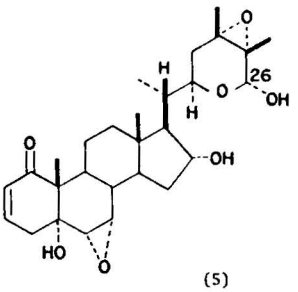
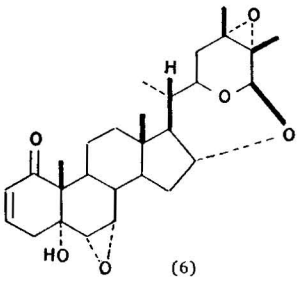


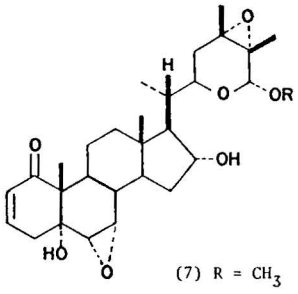
(1) 5β,6β-epoxy
(2) 4β-OH; 5β,6β-epoxy
(3) 2,3-dihydro; 5β,6β-epoxy
(4) 5α-OH; 6β-OH



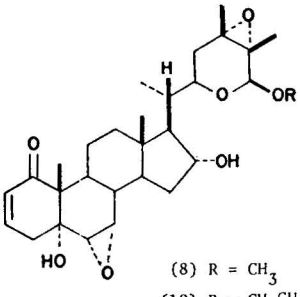
(5)



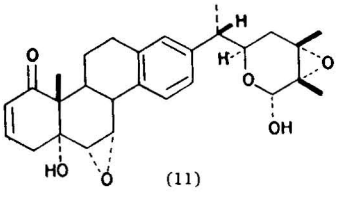
(6)



(7) R = CH_3
(9) R = CH_2CH_3



(8) R = CH_3
(10) R = CH_2CH_3



(11)

Fig. 1. Steroid structures.

(Merck, Darmstadt, G.F.R.) and the top half with an equal amount of basic alumina (Woelm, Eschwege, G.F.R.). This treatment was necessary for detection at 225 nm with air as a reference.

The extracts and compounds used were obtained and isolated from the leaves of *Physalis peruviana* L. and *Nicandra physaloides* (L.) Gaerth var. *albiflora*. These plants were raised from seeds at the experimental farm of the Faculty of Agriculture, Hebrew University of Jerusalem, Rehovot, Israel.

RESULTS AND DISCUSSION

We used high-sensitivity detection at 225 nm as described by Hunter *et al.*[11], who reported a detection limit of 5 ng for the withanolides.

Owing to the high polarity of the compounds separated in this study and in order to keep the elution times to the minimum, highly polar solvents were used. With nicalbins, methylene chloride plus 6% of 2-propanol gave an excellent separation of all four components (Fig. 2). The same solvent system was therefore used for the analysis of the plant extracts (Figs. 3 and 4).

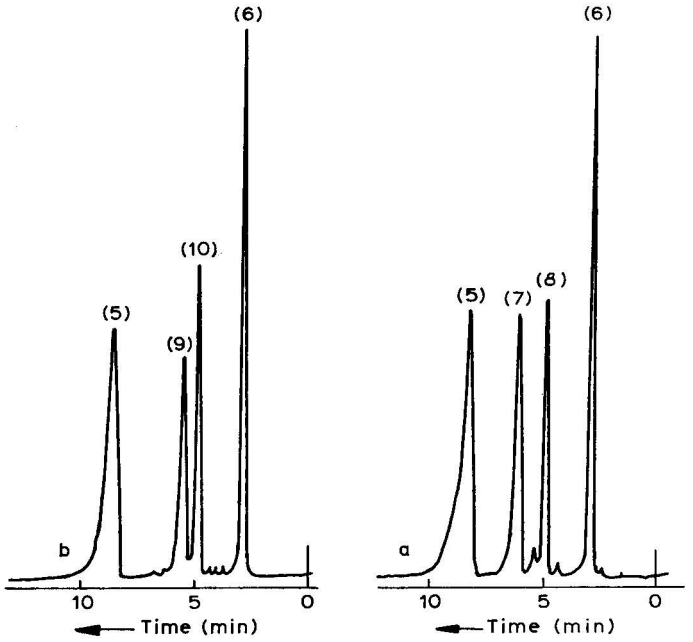
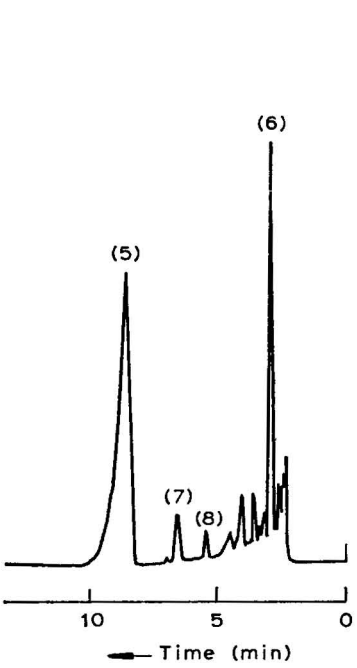


Fig. 2. (a) Chromatogram of standard mixture of nicalbin A (5), nicalbin A (26*R*)-methyl acetal (7), nicalbin A (26*S*)-methyl acetal (8) and nicalbin B (6). (b) Chromatogram of standard mixture of nicalbin A (5), nicalbin A (26*R*)-ethyl acetal (9), nicalbin A (26*S*)-ethyl acetal (10) and nicalbin B (6). Column: LiChrosorb SI-100, 5 μm. Solvent, methylene chloride–6% 2-propanol; flow-rate, 1.0 ml/min; detection, 225 nm.

The absence of Nic-1 (nicandrenone) was proved by the addition of a trace amount of the substance to the steroid mixture obtained by extraction of the leaves with ethanol (Fig. 5).

In the analysis of the constituents of *Physalis peruviana* we were faced with more polar compounds. Using the above mobile phase, withanolide E (1) and its 2,3-dihydroderivative (3) could not be separated* and 4β-hydroxywithanolide E (2) was eluted after a very long time. However, when acetonitrile–methylene chloride (1:1) was used, an excellent separation of steroidal lactones (1), (2) and (3) was

* At the time[7], the separation of this pair was extremely complicated.



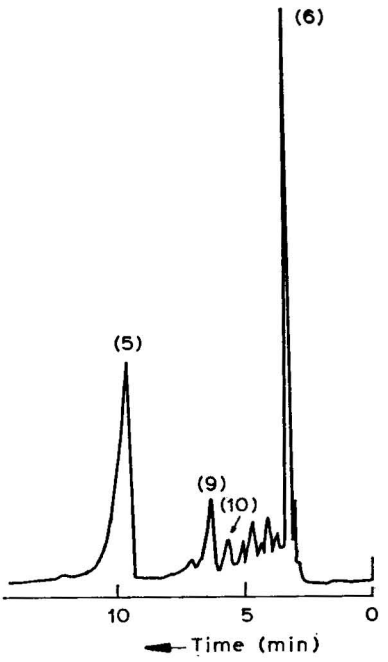


Fig. 3. Chromatogram of a crude methanolic extract obtained from leaves of *Nicandra physaloides* var. *albiflora*. For experimental conditions see Fig. 2.

Fig. 4. Chromatogram of a crude ethanolic extract obtained from leaves of *Nicandra physaloides* var. *albiflora*. For experimental conditions see Fig. 2.

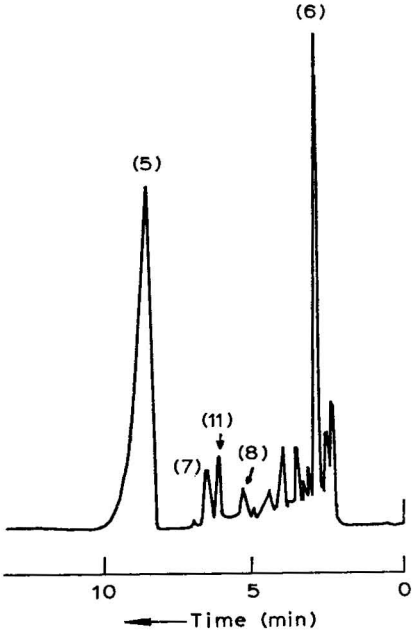


Fig. 5. Chromatogram obtained when a trace amount of Nic-1 (nicandrenome) (11) was added to the crude methanolic extract obtained from leaves of *Nicandra physaloides* var. *albiflora* (compare with Fig. 3).

achieved. This system was also applied to the crude plant extract (Fig. 6). Total separation, including withanolide S (4), was not possible in less then 30 min. The latter was eluted in a reasonable time when 2-propanol (5%) was added to the acetonitrile–methylene chloride (1:1) mixture.

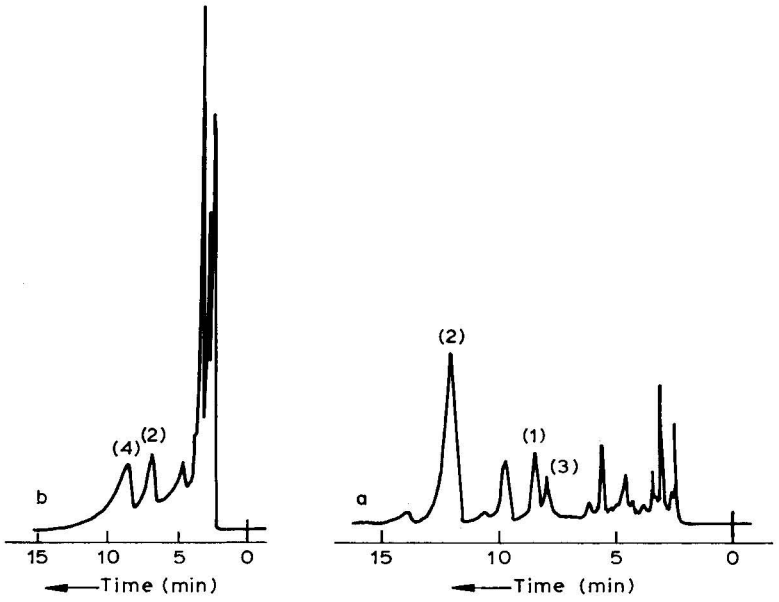


Fig. 6. (a) Chromatogram of a crude extract obtained from leaves of *Physalis peruviana*. Solvent: methylene chloride–acetonitrile (1:1). For other experimental conditions see Fig. 2. (b) Chromatogram of the same extract as above with methylene chloride–acetonitrile–2-propanol (47.5:47.5:5) as the solvent system.

CONCLUSIONS

Extracts and components of *Physalis peruviana* L. and *Nicandra physaloides* (L.) Gaerth var. *albiflora* provide a good demonstration of the advantages of HPLC for the analysis of solanaceous plants containing highly oxygenated steroids. The rapid and unequivocal separation of the epimeric hemiacetals, derived from nicalbin A during the alcoholic extraction procedure, and the clear distinction between withanolide E and its 2,3-dihydro derivative in the raw plant material, are noteworthy. This technique has obvious advantages in biosynthetic studies, in following the development of plants for optimal harvest time, and for selecting the preferred genotype of a given plant when one of the steroidal constituents is preferentially required.

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Note

High-performance liquid chromatography of glucuronide and sulfate conjugates using ion-pair chromatography

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Few methods are available for qualitative and quantitative analysis of sulfate and glucuronide conjugates of drugs and chemicals metabolized by the body. The wide range of polarities of a parent molecule, a biological metabolite and of highly polar conjugates makes the use of a single chromatographic separation difficult. Some methods presently employed to separate conjugates use ion-exchange chromatography[1], ion-exclusion–partition chromatography[2], reversed-phase chromatography[3], counter-current liquid–liquid partition[4], and gas chromatography after chemical derivatization[5]. Ion-pair reversed-phase high-performance liquid chromatography (HPLC) has been used on some steroid conjugates and some organic sulfates and carboxylic acids[6,7].

For the purpose of developing a single separation of all species found after metabolism, ion-pair reversed-phase HPLC has been examined. The system was tested for its ability to separate the normal mixture found after metabolism. This consists of a separation of glucuronide and sulfate conjugates of the same substance from each other and from the unconjugated material (precursor). The system was also examined for its ability to separate glucuronide (or sulfate) conjugates of different substances.

EXPERIMENTAL

Chemicals

p-Nitrophenyl glucuronide, *p*-nitrophenyl sulfate, α-naphthyl glucuronide, α-naphthyl sulfate, phenolphthalein glucuronide, *p*-acetylphenyl sulfate, and *p*-nitrocatechol sulfate were used as supplied by Sigma (St. Louis, MO, U.S.A.). Tetrabutylammonium bromide and *p*-nitrophenol were obtained from Eastman-Kodak (Rochester, NY, U.S.A.), and α-naphthol was purchased from Matheson, Coleman & Bell (Norwood, OH, U.S.A.). Harmol glucuronide and harmol sulfate were obtained as a generous gift from Dr. Klaus Brendel (Pharmacology Department, University of Arizona). The solvents used were filtered through a 5.0-μm Millipore filter (Millipore, Bedford, MA, U.S.A.) and de-gassed before use. Samples for analysis were prepared as a 0.02% solution in water adjusted to pH 6–8 and the ion-pairing reagent, tetrabutylammonium bromide, was prepared as a 0.01 *M* solution in water and in methanol.

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Apparatus

A Spectra-Physics Model 3500 high-performance liquid chromatograph equipped with an ultraviolet detector at wavelength 280 nm was employed and the column was a Spectra-Physics 250 × 3 mm I.D. stainless-steel column packed with 10-μm Spherisorb ODS (octadecylsilane, C_{18}). The injection port volume was 10 μl.

Procedure

Isocratic solvent compositions between 30 and 50% methanol in water were required for separation. The system was flushed with 100% methanol at the conclusion of each day and columns were occasionally cleaned by purging the system successively with methanol, ethyl acetate, chloroform and heptane and then reversing the series. A solvent flow-rate of 0.8 ml/min was used in the experiments.

RESULTS AND DISCUSSION

Ion-pair reversed-phase HPLC, using isocratic solvent compositions, was found to be sufficient to cleanly separate the compounds studied. Solvent compositions between 30% and 50% methanol in water using the HPLC conditions described above resulted in separations of *p*-nitrophenol from its corresponding glucuronide and sulfate conjugates and of α-naphthol from its corresponding glucuronide and sulfate conjugates (Table I). This separation models the mixture of compounds that might be found in excreta after administration of a chemical or drug. All of the naphthol species should separate in the form of ion-pairs at pH 6–8 as the pK_a of α-naphthol is 3.7 (ref. 8) and the conjugates should be stronger acids than the parent compound[9]. *p*-Nitrophenol may be only partly ionized as its pK_a is 7.15 (ref. 8) and the pH of the water was not adjusted with any greater precision than 6–8.

TABLE I

CAPACITY FACTORS OF *p*-NITROPHENOL AND α-NAPHTHOL AND CONJUGATES IN DIFFERENT SOLVENT COMPOSITIONS

Compound	*Capacity factor*				
	Methanol (%)				
	50	*45*	*40*	*35*	*30*
p-Nitrophenyl glucuronide	0.75	1.1	1.4	1.8	4.9
p-Nitrophenyl sulfate	2.0	3.1	5.0	10.9	25.5
p-Nitrophenol	1.4	2.1	3.2	5.6	6.6
α-Naphthyl glucuronide	1.2	2.4	4.8	7.1	23.2
α-Naphthyl sulfate	2.8	5.8	12.2	33.0	78.5
α-Naphthol	3.4	5.8	10.1	17.8	27.6

Four glucuronide conjugates (Fig. 1) and four sulfate conjugates (Fig. 2) could be separated from one another demonstrating that the separation was dependent on the parent molecule as well as the conjugating moiety. The sulfate conjugates were structurally similar to one another and the method was selective enough to separate the mixture. These results are similar to the work of Wahlund and co-workers[6,7].

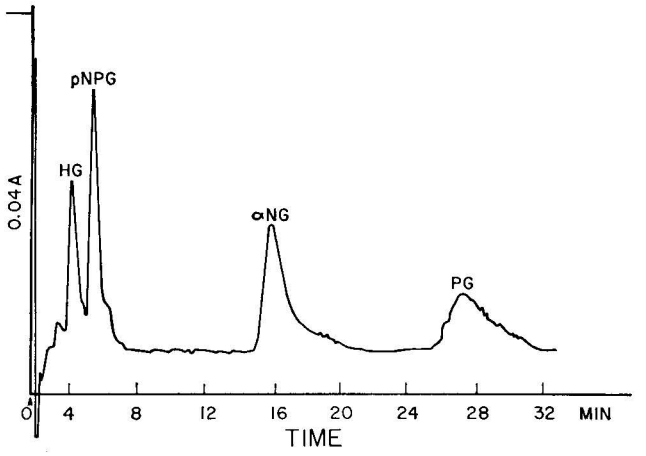

Fig. 1. Chromatography of 0.02% solutions of harmol glucuronide (HG), *p*-nitrophenyl glucuronide (pNPG), α-naphthyl glucuronide (αNG) and phenolphthalein glucuronide (PG). Eluent: 0.01 *M* tetrabutylammonium bromide in 35% methanol in water. Flow-rate, 0.8 ml/min and chart speed, 30 cm/h.

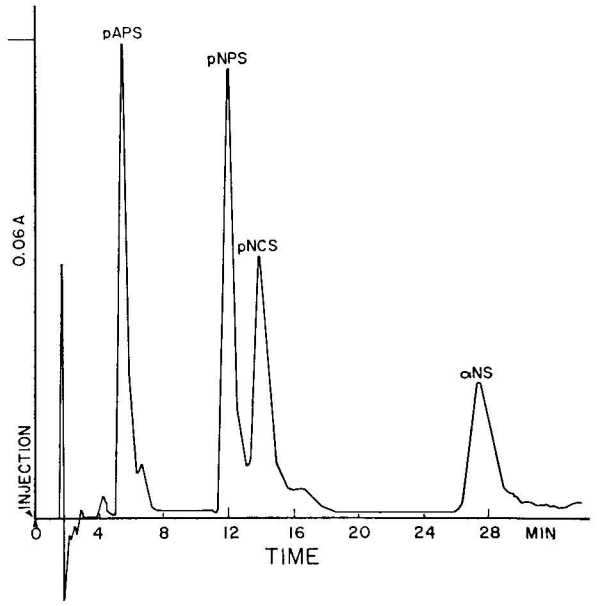

Fig. 2. Chromatography of 0.02% solutions of *p*-acetylphenyl sulfate (pAPS), *p*-nitrophenyl sulfate (pNPS), *p*-nitrocatechol sulfate (pNCS) and α-naphthyl sulfate (αNS). Eluent: 0.01 *M* tetrabutylammonium bromide in 40% methanol in water. Flow-rate, 0.8 ml/min and chart speed, 30 cm/min.

Ion-pair reversed-phase HPLC has been shown to separate readily model systems of a molecule and its glucuronide and sulfate conjugates and mixtures of different glucuronides and different sulfates. The system may be useful in estimating the concentration of conjugates in biological fluids in metabolite studies and clinical situations after appropriate separation from endogenous materials.

ACKNOWLEDGEMENTS

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Note

Quantitative analysis of polynitrophenols in water in the micro- to nanogram range by reversed-phase ion-pair liquid chromatography

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Environmental concerns dictate the need for reliable methods for the separation, identification and analysis of specific contaminants that might be present in process and related waters. This is certainly true in the explosives industry. In this regard, remarkable success has been achieved by several workers for the thin-layer chromatographic (TLC) separation of a wide variety of explosives[1]. Yasuda's work on the two-dimensional TLC separation and identification of trinitrotoluene impurities[2], the nitroso- and nitro-derivatives of diphenylamine[3] and ethyl centralite[4] are especially noteworthy. These procedures have depended on TLC separations on silica gel plates with organic solvents. The low-level detection of explosives in water has relied on solvent extraction followed by gas chromatographic (GC) analysis with a sensitive electron capture detector[5,6], or combination TLC–GC[7], and TLC–visible spectrometry[8]. Difficulties arise, however, with both the TLC and GC analyses of strongly ionizable compounds such as picric and styphnic acids. Extraction efficiencies from water into organic solvents are usually quite low, and often, these types of compounds cannot be analyzed by GC and either remain at the origin or tail on TLC plates, *e.g.*, trinitrobenzoic acid[2] and hexanitrodiphenylamine[3].

Reversed-phase paired-ion liquid chromatography (PIC) offers the possibility of directly analyzing water solutions containing these strongly ionizable compounds such as the polynitrophenols. This approach has recently been outlined in an excellent review by Tomlinson *et al.*[9]. These authors cite the work of Culbreth *et al.*[10] on the analysis of 4-nitrophenol in admixture with 4-nitrophenyl phosphate. We wish now to report the separation and analysis by PIC of synthetic mixtures of several types of polynitrophenols in water containing up to nine components. One compound, 2-amino-4,6-dinitrophenol, has recently been reported[11] as a biotransformation product from picric acid. A preconcentration step is outlined for the analysis of polynitrophenols at ng/ml concentrations.

EXPERIMENTAL

Polynitrophenol solutions

All nitrophenols were purified by recrystallization from the appropriate solvents, and melting points compared with known literature values. Standard aqueous solutions of each nitrophenol varying from 15 to 350 mg/l were made by

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dissolving the nitrophenol directly in water. Synthetic mixtures of nitrophenols were made from these standard solutions.

Chromatographic conditions

A high-performance liquid chromatograph (Hewlett-Packard, Model 1084A) equipped with a variable-wavelength detector (HP Model 1030) and variable-volume injector was used with a 10-μm RP-8 column (25 cm $\times$ 4.6 mm) maintained at 40°C. For isocratic elution, column flow was 2.0 ml/min, methanol–water (50:50, v/v) containing $5 \cdot 10^{-3}$ *M* tetrabutylammonium phosphate buffered at pH 7.5 (Pic-A reagent; Waters Assoc., Milford, MA, U.S.A.). Thirty milliliters of Pic-A reagent were dissolved in 1 l of distilled water, diluted with another liter of HPLC grade methanol, and filtered through a 0.45-μm filter (Millipore). For gradient elution, column flow was 1.0 ml/min, methanol–water (45:55, v/v) containing Pic-A reagent for 11 min, then increased to methanol–water (50:55, v/v) from 11 to 16 min. Solutions were degassed at 35°C for 30 min before establishing column flow. Standard injections were 100 μl.

Preconcentration of nitrophenols

The acidity of a 50–100 ml aqueous sample containing from 1 to 15 ng/ml each of a mixture of 2-amino-4,6-dinitrophenol and 2,4,6-trinitrophenol was adjusted to pH 2.0–2.5 with 0.1 *N* HCl. The acidified solution was passed in increments through a Sep-Pak C-18 Cartridge (Waters Assoc.) by means of a 10-ml glass syringe with Luer end-fitting. The adsorbed nitrophenols were extracted from the cartridge with 1–2 ml HPLC grade methanol and collected in a 10-ml beaker to which was added 0.5 ml distilled water. Most of the methanol was removed on a water-bath maintained between 60°C and 70°C. The residual volume was measured and analyzed by liquid chromatography (LC).

RESULTS AND DISCUSSION

Tables I and II show the detector responses for the PIC separation of five- and nine-component synthetic mixtures of polynitrophenols in water, respectively.

TABLE I

RETENTION TIMES AND DETECTOR RESPONSES FOR PIC SEPARATION OF FIVE POLYNITROPHENOLS

Concentrations, 1–15 mg/l; 100 μl injected. Isocratic elution. Detector wavelength, 254 nm.

Synthetic mixture	*Retention (min)*	*Detector response**	
		Area counts/ng	*mm/ng***
(water)	1.5	—	—
2-Amino-4,6-dinitrophenol	3.06	133 ± 3 (8)	1.56 ± 0.04 (8)
2,4-Dinitrophenol	4.26	104 ± 2 (8)	0.86 ± 0.02 (8)
2-Methyl-4,6-dinitrophenol	6.34	92 ± 4 (8)	0.54 ± 0.01 (8)
2,4,6-Trinitrophenol	7.67	103 ± 2 (6)	0.51 ± 0.01 (9)
3-Methyl-2,4,6-trinitrophenol	11.5	89 ± 3 (6)	0.30 ± 0.01 (8)

* Values in parentheses are number of determinations; ± values are standard deviations.

** Sensitivity, $8 \cdot 10^{-5}$ a.u./mm.

TABLE II

RETENTION TIMES AND RELATIVE RESPONSES FOR PIC SEPARATION OF NINE POLYNITROPHENOLS

Concentrations, 2–6 mg/l; 100 μl injected. Gradient elution. Detector wavelength, 254 nm.

Synthetic mixture	*Retention (min)*	*Relative response*	
		Area	*Height*
(water)	3.0	—	—
3-Hydroxy-2,4-dinitrophenol	5.32	0.44	0.49
3-Hydroxy-2,4,6-trinitrophenol	6.11	0.44	0.55
2-Amino-4,6-dinitrophenol	7.13	1.0*	1.0**
3-Hydroxy-4,6-dinitrophenol	8.52	0.32	0.28
2,6-Dinitrophenol	9.46	0.79	0.57
2,4-Dinitrophenol	10.5	0.79	0.50
2-Methyl-4,6-dinitrophenol	16.8	0.69	0.31
2,4,6-Trinitrophenol	20.0	0.78	0.39
3-Methyl-2,4,6-trinitrophenol	25.8	0.69	0.29

* 255 area counts/ng.

** 1.63 mm/ng at a sensitivity of $8 \cdot 10^{-5}$ a.u./cm.

The data from Table I indicated that integrated area or peak height responses could be used with equal analytical accuracy with a standard deviation of $3 \pm 1\%$ for repeated injections without internal standard. The somewhat better analytical results for the synthetic mixture shown in Table III were obtained with an internal standard and integrated areas of eluted phenols. Figs. 1 and 2 show actual LC traces for the phenol separations described in Tables I and II.

TABLE III

PIC ANALYSIS OF A SYNTHETIC MIXTURE OF POLYNITROPHENOLS

Synthetic mixture	*mg/l (Actual)*	*mg/l (Found)**	*%***
3-Hydroxy-2,4,6-trinitrophenol	4.84	4.81	99.4
3-Hydroxy-4,6-dinitrophenol	4.34	4.39	101
2,6-Dinitrophenol	3.84	3.86	101
2,4-Dinitrophenol	5.25	5.24	99.8

* Isocratic elution; 100 μl injected.

** Using integrated peak areas and 3-hydroxy-2,4-dinitrophenol as internal standard.

Detection limits

From the height responses given in Tables I and II, the detection limit for all the polynitrophenols was calculated to be 0.03 ± 2 mg/l. This limit was made assuming a signal/noise ratio of 2. Fig. 3 shows the LC separation of a two-component synthetic mixture of phenols at the 1 mg/l level.

Analyses at nanogram levels

The analyses of aqueous solutions containing 1–15 ng/ml concentrations of the polynitrophenols required a preconcentration step. In order to avoid solvent extrac-

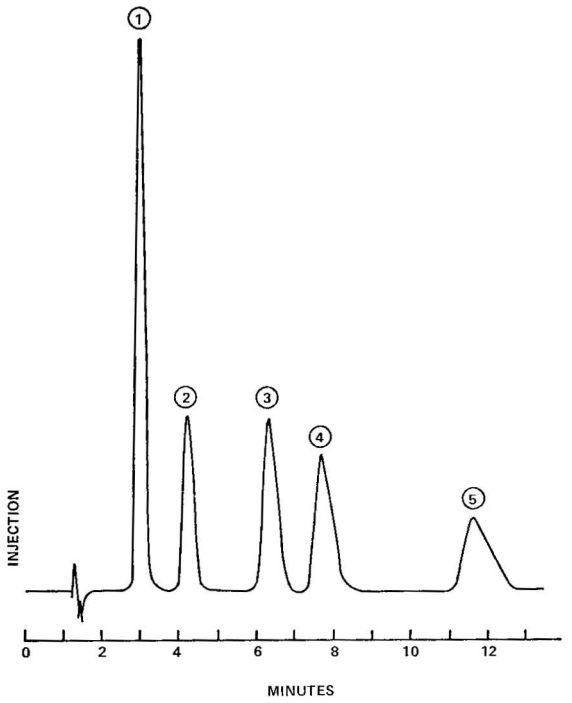

Fig. 1. PIC separation of 2-amino-4,6-dinitrophenol (1), 2,4-dinitrophenol (2), 2-methyl-4,6-dinitrophenol (3), 2,4,6-trinitrophenol (4) and 3-methyl-2,4,6-trinitrophenol (5).

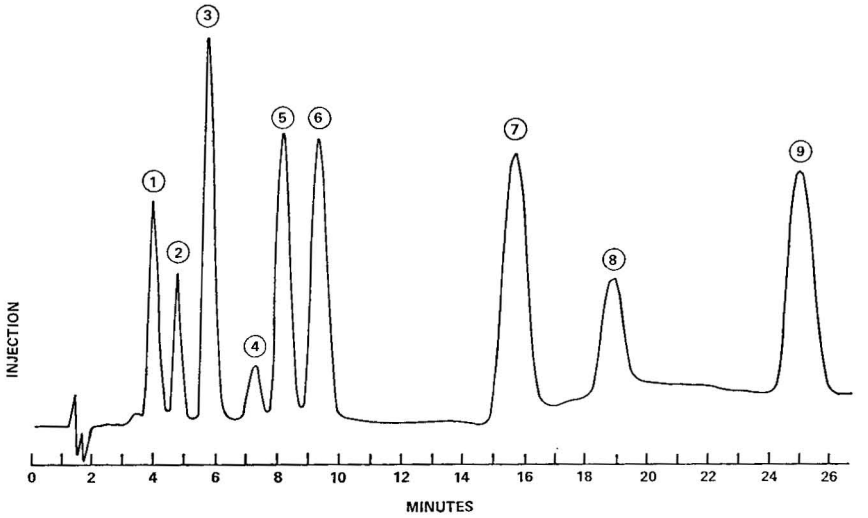

Fig. 2. PIC separation of 3-hydroxy-2,4-dinitrophenol (1), 3-hydroxy-2,4,6-trinitrophenol (2), 2-amino-4,6-dinitrophenol (3), 3-hydroxy-4,6-dinitrophenol (4), 2,6-dinitrophenol (5), 2,4-dinitrophenol (6), 2-methyl-4,6-dinitrophenol (7), 2,4,6-trinitrophenol (8) and 3-methyl-2,4,6-trinitrophenol (9).

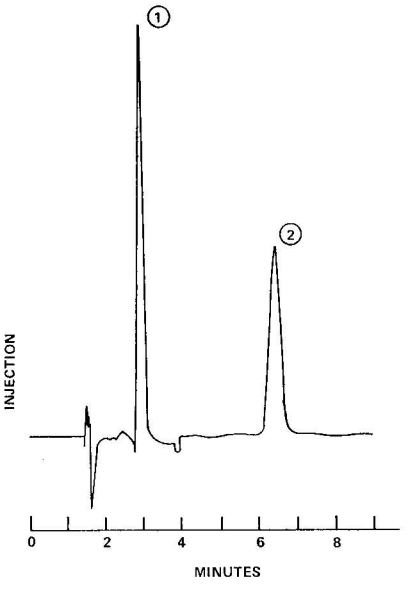

Fig. 3. PIC separation of 2-amino-4,6-dinitrophenol (1) and 2,4,6-trinitrophenol (2) at 1 mg/l concentration level.

tion procedures altogether, concentration on a stationary column, such as a Sep-Pak C-18 cartridge, followed by solvent extraction seemed ideal. The analyses of synthetic mixtures of 2,4,6-trinitrophenol and 2-amino-4,6-dinitrophenol at the ng/ml level are shown in Table IV. Recovery efficiencies for both nitrophenols were found to be markedly decreased at pH > 2, while recoveries of 2-amino-4,6-dinitrophenol were decreased at pH < 2. There was also a certain amount of "wash-out" of the adsorbed nitrophenols with samples larger than 100 ml. The addition of a small amount of water to the methanol extract was found necessary to prevent loss of nitrophenol. In addition, aqueous injections of the polynitrophenols gave superior LC traces.

TABLE IV

PRECONCENTRATION AND ANALYSES OF SYNTHETIC POLYNITROPHENOL MIXTURES AT THE ng/ml CONCENTRATION LEVEL

Concentrations, 1–15 ng/ml. Overall recovery from 50 to 100 ml of aqueous solution after concentration on Sep-Pak C-18 cartridge. Isocratic elution.

Synthetic mixture	*Recovery (%)* *
2-Amino-4,6-dinitrophenol	73 ± 12 (16)
2,4,6-Trinitrophenol	67 ± 10 (25)

* Values in parentheses are number of determinations; ± values are standard deviations.

Variable-wavelength analyses

2-Amino-4,6-dinitrophenol has two absorption maxima at 310 nm and 410 nm, while 2,4,6-trinitrophenol exhibits a single maximum at 355 nm in water. These maxima were not found to shift in the presence of Pic-A reagent. PIC separations of

a 1.0 mg/l synthetic mixture of these polynitrophenols were made with detector wavelength settings at 254 nm, 310 nm, 355 nm and 410 nm. Detector responses for 2-amino-4,6-dinitrophenol at 310 nm and 410 nm were found to be 0.81 and 0.91 times the responses at 254 nm, respectively. Detector responses for 2,4,6-trinitrophenol at 355 nm were 1.3 times the response at 254 nm. At wavelengths lower than 230 nm, absorption interferences with Pic-A reagent were observed. Although analyses of these compounds at wavelengths other than 254 nm did not markedly change their detection sensitivities, these measurable response differences could serve as a further means of phenol identification.

Application

A waste-water sample was filtered through a 0.45-μm filter and analyzed by the PIC procedure described. The LC traces showed the presence of a single peak corresponding to 40.7 $\pm$ 0.8 mg/l picrate ion. Spectrophotometric analysis (Cary 16) showed 41.6 $\pm$ 0.6 mg/l picrate ion. Since no other peaks were observed in the LC traces, it may be concluded that the sample contained none of the other nitrophenols reported in this study in concentrations greater than 0.03 $\pm$ 0.02 mg/l.

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Note

Simple thin-layer chromatography method for detection of pentachlorophenol in sawdust and woodshavings

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(Received March 10th, 1980)

Sawdust and woodshavings are extensively used in agriculture as a cheap source of litter for broiler chickens, turkeys, ducks, pigs and cattle. This material usually comes from the external layer of a tree that has been treated with pentachlorophenol (PCP) and its sodium salt which have excellent fungicidal properties and are used extensively in wood preserving solutions.

PCP is extremely toxic to young pigs[1], and its poisoning cases in cats have been reported in Perth[2], and in the Central Veterinary Laboratory[3]. Suspected PCP poisoning cases in chickens, pheasants and guinea pigs which have been reared on PCP treated sawdust and woodshavings beddings have also been found in the latter laboratory[4].

PCP in sawdust or woodshavings is usually determined by a gas–liquid chromatographic (GLC) method following an ion-exchange chromatography[5], which is not easily accessible and unsuitable for large batch analysis. A simple thin-layer chromatography (TLC) method is reported here for detection of PCP. PCP is extracted from sawdust into an acetic acid–methanol (1:9; v/v) mixture, the compound is then converted into chloranil by brief warming with concentrated nitric acid. The resulting chloranils are then spotted on thin-layer plates to separate from oxidised products of lower chlorophenols. The chloranils are detected by spraying a citric acid solution of tetramethyl-*p*-diaminodiphenylmethane (tetrabase). A blue oxidation product (quinoidal ion) of tetrabase is formed under heat, which colour intensity is proportional to the *O*- and *p*-chloranils formed.

EXPERIMENTAL

Materials

PCP was recrystallised from a technical product with ethanol–water mixture in the presence of decolorising carbon. A stock solution of 1 mg/ml was prepared in chloroform. Working standards of 10, 25, 50 and 100 μg/ml were prepared from the stock. Tetrabase reagent was prepared by dissolving 2.5 g of tetramethyl-*p*-diaminodi-

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phenylmethane and 10 g of citric acid in 10 ml of distilled water and dilute to 500 ml with distilled water. Silica gel G plates (20 × 20 cm^2) were used. All solvents and chemicals used were AR grade.

Method

An amount of 1 g of specimens was weighed into a 50-ml beaker and cut to fine pieces with scissors. For feedstuffs specimens, they were ground into powder form first before extraction. PCP was extracted from the specimens with 10 ml acetic acid–methanol mixture at 55°C for 10 min. The extracts were then filtered into 10-ml graduated centrifuge tubes with Whatman No. 1 filter paper. The specimens were washed with another 5 ml of the acetic acid–methanol mixture, filtered and added to the previous fractions. Both specimens and standards (1 ml working standards) were blown down to dryness under a gentle stream of nitrogen in a 37°C water bath. A 0.1-ml volume of concentrated nitric acid was then added to each tube, stoppered and boiled for 3 min in a water bath. The tubes were then cooled and the volume was made up to 0.5 ml with methanol. Samples of 10 μl were spotted on the thin-layer plate. The plates were run in dichloromethane for 30 min and dried. Then they were sprayed with tetrabase reagent, dried at ambient temperature and developed at 110°C for 10 min.

RESULTS AND DISCUSSION

Solvent systems

A few solvent systems had been tried to find a better separation between PCP and the lower chlorophenols. The systems tried were: acetone–hexane (20:80 and 30:70); acetone–toluene (20:80 and 30:70); dichloromethane; chloroform and a chloroform–acetone–diethylamine (5:4:1) mixture. The toluene system gave hazy spots and no colour was obtained with the diethylamine system. The results obtained for other systems in terms of R_F values of oxidised chlorophenols are shown in Table I.

TABLE I

R_F VALUES OF CHLOROPHENOLS IN DIFFERENT SOLVENT SYSTEMS AFTER NITRIC ACID OXIDATION

Standards of 100 μg/ml were used. Figures in brackets indicate very weak colour spots. Solvent systems: I = acetone–hexane (20:80) fraction, II = acetone–hexane (30:70) fraction, III = chloroform and IV = dichloromethane. 2,3,4-Trichlorophenol, *o*-chlorophenol, *p*-chlorophenol, *p*-nitrophenol and dinitrobenzene do not give any colour reaction even at 500 μg/ml level.

Chlorophenols	*Solvent systems*			
	I	*II*	*III*	*IV*
PCP	0.34	0.45	0.64	0.6
2,3,4,5-Tetrachlorophenol	(0.34)	(0.45)	(0.66, 0.7)	(0.61, 0.68)
2,3,4,6-Tetrachlorophenol	0.31	0.4	0.61, (0.17, 0.71)	0.54, (0.12, 0.61, 0.65, 0.68)
2,3,5,6-Tetrachlorophenol	long blue strip	0.45, (0.32, 0.39)	0.64, (0.71)	0.61, (0.68)
2,3,5-Trichlorophenol	(0.23)	(0.39)	(0.53)	(0.46, 0.53)
Tetrachlorohydroquinone	0.34	0.45	0.64	0.6

Specificity and sensitivity

The method was sensitive to detect down to 2 μg/g of PCP in the specimens when 20 μl oxidised extract was applied onto the plate. Sensitivity down to 80 ng was achieved by reducing the amount of methanol added to the tubes. Standards of concentrations 2–150 μg could be used to read against the specimens. Tests had been carried out on the lower chlorophenols, *p*-dinitrophenol and *o*-dinitrobenzene. The results show that the test is highly selective for PCP, and most of the lower chlorophenols do not give the blue colour even when 500 μg/ml standards were used (see Table I). Tetrachlorophenols gave blue spots with similar R_F values and could interfere with the detection of PCP.

A typical thin-layer chromatogram of chloranils obtained by this method is shown in Fig. 1.

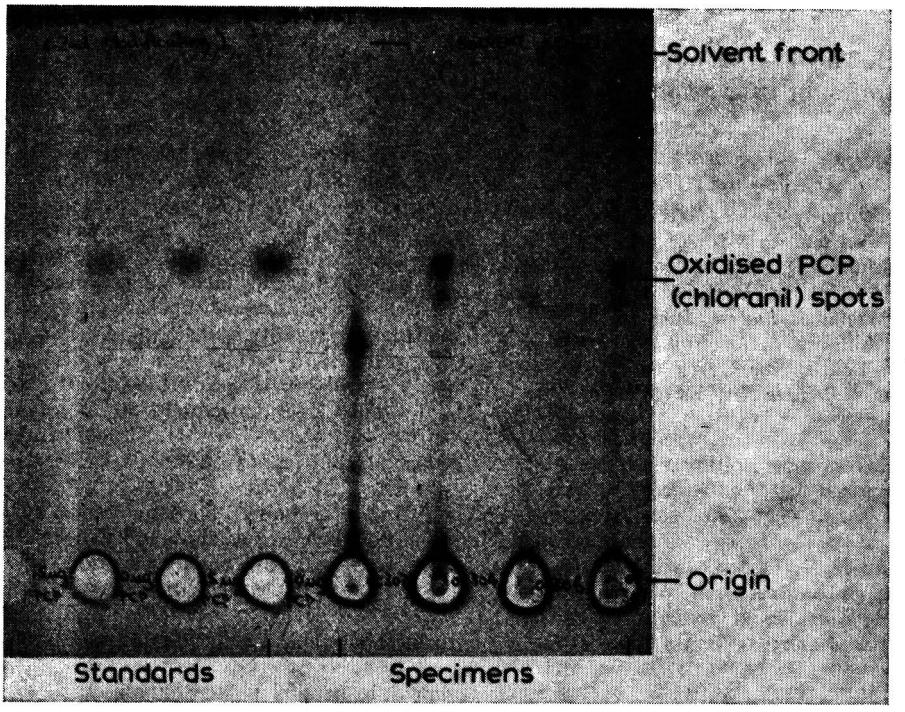

Fig. 1. A typical thin-layer chromatogram of oxidised PCP (chloranil) obtained by this method. Solvent dichloromethane.

PCP levels in different specimens had been obtained by both this method and the ion exchange–GLC method[5]. The results obtained were very similar ($t = 0.028$, $n = 14$). This method is much simpler and easier to carry out than the GLC method, and the whole procedure only takes about 90 min to give results. Though this method is less effective in separating tetrachlorophenols from PCP and the colour produced is only selectively good for PCP but not specific for it, this could be also an advantage for this method, as (1) tetrachlorophenols are also toxic to animals[6], the detection of

both compounds in the same specimen may be necessary; expecially when tetrachlorophenols are usually found in the technical PCP products, (2) tetrachlorophenols and other lower chlorophenols are metabolites of PCP in animals and bacteria in soil[7], the measurement of those break-down products at the same time may reflect the starting PCP level.

CONCLUSION

PCP can be separated and measured quantitatively by TLC. This TLC method is recommended for detection of PCP in sawdust, woodshavings and feedstuffs at field stations or veterinary investigation centres where no expensive instrumentation is installed, and also for batch analysis due to its low cost, rapidity and simplicity.

ACKNOWLEDGEMENTS

H. Ting is grateful to all the people in toxicology section, Central Veterinary Laboratories for their help when he was working there.

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Erratum

J. Chromatogr., 193 (1980) 181–195

Page 184, eqn. 6,

"$$\frac{Q_i}{w} = \int_{\text{peak}} c_{i,m} \cdot \mathrm{d}t = \int_0 \bar{c}_{i,m} \pm \{t_R(c_{i,m}) - t_R(\bar{c}_{i,m})\}\mathrm{d}c_{i,m}$$"

should read

"$$\frac{Q_i}{w} = \int_{\text{peak}} c_{i,m} \cdot \mathrm{d}t = \int_0^{\bar{c}_{,im}} \pm \{t_R(c_{i,m}) - t_R(\bar{c}_{i,m})\}\mathrm{d}c_{i,m}$$".

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journal of chromatography news section

MEETINGS

FIFTEENTH INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY
"CHROMATOGRAPHY '80"

The Fifteenth International Symposium on Advances in Chromatography will be held October 6–9, 1980, at the Astrohall in Houston, Texas.

A total of 51 papers and 22 poster sessions will be included at the Symposium representing contributions from 16 countries. A special feature of the meeting will be an exposition of the latest instrumentation and books.

Registrations should be made in advance. The programs, registration forms and hotel reservations can be obtained from:

Professor Albert Zlatkis,
Chemistry Department,
University of Houston,
Houston, Texas 77004, U.S.A.
Phone: (713) 749-2623

The detailed program of the Symposium is given below.

MONDAY, OCTOBER 6, 1980

CONTEMPORARY CHROMATOGRAPHY

L.S. Ettre, presiding

8:30 Welcome to Symposium
8:45 Presentation of the M.S. Tswett Chromatography Medals
9:00 I. Halász (Universität des Saarlandes, Saarbrücken, G.F.R. – Preparative separation of complex mixtures (vacuum residual of crude oil) by "extrography"
9:30 W. Melander, J. Stoveken and Cs. Horváth (Yale University, New Haven, CT, U.S.A.) – Stationary phase effects in reversed-phase chromatography: I. Energetics of retention on non-polar bonded phases
10:00 Intermission

A. Karmen, presiding

11:00 E. Bayer, H. Bauer, G.J. Nicholson, A.N. A. Rahman and W. Voelter (Universität Tübingen, Tübingen, G.F.R.) – Microscale HPLC instrumentation for open tubular capillary chromatography of dansyl-amino acids
11:30 J.L. Glajch, J.J. Kirkland, K.M. Squire and J.M. Minor (E.I. du Pont de Nemours and Company, Wilmington, DE, U.S.A.) – Optimization of solvent strength and selectivity for reversed-phase liquid chromatography using an interactive mixture-design statistical technique

Monday Afternoon

CAPILLARY COLUMN GAS CHROMATOGRAPHY

R.E. Kaiser, presiding

2:00 R.G. Jenkins and R.H. Wohleb (J & W Scientific, Inc., Orangevale, CA, U.S.A.) – The use of bonded phases in fused silica capillary gas chromatography

2:20 F.I. Onuska and R. Thompson (Canada Centre for Inland Waters, Burlington, Ontario, Canada) and W.A. Aue (Dalhousie University, Halifax, Nova Scotia, Canada) – Novel silylation procedures for glass capillary columns

2:40 S.R. Lipsky, W.J. McMurray, M. Hernandez and P. DiMauro (Yale University, New Haven, CT, U.S.A.) – Further observations on the utilization of fused silica glass capillary columns for gas chromatographic analyses

3:00 Intermission

W. Bertsch, presiding

4:00 W. Jennings (University of California, Davis, CA, U.S.A.) – Optimization of the GC system

4:20 R.G. Mathews, J. Torres and R.D. Schwartz (Pennzoil Products Co., Shreveport, LA, U.S.A.) – Applications of surface-modified porous silicas to glass capillary column preparation

4:40 B.M. Wright, M.L. Lee, S.W. Graham, L.V. Phillips and D.M. Hercules (Brigham Young University, Provo, UT, U.S.A.) –Glass surface analytical studies in the preparation of open tubular columns for gas chromatography

TUESDAY, OCTOBER 7, 1980

GAS CHROMATOGRAPHY DETECTORS

W. Jennings, presiding

8:30 R.E. Kaiser (Institute for Chromatography, Bad Dürkheim, G.F.R. – Modern calibration in chromatography

9:00 A. Karmen, S. Lam, G. Cummings and F. Chow (Albert Einstein College of Medicine, Bronx, NY, U.S.A.) – Specific detection in GLC effluents with on-line HPLC optical detectors

9:30 J.W. Birks, B. Shoemaker and E.A. Hill (University of Colorado, Boulder, CO, U.S.A.) – O_3 and $O_2(^1\Delta_g)$ as reagents for the chemiluminescent aerosol spray detector

10:00 Intermission

D.C. Fenimore, presiding

11:00 T. Gilfoil and H.H. Hill, Jr. (Washington State University, Pullman, WA, U.S.A.) – A selective ionization detector for gas chromatography based on atmospheric-pressure reactions with iodine

11:20 F.F. Andrawes (Lockheed Engineering and Management Services Co., Inc., and E.K. Gibson, SN7/Geochemistry Branch, Houston, TX, U.S.A.) – Helium ionization detector – a new approach in practice and applications

11:40 R.C. Leveson and N.J. Barker (Photovac, Inc., Thornhill, Ontario, Canada) – A portable multi-component air impurity analyzer having sub-part-per-billion capability without sample pre-concentration

12:00 J.N. Driscoll and L.F. Jaramillo (HNU Systems, Inc., Newton, MA, U.S.A.) – A new sensitive method for determination of polyaromatic hydrocarbons

GENERAL POSTER SESSIONS (Tuesday Afternoon, 1:00 P.M.)

1 J.R. Benson (Interaction Chemicals, Inc., Los Altos, CA, U.S.A.) – Variable capacity anion-exchange resins

2 D.M. Barends and A. Hulshoff (Rijksuniversiteit Utrecht, Utrecht, The Netherlands) – The determination of aminoglycoside antibiotics in serum by high-performance liquid chromatography with ultraviolet detection

3 T.H. Jupille, M.J. Gray and B. Black (Bio-Rad Laboratories, Richmond, CA, U.S.A.) – Reversed-phase HPLC on ion-exchange resins

4 J.H. Kindsvater, D.P. Lee and K.A. Weinberger (Hamilton Company, Reno, NV, U.S.A.) – Reversed-phase HPLC separations on the polymeric adsorbent PRP-1 for the analysis of analgesic and sulfa drug pharmaceuticals

5 J.S. Andrews and T.J. Good (Analytichem International, Inc., Harbor City, CA, U.S.A.) – The use of bonded phase extraction columns for rapid sample preparation of benzodiazepines and metabolites from serum for HPLC analysis

6 A.J. Varghese (The Ontario Cancer Institute, Toronto, Canada) – Reversed-phase high-pressure liquid chromatography of reduced misonidazole in urine samples

7 R.H.A. Sorel, S. Wiersema and A. Hulshoff (University of Utrecht, Utrecht, The Netherlands – A study on the retention mechanisms in dynamic anion-exchange high-performance liquid chromatography

8 S. Kapila and C.R. Vogt (University of Missouri, Columbia, MO, U.S.A.) – Analysis of tar by-products from in situ coal gasification

9 D. Westerlund, L.B. Nilsson and Y. Jaksch (Astra Läkemedel AB, Södertälje, Sweden) – Straight-phase ion-pair chromatography of zimelidine and similar divalent amines

10 S.A. Wise, W.J. Bonnett, F.R. Guenther and W.E. May (National Bureau of Standards, Washington, DC, U.S.A.) – A relationship between reversed-phase C_{18} liquid chromatographic retention and the Shape of polycyclic aromatic hydrocarbons

11 J.W. Anderson (Tracor Instruments, Austin, TX, U.S.A.) – An improved photoionization detector

12 L.F. Jaramillo and J.N. Driscoll (HNU Systems, Inc., Newton, MA, U.S.A) – Advantages of coupling a photoionization detector and a flame-ionization detector in series: improved selectivity and detection

13 J.A. MacDonald (Tracor Instruments, Austin, TX, U.S.A.) – Detection of permanent gases and hydrocarbons using the ultrasonic detector in gas chromatography

14 C.D. Pfaffenberger, A.J. Peoples and J. Guerra (University of Miami School of Medicine, Miami, FL, U.S.A.) – Drinking water as a source of serum chloroform

15 J. Novak, J. Verjrosta, M. Roth and J. Janák (Czechoslovak Academy of Sciences, Brno, Czechoslovakia) – Reduction of retention data measured for homologous compounds at different temperatures

16 R.J.P. Goedknegt (Tracor Instruments, Austin, TX, U.S.A.) – The use of cold injection ports to avoid double peaks and peak ghosting in gas chromatography with packed columns

17 D. Smith and W. Schnute (Finnigan Corp., Sunnyvale, CA, U.S.A.) – Automated analysis of volatile organic compounds in drinking water with a dedicated GC–MS system

18 M. Story, C. Boitnott and U. Steiner (Finnigan Corp., Sunnyvale, CA, U.S.A.) – Triple quadrapole MS–MS

19 H. Kalasz and J. Nagy (Semmelweis University of Medicine, Budapest, Hungary) and E. Tyihak and E. Mincsovics (Research Institute for Medicinal Plants, Budakalasz, Hungary) – The existence of an optimal flow-rate at thin-layer chromatography

20 B.V. Ioffe (Leningrad State University, Leningrad, U.S.S.R.) – Present-day state and tendencies of the development of gas chromatographic head-space analysis

21 P. Gebauer, P. Boček, M. Deml and J. Janák (Czechoslovak Academy of Sciences, Brno, Czechoslovakia) – Isotachophoresis of kinetically labile complexes

22 K. Sakodynskii (Institute of Physical Chemistry, Moscow, U.S.S.R.) – Chromatographic and physical chemical properties of pyrazole polymer sorbents

Tuesday Afternoon

GAS CHROMATOGRAPHY – GENERAL

E.C. Horning, presiding

2:00 M. Verzele, P. Sandra, S. Qureshi and G. Redant (Rijksuniversiteit Gent, Gent, Belgium) – High temperature quantitative glass capillary gas chromatography. Analysis of piperine and kinine

2:20 F. Merli, M. Novotny and M.L. Lee (Indiana University, Bloomington, IN, U.S.A.) – Fractionation and gas chromatographic analysis of aza-arenes in complex mixtures

2:40 C.F. Poole, W.-F. Sye, S. Singhawangcha, F. Hsu and A. Zlatkis (University of Houston, Houston, TX, U.S.A.) and A. Arfwidsson and J. Vessman (AB Hässle, Mölndal, Sweden) – New electron-capturing pentafluorophenyldialkylchlorosilanes as versatile derivatizing reagents for gas chromatography

3:00 Intermission

M.G. Horning, presiding

4:00 J.K. Haken and D. Srisuhk (The University of New South Wales, New South Wales, Australia) – A stationary phase classification scheme independent of a reference phase using molecular retention indices

4:20 W. Chen and X. Chen (Chinese Academy of Sciences, Peking, China) – The retention behavior in nonlinear chromatography: determination of isotherms on polymeric stationary phases GDX-series and its application to qualitative analysis

4:40 H. Hernandez (Antek Instruments, Inc., Houston, TX, U.S.A.) – The analysis of nitrogen bearing additives in polyolefin

5:00 A.S. Said, R.S. Al-Amuri and Y. Al-Sultan (University of Kuwait, Kuwait, Kuwait) – Extracolumnar effects in nonlinear chromatography

WEDNESDAY, OCTOBER 8, 1980

ENVIRONMENTAL AND BIOMEDICAL GAS CHROMATOGRAPHY

R.E. Sievers, presiding

8:30 E.D. Pellizzari, A. Schindler, W. Penn and R. Banker (Research Triangle Institute, Research Triangle Park, NC, U.S.A.) – Synthesis and evaluation of new polymeric sorbents for collection of vapor-phase organics in ambient air

8:50 J.J. Richard, C.D. Chriswell and J.S. Fritz (Iowa State University, Ames, IA, U.S.A.) – Concentration and determination of organic acids in complex aqueous samples

9:10 W. Wang, X. Ding and X. Wu (Chinese Academy of Sciences, Peking, China) – Determination of water at ppm levels in *cis*-buta-1,3-diene and its solvents by gas chromatography

9:30 B.S. Middleditch and B.P. Basile (University of Houston, Houston, TX, U.S.A.) – Discharge of elemental sulfur and its distribution in surficial sediments in the region of the buccaneer oil field

10:00 Intermission

W.J.A. VandenHeuvel, presiding

11:00 H.-Ch. Curtius, H. Farmer and F. Rey (University of Zurich, Zurich, Switzerland) – *In Vivo* studies of the tryptophan-*tert.*-hydroxylase system – quantitation of serotonin and tryptamine using gas chromatography–mass fragmentography

11:20 H.M. Liebich, A. Pickert, U. Stierle and J. Wöll (Universität Tübingen, Tübingen, G.F.R.) – Gas chromatography–mass spectrometry of saturated and unsaturated dicarboxylic acids in urine and blood

11:40 W. Vogt, K. Jacob, A.-B. Ohnesorge and G. Schwertfeger (Universität München, München, G.F.R.) – A highly sensitive method for the quantitation of homovanillic acid in cerebrospinal fluid

12:00 R. Segura and X. Navarro (Universidad Autonoma de Barcelona, Barcelona, Spain) – The use of halides as fluorescence inducing reagents for thin-layer chromatography

Wednesday Afternoon

NEW DEVELOPMENTS IN LIQUID CHROMATOGRAPHY

E. Grushka, presiding

2:00 R.P.W. Scott (Hoffmann-La Roche Inc., Nutley, NJ, U.S.A.) and C.F. Simpson (Chelsea College, London, Great Britain) – Solute–solvent interactions on the surface of reversed-phases, Part II

2:30 V.V. Berry (Polaroid Corp., Cambridge, MA, U.S.A.) – "Sequential isocratic step" liquid chromatography to achieve high sensitivity, near-universal detection while separating wide polarity mixtures

3:00 Intermission

J.J. Kirkland, presiding

4:00 F.A. Dombrose and M.B. Randall (University of North Carolina, Chapel Hill, NC, U.S.A.) – High-performance dry column elution chromatography (CDCD): a rapid and economical alternative to liquid column adsorption chromatography

4:30 R.W. Frei, A.H.M.T. Scholten, P.L.M. Welling and U.A.Th. Brinkman (Free University, Amsterdam, The Netherlands) – The use of PTFE coils in post-column photochemical reactors for liquid chromatography – application to pharmaceuticals

5:00 T. Tsuda (Nagoya Institute of Technology, Nagoya, Japan) – Open tubular microcapillary liquid chromatography with 30–40 μm I.D. columns

THURSDAY, OCTOBER 9, 1980

REVERSED-PHASE LIQUID CHROMATOGRAPHY

Cs. Horváth, presiding

8:30 R. McCormick and B.L. Karger (Northeastern University, Boston, MA, U.S.A.) – The role of organic modifier sorption on retention phenomena in reversed-phase liquid chromatography

9:00 A.P. Halfpenny and P.R. Brown (University of Rhode Island, Kingston, RI, U.S.A.) – An optimized assay for purine nucleoside phosphorylase by reversed-phase high-performance liquid chromatography

9:30 E. Grushka (The Hebrew University, Jerusalem, Israel) and F.K. Chow (State University of New York at Buffalo, Buffalo, NY, U.S.A.) – High-performance liquid chromatography of nucleotide–Mg(II) complexes: separation and mechanism of separation

10:00 Intermission

H.-Ch. Curtius, presiding

11:00 W. Voelter, K. Zech, P. Arnold and G. Ludwig (Universität Tübingen, Tübingen, G.F.R.) – High-speed ion-pair liquid chromatographic determination of purines, pyrimidines and their metabolites in human serum and urine

11:20 R.P. Evershed, E.D. Morgan and L.D. Thompson (University of Keele, Keele, Staffordshire, Great Britain) – An efficient and economic separation of geometric isomers on a preparative scale by liquid chromatography

11:40 S. Lam, F. Chow and A. Karmen (Albert Einstein College of Medicine, Bronx, NY, U.S.A.) – Reversed-phase chromatographic resolution of D and L dansyl amino acids by mixed chelate complexation

12:00 M. Harvey and S.D. Stearns (Valco Instruments Co., Inc., Houston, TX, U.S.A.) – A ten port HPLC switching valve: applications in sample injection and column switching

Thursday Afternoon

REVERSED-PHASE AND GENERAL LIQUID CHROMATOGRAPHY

R.P.W. Scott, presiding

2:00 R.C. Kong, B. Sachok and S.N. Deming (University of Houston, Houston, TX, U.S.A.) – Combined effects of pH and surface active ion concentration in reversed-phase liquid chromatography

2:20 A.P. Goldberg (DuPont Instruments, Wilmington, DE, U.S.A.) – Influences on reversed-phase chromatography

2:40 J.F. Thoma (South Bend Medical Foundation, Inc., South Bend, IN, U.S.A.) – The use of C-18 bonded silica for extraction of drugs, environmental pollutants and other clinically important compounds from aqueous solutions

3:00 Intermission

P.R. Brown, presiding

4:00 C.L. Guillemin, F. Danion and C. Mayen (Rhone-Poulenc, Aubervilliers, France) – The deferred standard method, a solution for quantitative analysis in laboratory and process high-performance liquid chromatography

4:20 M.L. Vestal (University of Houston, Houston, TX, U.S.A.) – A new universal detector for liquid chromatography

4:40 N. Tanaka, K. Sakagami and M. Araki (Kyoto Technical University, Kyoto, Japan) – The effect of alkyl chain length of stationary phase on retention and selectivity in reversed-phase liquid chromatography. Participation of solvent molecules in stationary phase

5:00 H.D. Meyer, K. Jacob and W. Vogt (Universität Munich, Munich, G.F.R.) – Diagnosis of porphyrias by ion-pair HPLC

NEW INTENSIVE SHORT COURSES
October 4–5, 1980

ASTROHALL
HOUSTON, TEXAS

1 Capillary Gas Chromatography
Directors: Dr. L.S. Ettre, Perkin-Elmer Corp., Norwalk, CT, U.S.A.
Dr. W.G. Jennings, University of California, Davis, CA, U.S.A.

2 High-Performance Liquid Chromatography
Director: Dr. Cs. Horváth, Yale University, New Haven, CT, U.S.A.

3 Gas Chromatography–Mass Spectrometry
Directors: Dr. B.S. Middleditch, University of Houston, Houston, TX, U.S.A.
Dr. C.J.W. Brooks, University of Glasgow, Glasgow, Scotland

4 High-Performance Thin-layer Chromatography
Director: Dr. D.C. Fenimore, Texas Research Institute for Mental Sciences, Houston, TX, U.S.A.

For details and registration forms write to: Dr. Albert Zlatkis, Chemistry Department, University of Houston, Houston, Texas 77004, U.S.A.

ANALYTICA 80

The 7th International Exhibition of Biochemical and Instrumental Analysis was held this year in the Bavarian capitol Munich, G.F.R. The Analytica 80 was organized in connection with the European Conference 'Biochemische Analytik' by the Münchener Messe- und Ausstellungsgesellschaft mbH (the Fair and Exhibition Company, Inc. of Munich) from April 29 to May 2.

The Analytica, being held biennially, has developed into an important European communication centre for biochemical and instrumental analysis. More than 20,000 people visited the exhibition, where 325 exhibitors from 30 countries were represented. The number of exhibitors at the Analytica 78 was 283 and there were less than 20,000 visitors on that occasion.

So soon after the 1980 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, spectacular new instruments in the field of chromatographic analysis were not expected; particularly not from the U.S. manufacturers who tried to introduce their new products in Atlantic City, NJ, U.S.A. Nevertheless a few instruments and accessories were totally new and many were new to the European and especially the German markets. The presentation of the new items on Analytica is sometimes confusing. Some manufacturers call an instrument or accessory new when it is shown for the very first time; others call their products new when they are shown for the first time in Europe, in Germany or on an Analytica exhibition.

Biotronik AG showed their new Model LC 7000 amino acid analyzer, an instrument clearly built to compete with LKB's new LC 4400, also shown on the Analytica 80, as well as with the already known Carlo Erba amino acid analyzer. This new generation of amino acid analyzers is characterized by much smaller overall dimensions. From the Japanese firm Jasco, Biotronik AG showed a complete HPLC-system, especially designed for the analysis of small amounts of sample material. The modular Jasco system can be used for the analysis of samples as small as 0.1 µl. Kratos' Schoeffel division exhibited the HPLC LC 250 instrument with the new modules KLIC, SF 770, FS 950 and MM 700. The last component is a memory module designed for the scanning of UV-Vis spectra between 190 and 700 nm with the variable-wavelength detector.

Kontron AG showed their new HPLC-system, very similar to one of the systems built by the West German manufacturer Gynkotec.

Varian AG, the local Varian subsidiary, exhibited the new ternary gradient system 5060. Du Pont de Nemours (Deutschland) GmbH showed the new Trirotor Model 870 with the three pump heads in one line. The new pump is lower than the old model and can be fitted more easily in a modular system. Spectra-Physics GmbH introduced their new SP-8700 HPLC-system with a newly designed reciprocating pump and the new SP-8400 variable-wavelength detector. The new system replaces the old modular one. The SP-8000 remains in the Spectra-Physics HPLC-program, the top-of-the line model. The company also showed the new B-version of the SP-8000 equipped with the video unit.

Siemens AG surprised the analytical world with the introduction of their new modular microprocessor-based gas chromatography system SiCHROMAT. The manufacturer already has the most common detectors for the new system available.

Kipp & Zonen GmbH exhibited the Model 8200 gas chromatograph from their Dutch manufacturer, Kipp Analytica BV.

Philips GmbH Unternehmensbereich Elektronik für Wissenschaft und Industrie, representing Pye Unicam Ltd. in the G.F.R., introduced the new microprocessor-based Model 304 gas chromatograph.

Carlo Erba Strumentazione S.p.A. showed their new 4200 series of GC instruments. The Italian manufacturers of gas chromatography equipment, Dani S.p.A. and Carlo Erba S.p.A. use the same "high resolution" label to indicate their capillary gas chromatographs.

Kontron AG showed the HNU GC 401 gas chromatograph equipped with the photoionization detector. Shimadzu (Europe) GmbH exhibited the electron-capture version of the very small GC-Mini-2 and the combination GC-RIA of a gas chromatograph and the well-known computing integrator. The combination is controlled via the integrator keyboard. Hewlett-Packard's 5880A gas chromatograph and Packard-Becker's Model 433 were new to most of the visitors on the Analytica 80.

Perkin-Elmer showed their black labelled B-versions of the SIGMA-series.

In the field of combination techniques, recently called "hyphenated" methods by Tom Hirschfeld, the small new manufacturer Dr. Franzen Analysen-Technik GmbH & Co. KG, from the North German town of Bremen, displayed their new, small and very much integrated gas chromatograph–mass spectrometer, especially designed for quantitative analysis. The instrument is equipped with a gas chromatograph especially suited for this purpose, and a mass quadrupole filter remarkably made of an exactly formed glass tube, coated with metal. Hewlett-Packard exhibited only their small 5990-type GC–MS instruments.

Kratos exhibited their model MS-25, also shown at the Analytica 78, and not their new successful MS-80.

Finnigan GmbH displayed their small combination 1020 with a Perkin-Elmer SIGMA-3 gas chromatograph. Vacuum Generators GmbH showed the Model 12000 GC–MS combination fitted with a Carlo Erba Fractovap 2900 gas chromatograph.

Bruker Analytische Messtechnik GmbH introduced their Model IFS 110 FT–IR–spectrometer, a combination of the FT–IR interferometer and a level three Hewlett-Packard 5880A gas chromatograph.

Several new instruments and accessories, were exhibited in the field of data collection, processing and presentation. Bryans Southern Instruments Ltd. and Watanabe GmbH introduced new and remarkably low built printer/plotters and BBC's Goerz division introduced their new Servogor 460 and 300 series potentiometric recorders.

The Analytica 80 exhibition emphasized in Europe the trends shown in Atlantic City, NJ, U.S.A. during the Pittsburgh Conference 1980: nearly all new instruments are microprocessor-based, most of them are controlled via alphanumerical keyboards and via a limited number of dedicated function keys, and the video tube is a rapidly gaining field in scientific instrumentation.

KLAAS H.P. BROER

NEW BOOKS

Factor analysis in chemistry, by E.R. Malinowski and D.G. Howery, Wiley, New York, Chichester, 1980, *ca.* 304 pp., price *ca.* US$ 31.25, £ 14.30, ISBN 0-471-05581-5.

Aquametry: A treatise on methods for the determination of water, Part 3, by J. Mitchell, Jr. and D.M. Smith, Wiley, New York, Chichester, **2nd ed.,** 1980, *ca.* 795 pp., price *ca.* US$ 57.10, £ 26.20, ISBN 0-471-02266-7.

Microweighing in vacuum and controlled environments, edited by A.W. Czanderna and S.P. Wolsky, Elsevier, Amsterdam, Oxford, New York, 1980, XIV + 404 pp., price Dfl. 160.00, US$ 78.00, ISBN 0-444-41868-7.

Numerical methods in chemistry, by K.J. Johnson, Marcel Dekker, New York, Basel, 1980, IX + 503 pp., price SFr. 62.00, ISBN 0-8247-6818-3.

Biochemical applications of mass spectrometry, First supplementary volume, by G.R. Waller and O.C. Dermer, Wiley, New York, Chichester, 1980, *ca.* 1000 pp., price *ca.* US$ 159.60, £73.20, ISBN 0-471-03810-5.

Physical chemistry, by R.A. Alberty and F. Daniels, Wiley, New York, Chichester, **5th ed., SI version,** 1980, *ca.* 750 pp., price *ca.* US$ 23.90, £ 10.95.

Analytical chemistry, by G.D. Christian, Wiley, New York, Chichester, **3rd. ed.,** 1980, *ca.* 736 pp., price *ca.* US$ 26.50, £ 12.15, ISBN 0-471-05181-0.

Inositol phosphates – Their chemistry, biochemistry and physiology, by D.J. Cosgrove and G.C.J. Irving, Elsevier, Amsterdam, Oxford, New York, 1980, XII + 191 pp., price Dfl. 95.00, US$ 46.25, ISBN 0-444-41874-1.

PUBLICATION SCHEDULE FOR 1980

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	D 1979	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	185 186	187/1 187/2 188/1	188/2 189/1 189/2	189/3 190/1	190/2 191 192/1	192/2 193/1 193/2 193/3	194/1 194/2 194/3	195/1 195/2 195/3	196/1 196/2 196/3	197/1 197/2 198/1	The publication schedule for further issues will be published later.		
Chromatographic Reviews			184/1	184/2					184/3				
Biomedical Applications		181/1	181/2	181/ 3-4	182/1	182/2	182/ 3-4	183/1	183/2	183/3	183/4		

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 193, No. 3, pp. 529–532. A free reprint can be obtained by application to the publisher)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc. should be on separate sheets.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

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References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the lay-out of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

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Contents of Volume 1, Nos. 5-6:

Publication Schedule:

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