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Column Liquid Chromatography

(Continued overleaf)

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Selective Sample Handling and Detection in High-Performance Liquid Chromatography

Journal of Chromatography Library, 39

part A

edited by **R.W. Frei**†, *Free University, Amsterdam, The Netherlands, and* **K. Zech**, *Byk Gulden Pharmaceuticals, Konstanz, FRG*

Part A of this two-volume project attempts to treat the sample handling and detection processes in a liquid chromatographic system in an integrated fashion. The need for more selective and sensitive chromatographic methods to help solve the numerous trace analysis problems in complex samples is undisputed. However, few workers realize the strong interdependence of the various steps - sample handling, separation and detection - which must be considered if one wants to arrive at an optimal solution. By introducing a strong element of selectivity and trace enrich- ment in the sample preparation step, fewer demands are placed on the quality of the chromatography and often a simple UV detector can be used. By using a selective detection mode, i.e. a reaction detector, the sample handling step can frequently be simplified and more easily automated. The impact of such a "total system" approach on handling series of highly complex samples such as environmental specimens or biological fluids can be easily imagined.

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part B

edited by **K. Zech**, *Byk Gulden Pharmaceuticals, Konstanz, FRG, and* **R.W. Frei**†, *Free University, Amsterdam, The Netherlands*

Part B completes the treatment of the handling, separation and detection of complex samples as an integrated, interconnected process. On the basis of this philosophy the editors have selected those contributions which demonstrate that optimal sample preparation leads to a simplification of detection or reduced demands on the separation process. Throughout the book emphasis is on chemical principles with minimum discussion of the equipment required - an approach which reflects the editors' view that the limiting factor in the analysis of complex samples is an incomplete knowledge of the underlying chemistry rather than the hardware available. This lack of knowledge becomes more evident as the demands for lower detection limits grow, as solving complex matrix problems requires a greater understanding of the chemical interaction between the substance to be analysed and the stationary phase.

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Computer-Assisted Method Development for High-Performance Liquid Chromatography

edited by **J.L. Glajch** *and* **L.R. Snyder**

(Spin-off from the Journal of Chromatography Vol. 485 plus an additional chapter, index and glossary)

This book deals with the use of the computer as an aid in selecting adequate or optimum conditions for a given analytical separation. Originally published as Volume 485 of the Journal of Chromatography, it has now been reprinted in book form, since the information is so useful that many chromatographers want a copy readily available in the lab.
An extensive Introduction is added to the book edition. This surveys the field and refers to the pages where particular items are discussed in the book. The addition of a Glossary of Terms, an Author Index and a Subject Index make this book an invaluable source of easily consulted information for the practising chromatographer.
For the purpose of this book, computer-assisted method development will be limited to specific procedures which are intended to be used with a computer - rather than their manually applied precursors. In that sense, the subject can be considered to have begun around 1980.
The ongoing, intense research activity into various forms of computer assisted HPLC method development provides the assurance that this approach can really assist the practical chromatographer working in an industrial laboratory.

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High-performance liquid chromatography retention index and detection of nitrated polycyclic aromatic hydrocarbons

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(First received June 6th, 1990; revised manuscript received September 24th, 1990)

ABSTRACT

Based on reversed-phase isocratic experiments and gradient optimization modeling, acetonitrile was found to provide optimum separation of nitrated polycyclic aromatic hydrocarbons (nitro-PAHs). A 31-min linear gradient between 24% and 80% acetonitrile in water at 35°C and 0.5 ml/min flow-rate was established. Nitro-PAH retention indices, *I*, were measured under these conditions. It was found that retention index values varied with changing column temperature and/or mobile phase compositions.

Diode-array, fluorescence (FD) and chemiluminescence (CD) detection were studied for nitro-PAHs. Diode-array detection responded linearly with detection limits between 2 and 12 ng/compound injected. In addition, dual-wavelength UV absorbance ratio (A_{230}/A_{254}, A_{330}/A_{254} and A_{230}/A_{330}) measurements at these wavelength pairs were reported. Fluorescence and chemiluminescence provided increased selectivity and sensitivity. Four orders of magnitude linear range were found for both detection methods with detection limits between 10 and 15 pg and 50 pg (on an NO_2/compound mole basis), respectively.

INTRODUCTION

Over the last decade, much research has been directed toward the development of analytical methodologies for the detection, identification, and quantification of nitrated polycyclic aromatic hydrocarbons (nitro-PAHs). Much of this effort is due to the fact that many nitro-PAHs produce mutagenic as well as carcinogenic activity [1–11]. Moreover, some isomeric nitro-PAHs have been shown to be genotoxic while others have not [1–4,8]. Thus, the requirement that analytical tools be capable of providing isomeric identification is self-evident. To date, total nitro-PAH concentrations found in environmental samples such as aerosols of ambient air; air, diesel exhaust, and wood smoke particulate matter; carbon black; grilled foods such as sausage, chicken, and fish; as well as nitro-PAH metabolites in biological tissue are typically < 10 ppm. This makes identification of isomers extremely difficult requiring well-defined chromatographic operating conditions concomitant with selective detection.

Most nitro-PAH methods have centered on gas chromatography (GC). Principally, this is due to the higher column separation factors achieved with capillary GC and the wide variety of gas-phase, specific detectors that are commercially

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available as compared to high-performance liquid chromatography (HPLC) [12–27]. For example, over fifty nitrated PAHs have been separated and identified with a 25 m × 0.31 mm I.D. fused-silica capillary coated with a 0.25-μm film of SE-52 and detected by an $NO\cdot/O_3$-specific chemiluminescence detector. Models were developed that both predicted retention characteristics of nitro-PAHs for standards that were not available and for a set of descriptors used to confirm nitro-PAH identity from actual GC retention obtained from a diesel exhaust particulate extract [21–23]. Many publications [12–20] have documented the presence of highly toxic OH–NO_2–PAHs, multi-condensed ring nitro-PAHs, and their metabolites present in the above-mentioned samples. Although capillary GC provides unmatched resolving power compared to HPLC, it is not amenable to thermally labile or low volatility organic compounds. High-resolution HPLC currently provides a sufficient alternative to GC for these types of compounds. Published reports illustrate reversed-phase gradient HPLC with UV, fluorescence, peroxyoxalate and $NO\cdot/O_3$ chemiluminescence, and electrochemical detection [28–36]. These studies have focused primarily on demonstrating nitro-PAH detector applicability (in particular for 1-nitropyrene) and not on nitro-PAH HPLC retention characteristics.

In a recent paper, the effect of temperature (T) (35–65°C) and organic modifier (acetonitrile, methanol) on the isocratic retention characteristics of nitro-PAHs on a reversed-phase C_{18} column were studied. The log k' (capacity factor) values were linearly dependent on organic–water volume fraction (φ) with plot slopes solvent-dependent. Van 't Hoff plots, log k' *vs.* $1/T$, revealed nitro-PAH transfer between the mobile and stationary phases were organic modifier and volume fraction dependent as well as compound specific [37]. The results from this study in combination with a commercially available software package (DryLab G) for optimizing gradient reversed-phase HPLC conditions were used to generate nitro-PAH retention index values I. Diode-array, fluorescence and chemiluminescence detection under optimum gradient conditions were evaluated, as well as nitro-PAH retention characteristics. The results of this study are presented in this paper.

EXPERIMENTAL

Chemicals and materials

The mobile phase consisted of Milli-Q purified water (Millipore, Milford, MA, U.S.A.) with HPLC-grade acetonitrile and methanol (Fisher Scientific, Medford, MA, U.S.A.) as the organic modifier. The mobile phase was filtered through a 0.45-μm nylon-66 filter (Rainin, Woburn, MA, U.S.A.), ultrasonicated and vacuum degased prior to use. The mobile phase was purged with helium throughout the HPLC experiment. Sources of nitro-PAH compounds have been identified elsewhere [38]. Nitro-PAHs were used without further purification. Standard solutions consisted of *ca.* 1 mg of nitro-PAH in 1 ml of acetonitrile. After preparation, samples were stored in the dark and refrigerated.

HPLC instrumentation

Reversed-phase HPLC separations were performed using a Hewlett-Packard (Palo Alto, CA, U.S.A.) 1090M liquid chromatography system equipped with a DR5 ternary delivery solvent system, diode-array and fluorescence detectors (standard flow

cell), temperature-controlled autosampler and column compartments. The Hewlett-Packard HPLC ChemStation controlled experimental conditions, collected and evaluated retention data as well as UV and fluorescence spectra. Sample injections were made using the autosampler and autoinjector system. A Rheodyne 3 mm × 0.5 μm column inlet filter preceded the analytical column, a 250 × 2.1 mm, 5 μm particle size, octadecylsilane, C_{18}, column (Alltech, Deerfield, IL, U.S.A.). Sample injections were made after the analytical column equilibrated with the mobile phase and the column temperature stabilized (35–55°C). HPLC flow-rate was 0.5 ml/min.

Diode-array UV detection. Chromatographic signals were simultaneously monitored at 230, 254 and 330 nm. Dual-wavelength absorbance ratios for each nitro-PAH were calculated by dividing the peak height measured at λ_1/λ_2, *viz.*, A_{230}/A_{254}, A_{330}/A_{254} and A_{330}/A_{230}. Four nitro-PAHs were used to evaluate the dynamic range and detection limit at 254 nm and 330 nm. The standard solutions, 200 ng/μl of each compound, were serially diluted until analyte signals were no longer observable.

Fluorescence detection. Nitro-PAHs were reduced to their amino analogues by a silica gel–zinc metal mixture (< 200 mesh; 1:1, w/w) contained in a 40 mm × 2.1 mm I.D. stainless-steel tube. The mobile phase consisted of 24% to 80% acetonitrile with 30 m*M* aqueous ammonium acetate (Aldrich, Milwaukee, WI, U.S.A.) solution. Nitro-PAH reduction efficiencies were evaluated in the pre-column derivatization mode, *i.e.*, the reducing column was placed before the analytical column. The conversion yield was calculated by measuring the peak area of unreacted nitro-PAHs with and without catalyst. The stop-flow scanning technique combined with pre-column reduction was used to obtain the optimal pair of excitation/emission ($\lambda_{ex}/\lambda_{em}$) wavelengths by stopping the mobile phase flow and trapping the reduced nitro-PAHs in the flow cell. Excitation and emission spectra were obtained separately to determine optimum $\lambda_{ex}/\lambda_{em}$ wavelength pair for each compound.

Chemiluminescence detection. Details of the NO·/O_3 chemiluminescence detection [21,30] and the interface [39] (used to separate the mobile phase from the analyte before the latter reached the detector) have previously been described.

HPLC retention index system

Nitro-PAH retention behavior under varying HPLC operating conditions was evaluated. Retention index, *I*, provides a calculated experimental measurement of solute retention relative to a pair of internal standards:

$$I = 100\,[n + (t_{r(s)} - t_{r(n)})/(t_{r(n+1)} - t_{r(n)})] \quad (1)$$

where $t_{r(s)}$ is the measured solute retention for which *I* is to be calculated, $t_{r(n)}$ and $t_{r(n+1)}$ are the bracketing compound retention times that elute immediately prior to and following the solute, while *n* is the number of rings present in the standard $t_{r(n)}$. The bracketing compounds used in this study were: nitrobenzene, 1-nitronaphthalene, 9-nitroanthracene, 6-nitrochrysene and 6-nitrobenzo[*a*]pyrene. For those compounds that eluted before nitrobenzene, *I* was calculated by dividing $t_{r(s)}$ by $100 \cdot t_{r(\text{nitrobenzene})}$. Nitro-PAH retention times were obtained at 254 nm.

DryLab G software (LC Resources, Lafayette, CA, U.S.A.) was used to determine optimum reversed-phase nitro-PAH separation conditions on the C_{18}

column as a function of linear gradient ramp and organic modifier (acetonitrile and methanol). Relative resolution maps and predicted chromatograms were calculated and plotted using this program.

RESULTS AND DISCUSSION

The objective of this paper was to study nitro-PAH retention characteristics under reversed-phase gradient HPLC conditions and to establish their retention indices at optimum experimental conditions. A secondary objective was to demonstrate applicability of these conditions with some nitro-specific detection methods. Toward this end, we reported earlier on the isocratic reversed-phase retention characteristics of nitro-PAHs as a function of organic modifier (acetonitrile and methanol), volume fraction (50:50, 60:40 and 70:30, v/v) and temperature (35–55°C). It was found that under optimum conditions, many of the nitro-PAHs exceeded "practical" analytical elution times (>1 h) with k' values greater than 10. For the purposes of this study, advantage was taken of the linear relationships found between log k' and φ to predict the optimum organic modifier for gradient separation of nitro-PAHs. The relative variation in S between each solvent type over the entire data set should provide the mechanism for such prediction. (S is the slope value for the log k' *vs.* φ plot and is related to the solvent strength of the organic modifier in the mobile

TABLE I

RELATIVE VARIATION IN S VALUES ($S=\delta S/S_{av}$) AS A FUNCTION OF ACETONITRILE–WATER AND METHANOL–WATER COMPOSITIONS

See ref. 37 for experimental data. Notes: (1) nitro-PAH retention times exceeded 1.5 h for compounds 38, 39, 43, 44 and 45 in methanol–water mobile phase compositions; (2) the remaining compounds listed in Table II were not studied; (3) $\delta S/S_{av}$ values were calculated by subtracting S_{exp} (slope of log k' *vs.* φ) from S_{cal} (calculated from S *vs.* log k_0 plots) divided by $100 \cdot S_{av}$ (average experimental S value for the corresponding compounds in a given solvent system).

No.	$\delta S/S_{av}$		No.	$\delta S/S_{av}$	
	Acetonitrile	Methanol		Acetonitrile	Methanol
2	− 2.23	1.58	23	− 5.77	6.58
3	8.21	− 3.44	24	3.40	−0.33
4	10.45	− 1.95	25	− 7.09	5.81
7	3.38	− 0.33	27	− 4.96	1.00
9	1.21	5.69	28	− 1.58	−0.68
10	0.79	− 2.77	29	− 9.33	7.19
11	−16.30	3.11	30	2.01	−7.22
12	0.74	0.18	31	− 3.54	−1.23
14	6.53	− 3.73	33	6.59	−5.03
15	− 9.30	6.44	34	− 2.28	2.18
16	5.13	−13.35	35	2.11	−7.60
17	−12.29	3.22	38	− 3.24	
18	− 0.23	3.00	39	12.65	
19	−12.15	− 0.22	43	8.78	
20	3.50	− 1.25	44	22.61	
21	− 7.43	− 0.24	45	9.22	

TABLE II

NITRATED POLYCYCLIC AROMATIC HYDROCARBONS

No.	Compound	No.	Compound
1	4-Nitroquinoline-N-oxide	24	1-Nitro-2-methylnaphthalene
2	8-Nitroquinoline	25	2,7-Dinitrofluorene
3	6-Nitroquinoline	26	2,8-Dinitrodibenzothiophene
4	5-Nitroquinoline	27	4-Nitrobiphenyl
5	Nitrobenzene	28	3-Nitrobiphenyl
6	2,4,5,7-Tetranitro-9-fluorenone	29	2,2′-Dinitrobenzyl
7	5-Nitro-6-methylquinoline	30	3-Nitrodibenzofuran
8	8-Nitro-7-methylquinoline	31	2-Nitrofluorene
9	1,8-Dinitronaphthalene	32	2-Nitrodibenzothiophene
10	1,3-Dinitronaphthalene	33	9-Nitrophenanthrene
11	2,4,7-Trinitro-9-fluorenone	34	9-Nitroanthracene
12	1,5-Dinitronaphthalene	35	3-Nitrophenanthrene
13	2,2′-Dinitrobiphenyl	36	1,3-Dinitropyrene
14	1-Nitronaphthalene	37	1,6-Dinitropyrene
15	1,3,6,8-Tetranitronaphthalene	38	1-Nitropyrene
16	9,10-Dinitroanthracene	39	3-Nitrofluoranthene
17	2,7-Dinitro-9-fluorenone	40	1,8-Dinitropyrene
18	2-Nitro-9-fluorenone	41	7-Nitro-3,4-benzocoumarin
19	2,6-Dinitro-9-fluorenone	42	4-Nitro-*p*-terphenyl
20	2-Nitronaphthalene	43	6-Nitrochrysene
21	3-Nitro-9-fluorenone	44	3-Nitroperylene
22	5-Nitroindan	45	6-Nitrobenzo[*a*]pyrene
23	2-Nitrobiphenyl		

phase; k' is the nitro-PAH capacity factor and φ the volume fraction of the organic modifier). The relative variation of S, ($\delta S/S_{ave}$), was calculated from S_{exp} from the slope of $\log k'$ *vs.* φ plots, minus S_{cal} from S *vs.* $\log k_0$ (k' at 100% H_2O) plots, divided by the average S_{exp} for each solvent type times one hundred. (See Tables II and VI in ref. 37 for details). The results of the $\delta S/S_{av}$ calculation for both organic modifiers are summarized in Table I (see Table II for compound No. and identity). As anticipated, acetonitrile was predicted to provide optimum solvent type for gradient nitro-PAH separation in general, evidenced by the wider $\delta S/S_{av}$ compound variations as compared to methanol.

In order to substantiate these findings and to reduce development time, a computer program (DryLab G) capable of calculating relative peak resolution under differing gradient conditions was employed. Twenty-two model compounds were selected representing a wide range of nitro-PAHs. Two experiments were performed for each solvent type linearly programmed between 5 and 100% organic modifier in water at 30 and 90 min, respectively. The flow-rate through the column was 0.5 ml/min. Table III denoted the retention times obtained and bands where peak valleys exceeded 50% (resolution, $R_s < 1.1$). Plots of the relative band resolution for each organic modifier as a function of gradient time suggested an optimum linear mobile phase program of 24% to 80% acetonitrile in water in 31 min. The operating conditions were established after simulation to reduce the overall elution time by decreasing the gradient range (*i.e.*, from 5–100% acetonitrile to 24–80% acetonitrile)

TABLE III

NITRO-PAH RETENTION TIMES

Separation conditions: 5% to 100% organic solvent in water in 30 and 90 min, respectively, at 35°C and 0.5 ml/min flow-rate.

No.	Acetonitrile		Methanol	
	30 min	90 min	30 min	90 min
3	11.63	19.68	12.77	21.25
4	12.82	23.42	14.39	28.03
7	14.62	28.23	17.29	36.40
8	15.03	30.02	18.28	39.30
9	16.23	33.85	19.89	44.22
12	17.68	37.72	20.92	46.20
14	18.28	39.02	22.25[b]	50.12
20	18.73[a]	40.28	22.25[b]	51.57
23	19.10[a]	42.21	22.68	55.85[a]
24	19.85[a]	44.05	23.75	56.18[a]
25	19.92[a]	45.23	24.70	58.80
27	20.58	46.19	25.73	61.82
31	21.24	47.88	26.35	63.99
33	21.89	49.75	27.21	66.85
34	22.17[a]	50.55	28.29[a]	68.82
35	22.56[a]	51.97	28.60[a]	69.88
37	23.30[a]	53.77	28.92[a]	70.99
38	23.72[a]	54.30	29.61[a]	73.32
42	24.79	58.12	29.99[a]	74.62
43	25.23	59.02	30.76[b]	78.51[b]
44	26.89	63.47	30.89[b]	78.51[b]
45	27.21	64.49	30.76[a]	89.50

[a] Indicating band pairs where more than 50% valley was found.
[b] Indicating that two nitro-PAH compounds coeluted.

while maintaining gradient slope. The gradient program yielded a minimum resolution compared to all other model compounds for band pair 33/34, *viz.*, $R_s = 1.9$. Table IV illustrated retention time reproducibility and the percent difference between experimental and predicted retention times at optimum gradient conditions (see Fig. 1 for band spacing comparisons; R_s for 33/34 and 42/43 were 2.1 and 1.7, respectively).

Evident from Table III and supported by chromatographic modelling and simulation was the relatively poorer peak resolutions obtained for the model compounds with methanol–water. For example, the maximum resolution for the most poorly resolved band pair was less than 0.13 for band pairs 43/44 as might be expected from Table II findings.

High back pressures were observed at 25°C for volume fractions less than 60% organic with the narrow bore C_{18} column. The high back pressure effectively restricted the useful gradient profile working range for study. Increasing the column temperature resulted in much earlier elution of the model compounds. For example, compound 45 eluted at 31.2 min (35°C) *versus* 28.6 min (55°C) concomitant with coelution of compounds 24/25 and 42/43 with acetonitrile–water mobile phase conditions.

TABLE IV

EXPERIMENTAL AND PREDICTED RETENTION TIMES

Separation conditions: 31-min gradient from 24% to 80% acetonitrile in water at 35°C and 0.5 ml/min flow-rate. Experimental and predicted chromatograms were shown in Fig. 1.

No.	Retention time (min) ± S.D.		
	Experimental ($n=3$)	Predicted	% Difference
3	6.64 ± 0.01	5.94	11.8
4	7.98 ± 0.02	7.54	5.8
7	10.45 ± 0.02	10.28	1.7
8	11.06 ± 0.02	11.01	0.5
9	13.10 ± 0.02	13.06	0.3
12	15.38 ± 0.03	15.43	0.3
14	16.23 ± 0.02	16.33	0.6
20	16.96 ± 0.03	17.09	0.8
23	17.76 ± 0.02	17.93	1.0
24	18.98 ± 0.02	19.13	0.8
25	19.47 ± 0.04	19.51	0.2
27	20.15 ± 0.03	20.38	1.1
31	21.18 ± 0.04	21.45	1.3
33	22.26 ± 0.04	22.56	1.3
34	22.73 ± 0.04	23.04	1.4
35	23.49 ± 0.06	23.79	1.3
37	24.64 ± 0.05	24.96	1.3
38	25.12 ± 0.10	25.49	1.5
42	27.50 ± 0.02	27.53	0.1
43	27.92 ± 0.02	28.18	0.9
44	30.62 ± 0.02	30.92	1.0
45	31.19 ± 0.02	31.50	1.0

Increasing column temperature lessened optimum resolution between band pairs to point where it made little sense for analytical applications. In contrast, relative separation remained the same along with reduced elution times when 10% methanol was added to the acetonitrile–water gradient. For example, compound 45 eluted at 27.5 min as compared to 31.2 min for the binary system.

Retention index experiments were performed for Table II nitro-PAHs. Listed in Table V are the retention index values when the 24% to 80% acetonitrile in water gradient was employed at 35°C, 45°C and at 35°C with a constant 10% methanol added. It was apparent that index values varied greatly as a function of HPLC operating conditions. The amount that a particular I value differed between operating conditions depended largely on the competitive differences in the interactions between the bracketing compounds *and* the target compound with the mobile phase.

Experiments were performed to evaluate three HPLC nitro-specific detection methods. Figs. 1 and 2 illustrate typical diode-array detection responses for the model compounds at 254 nm as well as 230 and 330 nm, respectively. Although nitro-PAH detection limits were wavelength-dependent and moderate (2 to 12 ng injected; signal-to-noise ratio 3:1), dual-wavelength absorbance ratio measurements (see Table VI) can be used for compound identification at the concentration levels found for the

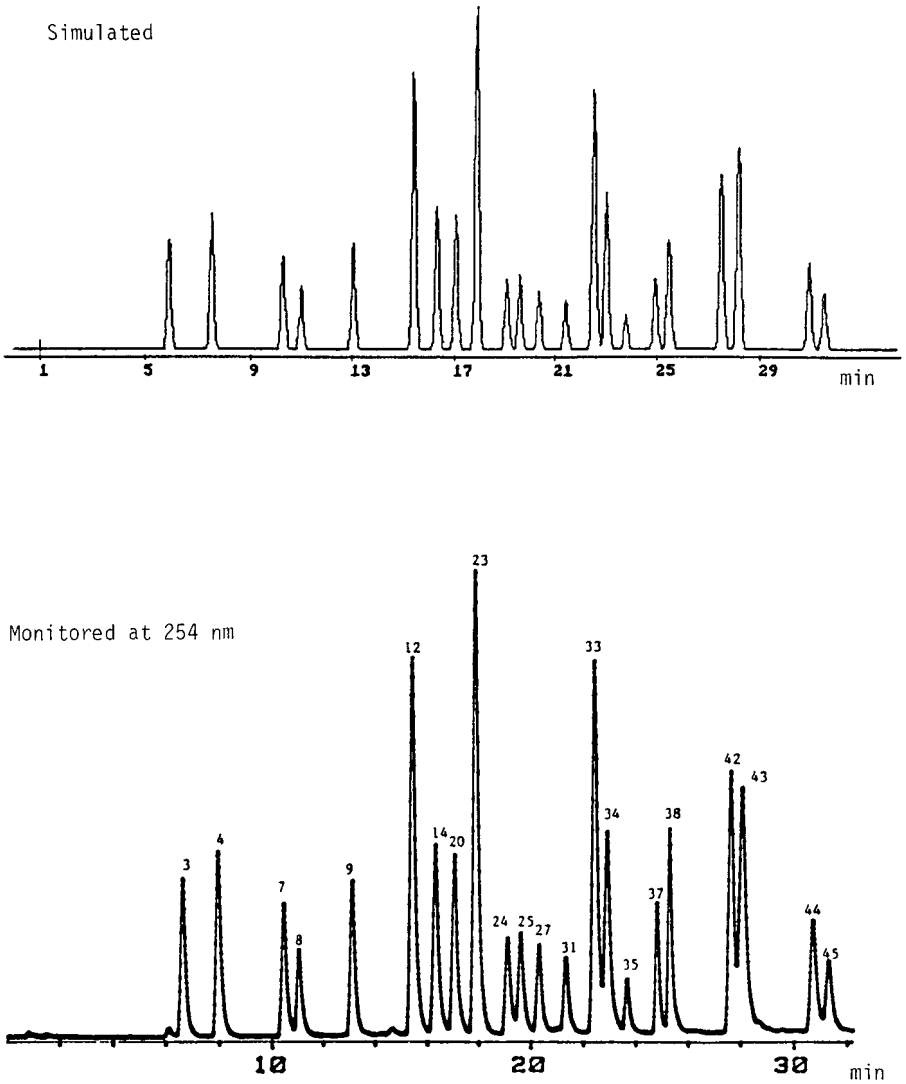

Fig. 1. Simulated and experimental (at 254 nm) chromatograms.

most prevalent nitro-PAHs in environmental samples, *e.g.*, 1-nitropyrene, 1-nitronaphthalene, 2-nitrofluorene. Advantage of UV spectra and chromatographic peak signal superimposition at the three wavelengths, respectively, provided differentiation between closely eluting compounds.

Post-column fluorescence detection was evaluated employing a 4 cm × 2.1 mm reducer column containing a zinc–silica gel (1:1, w/w) mixture. Ammonium acetate (30 m*M*) was added to the acetonitrile–water mobile phase to ensure quantitative

TABLE V

RETENTION INDEX, *I*, OF NITRO-PAHs ± S.D. ($n=3$)

Conditions: (A) 31-min linear gradient between 24 and 80% acetonitrile in water at 0.5 ml/min flow-rate and 35°C; (B), as (A) except 45°C; (C), as (A) except 10% methanol added.

No.	*I*		
	Conditions A	Conditions B	Conditions C
1	57.90 ± 0.16	55.90 ± 0.16	61.20 ± 0.13
2	78.96 ± 0.17	81.52 ± 0.10	76.92 ± 0.31
3	94.82 ± 0.22	99.16 ± 0.27	96.28 ± 0.32
4	95.73 ± 0.21	99.20 ± 0.23	99.31 ± 0.15
5	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
6	109.30 ± 0.24	117.08 ± 0.26	104.79 ± 0.21
7	126.12 ± 0.21	126.39 ± 0.21	121.39 ± 0.27
8	133.85 ± 0.24	138.34 ± 0.24	134.03 ± 0.28
9	159.97 ± 0.30	158.34 ± 0.30	152.57 ± 0.24
10	185.27 ± 0.18	172.93 ± 0.18	174.61 ± 0.26
11	187.89 ± 0.23	179.90 ± 0.22	184.01 ± 0.31
12	189.08 ± 0.23	181.29 ± 0.34	187.11 ± 0.28
13	190.95 ± 0.04	188.44 ± 0.04	189.50 ± 0.30
14	200.00 ± 0.00	200.00 ± 0.00	200.00 ± 0.00
15	202.49 ± 0.10	204.17 ± 0.10	201.16 ± 0.32
16	205.34 ± 0.59	206.31 ± 0.57	207.57 ± 0.37
17	205.49 ± 0.10	206.34 ± 0.09	210.43 ± 0.32
18	210.43 ± 0.11	208.29 ± 0.28	211.42 ± 0.25
19	210.80 ± 0.30	208.29 ± 0.28	211.45 ± 0.43
20	211.24 ± 0.34	210.73 ± 0.34	212.15 ± 0.32
21	216.55 ± 0.28	216.27 ± 0.28	216.13 ± 0.29
22	219.02 ± 0.11	219.35 ± 0.11	219.34 ± 0.22
23	223.56 ± 0.29	224.79 ± 0.29	219.78 ± 0.33
24	242.20 ± 0.31	242.57 ± 0.32	240.33 ± 0.36
25	249.84 ± 0.57	248.41 ± 0.37	250.98 ± 0.33
26	257.91 ± 0.03	254.84 ± 0.03	259.55 ± 0.13
27	260.23 ± 0.39	255.07 ± 0.39	259.92 ± 0.34
28	261.85 ± 0.13	258.57 ± 0.10	260.19 ± 0.34
29	269.71 ± 0.11	260.42 ± 0.39	265.84 ± 0.35
30	271.12 ± 0.32	271.78 ± 0.34	274.46 ± 0.08
31	276.06 ± 0.11	275.59 ± 0.13	277.34 ± 0.36
32	283.13 ± 0.03	281.61 ± 0.03	284.59 ± 0.06
33	292.77 ± 0.05	292.83 ± 0.05	294.00 ± 0.32
34	300.00 ± 0.00	300.00 ± 0.00	300.00 ± 0.00
35	309.89 ± 0.27	297.04 ± 0.37	312.33 ± 0.09
36	314.57 ± 0.41	314.05 ± 0.42	318.67 ± 0.38
37	336.82 ± 0.37	337.94 ± 0.03	340.85 ± 0.37
38	346.01 ± 0.23	346.51 ± 0.24	347.66 ± 0.42
39	347.70 ± 0.03	337.47 ± 0.03	348.56 ± 0.21
40	349.23 ± 0.17	337.39 ± 0.17	353.14 ± 0.04
41	390.82 ± 0.08	378.39 ± 0.08	390.17 ± 0.15
42	391.93 ± 0.24	393.49 ± 0.24	390.90 ± 0.23
43	400.00 ± 0.00	400.00 ± 0.00	400.00 ± 0.00
44	482.45 ± 0.19	479.43 ± 0.19	484.23 ± 0.29
45	500.00 ± 0.00	500.00 ± 0.00	500.00 ± 0.00

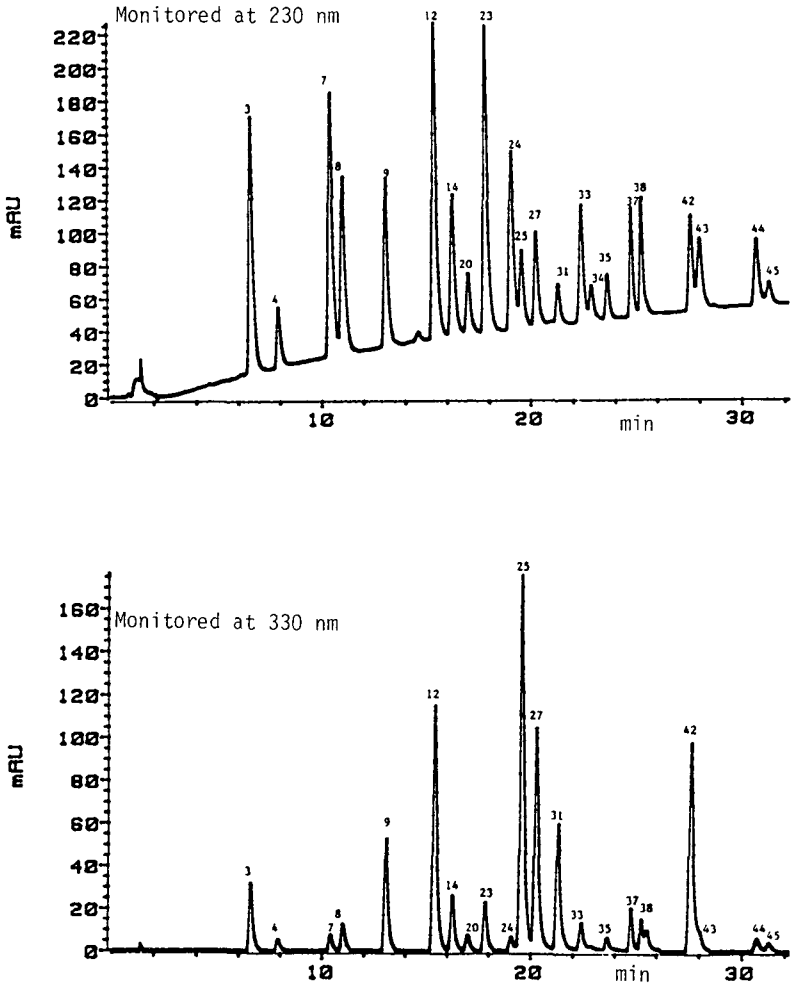

Fig. 2. Typical diode-array detection responses at 230 nm and 330 nm.

conversion (> 99%) of nitro-PAHs to the corresponding amino analogues. Fig. 3 illustrates the fluorescence response for the model compounds at 260/430 nm ($\lambda_{ex}/\lambda_{em}$). It was evident that compared to diode-array detection, fluorescence detection provided much better selectivity and sensitivity. The fluorescence dynamic range spanned four orders of magnitude for most of the model compounds with minimum detectable quantities between 10 and 15 pg of compound injected (see Table VII). Shown in Table VIII are the excitation and emission wavelengths for twenty-six nitro-PAHs (see ref. 35 for the remaining 19 nitro-PAHs).

Lastly, it was demonstrated that gas phase NO · /O_3-specific chemiluminescence detection can be employed with acetonitrile-water mobile phase compositions

TABLE VI

DUAL-WAVELENGTH ABSORBANCE RATIO VALUES FOR NITRO-PAHs ± S.D. ($n=3$)

ND = At least one signal not detected (100 ng/μl compound injected) at the indicated wavelength pair.

No.	A_{230}/A_{254}	A_{330}/A_{254}	A_{230}/A_{330}
1	0.776 ± 0.002	0.306 ± 0.001	2.536 ± 0.010
2	3.481 ± 0.153	0.792 ± 0.002	4.395 ± 0.023
3	0.769 ± 0.002	0.111 ± 0.001	6.928 ± 0.048
4	1.936 ± 0.012	1.860 ± 0.004	1.041 ± 0.002
5	0.375 ± 0.002	ND	ND
6	0.535 ± 0.004	ND	ND
7	4.174 ± 0.056	0.216 ± 0.004	19.324 ± 0.090
8	4.492 ± 0.031	0.566 ± 0.011	7.936 ± 0.035
9	2.544 ± 0.084	1.359 ± 0.008	1.872 ± 0.034
10	0.807 ± 0.004	0.163 ± 0.000	4.951 ± 0.012
11	1.244 ± 0.014	1.190 ± 0.011	1.045 ± 0.002
12	1.890 ± 0.023	1.216 ± 0.003	1.554 ± 0.015
13	1.175 ± 0.002	0.263 ± 0.000	4.468 ± 0.008
14	1.676 ± 0.038	0.545 ± 0.003	3.075 ± 0.050
15	0.854 ± 0.003	0.019 ± 0.000	44.948 ± 0.241
16	ND	0.121 ± 0.005	ND
17	0.893 ± 0.004	0.416 ± 0.004	2.129 ± 0.004
18	0.969 ± 0.002	ND	ND
19	0.978 ± 0.003	0.407 ± 0.001	2.404 ± 0.007
20	0.822 ± 0.004	0.173 ± 0.000	4.751 ± 0.023
21	0.568 ± 0.021	0.103 ± 0.002	5.515 ± 0.040
22	1.856 ± 0.009	1.200 ± 0.001	1.547 ± 0.012
23	1.566 ± 0.011	0.192 ± 0.002	8.114 ± 0.015
24	3.746 ± 0.100	0.271 ± 0.003	13.823 ± 0.213
25	1.872 ± 0.036	7.237 ± 0.004	0.259 ± 0.004
26	0.572 ± 0.003	ND	ND
27	2.569 ± 0.052	4.736 ± 0.049	0.542 ± 0.006
28	0.639 ± 0.002	0.049 ± 0.000	13.041 ± 0.041
29	1.011 ± 0.004	0.171 ± 0.000	5.912 ± 0.023
30	1.791 ± 0.013	2.873 ± 0.061	0.623 ± 0.011
31	1.325 ± 0.011	3.178 ± 0.042	0.417 ± 0.003
32	1.102 ± 0.001	ND	ND
33	0.767 ± 0.002	0.127 ± 0.001	6.039 ± 0.031
34	0.443 ± 0.006	ND	1.073 ± 0.009
35	0.457 ± 0.000	0.413 ± 0.002	21.762 ± 0.002
36	2.061 ± 0.043	0.021 ± 0.001	3.412 ± 0.050
37	2.007 ± 0.019	0.604 ± 0.002	7.017 ± 0.067
38	1.406 ± 0.012	0.286 ± 0.003	ND
39	1.532 ± 0.011	0.233 ± 0.020	5.560 ± 0.010
40	1.068 ± 0.043	0.312 ± 0.005	3.423 ± 0.082
41	0.877 ± 0.000	1.489 ± 0.003	0.589 ± 0.001
42	0.887 ± 0.001	1.499 ± 0.001	0.592 ± 0.001
43	0.728 ± 0.004	ND	ND
44	1.411 ± 0.015	0.196 ± 0.000	7.199 ± 0.025
45	0.770 ± 0.004	0.230 ± 0.000	3.348 ± 0.0.17

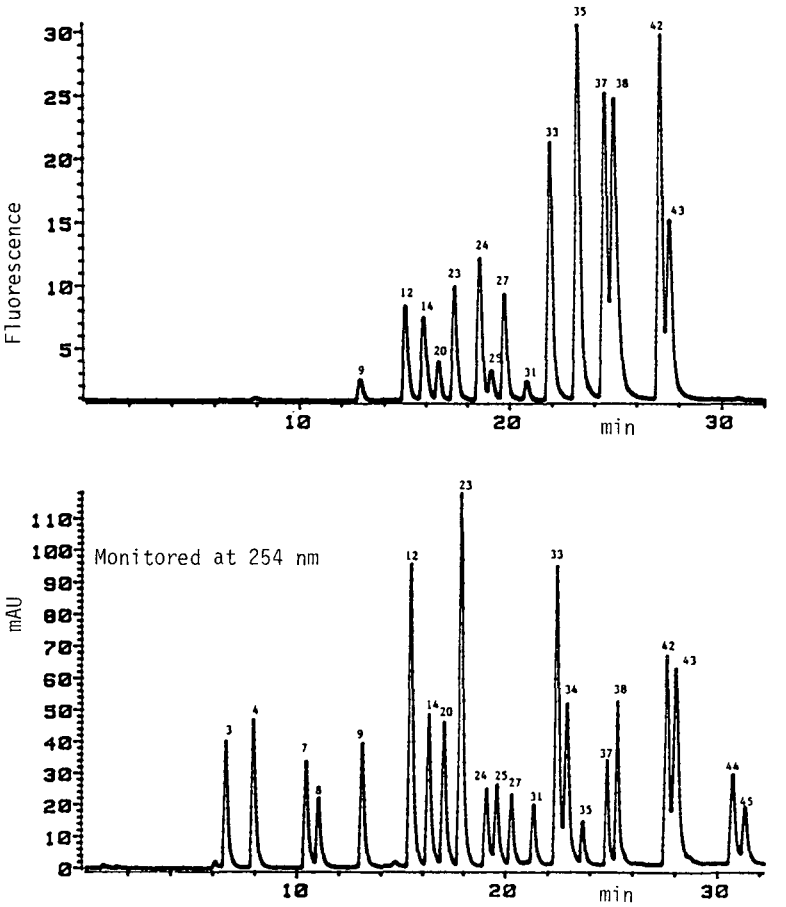

Fig. 3. Fluorescence detection responses at 260/430 nm ($\lambda_{ex}/\lambda_{em}$).

TABLE VII

FLUORESCENCE DETECTION LINEAR DYNAMIC RANGE AND MINIMUM DETECTABLE AMOUNTS

DL = Minimum detectable amount.

Compound	Intercept	Slope	Correlation	DL (pg)
1-Nitronaphthalene	−0.21	3.55	0.999	10
2-Nitrofluorene	0.38	1.54	0.999	15
9-Nitroanthracene	0.17	0.51	0.999	15
1-Nitropyrene	0.33	0.59	0.999	10

TABLE VIII

EXCITATION AND EMISSION WAVELENGTHS FOR NH_2-PAH ANALOGUES

No.	$\lambda_{ex}/\lambda_{em}$ (nm)	No.	$\lambda_{ex}/\lambda_{em}$ (nm)
2	245/432	22	232/350
3	245/432	23	227/394
4	230/485	24	244/414
7	245/490	25	292/387
8	245/432	26	231/407
9	229/417	27	285/367
10	247/420	28	232/399
13	228/372	29	230/335
15	264/417	30	227/359
16	264/495	31	285/370
17	232/387	32	243/407
18	290/365	33	247/430
19	232/387	42	295/425
21	245/407	44	227/540

[30]. Chemiluminescence detection responded linearly (between 70 and 1000 ng/compound injected) *and* on a per mole of NO_2 per mole of compound basis. It was shown that the chemiluminescence detection limit was both volume-fraction dependent as well as flow-rate dependent presumably due to pressure variations within the reaction chamber relative to the $NO \cdot /O_3$ reaction. An interface specifically designed to separate solvent-from-analyte before the latter reached the detector had been demonstrated for nitro-PAHs [39]. For the compounds evaluated, the HPLC–interface–chemiluminescence detection yielded nitro-PAH linear dynamic ranges comparable to the GC–chemiluminescence detection [21] with minimum detection levels of *ca.* 50 pg/compound injected; resulting in two orders of magnitude greater sensitivity than HPLC-chemiluminescence detection.

The gradient system described above, the HPLC- and GC-derived nitro-PAH retention indexes (and GC retention prediction models) in combination with nitro-specific detection capability should provide unparalleled capability for the identification and quantification of nitro-PAHs in complex environmental samples.

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Semi-preparative separation of polyhydroxylated sterols using a β-cyclodextrin high-performance liquid chromatography column

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ABSTRACT

A series of closely related polyoxygenated sterols from the sponge *Dysidea etheria* was resolved by high-performance liquid chromatography on a β-cyclodextrin column, a stationary phase apparently not used previously for the preparative separation of natural products. Analysis of the chromatographic behavior of the various sterols on the β-cyclodextrin column indicated that formation of an inclusion complex was not the separation mechanism involved in this case. Rather, the comparative analysis suggested that hydrogen bonding between the solute molecules and the hydroxyl groups on the rim of the β-cyclodextrin cavities best explained the results observed.

INTRODUCTION

Cyclodextrins are D(+)-glucopyranose units connected by α-(1,4) bonds to form cyclic oligosaccharides [1]. An interesting high-performance liquid chromatography (HPLC) column stationary phase has been produced by covalently bonding these molecules to silica gel via a seven to nine atom spacer [2]. β-Cyclodextrin is a non-ionic, cyclic, chiral carbohydrate that has the shape of a hollow truncated cone in which the side of the torus with the greater circumference has fourteen secondary hydroxyl groups, while that of the smaller side has seven primary hydroxyl groups [3]. Due primarily to favorable hydrophobic (interior of cavity) and/or hydrogen bonding interactions (hydroxyl containing faces), guest molecules can bind to and form inclusion complexes with β-cyclodextrin. This host–guest spatial interaction can result in appreciably different complexes from a pair of guests which differ only slightly in structure.

β-Cyclodextrin HPLC stationary phases have probably found their most important utility as chiral stationary phases (CSPs) [4–6]. The chiral recognition has been rationalized in term of several possible interactions between the β-cyclodextrin

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host and guest racemates [7]. Guest solutes can interact via Van der Waals–London dispersion forces with the hydrophobic cavity. In order for this to be effective, however, the molecule must fit tightly within the cavity. In most cases, the cylindrical binding characteristics to a racemic guest molecule are too symmetrical to induce large enantioselectivities. Consequently, there must be other points of interaction in order to achieve chiral recogition. The fourteen chiral secondary alcohols situated around the mouth of the cavity, as well as the seven primary hydroxyl groups situated around the opposite rim provide a number of these possible stereospecific interactions.

Numerous other types of closely related compounds that are not enantiomers, but which are also poorly resolved on more traditional columns, can be readily separated on this stationary phase. A variety of structural isomers has been resolved, including positional and geometrical (*cis–trans*) isomers of prostaglandins [8,9], polycyclic aromatic hydrocarbons, and steroid epimers [10]. These results encouraged us to apply the β-cyclodextrin column to the separation of the closely related polyhydroxylated sterols from the sponge *Dysidea etheria*. To the best of our knowledge, this stationary phase had not been used previously for the preparative separation of natural products.

EXPERIMENTAL

Instrumentation

HPLC separations were performed on a Perkin–Elmer Series 3B liquid chromatograph using a Knauer differential refractometer for detection. The flow-rate was either 1.0 or 3.0 ml/min as noted; the injection volume was 40 μl.

Column

A Hamilton PRP-1 column (300 mm × 7 mm I.D.) was used for the reversed-phase separations. An ASTEC β-cyclodextrin column (250 mm × 4.6 mm I.D.) was used for the β-cyclodextrin separations.

Samples

All sterols were naturally occurring sterols isolated from the sponge *Dysidea etheria*. Their structures were solved by spectroscopic methods [11,12]. Injection concentrations were approximately 10 mg/ml for all sterols; 0.5–1.0 mg of sterol mixtures were injected per run.

RESULTS AND DISCUSSION

The extremly polar polyhydroxylated sterols which we encountered in extracts of *D. etheria* were not amenable to normal-phase chromatography. While some of the sterols could be purified on octadecylsilyl bonded phase columns, some mixtures remained unresolved. The β-cyclodextrin column, however, using acetonitrile–water (1:1) as the mobile phase, gave nearly baseline separation of sterols **1** and **3** (Fig. 1). This initial success was followed by the separations of **4** and **5** (Fig. 2), **2** and **6** (Fig. 3), and **7** and **8** (Fig. 4) using the β-cyclodextrin column with various ratios of acetonitrile–water. What these separations had in common was that the nuclear framework and functional group substitution patterns on the sterol nuclei were the

Fig. 1. β-Cyclodextrin separation of **1** and **3**. Acetonitrile–water (1:1); 1.0 ml/min.

same for both members of the separated pairs, while the differences resided only in the side chains of each pair (Table I).

Once the sterols were purified by these β-cyclodextrin chromatographies and their structures determined, it became obvious that we had not separated epimeric pairs as we had originally assumed, but rather analogues in which the side chains of each member of a pair differed only slightly from one another. The sterols separated in the chromatographies shown in Figs. 1, 3, and 4 differed in that one member of each set contained a carbon–carbon double bond at C-22 and an extra methyl group at C-24 in the C_8 sterol side chain. The fact that these congeners co-eluted by reversed phase chromatography in all three cases (non-acetylated, mono-acetylated, and di-acetylated) with sterols possessing a normal C_8H_{17} side chain indicated that the apparent polarity of these two very different side chains must be similar. It was interesting to note that whereas the reversed-phase column could separate sterols differing only by

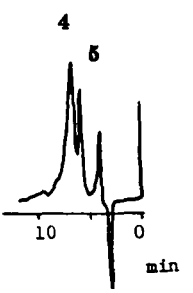


Fig. 2. β-Cyclodextrin separation of **4** and **5**. Acetonitrile–water (9:11); 1.0 ml/min.

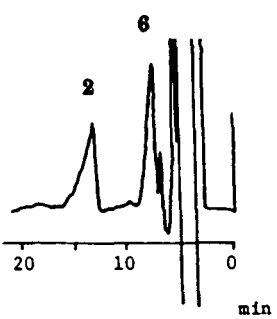


Fig. 3. β-Cyclodextrin separation of **2** and **6**. Acetonitrile–water (4:1); 1.0 ml/min.

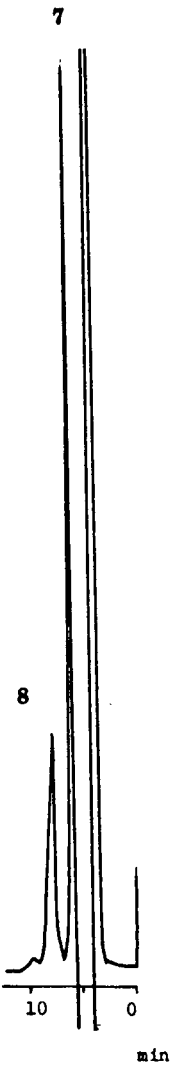
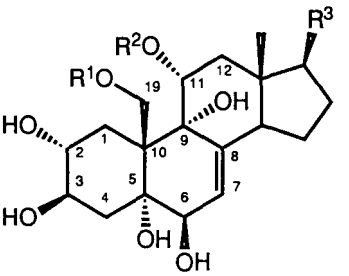
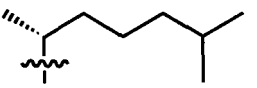
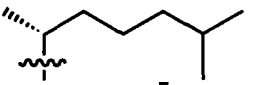
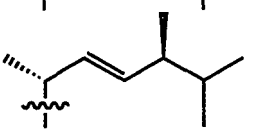
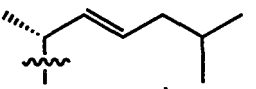
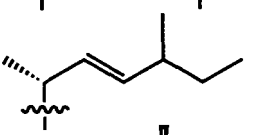
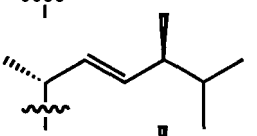
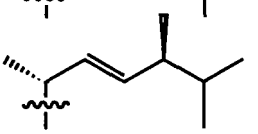
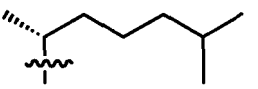


Fig. 4. β-Cyclodextrin separation of **7** and **8**. Acetonitrile–water (5:1); 1.0 ml/min.

Δ^{22} double bond from those having a normal, saturated side chain, the addition of a methyl group at C-24, along with the unsaturation, posed a very difficult problem for a reversed phase separation.

The remaining β-cyclodextrin separation, which is shown in Fig. 2, was interesting in that it represented a separation between two much more closely related sterols than the other three separations. Sterols **4** and **5** were positional isomers of one another, since **4** possessed a normal C-26, 27 isopropyl group, whereas **5** was missing the C-27 methyl, but had a methyl branch at C-24 instead. Djerassi and co-workers [13,14] have reported the separation of sterols differing by these same side chains situated on



	R^1	R^2	R^3
1	Ac	Ac	
2	H	H	
3	Ac	Ac	
4	Ac	Ac	
5	Ac	Ac	
6	H	H	
7	H	Ac	
8	H	Ac	

identical sterol nuclei. Only relative retention time (RRT) data were given in both instances, which makes it difficult to determine how well resolved the two components were. In both cases, though, the RRT values were close enough (0.72 to 0.76, and 0.70 to 0.75) to indicate that these closely related compounds were not as well resolved using reversed-phase chromatography as **4** and **5** were on the β-cyclodextrin column.

TABLE I

EXAMPLES OF β-CYCLODEXTRIN SEPARATIONS OF 5α-CHOLESTA-7-ENE-2α, 3α, 5α, 6β, 9α, 1α, 19-HEPTOL STEROLS

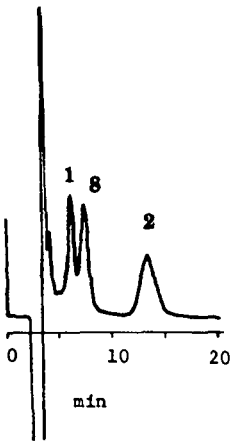
t_R = Retention time; k' = capacity factor; R_s = resolution.

Eluent: acetonitrile–water	Sterol	No. of acetates	Side chain substitutions	t_R (min)	k'	R_s
1:1	1[a]	11,19-Diacetate	(Normal) C_8H_{17}	8.6	3.3	0.93
	3[a]	11,19-Diacetate	Δ^{22}, 24-Methyl	6.5	2.3	
9:11	4[b]	11,19-Diacetate	Δ^{22}	6.4	1.3	0.71
	5[b]	11,19-Diacetate	Δ^{22}, 27-Nor, 24-methyl	5.6	1.0	
4:1	2[c]		(Normal) C_8H_{17}	13.5	3.8	3.0
	6[c]		Δ^{22}, 24-Methyl	7.5	1.7	
5:1	7[d]	11-Acetate	Δ^{22}, 24-Methyl	6.1	0.9	1.05
	8[d]	11-Acetate	(Normal) C_8H_{17}	7.9	1.4	

There seems to be general agreement that when aqueous–organic mobile phases are used, the mechanism of separation on the β-cyclodextrin column is the formation of inclusion complexes with solute molecules [1–3,15–17]. Armstrong *et al.* [18] have suggested that in order for chiral recognition to be effective a compound needs at least one aromatic ring, preferably two, and that the chiral center must be in close proximity to the ring moieties. The formation of an inclusion complex, however, has also been invoked to explain the separation mechanism of diastereomers and geometric isomers which lack aromatic rings such as steroid epimers [3,19] and prostaglandins [8,9]. In the separation of *cis–trans* isomers of prostaglandins [9], for example, it was assumed that an incluson complex would form in such a way that the polar functional groups would stay away from the hydrophobic center and/or allow hydrogen bonding with the hydroxyl groups on the edge of the cavity. The elution order of *cis* isomer, followed by *trans,* was explained by the more compact conformation of the *cis* isomer, which allowed for a tighter complex.

It appeared to us, however, that even under typical reversed-phase conditions (acetonitrile–water) the separations of the polyhydroxylated sterols did not proceed by an inclusion complex mechanism. We decided to perform some simple experiments using the sterols already isolated and characterized in order to determine what interactions were responsible for separating these compounds.

The first series of these polyhydroxylated sterols had identical oxidation patterns and side chains, but varying degrees of acetylation. The elution order on the β-cyclodextrin column, shown in Fig. 5, was: di-acetate (**1**), followed by mono-acetate (**8**), followed by the non-acetylated sterol (**2**). This was the opposite of the elution pattern observed for the same compounds injected onto a reversed-phase column where the elution order was most polar to non-polar, as expected (Fig. 6). These data seemed to indicate that the non-polar side chain of the sterols was not necessarily included into the hydrophobic cavity, if this were the case, the elution order should have approximated more closely that of the reversed-phase separation.



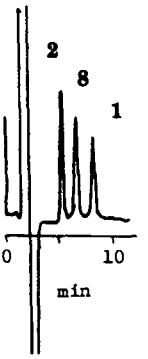


Fig. 5. β-Cyclodextrin separation of **1**, **2** and **8**. Acetonitrile–water (1:1); 1.0 ml/min.

Fig. 6. Reversed phase separation of **1**, **2** and **8**. Acetonitrile–water (1:1); 3.0 ml/min.

The next experiment involved the coinjection (Fig. 7) of a 5α- (**2**), and a 5β-polyhydroxysterol (**9**), both with the same substitution, acetylation, and side chain alkylation patterns. The conformations of these two molecules are so different about the A and B rings that an inclusion complex formed with this region of the molecule should have dramatically different characteristics for the two epimers. What was found, however, was that the two compounds had virtually the same retention times, and hence were not resolved at all. These two compounds were well separated on the reversed-phase column, with the *cis* compound eluting after the *trans*. It appeared that the hydroxyl groups of the sterols were hydrogen-bonding with the hydroxyl groups around the perimeter of the β-cyclodextrin molecule rather than forming

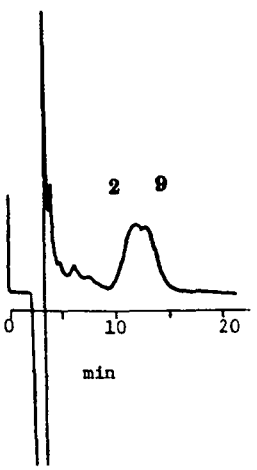
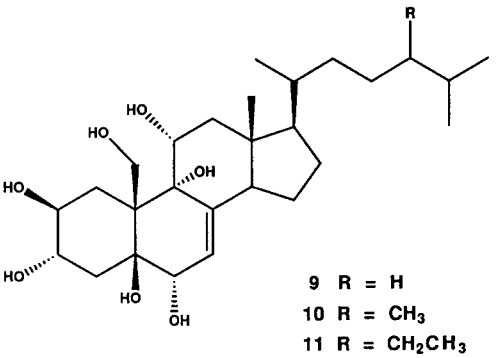
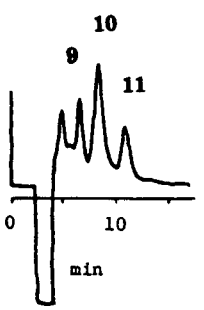


Fig. 7. β-Cyclodextrin separation of **2** and **9**. Acetonitrile–water (1:1); 1.0 ml/min.



inclusion complexes. Both of these experiments, therefore, pointed to the conclusion that polyhydroxylated sterols are retained on the β-cyclodextrin column through relatively non-specific hydrogen bonding interactions, and that inclusion complex formation with either the hydrophobic side chain or the sterol nucleus was not occurring. In this regard, the separation mechanism was analogous to that of a diol column. This behavior has already been observed for the normal-phase operation of β-cyclodextrin columns [15].

The third experiment involved the elution of a homologous series of 5β-hydroxysterols with the same ring substitutions but with slightly different side chains. The three sterols (**9**, **10** and **11**) differed by the degree of alkylation at the C-24 position. The sterols eluted in the order of increasing alkylation (Fig. 8), which was the same order as that observed for separation on a reversed phase column (Fig. 9). This observation was also in agreement with the separation mechanism proposed above, since hydrogen bonding of the hydroxyl groups of the sterols to the hydroxyl groups on the β-cyclodextrin molecule would allow the non-polar side chain to determine the elution order by partitioning into the mobile phase. If this were the case, then the more highly branched side chain would elute last in the polar mobile phase, as was observed. This by itself, however, does not preclude the possibility that the non-polar side chains are included into the hydrophobic cavity. When taken in conjunction with the previous



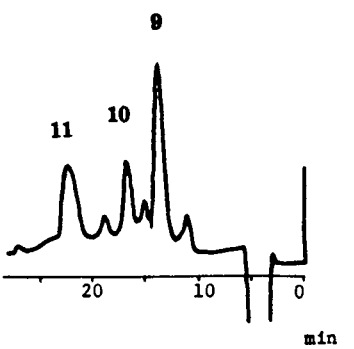


Fig. 8. β-Cyclodextrin separation of **9**, **10** and **11**. Acetonitrile–water (1:1); 1.0 ml/min.

Fig. 9. Reversed phase separation of **9**, **10** and **11**. Acetonitrile–water (2:1); 3.0 ml/min.

observations, though, the evidence seems to support overwhelmingly hydrogen bonding to the polar portions of the sterol molecules as the primary separation mechanism.

In the light of these data, it would be prudent to reexamine the interactions responsible for separations of other compounds by this versatile stationary phase, since insight into this might allow an even greater array of substances to be resolved. While there is no doubt that many separations, particularly those of enantiomers, are the result of inclusion complexing, it would be interesting to find out if enantiomeric or diastereomeric separations can occur in the absence of this mechanism. The β-cyclodextrin column would certainly seem to be another powerful tool for the natural products chemist confronted with challenging separation problems.

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Chromatographic resolution of 1,2-amino alcohols on a chiral stationary phase containing N,N′-(3,5-dinitrobenzoyl)-*trans*-1,2-diaminocyclohexane

Theoretical and practical aspects

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ABSTRACT

Optical resolution of several 1,2-diamino alcohols including some β-adrenergic blocking agents (β-blockers) was obtained by high-performance liquid chromatography on a chiral stationary phase containing 3,5-dinitrobenzoyl derivatives of *trans*-1,2-diaminocyclohexane (DACH-DNB) as chiral selectors. After formation of oxazolidin-2-one derivatives, racemic amino alcohols were completely resolved (α values ranging from 1.14 to 1.55 and R_s from 1.2 to 3.3) on a 250 × 4.0 mm I.D. stainless-steel column. Further, some separations on chiral and achiral, coupled columns are reported: they show diastereo- and enantioselectivity for amino alcohols with more than one chiral centre. The method allows the utilization of both spectrophotometric and spectrofluorimetric detectors; moreover the availability of the (*R*,*R*), (*S*,*S*) selectors makes it possible to evaluate enantiomeric excesses higher than 99.9%. Some separations were also carried out with microbore columns (2.0 mm I.D.), which afforded the same performance.

INTRODUCTION

Many drugs or physiologically important compounds are chiral molecules and the optical isomers of these compounds may differ in their pharmacological activities. In some instances differences in the undesirable side-effects of these enantiomers are important.

One of the major goals in biochemical and pharmaceutical research is the provision of optically pure compounds. Nevertheless, it may be worth noting that 467 of the existing 528 chiral synthetic drugs are marketed as racemates and only the remaining 61 are sold as single enantiomers. Indeed, the production of optically pure compounds either by asymmetric synthesis or by crystallization of diastereoisomeric mixtures is usually expensive. Therefore, rapid and sensitive methods for the

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resolution and quantification of the individual optically pure enantiomers are of great importance; chromatographic methods on both the analytical and preparative scale have become of considerable interest in recent years.

Basically, two different approaches are available for the liquid chromatographic resolution of enantiomers: the formation of non-transient diastereoisomeric derivatives by reaction with a chiral derivatizing agent (CDA) followed by chromatography on a non-chiral column, and the formation of transient diastereoisomers by means of a chiral mobile phase additive (CMPA) or by the use of chiral stationary phases (CSPs) [1].

Direct high-performance liquid chromatographic (HPLC) separation on a column packed with a chiral stationary phase is more rapid and suitable for the resolution of a racemic mixture. Further, in many instances this approach allows the recovery of the enantiomorph with a high degree of optical purity [1–4].

Adrenergic blocking agents (β-blockers) are amino alcohols and are widely used as drugs for the treatment of hypertension and angina pectoris. It is well known that the pharmacological properties of these compounds are mainly due to the (*S*)-(−) isomer. So, for example, the (*S*)-(−)-propranolol is 100 times more active than the (*R*)-(+) enantiomer.

Racemic mixtures of β-blockers have been separated as oxazolidin-2-one derivatives using α_1-acid glycoprotein [5] bonded stationary phase or swollen microcrystalline triacetylcellulose [6] on a semipreparative scale. The same racemic mixtures were also successfully resolved using as chiral selectors (*R*)-(−)-(3,5-dinitrobenzoyl)phenylglycine [7] or monosaccharide derivatives chemically bonded to the stationary phase [8].

Optical resolution of β-blocking agents was also performed without derivatization on columns packed with cellulose triphenylcarbamate derivatives supported on silica gel [9] or α_1-acid glycoprotein bonded stationary phase [10]. Taking into account the wide interest in this type of compound, we have carried out the resolution of several chiral amino alcohols, including the most common β-blockers, by using standard and microbore columns packed with the N,N′-3,5-dinitrobenzoyl derivative of *trans*-1,2-diaminocyclohexane as a chiral selector. The above CSP has been successfully used for the separation of racemic sulphoxides, selenoxides [11], anti-inflammatory agents and some β-blocking agents [12,13].

In this paper we present the results of an HPLC study of the above compounds.

EXPERIMENTAL

Apparatus

Analytical liquid chromatography was performed on a Waters Assoc. (Milford, MA, U.S.A.) chromatograph equipped with a Model U6K injector, two Model M510 solvent delivery systems and a temperature control module (TCM). Different detectors were used, including a Model M490 programmable multi-wavelength detector (Waters Assoc.) and a Model LS-5 luminescence spectrometer (Perkin-Elmer, Norwalk, CT, U.S.A.).

Microbore HPLC was performed with a Carlo Erba (Milan, Italy) System 20 equipped with a micro-UVIS 20 UV–visible detector (2-mm optical path length, 250-nl cell volume, time constant τ = 100 ms) and a Rheodyne Model 8125 5-μl loop injector.

Chromatographic data were collected and processed on a Waters Assoc. Model 840 data and chromatography control station.

^1H and ^{13}C NMR spectra were recorded on a Varian (Palo Alto, CA, U.S.A.) XL 300 spectrometer; all resonances are reported in ppm relative to tetramethylsilane (TMS). Mass spectra were recorded with a VG Tritech, (Manchester, U.K.) TS250 mass spectrometer (electron impact 70 eV). IR spectra were recorded as potassium bromide pellets on a Nicolet (Madison, WI, U.S.A.) 5DX Fourier transform (FT)-IR spectrometer.

Chemicals

LiChrosorb Si 100 (5 μm) and HPLC-grade solvents were obtained from Merck (Darmstadt, F.R.G.), 3-glycidoxypropyltrimethoxysilane (GOPTMS) from Janssen (Beerse, Belgium), (1*R*,2*R*)-diaminocyclohexane [(1*R*,2*R*)-DACH], (1*S*,2*S*)-diaminocyclohexane [(1*S*,2*S*)-DACH], 3,5-dinitrobenzoylchloride (DNB-Cl), 1,1′-carbonyldiimidazole (CDI), 20% phosgene solution in toluene, (*R*)- and (*S*)-propranolol (**9**) hydrochloride, (1*R*,2*S*)-ephedrine (**2**), (1*S*,2*R*)-ephedrine (**2**) hydrochloride, (1*R*,2*S*)-norephedrine (**4**) and (1*S*,2*R*)-norephedrine (**4**) hydrochloride from Fluka (Buchs, Switzerland) and (1*R*,2*R*)-ψ-ephedrine (**3**) and (1*S*,2*S*)-ψ-ephedrine (**3**) from Sigma (St. Louis, MO, U.S.A.). Racemic β-blockers were supplied by the following manufacturers: pronethalol (**7**) hydrochloride and atenolol (**14**) from ICI (Macclesfield, U.K.), oxprenolol (**5**) hydrochloride and metoprolol (**8**) tartrate from Ciba-Geigy (Varese, Italy), acebutolol (**12**) hydrochloride from Bayer (Milan, Italy), RBS Pharma (Milan, Italy), SIT (Pavia, Italy) and SPA (Milan, Italy), pindolol (**13**) from Sandoz (Milan, Italy), sotalol (**11**) hydrochloride from Bristol (Latina, Italy) and alprenolol (**1**) hydrochloride from Bik Gulden (Milan, Italy). 1-(2′5′-Dimethoxyphenyl)-2aminoethanol (**10**) was kindly provided by Prof. F. Macchia (Istituto di Chimica Organica, Università di Pisa, Italy). Betaxolol (**6**) was extracted from pharmaceutical preparations. Other chemicals were of analytical-reagent grade and used as received.

Fig. 1 shows the stuctures of the racemic compounds used.

Preparation of CSP

The CSP was prepared as shown in Fig. 2 [14].

Preparation of oxazolidin-2-one derivatives

Cyclization with 1,1-carbonyldiimidazole (CDI). A 1.1-mmol amount of CDI and 0.5 ml of anhydrous pyridine were added to a solution of 1.0 mmol of racemic amino alcohol in 10 ml of anhydrous tetrahydrofuran and then refluxed for 1 h. The mixture was diluted with diethyl ether (30 ml) and the organic layer was washed with acidified water (3 × 20 ml), dried over magnesium sulphate and evaporated to dryness under reduced pressure (*ca.* 23 mmHg). The residue was chromatographed on a silica gel column and afforded the desired products in 80% yield (not optimized).

Cyclization with phosgene [15]. A stirred mixture of amino alcohol (5 mmol), 10% sodium hydroxide solution (15 ml) and diethyl ether (25 ml) (dichloromethane for acebutolol derivatization) was cooled to 0°C and 20% phosgene solution (5 ml) in toluene was added dropwise. The mixture was stirred for 1 h, then the organic layer was collected, dried over magnesium sulphate and evaporated to dryness under reduced pressure (*ca.* 23 mmHg).

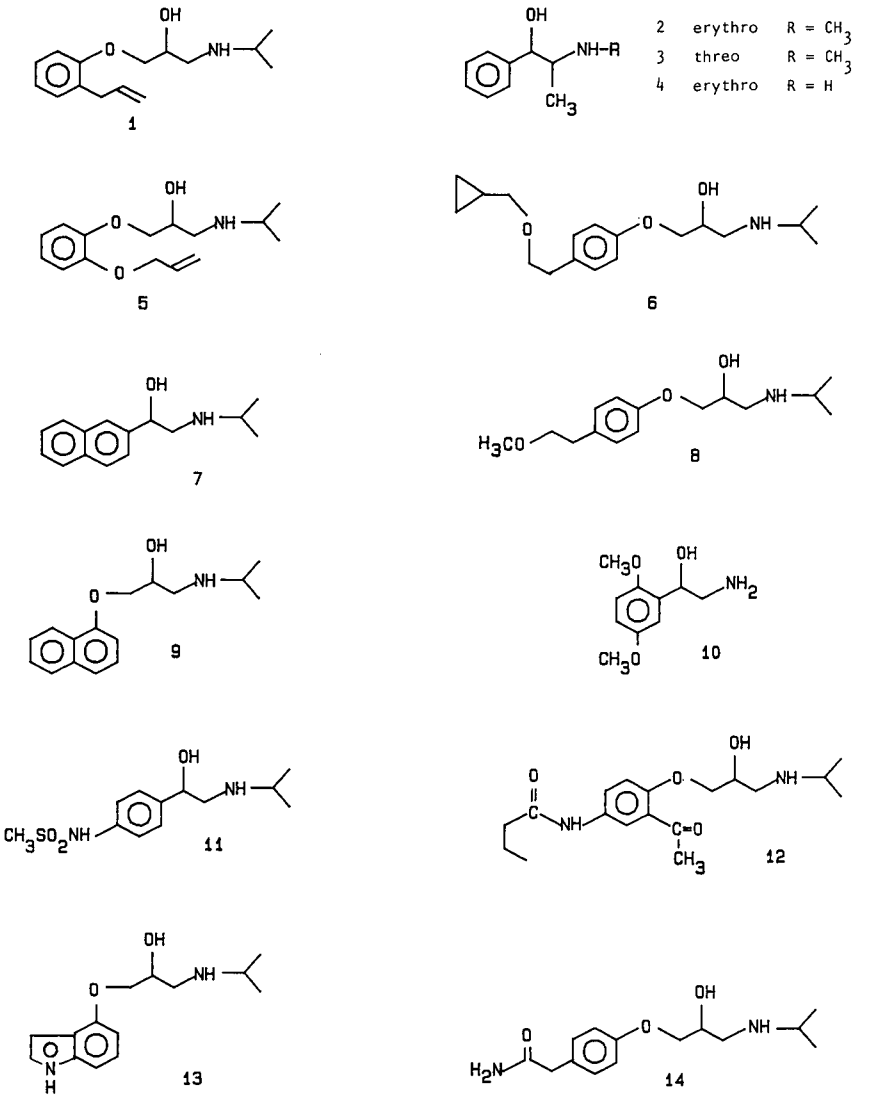

Fig. 1. Structures of amino alcohols resolved as oxazolidin-2-ones on DACH-DNB CSP.

Racemic mixtures of oxazolidin-2-ones were recrystallized from ethyl acetate–*n*-hexane giving pure products in 90% yield (not optimized), and the non-racemic mixtures were purified by filtration on a small column and eluted with a solvent mixture compatible with the substrate polarity.

The physico-chemical characterization of the above derivatives was realized on the basis of ^{1}H NMR and ^{13}C NMR (noise decoupling) signals and DEPT, mass and FT-IR spectra and the results were in agreement with the proposed structures.

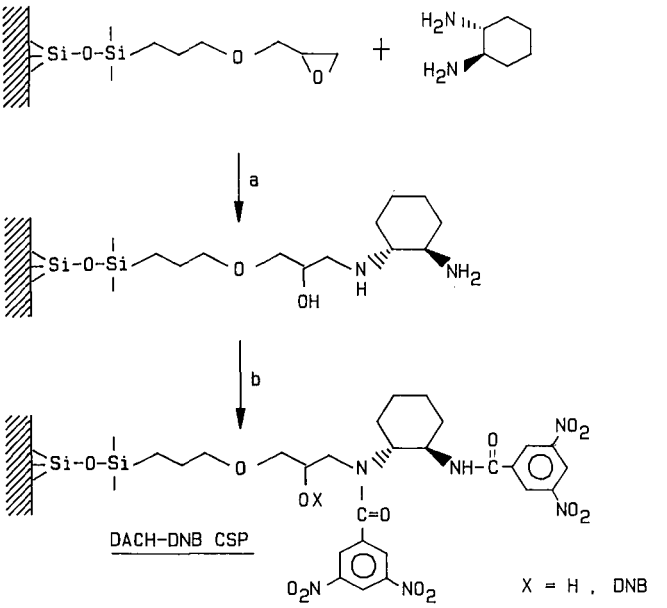

Fig. 2. Synthetic pathway for the preparation of DACH-DNB CSP. (a) (*R,R*)-(−)-diaminocyclohexane, dimethylformamide, 96 h, room temperature; (b) dinitrobenzoyl chloride, tetrahydrofuran, triethylamine, 2 h reflux.

Treatment of samples extracted from whole blood

In this procedure, as already described [7], phosgene was utilized as the derivatizing agent.

Column packing and column evaluation

The stainless-steel column (250 × 4 mm I.D. or 150 × 2.0 mm I.D.) was packed with LiChrosorb Si 100–DACH-DNB (5 μm) using a slurry packing procedure improved with respect to that already described [12]. Grafted silica (3.300 or 0.700 g) was dispersed in chloroform–isopropanol (50:50, v/v) (60 and 10 ml, respectively) and then treated ultrasonically for 5 min. The slurry obtained was packed with a Haskel DSTV-122 pump using *n*-hexane as pressurizing agent (8000 p.s.i., 15 min).

n-Hexane–isopropanol (99:1, v/v) was the eluent used in the evaluation of the kinetic performance of the chiral columns of different inner diameter and *n*-hexane was the solvent for the test mixture (naphthalene and anthracene).

Dimensionless parameters for the two columns such as reduced plate height (h), reduced velocity (υ), flow resistance parameter (φ) and separation impedance (E), were calculated according to Bristow and Knox [16] (column of 4.0 mm I.D., $h_{min} = 2.17$, $\upsilon_{opt} = 2.01$, $\varphi = 632$, $E = 2976$; column of 2.0 mm I.D., $h_{min} = 2.23$, $\upsilon_{opt} = 1.61$, $\varphi = 620$, $E = 3083$; for naphthalene, $k' = 0.50$).

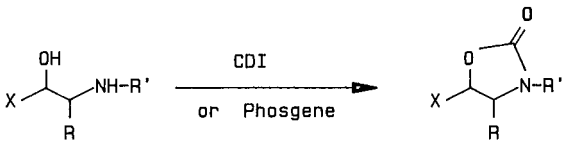

Fig. 3. Scheme of the derivatization procedures.

Chromatographic conditions

Racemic oxazolidin-2-ones were eluted with different mobile phases of increasing polarity (see Table I) at a flow-rate of 2.0 ml/min at 25°C (column 250 × 4.0 mm I.D.). UV absorbances were recorded at 254 nm unless stated otherwise.

RESULTS AND DISCUSSION

The racemic amino alcohols were analysed by HPLC as oxazolidin-2-one derivatives, which reduced the polarities of the analytes and made possible elution with solvents of medium polarity. Moreover, the derivatization (Fig. 3) enhances the

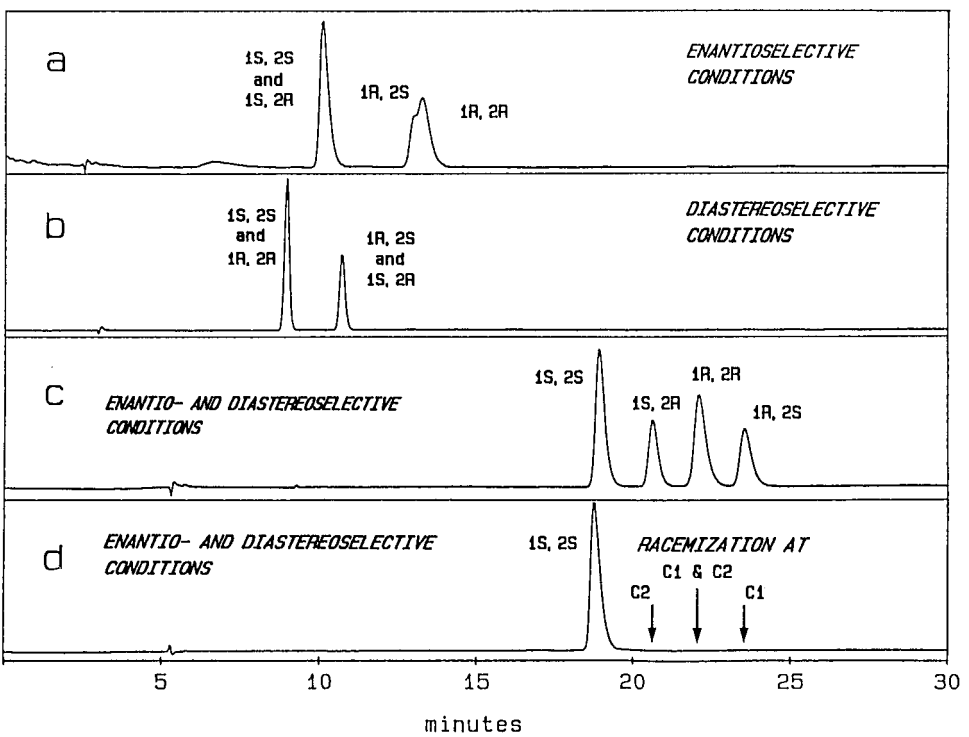

Fig. 4. Analysis of a mixture of racemic ψ-ephedrine and racemic ephedrine. (a) Chiral column, (*R,R*)-DACH-DNB (250 × 4 mm I.D.); (b) achiral column, LiChrosorb Si 60 (250 × 4 mm I.D.); (c) chiral and achiral columns connected in series; (d) coupled columns, racemization test for (1*S*,2*S*)-ψ-ephedrine. Detector, UV at 260 nm; flow-rate, 1.0 ml/min; other chromatographic conditions as in Table I.

recognition ability of the CSP because of the considerable and favourable conformational rigidity of the cyclization products.

The derivatization methods employed (CDI or phosgene, see Experimental) proceed without appreciable racemization of the substrates. Thus, mixtures having a well defined enantiomeric ratio (e.r.) do not exhibit variations of their enantiomeric composition after derivatization and analysis by means of the chiral column; *e.g.*, a commercial sample of (*S*)-propranolol (**9**) (Fluka; labelled e.r.: *S*:*R* = 99.5:0.5) derivatized as reported above and chromatographed on a DACH-DNB column showed an e.r. of *S*:*R* = 99.474:0.526 as revealed by digital electronic integration (mean of five measurements).

The stereochemical stability of both chiral centres of compounds such as **2** and **3** during the derivatization reaction was checked for the oxazolidin-2-one derivatives of (1*R*,2*S*)-ephedrine and (1*S*,2*S*)-ψ-ephedrine by ^{1}H NMR spectroscopy[a] and by HPLC on tandem columns (chiral DACH-DNB column plus achiral silica gel column) (Fig. 4). The reported results clearly indicate that whereas the chiral DACH-DNB column shows a very high enantioselectivity (Fig. 4a), the achiral silica column shows a good diastereoselectivity (Fig. 4b). Hence the connection of the two columns in series allows both enantioselectivity and diastereoselectivity values to be obtained that are suitable for the total separation of the two diastereoisomeric pairs, corresponding to compounds **2** and **3**, into four isolated stereoisomers (Fig. 4c),

Fig. 4d shows the results obtained using the same two columns in series for the analysis of the oxazolidin-2-one derived from the derivatization reaction of (1*S*,2*S*)-ψ-ephedrine. Also in this instance no peak assignable to racemization products was detected.

The chromatographic results for the amino alcohols examined are summarized in Table I; in addition, some examples showing different values of the resolution factor (R_s) are reported in Fig. 5. It can be assumed that the observed high chromatographic resolution values depend both on the great efficiency of the column and on its high enantioselectivity, as the kinetics and thermodynamics of the process are in an optimum state under the selected chromatographic conditions.

The low values of h_{min} show that the introduction of polar functional groups on the silica surface does not significantly influence the ability of the chiral support to establish rapid interactions with solutes (a necessary condition for obtaining highly efficient chiral columns); moreover, the low flow resistance observed guarantees adequate reproducibility of the original performance of silica gel.

With regard to thermodynamic aspects of the stereoselective molecular recognition process, DACH-DNB CSP is characterized by the precence of aromatic groups capable of establishing π–π donor–acceptor interactions with analytes containing π-basic aromatic portions: both π-acid (dinitrobenzoyl) and π-basic group in the

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[a] ^{1}H NMR spectra of (1*R*,2*S*)-ephedrine (**2**) oxazolidin-2-one and (1*S*,2*S*)-ψ-ephedrine (**3**) oxazolidin-2-one did not show evidence of any contamination derived from partial racemization during the derivatization process. ^{1}H NMR of (1*R*,2*S*)-ephedrine (**2**) oxazolidin-2-one (300 MHz, $CDCl_3$): δ 0.781 (3H, d, J = 6.3 Hz, CH_3CH), 2.877 (3H, s, CH_3N), 3.900–4.200 (1H, m, CH_3CH), 5.585 (1H, d, J = 8.1 Hz, C_6H_5CH), 7.200–7.400 ppm (5H, m, C_6H_5). ^{1}H NMR of (1*S*,2*S*)-ψ-ephedrine (**3**) oxazolidin-2-one (300 MHz, $CDCl_3$): δ 1.362 (3H, d, J = 6.3 Hz, CH_3CH), 2.804 (3H, s, CH_3N), 3.500–3.600 (1H, m, CH_3CH), 4.901 (1H, d, J = 7.8 Hz, C_6H_5CH), 7.341–7.500 ppm (5H, m, C_6H_5).

TABLE I

CHROMATOGRAPHIC RESULTS

CSP, (*R,R*)-DACH-DNB–LiChrosorb Si 100, 5 μm, (250 × 4.0 mm I.D.); flow-rate, 2.0 ml/min; temperature, 25°C; detector, UV, 254 nm. k' = Capacity factor; α = separation factor; R_s = resolution.

No.	Compound	k'	α	R_s	Eluent
1	Alprenolol	3.64	1.51	3.3	CH_2Cl_2–*n*-hexane (80:20)
2	Ephedrine	3.00	1.38	2.6	CH_2Cl_2–*n*-hexane–2-propanol (89:19:1)
3	ψ-Ephedrine	3.00	1.40	2.7	
4	Norephedrine	2.04	1.14	1.2	CH_2Cl_2–CH_3OH (99:1)
5	Betaxolol	1.21	1.39	2.8	
6	Oxprenolol	1.21	1.45	3.2	
7	Pronethalol	1.34	1.38	3.0	
8	Metoprolol	1.48	1.39	2.4	
9	Propranolol	1.92	1.40	3.2	
10	1-(2',5'-Dimethoxyphenyl)-2-aminoethanol	2.34	1.21	1.4	
11	Sotalol	5.35	1.55	3.3	
12	Acebutolol	5.56	1.24	1.7	
13	Pindolol	1.00	1.40	2.6	CH_2Cl_2–CH_3OH (90:10)
14	Atenolol	1.51	1.26	1.7	

diastereoisomeric CSP–solute complex are spatially locked in close parallel planes and disposed in such a way as to make simultaneously possible further interactions necessary for chiral recognition (mainly hydrogen-bond and/or dipole–dipole interactions).

The chiral recognition is expected to be more operative if the number of allowed conformations for both analytes and the CSP is reduced; as a consequence, derivatization of racemic solutes leading to a rigid cyclic system was found to produce the highest degree of enantioselectivity (*e.g.*, simple N- or N-,O-acylation products of racemic propranolol were not resolved on DACH-DNB CSP).

Compounds **1, 5, 6, 8, 9, 12, 13** and **14** are structurally similar, having the same propanolamine side-chain and differing only in the aromatic moiety; the highest selectivities were obtained with solutes containing either good π-basic aromatic rings (propranolol and pindolol) or aromatic rings bearing non-polar substituents (oxprenolol, alprenolol, betaxolol, metoprolol). Acebutolol and atenolol, with a polar amide group, distant from the chiral centre, gave lower resolutions, probably because of the strong, non-stereospecific interactions between this group and CSP. Addition of a second stereogenic centre (compounds **2**, **3** and **4**) or direct connection of the aromatic portion to the alcoholic chiral carbon (compounds **7**, **10** and **11**) did not result in significant variations of α values. Finally, the low degree of stereoselectivity and peak tailing shown by the oxazolidin-2-ones derived from N-unsubstituted amino alcohols (compounds **4** and **10** gave the lowest α and R_s values) can be attributed to strong hydrogen bonding between the analytes and CSP amide groups.

Precision of analysis and practical considerations

The high speed of analysis, the high values of enantioselectivity and, therefore, of

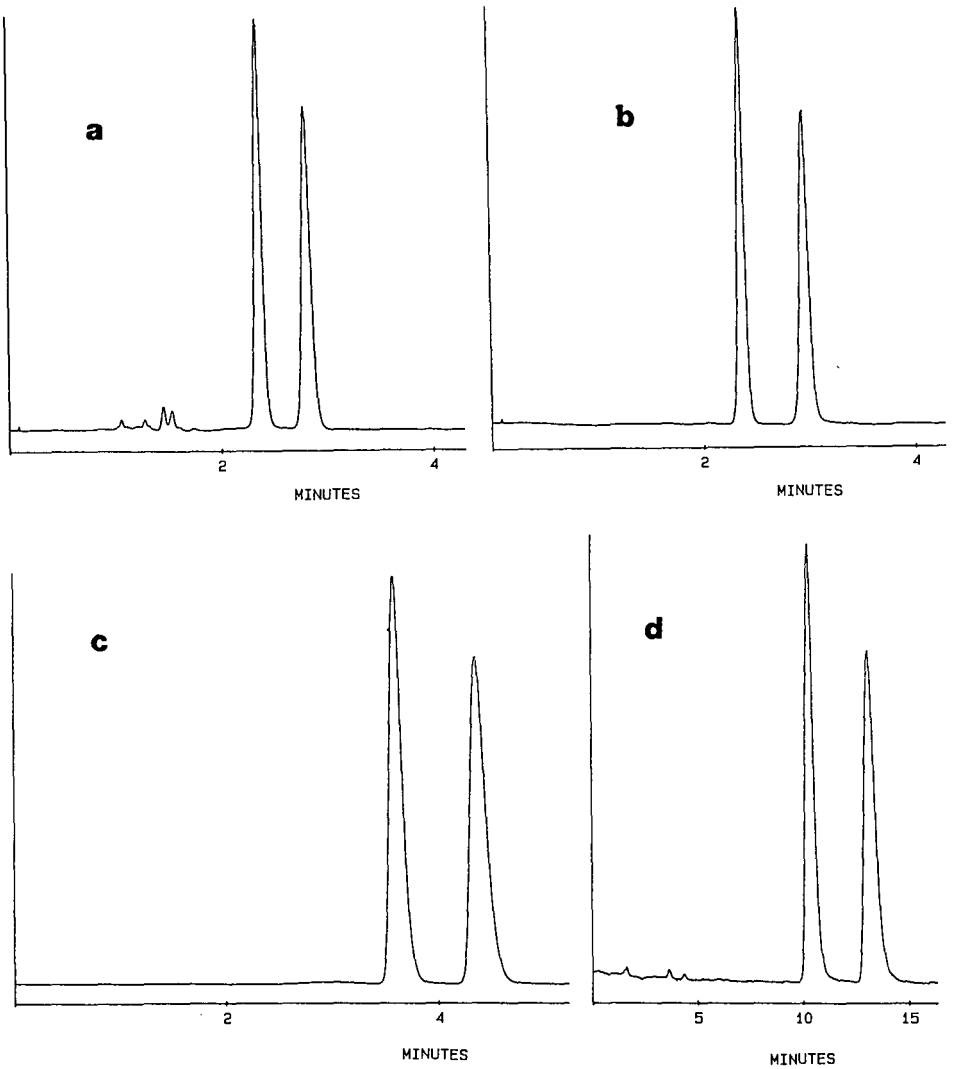

Fig. 5. Resolutions of racemic β-blockers as oxazolidin-2-one derivative. (a) Betaxolol; (b) oxprenolol; (c) metoprolol; (d) sotalol. Detector, UV at 275 nm; other chromatographic conditions as in Table I.

the chromatographic resolution allow the enantiomeric excess (e.e.) to be determined very precisely and accurately also if only very small amounts of product are available. With regard to this point, samples of propranolol (as the oxazolidin-2-one) at different enantiomeric excesses, (prepared *ad hoc* by mixing known amounts of the pure enantiomers) were analysed on a microbore column of 2.0 mm I.D.

Fig. 6a and b show the results obtained by injecting the same sample of (*S*)-propranolol as the oxazolidin-2-one (e.e. 99%) on two columns of DACH-DNB of the same size but of opposite chirality. The reversal of the order of peak elution for enantiomerically enriched selectands by inverting the chirality of the selector is a useful

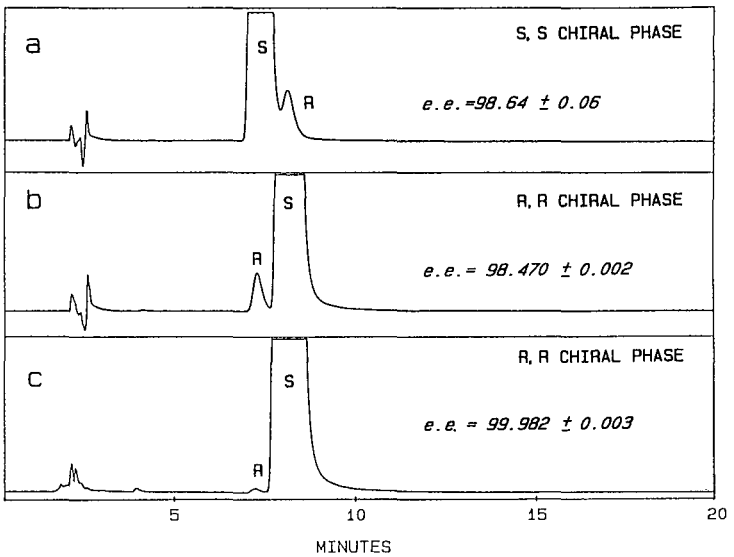

Fig. 6. Enantiomeric trace analysis of (*S*)-propanolol. Columns, 150 × 2 mm I.D. packed with (a) (*S,S*)-DACH-DNB and (b and c) (*R,R*)-DACH-DNB CSP; eluent, *n*-hexane–2-propanol–methanol (40:30:30, v/v/v); flow-rate, 200 μl/min; detector, UV at 230 nm; k'_1 = 2.79; α = 1.20.

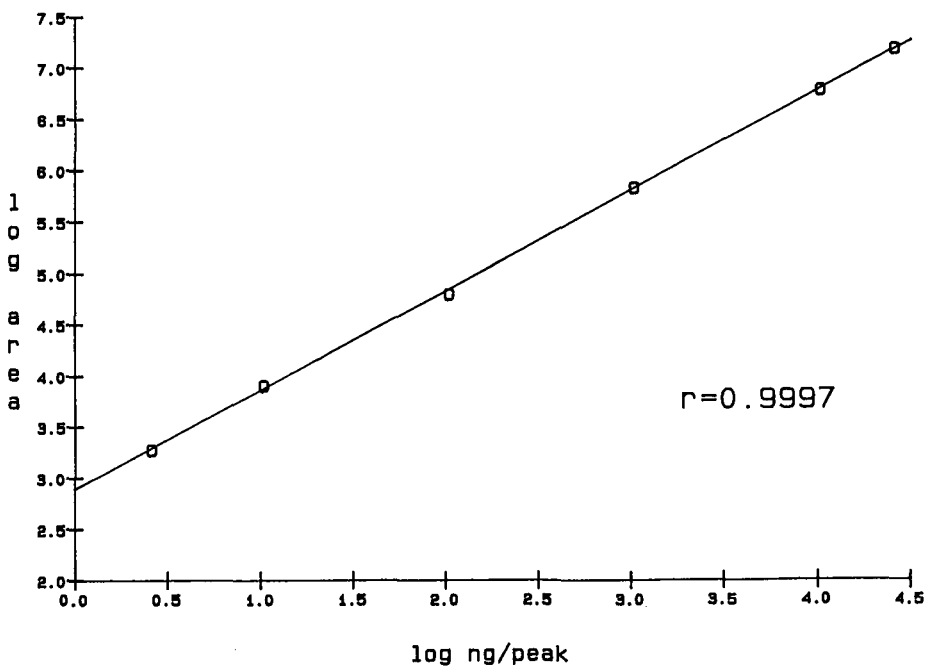

Fig. 7. Variation of peak area (UV detection) as a function of sample size for racemic propranolol. The limit of detection was 1.0 ng for each peak, with a signal-to-noise ratio of 3. Chromatographic conditions as in Fig. 6.

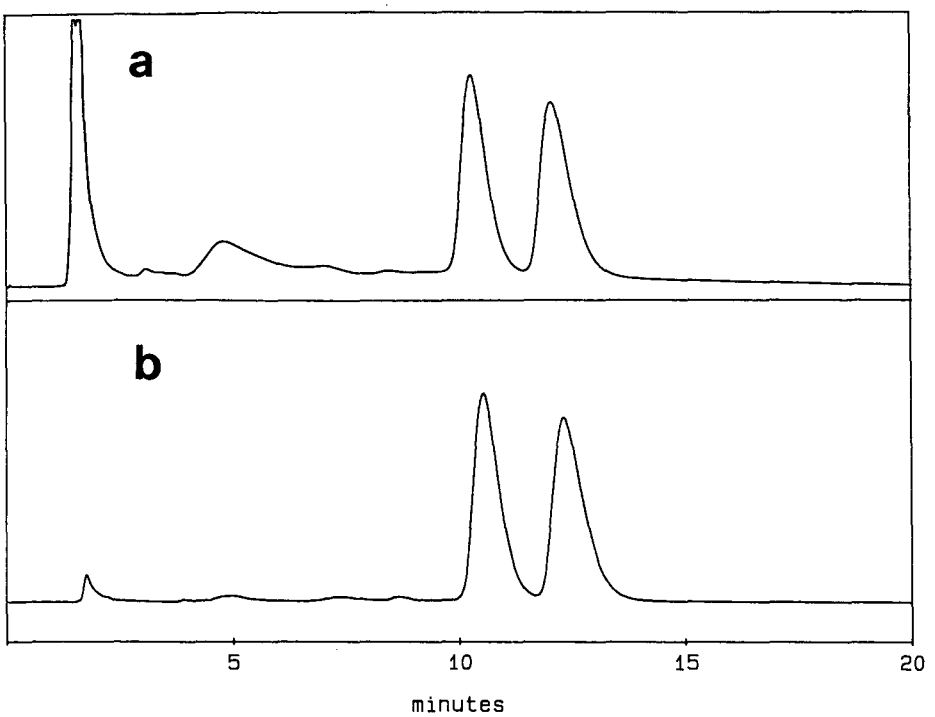

Fig. 8. Comparison between (a) UV (275 nm) and (b) fluorimetric (λ_{ex} = 275 nm, λ_{em} = 300 nm) detection for racemic atenolol extracted from human plasma. Eluent, *n*-hexane–2-propanol–methanol (40:30:30, v/v/v); k'_1 = 4.98; α = 1.21.

diagnostic tool for verifying an enantiomer separation but it may also have an important role in quantitative enantiomer analysis. In the above example, the reversal of the chirality of the selector allows the deficient enantiomer [(*R*)-propranolol oxazolidin-2-one] to be eluted as the peak preceding that of the main component. The digital electronic integration is easy, accurate and precise in the separation reported in Fig. 6b, where the deficient enantiomer is eluted first (e.e. = 98.470 ± 0.002).

Systematic errors in chromatographic enantiomer analysis may be recognized when the e.e. determination is performed on two phases of opposite chirality [17]. Thus, if a correct integration method (tangents method) is applied (Fig. 6a), a value closer to the real one can be obtained also if the precision of the measurement is low. Thus, this technique should be employed for the evaluation of trace amounts, especially when the deficient enantiomer is eluted first.

Fig. 6c shows the determination of the e.e. of another sample of (*S*)-propranolol oxazolidin-2-one. The value obtained was 99.982 ± 0.003 for the (*R*)-antipode as revealed by digital electronic integration carried out on five measurements of both enantiomeric peaks; in our particular case the detection response was linear over four orders of magnitude (Fig. 7).

Finally, we wish to point out that the above method can be successfully applied to the determination of β-blockers in blood plasma. Fig. 8 shows the results obtained;

both detection methods (spectrophotometric and spectrofluorimetric) gave fairly good results but the spectrofluorimetric detection proved to be more selective and sensitive.

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Column-friendly reversed-phase high-performance liquid chromatography of peptides and proteins using formic acid with sodium chloride and dynamic column coating with crown ethers

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ABSTRACT

Reversed-phase high-performance liquid chromatography of proteins, traditionally carried out with strong acids like trifluoroacetic acid or phosphoric acid, which can damage reversed-phase columns, can be performed with excellent results using the far milder formic acid in the presence of salt. For certain separations, dynamic coating of the column with crown ethers can bring added resolution. Examples given are for peptides from a digest of methionine growth hormone, protein separations from whey proteins containing α-lactalbumin and the β-lactoglobulins A and B, and bovine and porcine insulins. The separation of methionine-growth hormone from growth hormone is also described.

INTRODUCTION

In a previous publication [1] we demonstrated the fact that it is not necessary to use a strong acid like trifluoroacetic acid (TFA) to elute peptides successfully from a reversed-phase column, but that a weaker acid (formic acid) which is less damaging to that column [2] can be used instead and may, in fact, give a better resolution. In this publication we will show that separations can be further improved by the addition of a moderate amount of salt. While for work with small peptides this may not always be necessary, it is demonstrated to be necessary in many cases for the separation of the larger peptides and proteins when using the formic acid system.

For especially difficult separations it has proven useful to change the character of the column somewhat by coating it with some compound of greater hydrophobicity than acetonitrile or some other organic eluent. If a compound less polar than the eluting solvent is added to this eluent, the column will become impregnated with it since the hydrophobic interaction between the column and the "non-polar" additive is stronger than that between the column and the bulk of the organic solvent. Among the solvents which have a relatively large effect even if added in small quantities are the ethers tetrahydrofuran and dioxan [3]. To a large extent the influence of the additive

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can be attributed to the hydrophobic interactions with the alkyl groups of the reversed-phase stationary phase, but they can also be bound to the free silanol groups of the stationary phase by a strong hydrogen bond formation (silanophilic interaction) [4]. This effect could perhaps be called "dynamic endcapping". The result of these two effects is that the column retains as much of the non-polar additive as it possibly can in a dynamic equilibrium with the surrounding buffer. Since this additive although non-polar is still more polar than the alkyl groups of the stationary phase the character of the column has now been modified. It may be unnecessary to add more than 0.1% of such a compound to obtain the maximum effect. If the organic modifiers are added in larger quantities, the unique difference between column and buffer would be diminished.

Other cyclic ether compounds of interest are the crown ethers, which have been used for dynamic coating of reversed-phase columns for the separation of alkali metals [5] and racemic amino acids [6]. Crown ethers have also been used for the chromatography of proteins and nucleic acids. In this case [7] the crown ethers were chemically bound to the silica. The authors describe the importance of the choice of salt in connection to the metal-binding properties of the crown ether. They demonstrate that to achieve the desired chromatographic effect it is imperative to use potassium chloride instead of the sodium salt. Another family of compounds displaying the dynamic coating phenomenon is the paired ion chromatographic (PIC) reagent group [8]. This paper presents the results from a comparison of crown ether addition *versus* sodium chloride addition to a mobile phase containing formic acid.

EXPERIMENTAL

Apparatus

Two sets of apparatus were used in this study.

A Waters Assoc. high-performance liquid chromatography (HPLC) system was used and consisted of two M-6000A pumps, a Model 680 gradient programmer, a Rheodyne Model 7125 injector with 2-ml loop, a 450 variable-wavelength detector and an Omniscribe (Houston Instruments) recorder.

An LKB HPLC system was also used interchangeably and consisted of a LKB 2249 gradient pump, a LKB 2141 variable-wavelength monitor, a Rheodyne Model 7125 injector and a Kipp & Zonen (Delft, The Netherlands) Model BD 41 recorder.

In all cases presented here a Vydac-Protein C_4 (250 × 4 mm) HPLC column was used.

Reagents

The following reagents were used: Milli Q water, acetonitrile (Mallinckrodt, ChromAR HPLC grade), sodium chloride (May & Baker, reagent grade), formic acid (May & Baker, Pronalys AR grade), 12-crown-4 (Aldrich), 18-crown-6 (Sigma), dioxan and tetrahydrofuran (Ajax, Unilab), diglyme, diisopropyl ether, methyl-cellosolve, glycol and *tert.*-butanol (BDH).

The following proteins were studied: methionine human growth hormone (Met-hGH) and recombinant human growth hormone (rhGH, Genentech), albumin (bovine, Sigma) and insulin (bovine and porcine, Sigma), acid whey protein isolate

containing mainly α-lactalbumin and β-lactoglobulins A and B was obtained from D. F. Elgar [9].

Buffer preparation

When buffer systems without salt were used the composition was as follows. Buffer A: 0.1% formic acid in water–acetonitrile (49:1) for peptides (digests) or 0.1% formic acid in water–acetonitrile (9:1) for proteins, to which the small amount of crown ether or other coating material may be added. Buffer B: 0.1% formic acid in water–acetonitrile (1:9) (+ the additive).

Salt-containing buffers were prepared as follows. A 10.0-g amount of sodium chloride was dissolved in water, 1.0 ml of formic acid and the desired quantity of crown ether or other modifier were added after which the volume was made up to 100 ml. Buffer B (500 ml) was prepared by filtration of 300 ml of acetonitrile through a 0.2-μl filter, followed by 150 ml of water and 50 ml of the above salt solution.

Attention: During the preparation of this buffer the mixture will become quite cold and will separate into two layers. Gentle warming will bring it to room temperature where the solution will be homogeneous. The buffer containing 1% sodium chloride can not contain more than 60% acetonitrile if a single layer at room

TABLE I

CONDITIONS FOR CHROMATOGRAMS

Separation of	Chromatogram No.	Buffers used for gradient	Linear gradient			
			Initial % B	Duration of the programme (min)	Final % B	Flow-rate (ml/min)
Tryptic digest of Met-hGH	1A	IA and IB	0	60	70	1
	1B	IIA and IIB	0	60	70	1
	1C	IIIA and IIIB	0	60	100	1
	1D	IVA and IVB	0	45	100	1
rhGH and Met-hGH	2A	VA and VB	100% B[a]			
	2B	VIA and VIB	60	45	80	1[b]
	2C	VIIA and VIIB	70	45	90	1
	2D	VIIIA and VIIIB	75	45	85	1
Bovine and porcine insulin	3A	VA and VB	20	45	30	1
	3B	VIA and VIB	15	45	30	1
	3C	VIIA and VIIB	30	45	50	1
	3D	VIIIA and VIIIB	30	45	50	1
Whey proteins	4A	VA and VB	30	45	50	1
	4B	VIA and VIB	25	45	50	1
	4C	VIIA and VIIB	60	45	80	1
	4D	VIIIA and VIIIB	60	45	80	1
All proteins	5	VIIIA and VIIIB	30	60	90	1
Whey proteins	6	IXA and IXB	50	45	70	1

[a] Isocratic.

[b] Gradient changed to 100% B after 15 min.

temperature is to be maintained. Buffer A is made similarly but does not require warming up.

Final composition of buffer A (for the chromatography of the digests): 0.1% formic acid + 1% sodium chloride (+ x% of the desired additive) in water–acetonitrile (49:1); for chromatography of the proteins the preferred ratio of water–acetonitrile is 1:9.

Composition of buffer B (for peptides as well as proteins): 0.1% formic acid + 1% sodium chloride (+ x% of the desired additive) in water–acetonitrile (2:3).

The gradients used were all linear and are described in Table I. Whenever possible for comparisons of chromatograms with or without an additive, comparable gradients were used. This however was not always possible because of the changed characteristics of the column. In general, as may be expected, the more polar the additive tested for dynamic coating, the less acetonitrile is needed to elute the proteins.

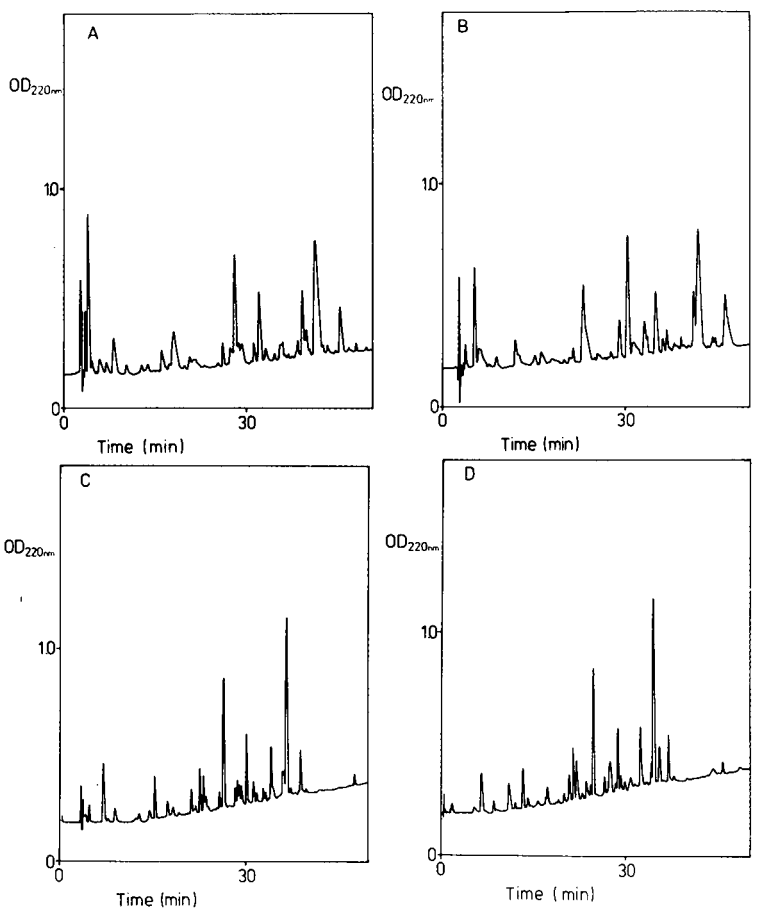

Fig. 1. Tryptic digest of Met-hGH. Elution (A) 0.1% formic acid, (B) + crown ether, (C) + NaCl, (D) + crown ether + NaCl. For buffer composition and gradient see Tables I and II.

Digests

The tryptic digests were prepared as previously described [1].

RESULTS AND DISCUSSION

Hodgin *et al.* [3] have reported the use of tetrahydrofuran and dioxan in reversed-phase HPLC. Previous uses of crown ether have involved the chromatography of alkali metals [5] and amino acids [6] or the immobilization of the crown ether [7]. The following results illustrate further potential uses of crown ethers with and without the addition of sodium chloride.

A tryptic map of Met-hGH (Fig. 1) illustrates the effectiveness of 0.1% formic acid plus NaCl (Fig. 1C) or NaCl with 18-crown-6 ether (Fig. 1D) in the separation of the peptides. The crown ether with 0.1% formic acid (Fig. 1B) effects little improvement over 0.1% formic acid alone (Fig. 1A).

A similar trend is seen in the attempted separation of Met-hGH and rhGH (Fig. 2) except in the case of intact proteins the combined effect of crown ether and NaCl produces the best separation (Fig. 2D). Note that formic acid alone does not appear to elute these proteins in the times used in this study (60 min or less).

In the examples of bovine and porcine insulin separations (Fig. 3) the effect is reversed, *i.e.*, even with identical gradients the better separation is achieved in the 0.1% formic acid plus NaCl case (Fig. 3C).

The situation reverses again for the chromatograms of a whey protein isolate containing largely α-lactalbumin and β-lactoglobulins A and B, with both additives effecting the best separation in the presence of formic acid (Fig. 4D). All the proteins

TABLE II

COMPOSITION OF BUFFERS

Buffer	Formic acid (%)	Sodium chloride (%)	18-Crown-6 (%)	Water–acetonitrile
IA	0.1	–	–	49:1
IB	0.1	–	–	1:9
IIA	0.1	–	0.05	49:1
IIB	0.1	–	0.05	1:9
IIIA	0.1	1	–	49:1
IIIB	0.1	1	–	2:3
IVA	0.1	1	0.05	49:1
IVB	0.1	1	0.05	2:3
VA	0.1	–	–	9:1
VB	0.1	–	–	1:9
VIA	0.1	–	0.05	9:1
VIB	0.1	–	0.05	1:9
VIIA	0.1	1	–	9:1
VIIB[a]	0.1	1	–	2:3
VIIIA	0.1	1	0.05	9:1
VIIIB[b]	0.1	1	0.05	2:3
IXA	0.1	1 (KCl)	0.05	9:1
IXB	0.1	1 (KCl)	0.05	2:3

[a] Identical to IIIB.

[b] Identical to IVB.

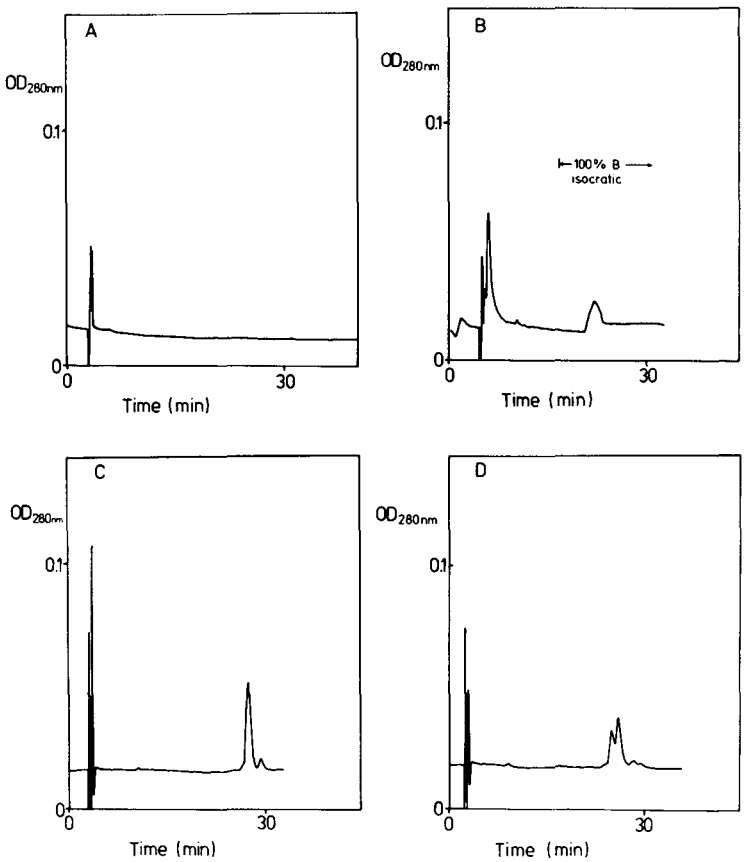

Fig. 2. Met-hGH and rhGH. Elution as in Fig. 1. For buffer composition and gradient see Tables I and II.

described in this publication were combined and chromatographed together (Fig. 5). This chromatogram shows the effectiveness of the combination of 0.1% formic acid, 18-crown-6 ether and sodium chloride in separating a mixture of proteins. For a good separation of proteins of very close polarity like the β-lactoglobulins or the two growth hormones, it is often not only important to use a shallow gradient, but also to start the chromatogram at the highest possible percentage of buffer B that still allows retention. Too low a percentage of buffer B will tend to bind the proteins too tightly to the column and may not allow quantitative and/or discreet elution.

Crown ethers react strongly with the different alkali and earth alkali metals [5,7]. This has prompted us to investigate the differences between sodium chloride and potassium chloride in the reversed-phase chromatography as well. Instead of the normal 1% of sodium chloride in the buffers, 1% of potassium chloride was used in one set of experiments. The results (Fig. 6) indicate that the proteins elute at a lower % buffer B, although the resolution is comparable.

Whether the dynamic coating will improve a certain separation is unpredictable from the examples shown here. The combination of the crown ether and sodium

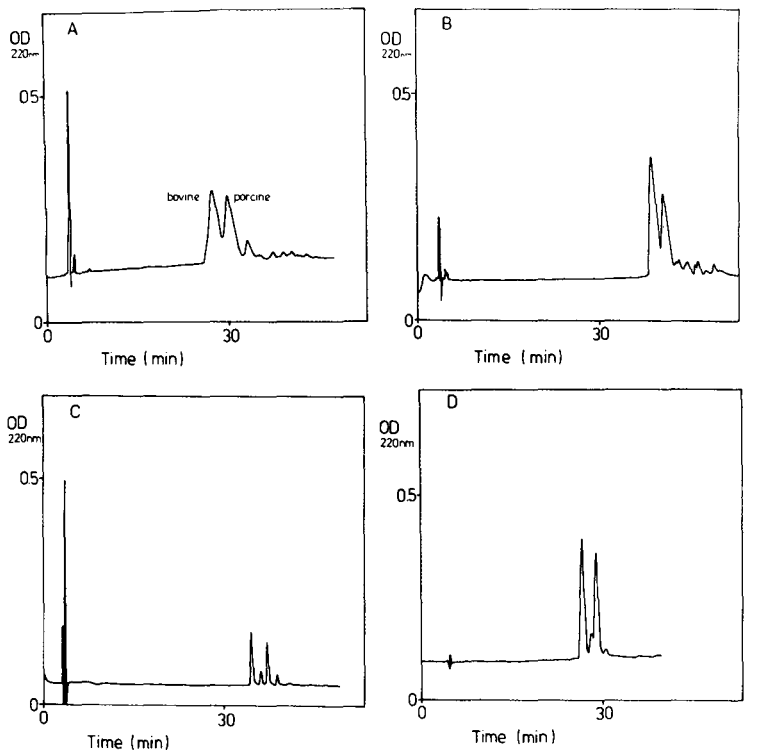

Fig. 3. Bovine and porcine insulin. Elution as in Fig. 1. For buffer composition and gradient see Tables I and II.

chloride certainly seems to be a powerful one in some cases. While it definitely improved the separation of the β-lactoglobulins and the surrounding small peaks (Fig. 4D) and of the growth hormones (Fig. 2D), the situation is reversed for the separation of the insulins (Fig. 3D). In the chromatograms with formic acid and crown ether but without sodium chloride, the effect of the crown ether is noticeable but nowhere does it seem as beneficial as the addition of the salt with or without crown ether.

All the examples of chromatograms given here containing a crown ether were carried out with 18-crown-6. Chromatograms prepared in the presence of 12-crown-4 were virtually identical to the 18-crown-6 examples shown here. The amount of crown ether added has been studied in a number of cases and it was found that there was no significant advantage to addition of more than 0.05% of these compounds. All other compounds tested for "dynamic coating", tetrahydrofuran, dioxan, diglyme, diisopropylether, methylcellosolve, glycol and *tert.*-butanol did not produce the beneficial effect shown here when used in conjunction with sodium chloride. In a few cases they could be used in separations without any salt and could effect improved separations when used in very low concentration (0.05%). At higher concentrations the effects were again counter-productive.

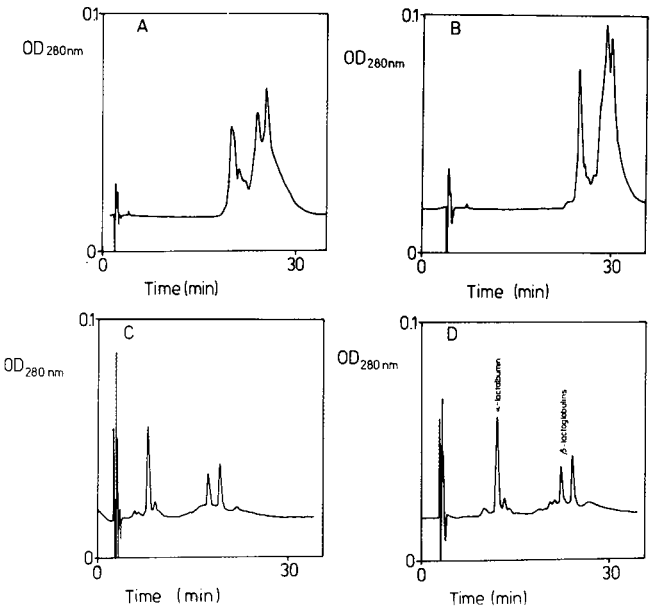

Fig. 4. Whey proteins. Elution as in Fig. 1. For buffer composition and gradient see Tables I and II.

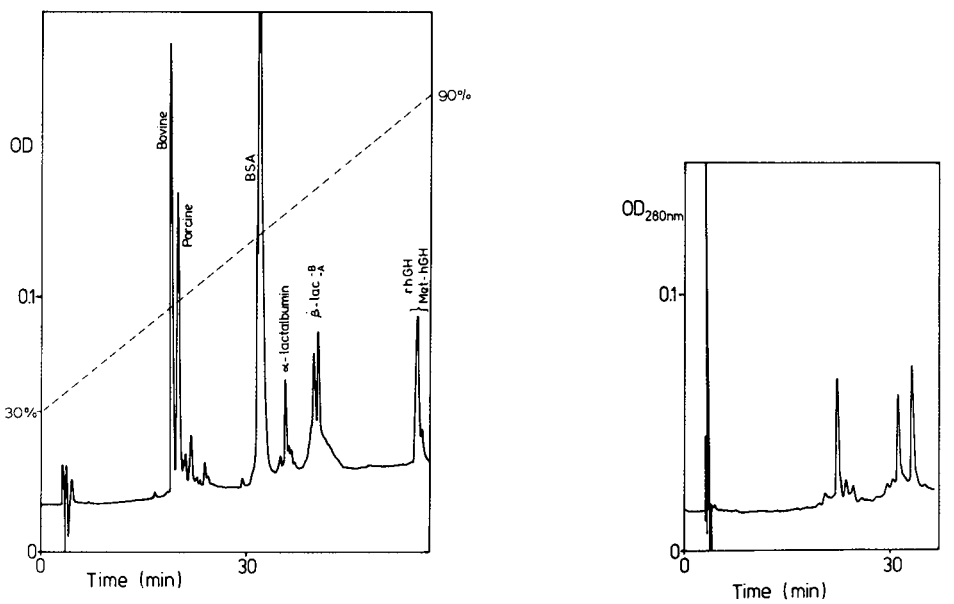

Fig. 5. All proteins. Elution as in Fig. 1D. For buffer composition and gradient see Tables I and II. lac = Lactoglobulin.

Fig. 6. Whey proteins. Elution as for Fig. 1D except KCl replaces NaCl. For buffer composition and gradient see Tables I and II.

The possible use of guanidine hydrochloride instead of sodium chloride was also investigated. Guanidine hydrochloride has the advantage that it is much more soluble in the acetonitrile than sodium chloride, to the extent that solutions of 2% of this compound can be prepared in 80% acetonitrile. (A maximum of 1% of sodium chloride in 60% acetonitrile is possible!) The examples studied above, when run with guanidine hydrochloride buffer did not show the resolution that the sodium chloride containing buffers were capable of. With guanidine hydrochloride it was found that the addition of 1% of the salt is necessary to effect elution. A composition of 0.5% was not enough while there was no significant difference between additions of 1% or 2%.

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Determination of organophosphorus and carbamate pesticide standards by liquid chromatography with detection by inhibition of immobilized acetylcholinesterase

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ABSTRACT

A sensitive method for post-column reaction detection of carbamate and organophosphorus pesticides is described, based on the inhibition of immobilized acetylcholinesterase. The compounds are separated by reversed-phase liquid chromatography with tetrahydrofuran–water as the mobile phase in a isocratic system. The reactor used for detection comprises a conventional flow injection assembly for monitoring activity of acetylcholinesterase immobilized on controlled pore glass in a mini-column with spectrophotometric detection. The detection limits and linear calibration ranges are 2.6 and 5–40 ng for paraoxon, 0.04 and 0.1–1.6 ng for diisopropylfluorophosphate, 18 and 20–100 ng for isopropyl N-phenylcarbamate and 29 and 40–400 ng for isopropyl N-(3-chlorophenyl)carbamate. Only 16 min is required for the determination of a mixture of the above pesticides, and each enzyme column can be used for 90 determinations.

INTRODUCTION

Organophosphorus compounds are widely used in agriculture, medicine and industry, and include some highly toxic chemical warfare agents. Carbamates and organophosphorus pesticides are frequently applied in agriculture. However, whereas the organophosphorus compounds are generally used alone, they may be also be applied in conjunction with a carbamate pesticide for the control of a specific pest showing resistance to the organophosphorus compound.

Carbamates and organophosphorus pesticides have been applied to a variety of crops including green vegetables. These uses leave residues on the crops and also contaminate surface waters draining from the cropland. The presence of carbamates and organophosphorus pesticides in water, food and animal feedstuffs presents a potential hazard owing to their high mammalian toxicity. Under environmental conditions carbamates and organophosphorus pesticides could persist at the mg l^{-1} level in water, food and feed for a number of days or weeks depending on temperature and pH [1]. This makes it necessary to develop analytical methods which offer high selectivity and sensitivity for both identification and quantitation.

Ideally, multiresidue methods should provide rapid identification and quantifi-

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cation of as many different pesticides as possible in a wide array of sample matrices. Although reversed-phase liquid chromatography (LC) has often been used for analysis of carbamates, this is not the case for the organophosphorus pesticides. This situation arises because carbamate pesticides are difficult to analyse by gas chromatography because of thermal instability and that LC detectors often lack the sensitivity required for organophosphorus pesticide analysis. Pre-column derivatization for improved UV detection of pesticides in LC is always a possibility, though this requires additional sample handling, treatment, and work-up and provides additional room for error. Post-column detectors based on specific chemical reactions offer an alternative method, with excellent selectivity and sensitivity, providing that the extracolumn peak broadening is kept to a minimum.

One of the most notable changes that is occurring in analytical chemistry in general and pesticide analysis in particular is the rapidly growing impact of biotechnology. Many workers [2–9] have reported the enzymatic determination of carbamate and organophosphorus pesticides. Unfortunately, these methods are of limited used because of a lack of specificity and do not allow the simultaneous determination of several pesticides.

The inhibition of cholinesterase enzymes has been widely applied for the detection of trace amounts of organophosphorus pesticides on thin-layer chromatograms [10–13]. Ramsteiner and Hormann [14] coupled a continuous-flow analyzer to a liquid chromatograph and determined organophosphate and carbamate insecticide residues in a plum-leaf extract. Moye and Wade [15] developed a fluorimetric enzyme-inhibition detector for carbamate pesticides and applied the system to the determination of these compounds in crop samples. It should be borne in mind that these methods are suitable only for polar compounds soluble in water. Sipponen [16] developed a spectrophotometric enzyme-inhibition detector for organophosphorus compounds not detectable in trace amounts with any conventional liquid chromatography detector, and explored the possibility of using organic solvents in the mobile phase. The major fault of all these systems is the need to pump relatively large amounts of enzyme solutions in the flow systems used.

Recently, we reported the development of an enzyme-inhibition detector [9] in a flow system for the sensitive determination of paraoxon. The procedure was based on acetylcholinesterase immobilized on controlled-pore glass, and spectrophotometric monitoring of the enzyme-catalyzed reaction. The detection limit for paraoxon was $8 \cdot 10^{-9}$ *M* in a stopped flow system, and $4 \cdot 10^{-7}$ *M* in a continuously flowing system. This paper describes the development of a method for the simultaneous determination of two carbamate and two organophosphorus pesticides using reversed-phase LC coupled with a detector based on that continuous flow system involving immobilized acetylcholinesterase.

EXPERIMENTAL

Reagents

Acetylcholinesterase (E.C. 3.1.1.7, AChE, from eel, Type VI-S, 200 U mg^{-1}) and controlled porosity glass (CPG-240, 80–120 mesh, 22.6 nm mean pore diameter) were obtained from Sigma. Diethyl-*p*-nitrophenylphosphate (paraoxon, 95% pure, Sigma), isopropyl N-phenylcarbamate (99% pure, Sigma) and isopropyl N-(3-chloro-

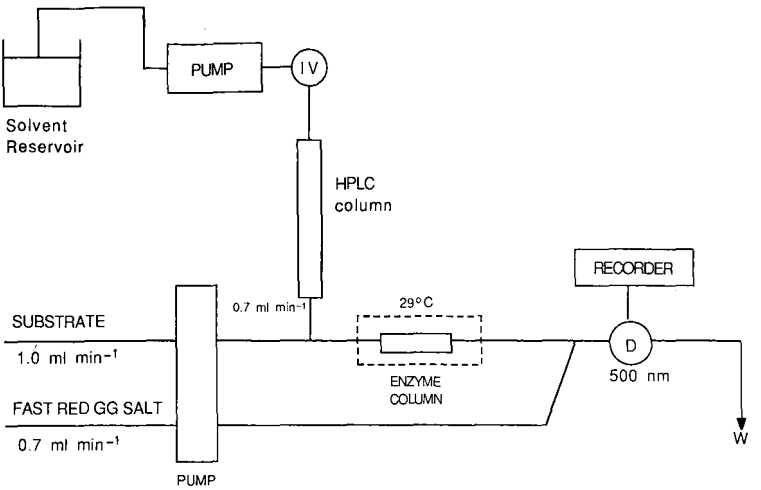

Fig. 1. Liquid chromatograph-flow detector assembly. Fast red GG salt is *p*-nitrobenzenediazonium fluoroborate; substrate is α-naphthyl acetate; enzyme is acetylcholinesterase. D = Detector; W = waste.

phenyl)carbamate (Sigma) were used for preparing 1000 mg l^{-1} stock solutions in methanol. Diisopropylfluorophosphate (DFP, Sigma) was used for preparing a 100 mg l^{-1} stock solution in methanol. No changes were observed in these solutions after 1 week at 4°C. Standard solutions of the pesticides were prepared by dilution of the stock solutions with water. A $5.0 \cdot 10^{-3}$ *M* stock solution of α-naphthyl acetate (Sigma) was prepared by dissolving 0.093 g in 100 ml of acetone–water (5:95). A $5.0 \cdot 10^{-3}$ *M* stock solution of *p*-nitrobenzenediazonium fluoroborate (Sigma) was prepared by dissolving 0.118 g in 100 ml of water.

The pH was adjusted with a 0.05 *M* phosphate buffer (pH 8.0) containing 45 m*M* sodium chloride and 12 μ*M* magnesium chloride. HPLC grade methanol, acetonitrile (May and Baker) and tetrahydrofuran (Aldrich) were used in preparing mobile phases. Distilled deionized water was used throughout.

The stock solutions were kept in a refrigerator. All chemicals used were of analytical or HPLC grade.

Enzyme immobilization

This was achieved on CPG following the procedure described earlier [9]. The product was packed in a glass tube (10 × 2.5 mm I.D.), so that the length of the immobilized enzyme zone was 10 mm. The beads were stored at 4°C in 0.05 *M* phosphate buffer, pH 6.0.

Apparatus and procedures

The liquid chromatographic system consisted of a RR/035 solvent pump (Milton Roy) equipped with a Rheodyne 7125 valve injector (20-μl loop) connected to a Spectromonitor (LDC) variable wavelength detector. A Labdata (Labdata Instrument Services Ltd.) recorder was connected to the system. Spherisorb S10 ODS2 or Spherisorb 5 Amino chromatographic columns (Phase Separations), 25 cm × 4.6 mm I.D., 10 or 5 μm particle size, respectively, were used.

The assembly of the liquid chromatograph and the spectrophotometric enzyme-inhibition detector is shown in Fig. 1. The absorbance was measured at 500 nm. The manifold and reaction coil tubing were 0.5 mm I.D. PTFE. The pump was a Gilson Minipuls 2 (Anachem). The effluent from the chromatographic column was merged with the buffered substrate solution ($3.0 \cdot 10^{-4}$ *M* α-naphthyl acetate in 0.05 *M* phosphate buffer containing 45 m*M* sodium chloride and 12 μ*M* magnesium chloride). The substrate was hydrolyzed by the immobilized enzyme to α-naphthol which reacts with *p*-nitrobenzenediazonium fluoroborate, providing a continuous baseline absorbance at 500 nm. In the presence of a cholinesterase inhibitor in the column effluent, enzyme activity is temporarily decreased in proportion to the inhibitor concentration, thus producing a decrease in absorbance (negative peak).

In order to avoid contamination, all apparatus exposed to pesticide solutions were carefully cleaned by contact with 2% (w/v) sodium hydroxide solution for a few hours to ensure hydrolysis of the pesticide.

RESULTS AND DISCUSSION

Effect of organic solvent on immobilized enzyme activity

A major problem in employing an enzyme in a LC detector is to find a mobile phase that allows analytical separation of the desired compounds but does not at the same time inactivate the enzyme and decrease sensitivity. Solvent systems of water mixed with methanol, acetonitrile or tetrahydrofuran are widely used in reversed-phase LC, and the influence of these three solvents on the activity of immobilized AChE and its inhibition was investigated.

This study was carried out using the flow injection procedure described earlier [9], except that the carrier solution contained up to 40% (v/v) organic solvent. Significantly higher concentrations of organic solvent completely inactivated the

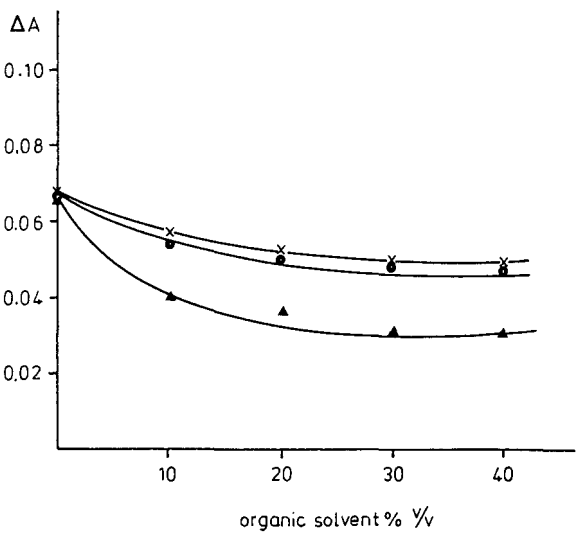

Fig. 2. Effect of organic solvent content on inhibition of immobilized acetylcholinesterase by paraoxon: × = methanol–water; ● = tetrahydrofuran–water; ▲ = acetonitrile–water.

enzyme. Up to 40% organic solvent had little effect on the baseline absorbance, but above 20% there is an appreciable increase in detector noise. Sipponen [16] reported that 8% acetonitrile totally inhibited soluble electric eel AChE. The same author reported that up to 20% methanol appeared to increase the activity of butyrylcholinesterase. Neither effect was observed in this work for the immobilized AChE.

The results obtained for inhibition by paraoxon are shown in Fig. 2. The peak height for $8.0 \cdot 10^{-6}$ *M* paraoxon decreases steadily in all organic solvents, but most appreciably in acetonitrile. Thus methanol or tetrahydrofuran would be more satisfactory solvents if high sensitivity is to be achieved. Reproducible responses to pesticides were achieved when the proportion of methanol or tetrahydrofuran in the reaction mixture did not exceed 20%. Thus, in subsequent experiments the flow-rates were adjusted to ensure that the concentration or organic solvent in the solution pumped to the enzyme was about 20%.

Selection of chromatographic conditions

Efficient separation conditions were established by using reversed-phase LC with UV detection at 254 nm. A Spherisorb S10 ODS2 or Spherisorb 5 Amino column was used with isocratic elution by 25, 40, 50, 75 or 90% organic solvent in the mobile phase. The best separations achieved are shown in Figs. 3 and 4. As would be expected, DFP solutions at concentrations as high as 200 mg l^{-1} could not be detected by UV spectrophotometry at 254 nm.

The chromatographic conditions used in Figs. 3 and 4 were chosen to study the possibility of combining the LC system with the enzymatic inhibition detection system. Although separation on Spherisorb 5 Amino with methanol–water (40:60) mobile

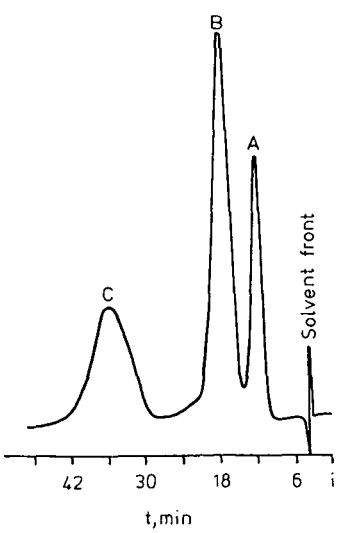

Fig. 3. Chromatogram showing the simultaneous separation of paraoxon and carbamate pesticides by isocratic elution. Column: Spherisorb 5 Amino. Mobile phase: methanol–water (40:60). Flow-rate: 1.0 ml min^{-1}. UV detection at 254 nm. Solute concentrations: 50 μg ml^{-1}. Peaks: A = isopropyl N-phenylcarbamate; B = paraoxon; C = isopropyl N-(3-chlorophenyl)carbamate. i = Injection.

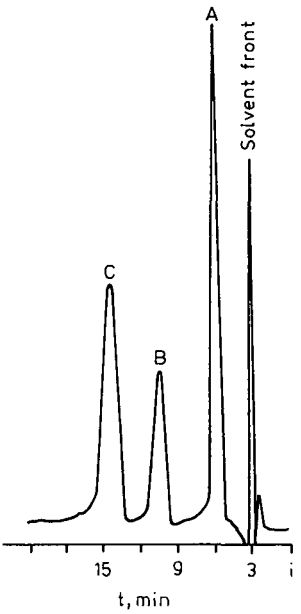

Fig. 4. Chromatogram showing the simultaneous separation of paraoxon and carbamate pesticides by isocratic elution. Column: Spherisorb S10 ODS2. Mobile phase: tetrahydrofuran–water (50:50). Flow-rate: 0.7 ml min^{-1}. UV detection at 254 nm. Solute concentrations: 10 μg ml^{-1}. Peaks: A = paraoxon; B = isopropyl N-phenylcarbamate; C = isopropyl N-(3-chlorophenyl)carbamate.

phase gave more sensitivity, this system was rejected because DFP has a retention time of 55 min under these conditions. Therefore, separation with tetrahydrofuran–water (50:50) on Spherisorb S10 ODS2 was chosen for further studies. Fig. 5 shows representative chromatograms of a four-pesticide sample obtained by using UV or enzymatic detection. The separation was achieved in 16 min. The enzymatic detector allows detection of traces of DFP, unlike the UV detector.

High sensitivity and good repeatability are achieved by keeping the concentration of tetrahydrofuran that passed through the enzyme column at *ca.* 20%. This amount is enough to keep in solution the α-naphthol produced by the enzymatic hydrolysis. It was found, however, that the presence of tetrahydrofuran decreases the useful life of the column; it was possible to make about 90 determinations of pesticides instead of the 150 reported earlier before significant loss of sensitivity occurred [9]. A change of enzyme column every 90 determinations is therefore recommended.

Analytical characteristics

Linear relationships between peak height and concentration were obtained in the range 5–40 ng for paraoxon, 0.1–1.6 ng for DFP, 20–100 ng for isopropyl N-phenylcarbamate and 40–400 ng for isopropyl N-(3-chlorophenyl)carbamate. The detection limits (amounts giving signal-to-noise ratio of 2) were 2.6 ng of paraoxon, 0.04 ng of DFP, 18 ng of isopropyl N-phenylcarbamate and 29 ng of isopropyl N-(3-chlorophenyl)carbamate. The detection limits for UV detection were 18, 23 and

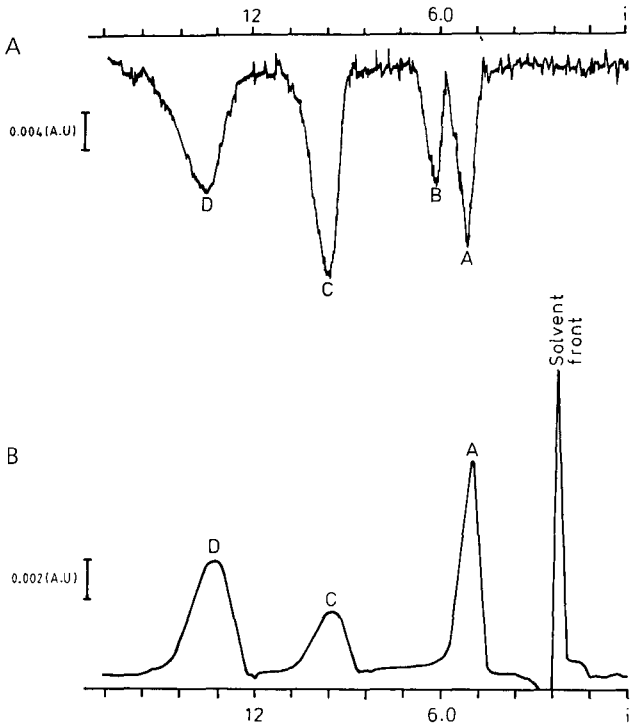

Fig. 5. Comparison of chromatograms of carbamate and organophosphorus pesticides. Column: Spherisorb S10 ODS2. Mobile phase: tetrahydrofuran–water (50:50). Flow-rate: 0.7 ml min^{-1}. Peaks: A = paraoxon; B = DFP; C = isopropyl N-phenylcarbamate; D = isopropyl N-(3-chlorophenyl)-carbamate. (A) Enzymatic detection; solute concentrations: 2 mg ml^{-1} for A, C, D, 0.04 mg ml^{-1} for B. (B) UV detection, solute concentrations: 20 mg ml^{-1}.

40 ng, respectively, which compare well with those reported by Lawrence and Turton [17]. Relative standard deviations ($n = 6$) were 1.2% (20 ng of paraoxon), 1.5% (0.2 ng of DFP), 1.7% (30 ng of isopropyl N-phenylcarbamate) and 1.9% [60 ng of isopropyl N-(chlorophenyl)carbamate].

The repeatability of retention times was studied by injecting 20 ng of paraoxon, 0.6 ng of DFP, 30 ng of isopropyl N-phenylcarbamate and 40 ng of isopropyl N-(3-chlorophenyl)carbamate on four successive days. The relative standard deviation of the retention times was 1.2%. Only 16 min were required for each complete determination.

CONCLUSIONS

Immobilized acetylcholinesterase can successfully be utilized in a post-column reactor system for detection of traces of organophosphorus and carbamate pesticides. The arrangement described allows the use of eluents containing organic solvents. High sensitivity and good repeatability are achieved by restricting the amount of the organic solvent to about 20%. Direct injection of sample is possible without the problems of

non-volatile compounds which are significant in gas chromatographic analyses. Mixtures of carbamates and organophosphorus pesticides can be analysed without changing the column or detector.

The proposed system should be applicable to the detection of any cholinesterase inhibitor. By using enzymes other than cholinesterase in an immobilized form, continuous assay of other compounds that are potent enzyme inhibitors should also be possible.

Sensitivity of detection depends crucially on the ability of the compound to inhibit the particular enzyme preparation. Several reports [5–8,11,12,16] have shown that organophosphorus and carbamate compounds tend to differ markedly in their ability to inhibit cholinesterases from different sources. Therefore, studies of cholinesterases from different sources could provide more sensitivity for certain cholinesterase inhibitors.

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Liquid and high-pressure carbon dioxide chromatography of β-blockers

Resolution of the enantiomers of nadolol[a]

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(First received April 19th, 1990; revised manuscript received June 19th, 1990)

ABSTRACT

The enantiomers of six chiral aryloxypropanolamine β-blockers were separated by high-performance liquid chromatography on a column of modified cellulose immobilized onto silica (Chiralcel OD), using mixtures of heptane or hexane with a polar modifier. The quality of the separations was improved and retention times were reduced by eluting under subcritical conditions with carbon dioxide containing 20% of methanol. Nadolol, which has two chiral centres, gave three peaks instead of four on Chiralcel OD. A separation of the four isomers was obtained on an α_1-acid glycoprotein column eluted with an aqueous buffer containing octanoate ions. A direct separation of the two diastereomers could not be obtained with achiral stationary phases, but the derivative formed by the base-catalysed reaction of nadolol with isopropyl isocyanate was well resolved on a column of amino-bonded silica eluted with carbon dioxide–methanol. This paper demonstrates the advantages of high-pressure carbon dioxide chromatography for the analysis of stereoisomers.

INTRODUCTION

The chirality of drugs is an important issue from the pharmacological, pharmacokinetic, toxicological, and regulatory points of view [1,2]. Despite the lack of a predictive mechanistic theory, direct chromatographic chiral separations have been described for compounds having numerous different functions [3]. The currently available chiral stationary phases can be classified according to their chemical properties and, in general, each type of phase is suitable for compounds having a limited range of functionalities [4,5]. The β-adrenoceptor antagonists (β-blockers) are a clinically important class of drugs used in the treatment of cardiovascular disorders and of ocular hypertension. They have in common a substituted aryloxypropanolamine function of which the hydroxy-substituted carbon is chiral (see

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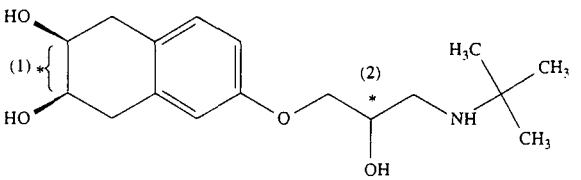


OPTICAL ROTATION		RACEMATE
Centre (1)	Centre (2)	
+	+	A
-	-	
+	-	B
-	+	

Fig. 1. Structure of nadolol.

nadolol, Fig. 1). Despite this structural similarity between members of the series, the racemic compounds display a wide variety of pharmacological properties.

Mode-of-action studies of the β-blockers reveal marked enantioselectivity (*e.g.*, refs. 6 and 7), and therefore the pharmacological profiles of racemic β-blockers are composites of the profiles of the enantiomers. As both enantiomers usually have some relevant activity, an optically pure β-blocker is not necessarily a "better" product than the racemate; it is rarely possible to describe a racemate as a mixture of an active substance and "isomeric ballast" [8]. Nevertheless, the synthesis and analysis of optically pure β-blockers represents a field of much commercial importance.

Chiral chromatographic separations, mostly with stationary phases of modified cellulose grafted onto silica (Chiralcel series), which are suitable for pharmaceutical analysis and pharmacokinetic studies, have been described for all of the important aryloxypropylamines [4]. Chiralcel columns are used in the normal-phase mode and they are usually eluted with a mixture of hexane and polar modifiers such as *n*-propanol [4]. Normal-phase separations are greatly improved in terms of speed and resolution by eluting with mixtures of carbon dioxide and methanol, in which solutes have relatively favourable diffusion coefficients [9,10]. This technique is generally referred to as supercritical fluid chromatography (SFC). However, as the state of the fluid used in this present study is not always supercritical, we shall use the abbreviation in quotation marks. "SFC" is now used routinely in many laboratories and it is perhaps time to adopt a convenient but more theoretically sound vocabulary. The essential feature of "SFC" is that a fluid that would be gaseous at pressures near atmospheric is compressed to a density at which it has significant solvating power. The principal advantages of "SFC" over conventional liquid chromatography are that diffusion coefficients are greater, and that the solvating power can be varied by means of pressure-induced density changes. Bearing in mind that the main constituent of the eluent is usually CO_2, we suggest that an expression such as "high-pressure carbon

dioxide chromatography (HPCDC)" would be more generally appropriate than "SFC" whenever the solvating power of the CO_2 plays a significant role in the chromatographic mechanism. This type of expression correctly applies to all applications of this particular eluent at pressures higher than those generally used in gas chromatography [11]. We have been unable to devise an expression that would cover all "SFC" mobile phases.

Previously, we described chiral separations of six β-blockers by high-performance liquid chromatography (HPLC) on a Chiralcel OD column [4]. In this paper, we evaluate the improvements that can be obtained by using "SFC" techniques with particular emphasis on the resolution of the isomers of nadolol. Among the clinically important β-blocking drugs, nadolol presents a particular chromatographic challenge as it possesses two chemically different chiral sites, the second being a *cis*-1,2-dihydroxy function (Fig. 1). The commercial drug substance is a mixture of approximately equal proportions of the two racemic diastereomers, the $+-/-+$ (*RS/SR*) mixture being designated racemate A and the $++/--$ (*RR/SS*) mixture racemate B [12]. The diastereomers are separable by fractional crystallization, but they are surprisingly difficult to separate chromatographically. One report mentions unsuccessful trials in which 202 different solvent systems were tested on 14 different thin-layer chromatographic supports [12]. A method using reversed-phase chromatography was reported in 1981 [13], but we were unable to reproduce this result using numerous currently available column packings. In 1984, an incomplete ion-exchange separation was described [14]; the analysis time was 20 min. In the absence of a robust chromatographic method for separating the diastereomers of underivatized nadolol, the official method for analysing the drug substance [15] is infrared spectroscopy, a relatively insensitive and inaccurate technique. In this paper, we show that the reaction of nadolol with isopropyl isocyanate [16,17] in the presence of a base yields a derivative whose diastereomers are well separated by normal-phase chromatography on a column of amino-bonded silica.

Nadolol gave at most three partially resolved peaks when analysed on the columns we tested during the study of chiral separations by HPLC on Chiralcel OD cited above [4]. In 1986, Schill *et al.* [18] described the chiral separations of the individual diastereomers on an α_1-acid glycoprotein (α_1-AGP) stationary phase. However, one member of each enantiomeric pair had the same retention time, so that a mixture of the diastereomers would, as on the Chiralcel OD column, have given three peaks instead of four. In addition, the original α_1-AGP columns were not ideally suited to pharmaceutical analysis; their loading capacity was limited, retention times were long and the peaks were generally broad. These factors impaired the detection of trace levels of the unwanted enantiomer. We now report that these difficulties are less severe with a "second generation" α_1-AGP column [19], and that, by carefully selecting the mobile phase, a complete separation of the four isomers of nadolol can be obtained.

EXPERIMENTAL

Reagents and chemicals

A racemic mixture containing approximately equal proportions of the diastereomers of nadolol was kindly supplied by Squibb. Isopropyl isocyanate (> 99%) was purchased from Fluka (Buchs, Switzerland). Liquid carbon dioxide, quality N 45 (< 7

TABLE I

CHIRAL AND NON-CHIRAL COLUMNS TESTED FOR THE CHROMATOGRAPHY OF β-BLOCKERS

Column	Supplier	Dimensions (mm)	Comments	No. of peaks[a]
Suplex pKb-100	Supelco	150 × 4.6	Reversed-phase	1
Erbasil 5 C_{18}/H	Carlo Erba	150 × 4.6	Reversed-phase	1
Zorbax Phenyl	DuPont	150 × 4.6	Reversed-phase	1
Spherisorb Mixed-Mode	Phase Separations	150 × 4.6	Reversed-phase, normal-phase (HPLC, SFC)	1
Spherisorb Phenyl	Phase Separations	150 × 4.6	Reversed-phase; chiral ion-pair with ZGP (HPLC, SFC)	1
Asahipak ODP-50	Asahi	150 × 4.6	Reversed-phase	1
Spherisorb S5 CN	Phase Separations	150 × 4.6	Normal-phase; chiral ion-pair with ZGP (HPLC, SFC)	1
Partisil 5	Whatman	150 × 4.6	Normal-phase	1
Zorbax BP-SIL	DuPont	150 × 4.6	Normal-phase	1
Spherisorb NH_2	Phase Separations	150 × 4.6	Normal-phase, derivatization with isopropyl isocyanate (HPLC)	1
Spherisorb NH_2	Phase Separations	150 × 4.6	Normal-phase, derivatization with isopropyl isocyanate (SFC)	2
LiChrospher Diol	Merck	150 × 4.6	Normal-phase	1
Chiral Pro-Cu-Si 100 Polyol	Serva	250 × 4.6	Ligand exchange	1
Cyclobond I (10 μm)	Astec	250 × 4.6	Reversed-phase, normal-phase (HPLC, SFC)	1
Chiralcel OD (10 μm)	Daicel	250 × 4.6	Normal-phase (HPLC, SFC)	3
Chiralcel OC (10 μm)	Daicel	250 × 4.6	Normal-phase (HPLC, SFC)	2
Chiralcel OG (10 μm)	Daicel	250 × 4.6	Normal-phase (HPLC, SFC)	2
Chiral-AGP	Baker	100 × 4.0	"Affinity chromatography" with ion-pairing (HPLC)	4
Pirkle covalent L-phenylglycine	Regis	250 × 4.6	Normal-phase, derivatization with isopropyl isocyanate (SFC)	2
Supelcosil-LC-(R)-urea	Supelco	250 × 4.6	Normal-phase, derivatization with isopropyl isocyanate (SFC)	2

[a] Number of peaks obtained for the racemic mixture of the two diastereomers of nadolol (four isomers).

ppm of water) was purchased from Alphagaz (Bois-d'Arcy, France). Other reagents and solvents were obtained from commercial sources.

Columns

The chiral and non-chiral columns used are listed in Table I.

Apparatus ("SFC" and HPLC)

Chromatography was performed using equipment manufactured by Jasco (Tokyo, Japan; supplied by Prolabo, Paris, France), except where indicated otherwise. The two pumps (Model 880-PU) are connected for gradient elution by means of a Model 801-SC controller and Model MX-50 dynamic high-pressure mixing unit. The head of one of the pumps is cooled to $-2°C$ when CO_2 is used. The mixing unit is connected to a standard injector (Model 7125; Rheodyne, Cotati, CA, U.S.A.) via a pressure relief valve (Rheodyne Model 7037). The column is mounted in a thermostatically controlled oven. The UV detector (Model 875-UV) is fitted with the standard high-pressure cell (4 μl; 5-mm path length). When CO_2 is used, the eluent is discharged via an automatic back-pressure regulator (Model 880-81). Chromatograms were recorded using an electronic integrator (Model C-R3A; Shimadzu, Kyoto, Japan; supplied by Roucaire, Vélizy-Villacoublay, France).

Chromatography of underivatized nadolol

Nadolol was dissolved in appropriate solvents at a concentration of 0.1% and 20 μl were injected. For each column, optimization of the "SFC" separation was attempted by varying the temperature between 20 and 45°C and the pressure between 100 and 280 bar and by varying the nature and concentration of the polar modifier.

Several hundred micrograms of nadolol were fractionated by repetitive injections onto the Chiralcel OD column, under the optimized conditions. The fractions corresponding to each of the three peaks were separately pooled and the solvent was evaporated in a vacuum centrifuge. The optical activities of methanolic solutions of the fractions were measured using a Jasco Model DIP-370 polarimeter (sodium D-line). Subsequently, the fraction corresponding to the first-eluted peak was re-evaporated and the residue was derivatized as described below.

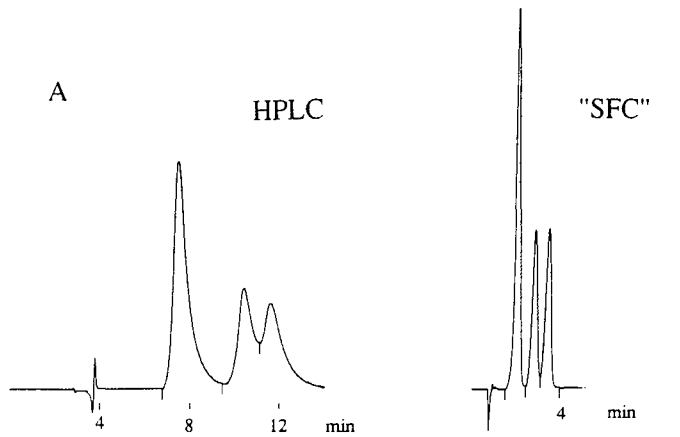
Derivatized nadolol

A suspension of nadolol (10 mg) in dichloromethane (350 μl), triethylamine (5 μl) and isopropyl isocyanate (150 μl) was prepared in a small conical glass tube fitted with a B-10 ground-glass stopper. The tip of the tube was heated at 60°C for 1 h, after which the solvent and excess of reagents were evaporated under a stream of nitrogen at room temperature. The pale brown residue was dissolved in a few drops of methanol, which was evaporated, and the derivative was taken up in methanol.

Exact experimental conditions for the separations that were studied in detail are given in the legends to the figures.

RESULTS AND DISCUSSION

The chiral and non-chiral columns used are listed in Table I, together with an indication of the mode of elution and the results obtained for nadolol. Chiralcel OD



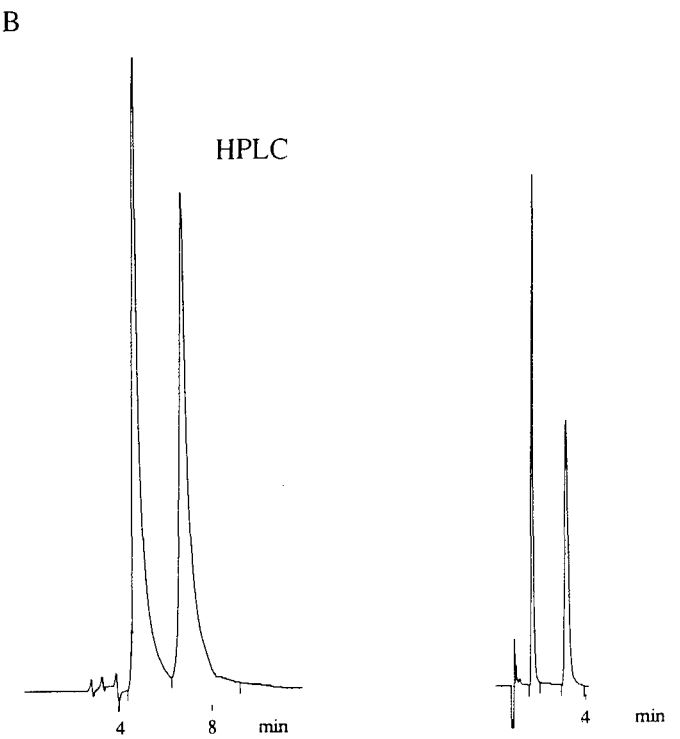


Fig. 2.

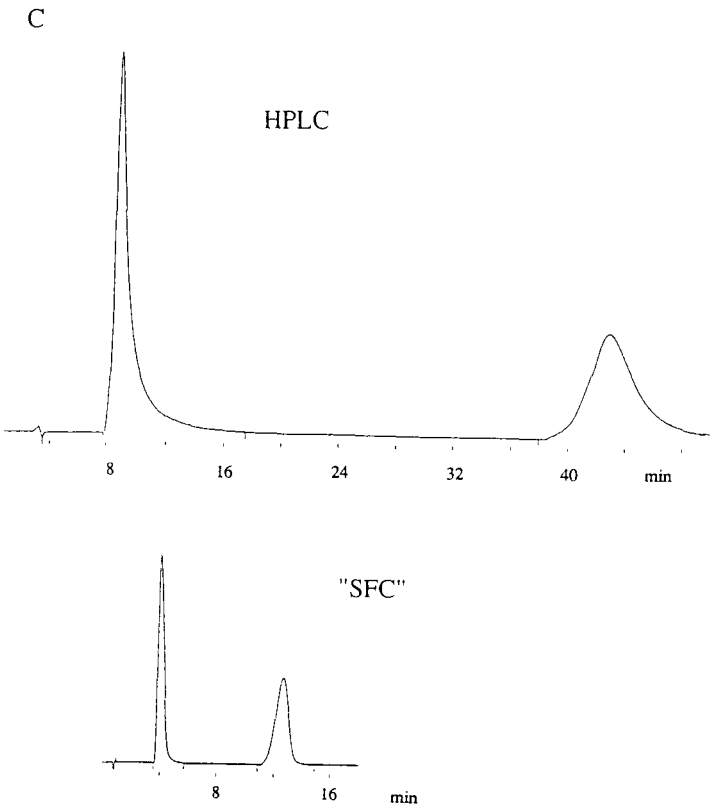


Fig. 2.

(*Continued on p. 62*)

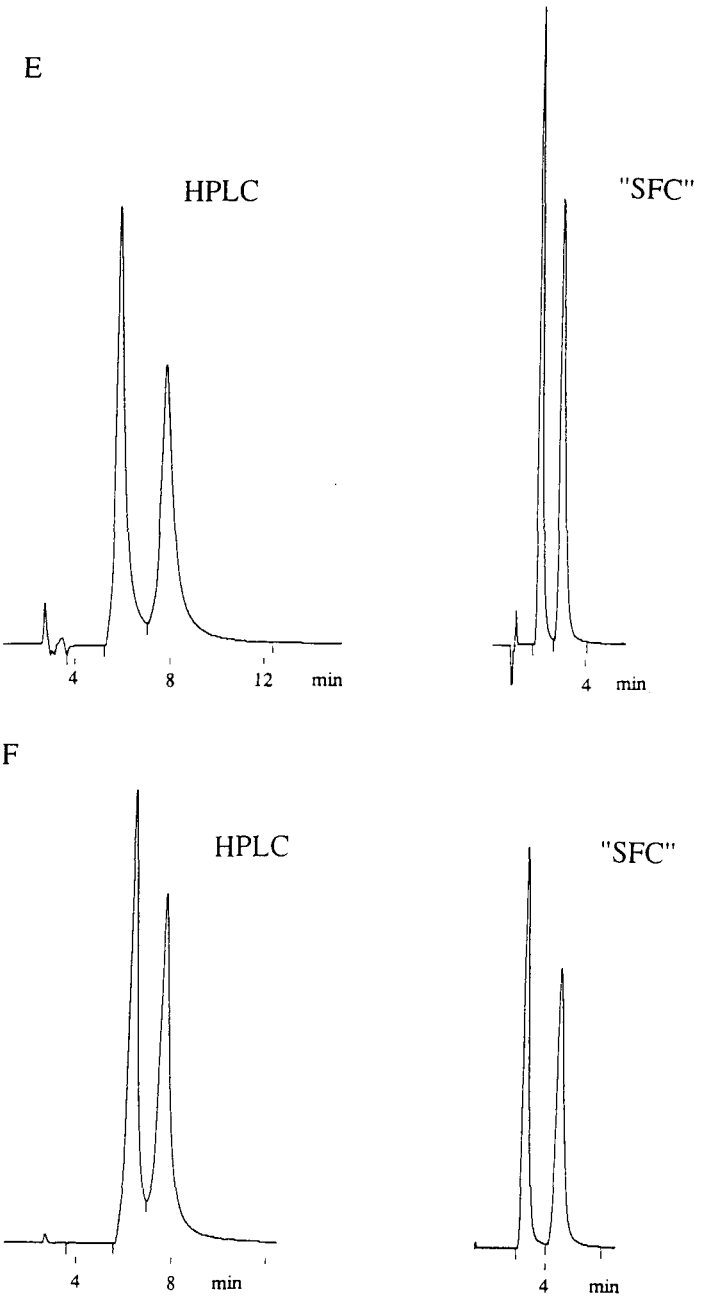


Fig. 2. Separations of β-blockers on Chiralcel OD (10 μm) column (250 × 4.6 mm I.D.) by HPLC and "SFC", with UV detection at 254 nm. (A) Nadolol; the mobile phase for HPLC was hexane–ethanol–2-propanol (80:15:5, v/v/v), temperature 22°C, flow-rate 1.0 ml/min; the mobile phase for "SFC" was CO_2–methanol (80:20, v/v), temperature 35°C, pressure 200 bar, flow-rate 4.0 ml/min. (B) Betaxolol hydrochloride; the HPLC mobile phase was hexane–2-propanol (83:1,7 v/v); the other chromatographic conditions were the same as in A. (C) Pindolol; the chromatographic conditions were the same as in A. (D) Metoprolol; the chromatographic conditions were the same as in A. (E) Cicloprolol hydrochloride; the chromatographic conditions were the same as in A. (F) Propranolol; the chromatographic conditions were the same as in A.

proved to be generally useful for all the β-blockers tested and, because this stationary phase functions in the normal-phase mode, we investigated the improvements that could be obtained by the use of "SFC".

Enantiomeric separations of β-blockers on Chiralcel OD: comparison between HPLC and "SFC"

Chromatograms of six β-blockers were obtained using the same Chiralcel OD column under standard HPLC conditions (elution with mixtures of hexane or heptane and propanol) and under "SFC" conditions (CO_2–methanol). The results, presented in Fig. 2, clearly indicate that, although the separations obtained by the two techniques are qualitatively similar, the "SFC" technique gives superior efficiencies and shorter retention times. The "SFC" traces were obtained under conditions that had been optimized, with a view to obtaining adequate separations with short retention times, by varying the temperature, pressure and flow-rate, as well as the concentration of methanol.

As expected for normal-phase chromatography, increasing the concentration of methanol led to decreased retention indices and a slight loss in efficiency. At the methanol concentration (20%) and temperature (35°C) finally chosen, it is most likely that the eluent is far below its critical temperature [20], although there appears to be no general agreement on the appropriate equations for calculating the critical parameters of mixtures [21]. The effects of varying, at a constant mass flow-rate, the temperature and the pressure at the column outlet are shown in Fig. 3 for betaxolol. Most interesting is a marked decrease in capacity factor (k') with increasing pressure, typical of separations usually described as "supercritical", although the pressure dependence was about the same at 20°C as at 45°C. The simplest explanation for this observation would be that the solvent mixture is more compressible than conventional solvents.

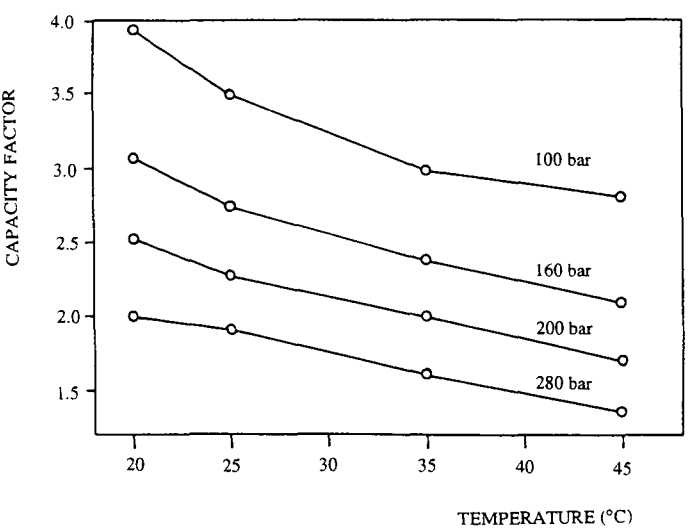


Fig. 3. Variation of k'_2 of betaxolol in "SFC" as a function of pressure and temperature. The chromatographic conditions were the same as in Fig. 2B.

The retention index decreased with increasing temperature, an effect that is always observed in liquid chromatography. For practical purposes, for the separations described here, the improvement afforded by the use of "SFC" can be attributed to the more favourable chromatographic properties (diffusion coefficients and viscosity) of CO_2–methanol compared with hexane [9,10]. The advantages of mobile phases that would be gaseous at atmospheric pressure extend to temperatures well below the

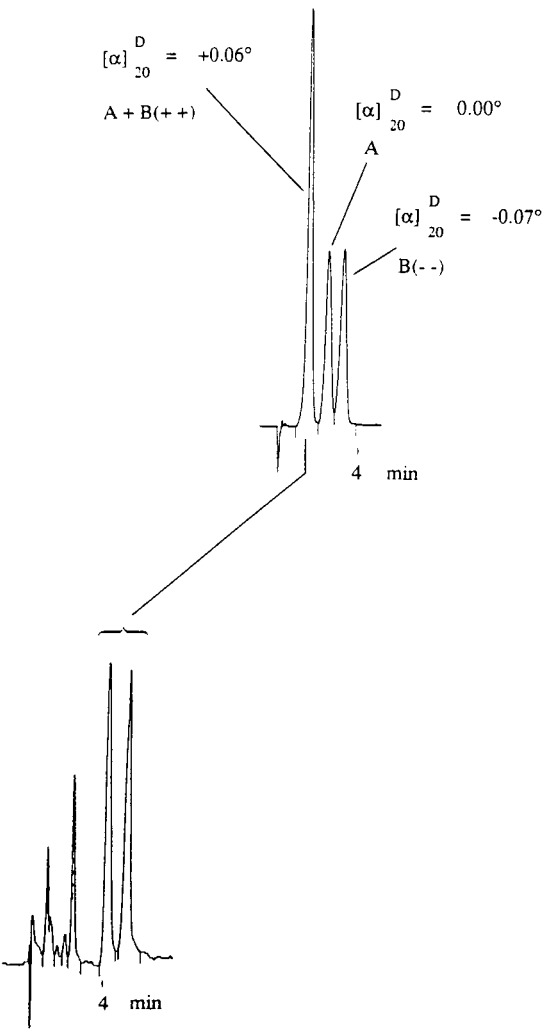


Fig. 4. Characterization of the stereoisomers of nadolol. Upper panel: optical activities and postulated configurations of the three peaks separated by "SFC" on a Chiralcel OD column. The chromatographic conditions were the same as in Fig. 2A. Lower panel: "SFC" separation of isopropyl isocyanate derivative of the first peak on the upper panel, collected and reanalysed by "SFC" with a Spherisorb-NH_2 (5 μm) column (100 × 4.6 mm I.D.); mobile phase CO_2–methanol (95:5, v/v), temperature 25°C, pressure 200 bar, flow-rate 3.0 ml/min.

critical point; at 20°C, the viscosity of liquid CO_2 is 0.071 cP, whereas that of hexane is 0.326 cP [22].

Nadolol: separations on Chiralcel OD

The Chiralcel OD column used gave three peaks for nadolol (Figs. 2A and 5A), whereas during an earlier study with another column of the same type we could obtain only two peaks. It is difficult to explain this batch-to-batch variation. Attempts to improve the separation further were unsuccessful. The first of the three peaks had twice the area of the other two, indicating that the two less intense peaks represent two of the four optically pure isomers that form the mixture (Figs. 4 and 5). As the separate diastereomers were not available to us, we collected fractions corresponding to the

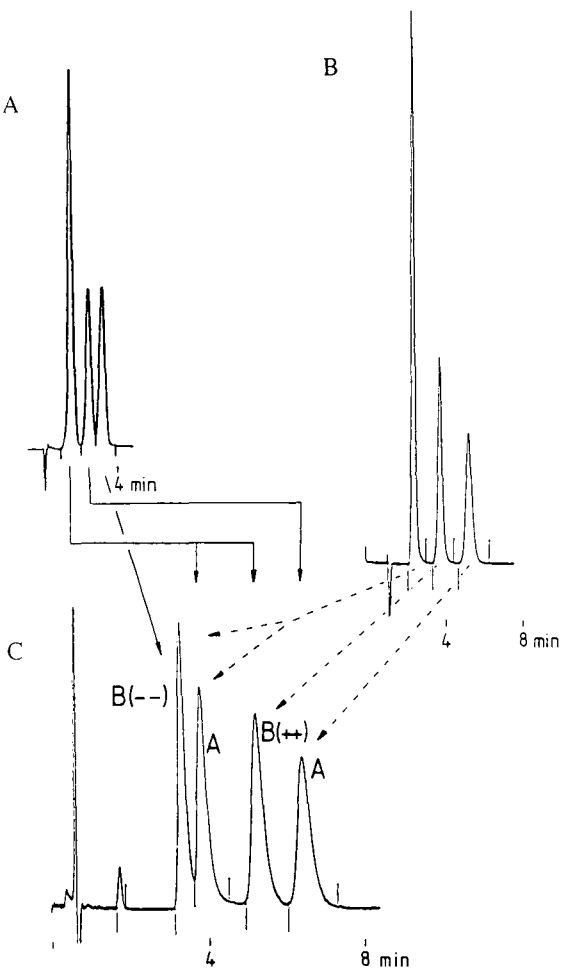


Fig. 5. Separations of the isomers of nadolol. (A) Chiralcel OD as in Fig. 2A. (B) α_1-AGP; the mobile phase was KH_2PO_4 (0.025 *M*) and tetrabutylammonium bromide (0.1%), adjusted to pH 7.5 with NH_3, temperature 22°C, flow-rate 0.9 ml/min. (C) α_1-AGP; the chromatographic conditions were as in (B), except that the mobile phase contained 0.05% octanoic acid instead of tetrabutylammonium bromide.

three peaks. These fractions gave optical activities of +0.06°, 0.00° and −0.07° respectively; although the measurements are close to the limit of detection of the polarimeter, it is clear that the activities of the second and third peaks are not of opposite sign. Therefore, they represent one enantiomer of each of the diastereomeric pairs. The other two enantiomers, which form the first peak, are also diastereomerically related. Further, since the enantiomers of the diastereomer designated racemate B [12] have the configurations (+ +) and (− −), the third peak, which has a larger absolute value of α than the second (Fig. 5A), can be assigned as the isomer B(− −). The second peak therefore represents one of the enantiomers of racemate A.

Nadolol: chiral separations on α_1-AGP

As the isomers of nadolol were not fully resolved on the Chiralcel columns, we reinvestigated the use of a new "second generation" α_1-AGP column. When eluted with a phosphate buffer containing tetrabutylammonium ion [18], three peaks were obtained for nadolol (Fig. 5B). The separation was qualitatively the same as that described by Schill *et al.* [18], but the efficiency, resolution and analysis time were all more favourable. In the absence of tetrabutylammonium ion, retention times were longer and the three peaks were broader and less well separated (not shown). The effects of different mobile phase additives were tested, and the results obtained with a buffer containing 0.05% of octanoic acid are shown in Fig. 5C. This chromatogram

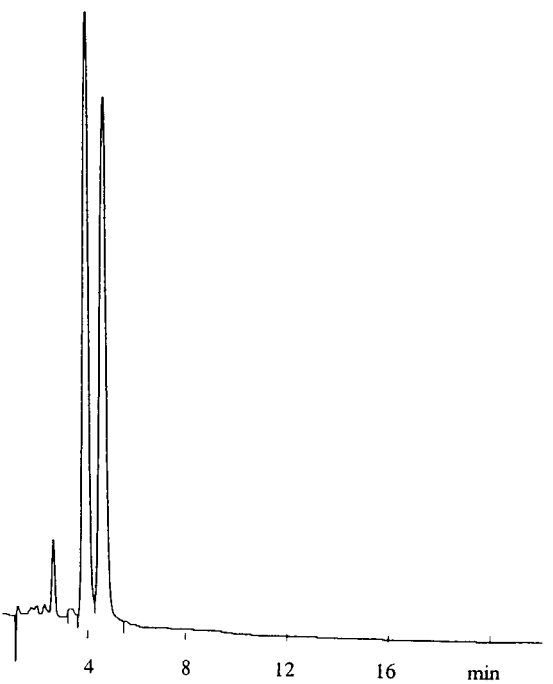


Fig. 6. "SFC" separation of the diastereomers of nadolol derivatised with isopropyl isocyanate in the presence of triethylamine, on a Spherisorb-NH_2 column. The chromatographic conditions were the same as in Fig. 4 (lower panel).

has four peaks of equal areas, separated almost to the baseline. With lower concentrations of octanoic acid, the first two peaks began to coalesce; at higher concentrations the second peak overlapped the third. Presumably, the octanoate modifies the relative retention times by forming ion pairs with the solutes, as both the additive and the stationary phase are negatively charged. The peaks in Fig. 4 were identified (1) by injecting the fractions (of known relative optical activities) from the Chiralcel OD column (Fig. 5A) onto the α_1-AGP column and (2) from the relative retention times of the A and B diastereomers given for α_1-AGP by Schill *et al.* [18]. There was insufficient information to distinguish the enantiomers of diastereomer A.

Nadolol: separation of the diastereomers on non-chiral columns

Because of doubts about the long-term availability and reproducibility of chiral columns, an achiral chromatographic method for separating the diastereomers would be of value as a replacement for the infrared spectroscopic method currently used for the quality control of nadolol. We attempted without success to separate the diastereomers of underivatized nadolol with the columns and eluents listed in Table I. Bearing in mind the unsuccessful efforts of other workers (see Introduction), there appears to be little further scope for this approach. We therefore turned to the use of a derivative. Isopropyl isocyanate was chosen as derivatizing reagent, because it reacts

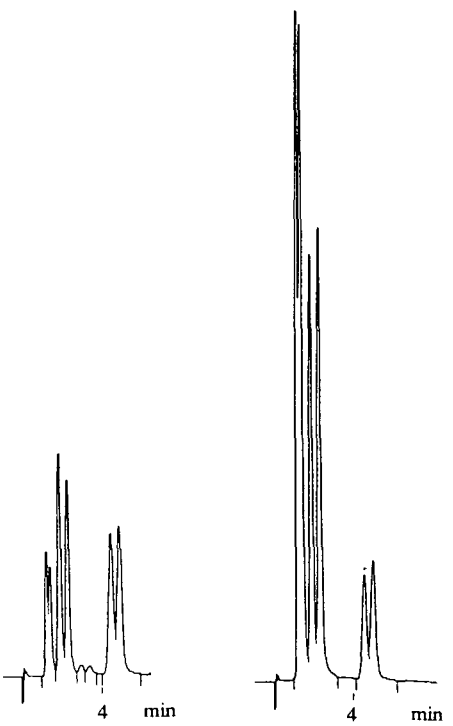


Fig. 7. Separations of the diastereomers of nadolol derivatised with isopropyl isocyanate in the absence of triethylamine (two preparations). The column was the same as in Fig. 4 (lower panel); mobile phase CO_2–methanol (90:10, v/v), temperature 25°C, pressure 200 bar, flow-rate 4.0 ml/min.

with most common functional groups that possess an active hydrogen to give neutral products, and because several cases of enhanced chromatographic stereoselectivity have been described by König's group [16,17]. Following reaction under the conditions described under Experimental, the racemic mixture of nadolol diastereomers gave two well resolved peaks of equal areas when chromatographed using "SFC" conditions on a non-chiral column (amino-bonded silica; Fig. 6). When the reaction was carried out in the absence of triethylamine, the chromatogram showed several pairs of peaks, presumably representing different incompletely derivatized products (Fig. 7), for each of which the diastereomeric pair was separated. As, when triethylamine was used, the products formed represent the least retained pair of derivatives, and as no other significant peaks appeared on the chromatogram, it can be assumed that the catalysed reaction was virtually quantitative.

Surprisingly, apart from the Spherisorb-NH_2 column, none of non-chiral columns listed in Table I separated the diastereomers of the isopropyl isocyanate derivative.

Isopropyl isocyanate derivative of nadolol: separation of the isomers on chiral columns

As the separation of the diastereomers of nadolol on an achiral column was facilitated by derivative formation, it was hoped that a chiral column might separate all four isomers of the derivative. All the chiral stationary phases (except α_1-AGP) listed in Table I were tested under "SFC" conditions, but the results were disappointing. The Pirkle-type covalently-bonded L-phenylglycine and the Supelcosil-LC(R)–urea columns gave two but not four peaks, and the other columns gave only one.

CONCLUSION

As the retention mechanism involved in chiral chromatographic separations on modified celluloses is essentially normal-phase, this is a field in which "SFC" can be used to advantage. All of the common β-blockers except nadolol are fully resolved by this approach. The direct chiral analysis of the four isomers of nadolol can now be carried out on the α_1-AGP stationary phase. Alternatively, Chiralcel OD may be used, provided that the diastereomeric ratio is also determined by chromatography of a derivative. The choice between these two methods may depend, ultimately, on the reproducibility of the commercially available columns.

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Reversed-phase high-performance liquid chromatographic separation of the enantiomers of trimetoquinol hydrochloride by derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate and application to the optical purity testing of drugs

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ABSTRACT

The enantiomers of the bronchodilator trimetoquinol hydrochloride were separated by reversed-phase high-performance liquid chromatography after derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (TAGIT) reagent. The corresponding diastereomeric thiourea derivatives were baseline resolved on an ODS column with a resolution of more than 2 within 10 min. The derivatization proceeded smoothly and quantitatively within 15 min at room temperature even in aqueous solution. The favourable UV adsorption of the derivatized enantiomers permitted the detection of the (*R*)-(+)-isomer in (*S*)-(−)-trimetoquinol hydrochloride down to the 0.2% level. The determination of the optical purity of trimetoquinol hydrochloride drug substance and in tablets and injections was succesfully achieved. The optimization of the derivatization procedures and separation conditions is described.

INTRODUCTION

The enantiomers of different drugs, which have one or multiple asymmetric centres, may differ widely in their biological activities and toxicological properties [1,2] and it is therefore important to establish methods for the enantiomeric purity testing of chiral drugs. Trimetoquinol hydrochloride [(*S*)-(−)-form], which was developed at Tanabe Seiyaku as a broncodilator for use in bronchospastic diseases, such as asthma, has been found to be therapeutically more effective than the (*R*)-(+)-isomer, which has weak cardiovascular and bronchodilator activity [3]. A method for the determination of the optical purity of trimetoquinol hydrochloride and that in preparations such as tablets and injections is required in order to certify the quality. It is also required for the method to be simple, sensitive and reproducible.

Chromatographic methods have been widely used to separate and quantify the enantiomers of compounds in mixtures, especially high-performance liquid chromatography (HPLC). Recently much work has been conducted to separate enantiomers

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directly using chiral stationary phases [4,5]. Numerous theoretical studies on chiral recognition have also been conducted to establish the mechanism of the separation of enantiomers and to select suitable chiral stationary phases according to their mode of separation mechanism and the type of chiral compounds, which have different functional groups and structures [4–6]. Although several commercially available chiral stationary phases have been used to achieve the successful separation of the enantiomers of the drug, it was unsuccessful for trimetoquinol hydrochloride. The chiral derivatization method was then employed to separate the enantiomers.

(*S*)-(−)-N-1-(2-Naphthylsulphonyl)-2-pyrrolidine carbonyl chloride (NSP-Cl), which can react with a hydroxy or an amino group, and which has been successfully applied to the separation of the enantiomers of diltiazem hydrochloride [7] and DL-amino acids [8], was first employed as a chiral reagent. Separation of the corresponding diastereomers of trimetoquinol with NPS-Cl was achieved by normal-phase HPLC and has been reported elsewhere [9]. The derivatization with NSP-Cl proceeds in non-aqueous media and with a small amount of alkali. This is a useful method for determining the optical purity of drug substances. However, it requires evaporation of the large volume of water used for the derivatization of the drug in injections, where the drug substance is dissolved in an aqueous solution. Sample treatment is very tedious and time consuming for routine analysis.

2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (TAGIT or GITC), which reacts with amino compounds even in aqueous media and has been successfully applied to the chiral separation of DL-amino acids [10,11] and β-blocking agents [12], was subsequently employed. Trimetoquinol reacted readily with TAGIT at room temperature and with a small amount of alkali in aqueous media. The corresponding diastereomers were successfully separated by an analytical ODS column within 10 min. We have been investigating the chiral separation or chiral recognition of drugs by reversed-phase HPLC. This mode is more useful than normal-phase HPLC for the determination of enantiomeric pairs of drugs in biological or aqueous solutions. Reversed-phase HPLC is also more reproducible than normal-phase HPLC, which is sensitive to moisture in the mobile phase.

This paper describes the reversed-phase HPLC chiral separation of the enantiomers of trimetoquinol hydrochloride. The optimization of the derivatization procedures and HPLC conditions is discussed. Applications to the determination of the optical purity of the drug substance and in tablets and injections are also described. Simple and rapid optical purity testing of trimetoquinol hydrochloride was accomplished.

EXPERIMENTAL

Equipment

The liquid chromatograph consisted of an LC-3A high-pressure pump, a CT-O column oven and an SPD-2A variable-wavelength UV detector (Shimadzu, Kyoto, Japan). A Shimadzu SPD-6MA photodiode-array detector was also used to monitor the UV spectra of peaks. Samples were applied to the column with a Rheodyne 7125 loop injector. An ODS column (150 mm × 4.6 mm I.D.) was used, slurry packed with Inertsil ODS-2 (particle size 5 μm) (Gasukuro Kogyo, Tokyo, Japan). Peak integration was carried out with a Chromotopac C-R5A dataprocessor (Shimadzu). Quanti-

fication of the (+)-antipode of trimetoquinol hydrochloride was effected using the equation

$$(+)\text{-antipode (\%)} = \frac{\text{peak area of (+)-antipode}}{\text{sum of peak areas of (+)-antipode and (−)-trimetoquinol}} \cdot 100$$

Materials

The chiral reagent TAGIT was purchased from Wako (Tokyo, Japan). Trimetoquinol hydrochloride [(*S*)-(−)-form], its racemate and Inolin tablets (3 mg of trimetoquinol per tablet), Inolin injections (0.1 mg in 1 ml and 0.05 mg in 20 ml) were obtained from the research laboratories at Tanabe Seiyaku (Osaka, Japan). The structure of (*S*)-(−)-trimetoquinol hydrochloride is shown in Fig. 1. HPLC-grade acetonitrile and methanol, and analytical-reagent grade N,N-dimethylformamide (DMF) were purchased frm Katayama Kagaku Kogyo (Osaka, Japan). Water was purified with a Millipore RO-60 water system (Millipore Japan, Tokyo, Japan). All other reagents and solvents were of analytical-reagent grade from Katayama Kagaku Kogyo. All quantitative volume transfer work at the microlitre level was performed with an Eppendorf digital pipetter (VWR, IL, U.S.A.) and a 10-μl syringe (Hamilton, NV, U.S.A.).

Chromatographic conditions

Optical resolution was carried out with the Inertsil ODS-2 column at a flow-rate of 1.0 ml/min and 40°C. The derivatized chiral drug was monitored with a UV detector at 250 nm, which is the wavelength of maximum absorption of the TAGIT reagent. The UV spectra of each derivatized enantiomer were also measured with a photodiode-array detector over the wavelength range 200–300 nm.

The mobile phase consisted of 0.05 *M* phosphate buffer (pH 3.0)–acetonitrile (29:21, v/v). The buffer was prepared by dissolving 6.8 g of potassium dihydrogenphosphate in 1000 ml of water, and the pH was adjusted to 3.0 with diluted phosphoric acid (*ca.* 10%). The mobile phases were passed through a membrane filter of 0.45-μm pore rize and 4.7-cm diameter (Fuji Photo Film, Tokyo, Japan) and were degassed by using a Branson Model B-2200 ultrasonic cleaner (Yamato, Tokyo, Japan) prior to use.

Derivatization of drug substances

About 1 mg of trimetoquinol hydrochloride or its racemate was dissolved in 1 ml of DMF. To 100 μl of this solution were added 100 μl of 0.5% (w/v) TAGIT solution in

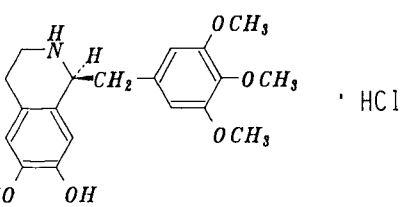

Fig. 1. Structure of (*S*)-(−)-trimetoquinol hydrochloride.

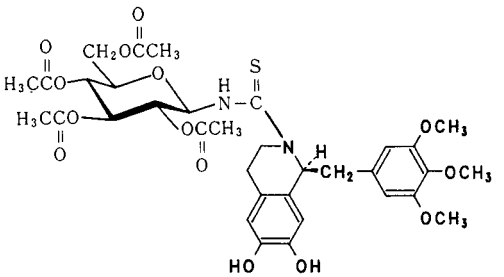

Fig. 2. Structure of TAGIT-derivatized (*S*)-(−)-trimetoquinol.

acetonitrile and 2 μl of triethylamine. The mixture was shaken vigorously and allowed to stand at room temperature for 15 min. The corresponding thiourea derivative was formed in this step and has the structural formula as shown in Fig. 2. After reaction, 2 μl of ethanolamine were added and the solution was shaken well and allowed to stand for 5 min to remove the excess of TAGIT reagent. Finally, 200 μl of 10% acetic acid were added and this acidic solution was used as a sample solution.

Derivatization of drug substances in tablets

Inolin tablets, each of which contains 3 mg of trimetoquinol hydrocloride, were extracted by DMF as follows. Twenty tablets were weighed accurately and ground, the powder, containing nearly 10 mg of trimetoquinol hydrochloride, was transferred into 10-ml volumetric flask and 7 ml of DMF were added. The flask was then sonicated for 10 min and warmed in a water bath (*ca.* 40°C) for 10 min with occasional shaking and cooled. After dilution to volume with DMF, the flask was shaken well, the solution was filtered and 100 μl of the filtrate were derivatized using the same procedure as described above.

Derivatization of drug substances in injections

Inolin injections contain 0.1 mg of trimetoquinol hydrochloride per ampoule (ampoule volume 1 ml) and those for intravenous injection contain 0.05 mg per ampoule (ampoule volume 20 ml). An ampoule of Inolin injection (0.1 mg) was cut and 200 μl of the solution were pipetted into a 2-ml vial and 200 μl of 0.5% (w/v) TAGIT solution in acetonitrile and 10 μl of 3% ammonia solution were added. The vial was shaken and allowed to stand at room temperature for 15 min. After reaction, the solution was subjected to the same procedure as for drug substances.

To 1 ml of Inolin intravenous injection were added 200 μl of 0.5% (w/v) TAGIT solution in acetonitrile and 10 μl of 3% ammonia solution, and the solution was subjected to the same procedure as for Inolin injection (0.1 mg), except for the addition of 10% acetic acid (100 μl).

Aliquots (10–20 μl) of these sample solutions were then injected into the HPLC system using a 25-μl microsyringe (Hamilton) and quantification of the antipode was effected by calculation from the peak areas of each enantiomer according to the above equation.

Preparation of blank

A blank for the derivatization of the enantiomeric drug was prepared by using the same procedure in the absence of drug substance.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Separation of the derivatized racemate of the chiral drug was investigated with the analytical ODS column (150 mm × 4.6 mm I.D.), using a mobile phase consisting of 0.05 *M* phosphate buffer (pH 3.0) and an organic solvent. Capacity factors (k'), separation factors (α) and resolution (R_s) of corresponding diastereomers obtained with various concentrations and types of organic solvent are summarized in Table I. The TAGIT-derivatized trimetoquinol racemate was baseline resolved by reversed-phase HPLC using acetonitrile or methanol as the organic solvent with a short analysis time. Methanol was more effective than acetonitrile for the resolution of the diastereomers, as judged from the resolution values and analysis times, although baseline resolution was achieved with both. With the use of methanol in the mobile phase, unreacted TAGIT reagent eluted near the peak of (*S*)-(−)-trimetoquinol and the column pressure drop was higher than with the acetonitrile-containing mobile phases. Acetonitrile was therefore selected as the organic modifier of the mobile phase. In the separation of TAGIT-derivatized DL-amino acids, acetonitrile was effective for the resolution of TAGIT–DL-serine, which was not resolved using methanol. On the contrary, methanol was effective for the resolution of TAGIT–DL-arginine, which was not baseline resolved with the acetonitrile-containing mobile phase. Thus, the simultaneous separation of common protein amino acids enantiomers was successfully achieved by gradient elution using a ternary mobile phase of methanol, acetonitrile and buffer solution [11].

A typical chromatogram of TAGIT-derivatized trimetoquinol racemate in a mobile phase of acetonitrile–phosphate buffer (pH 3.0) (21:29) is shown in Fig. 3. UV spectra of each peak monitored by using a Shimadzu SPD-6MA photodiode-array detector are shown in Fig. 4. Each diastereomer shows an identical spectrum of the

TABLE I

CAPACITY FACTORS, SEPARATION FACTORS AND RESOLUTION OF TRIMETOQUINOL RACEMATE USING METHANOL AND ACETONITRILE AS AN ORGANIC MODIFIER

Organic modifier	Content (%)	k'		α	R_s
		(−)-	(+)-		
Methanol	60	1.67	2.29	1.37	2.97
	57.5	2.27	3.12	1.37	3.42
	56	3.15	4.33	1.37	3.74
	55	4.14	5.66	1.37	4.14
Acetonitrile	44	2.63	3.03	1.15	1.79
	42.5	2.94	3.41	1.16	1.92
	42	3.49	4.06	1.16	2.10
	41	3.89	4.54	1.17	2.27

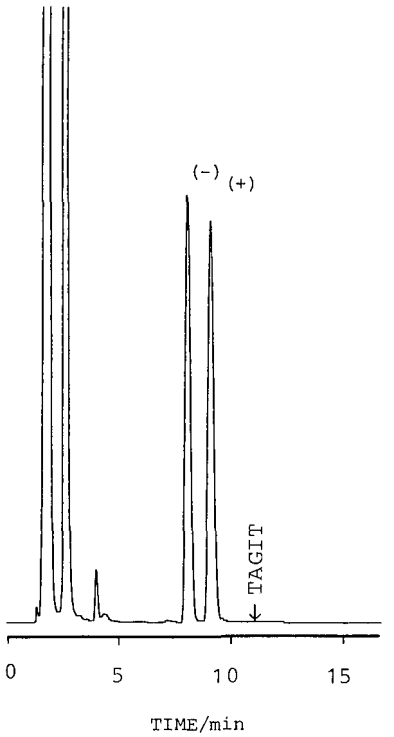

Fig. 3. Separation of the trimetoquinol racemate after derivatization with TAGIT. Mobile phase, 0.05 *M* phosphate buffer (pH 3.0)–acetonitrile (29:21), Column, Inertsil ODS-2 (150 mm × 4.6 mm I.D.); temperature, 40°C. Flow-rate, 1.0 ml/min. Detection, 250 nm.

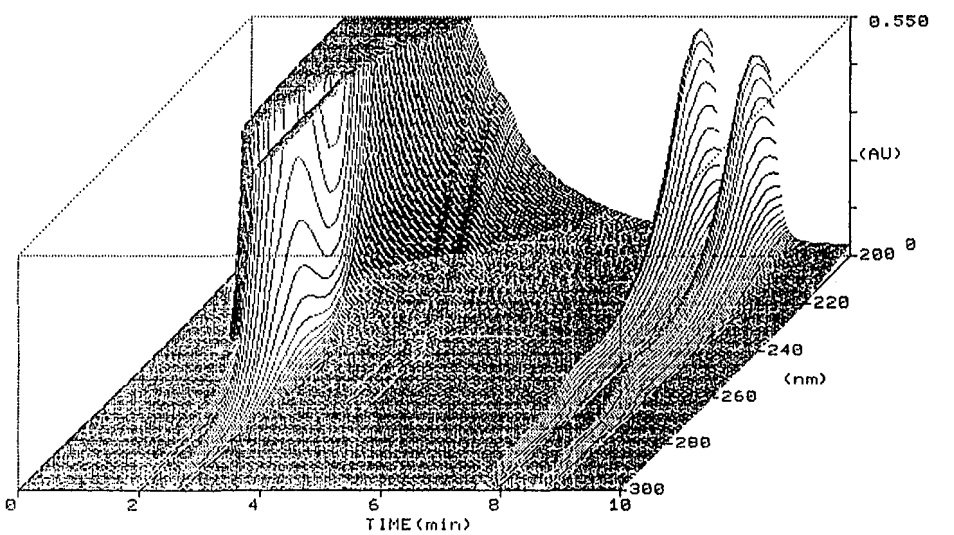

Fig. 4. UV spectra of each diastereomer monitored by a photodiode-array detector.

maximum absorption at 208 nm. Trimetoquinol hydrocloride itself shows the maximum absorption at 283 nm in 0.01 *M* hydrochloric acid solution [13]. Separation was successfully achieved within 10 min without interference from the TAGIT peak (see Fig. 3) or reagent blank peaks (see Figs. 5 and 6). Trimetoquinol [(*S*)-(−)-form] eluted faster than the antipode [(*R*)-(+)-form]. This elution order (*S*)-(−)-isomer > (*R*)-(+)-isomer corresponds to the results for other TAGIT derivatized drugs such as β-blocking agents [12] and antihypertensive agents [14]. However, for denopamine, which is a cardiotonic agent, the elution order was (*R*)-(−)-isomer > (*S*)-(+)-isomer [15]. The excellent resolution is probably due to the lipophilic nature of the tetraacetylglucopyranosyl residue of TAGIT and its conformational rigidity [10,11].

Optimization of derivatization conditions

Optimization of the derivatization of trimetoquinol hydrochloride with TAGIT was investigated by varying the reaction temperature (room temperature and 40°C), reaction time (from 5 to 60 min) and the amounts of TAGIT reagent (two, five and ten times that of of the drug substance) and alkali. The effect of the species of alkali on the reactivity was also investigated. The derivatization conditions were selected so as to result in the greatest peak height of the TAGIT-derivatized trimetoquinol. The derivatization proceeded rapidly within 15 min without warming with an excess of TAGIT (more than five times that of the sample) and a small amount of alkali. Concerning the type of alkali, reaction of the drug substance and those in tablets readily proceeded by using triethylamine, as in the derivatization of amino acids. However, for Inolin injections (aqueous solutions), dilute ammonia solution (*ca.* 3%) was more effective for the reactivity than triethylamine and a 1 *M* sodium hydroxide solution and its use gave the maximum peak height of the derivative, although ammonia itself can react with TAGIT. The use of dilute ammonia solution to catalyse the reaction was not a problem because unreacted TAGIT remained in sufficient amount even after reaction for 15 min, as judged from its peak area. The peak area was, however, reduced by half after 45 min in comparison with that after a 15-min reaction. The reaction blank in the chromatogram obtained with the use of ammonia solution (*ca.* 3%) was also smaller than that with other alkalis.

The peak areas of TAGIT-derivatized trimetoquinol hydrochloride in injections (0.1 mg) obtained with various reaction times at room temperature are summarized in Table II together with the data on the stability of the derivative in the reaction solution. With an excess of TAGIT reagent, the reaction proceeded rapidly and quantitatively, as judged from the constant peak areas of the derivatives for reaction times from 10 to 60 min. The peak obtained from a reaction mixture that was warmed at 40°C in a water-bath for 15 min had the same area as that obtained at room temperature.

The conditions selected are described under Experimental.

Linearity of response and limit of detection

Linearity of response for trimetoquinol hydrochloride [(*S*)-(−)-form] was investigated by derivatizing samples with a concentration range 20–120% of the concentration of drug substance in the sample (0.1%, w/v). The graph passed through the origin and the peak areas exhibited linearity over the tested range with a correlation coefficient $r = 0.996$.

The recovery of peak-area response of the (*R*)-(+)-isomer added to (*S*)-(−)-

TABLE II

PEAK AREA OF TAGIT-DERIVATIZED (*S*)-(−)-TRIMETOQUINOL AS A FUNCTION OF REACTION TIME AND STABILITY OF THE SAMPLE SOLUTION AT ROOM TEMPERATURE

	Reaction time (min)				Storage period (days)	
	5	10	15	60	1	4
Peak area (μV s × 10^4)[a]	48.2	50.9	50.4	49.9	49.1	49.4

[a] Average value ($n=3$).

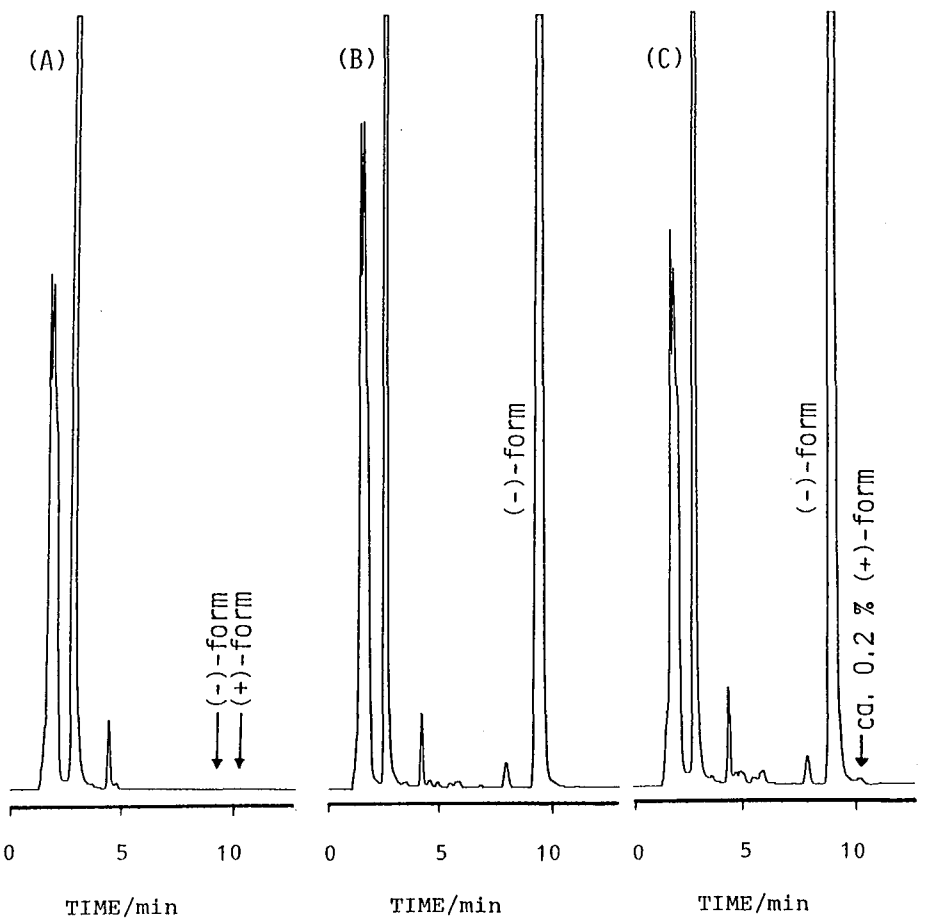

Fig. 5. Chromatograms of (A) a blank, (B) standard (*S*)-(−)-trimetoquinol and (C) standard trimetoquinol with *ca.* 0.2% of (+)-isomer added. Conditions in Fig. 3.

trimetoquinol hydrochloride was also examined over the range 0.2–2.0% (w/w) added. The recovery of the antipode averaged 98.2% over this addition range. The graph also passed through the origin with a slope of 1.07, an intercept of 0.03 and a correlation coefficient $r = 0.994$.

These results indicate that the extent of derivatization reaction of the (*R*)-(+)- and (*S*)-(−)-isomers with TAGIT is independent of the amount of each enantiomer presented in the reaction mixture or whether the reactivity of each enantiomer with TAGIT is the same. That is, the peak-area ratio of the derivatized product is equal to the weight ratio of the underivatized (+)- and (−)-isomers. Chromatograms of optically pure (*S*)-(−)-trimetoquinol hydrochloride, (*S*)-(−)-trimetoquinol with *ca.* 0.2% of the (*R*)-(+)-antipode added and a blank are shown in Fig. 5. It was found that the limit of detection of the (+)-isomer under these conditions was down to the 0.2% level.

Stabilities of sample and reagent solutions

The excess of TAGIT reagent that remains after the derivatization of the drug was removed by addition of ethanolamine, resulting in a faster elution than that of the TAGIT-derivatized drugs. The peak of TAGIT-derivatized ethanolamine appeared at *ca.* 2.5 min (see Figs. 3 and 4). Finally, the solution was acidified to *ca.* pH 3.0 by the addition of 10% acetic acid as described above. The acidic sample solutions were stable for at least 4 days at room temperature, as judged from the constant peak areas of the TAGIT-derivatized drug shown in Table II. This will allow overnight analysis by using an auto-sampler in the routine optical purity testing of many samples. The reactivity of TAGIT reagent in acetonitrile also did not decrease for at least 1 week.

Optical purity testing of trimetoquinol hydrochloride and those in preparations

Determination of the optical purity of drug substances and those in tablets was performed according to the described procedure. Trimetoquinol hydrochloride was extracted from the tablet matrix quantitatively by using DMF with sonication and warming, owing to its good solubility in DMF. The (*R*)-(+)-isomer was not detected in all batches of drug substances and Inolin tablets tested. These have excellent optical purity of more than 99.8%, as shown in Table III.

TABLE III

RESULTS OF OPTICAL PURITY TESTING OF TRIMETOQUINOL HYDROCHLORIDE AND THAT IN PREPARATIONS

Sample	Batch	Optical purity (%)
Trimetoquinol hydrochloride	AQ-1	>99.8
	AQ-2	>99.8
Inolin tablets (3 mg)	IJ-1	>99.8
	IJ-2	>99.8
Inolin injection (0.1 mg)	IC-1	>99
Inolin injection (0.05 mg)	IC-2	>95

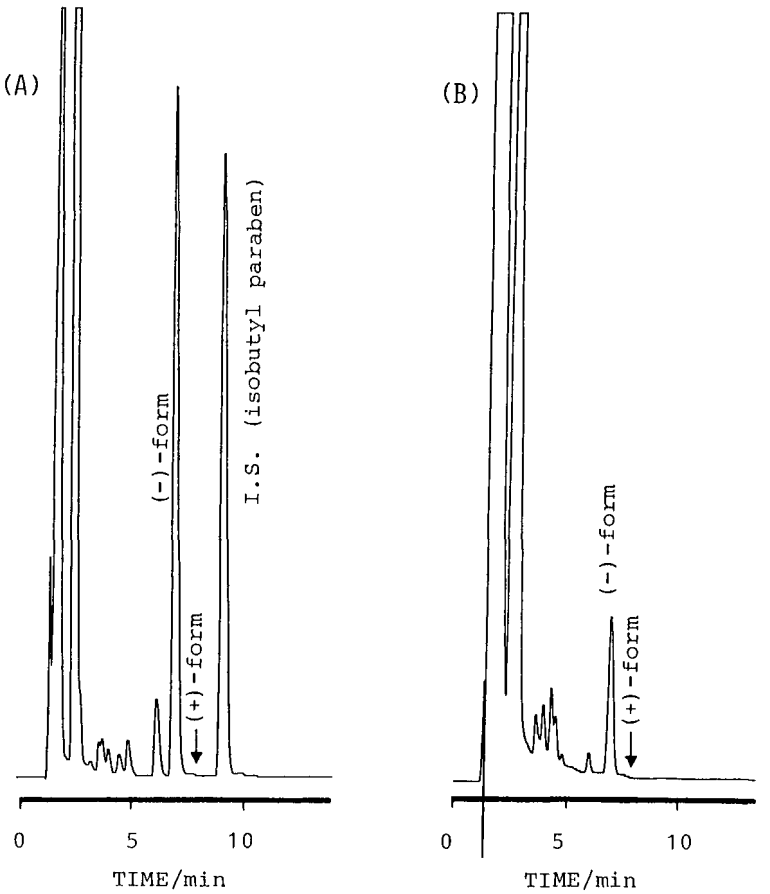

Fig. 6. Chromatograms for (A) the assay and optical purity testing of Inolin injection (0.1 mg) and (B) optical purity testing of Inolin intravenous injection. Conditions in Fig. 3 except that the flow-rate was 1.1 ml/min.

Assay and optical purity testing of Inolin injections (0.1 mg) were successfully performed by using *p*-hydroxyisobutyl benzoate as an internal standard (I.S.). A typical chromatogram is shown in Fig. 6A. An I.S. solution was prepared by dissolving the substance in 10% acetic acid, the solution obtained being added to the reaction mixture to acidify the solution in the last step of the derivatization (100 or 200 μl) to give a peak-area ratio of the drug to I.S. of *ca.* 1. A standard solution for the assay was prepared by dissolving a known amount of the drug and adding the I.S. solution using the same procedure as for the sample solution preparation. The averaged assay value for ten ampoules of Inolin injection (0.1 mg) was 103.6% with a relative standard deviation of 2.9%. The (*R*)-(+)-isomer was not detected (Table III). Both assay and optical purity testing could be performed simultaneously in one analysis. The limit of detection for this preparation was almost 1%. The optical purity of the Inolin injections (0.1 mg) was more than 99% in all instances. The optical purity of Inolin

intravenous injections could also be successfully determined by the same method, as shown in Fig. 6B, although the limit of detection was about 5%.

CONCLUSIONS

The reversed-phase HPLC method presented here is simple, relatively sensitive and reproducible. The derivatization of trimetoquinol hydrochloride with TAGIT proceeded rapidly and quantitatively at room temperature. The derivatized drug is stable in acidic solution and the TAGIT reagent solution. Baseline resolution of the diastereomers was achieved within 10 min on an analytical ODS column. Optical purity testing of the drug substance and in tablets and injections was also successfully achieved. This method may be useful for separating the enantiomers of other drugs or compounds which have functional groups that react with TAGIT, with minor alterations to the chromatographic and derivatization conditions.

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(*R*)- and (*S*)-Naphthylethylcarbamate-substituted β-cyclodextrin bonded stationary phases for the reversed-phase liquid chromatographic separation of enantiomers

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ABSTRACT

(*R*)- and (*S*)-naphthylethylcarbamate-β-cyclodextrin bonded phases were originally developed for the normal-phase separation of enantiomers. Although their selectivity resembled that of some of the earlier substituted cellulosic phases, the functionalized cyclodextrin stationary phases were much more stable as they were bonded to the silica support and not adsorbed. Because of their stability, the naphthylethylcarbamate-β-cyclodextrin stationary phase was utilized in reversed-phase separations. It was found that a completely different set of enantiomers was resolved by this column in the reversed-phase mode. This included racemic pesticides such as Dyfonate, Ruelene, Ancymidol and Coumachlor; as well as a variety of pharmacologically active compounds such as Tropicamide, Indapamide, Althiazide, Tolperisone, a sulfonamide from Merck Sharp & Dohme that has been resolved only by indirect methods, and over twenty others. It appears that the naphthylethylcarmabate-β-cyclodextrin bonded phase is a highly effective multimodal chiral stationary phase. The concept of a multimodal chiral stationary phase is discussed. The effect of pH and the configuration of the naphthylcarbamate group is considered as well.

INTRODUCTION

Recently a series of functionalized, cyclodextrin (CD) bonded, stationary phases was introduced for the normal-phase liquid chromatographic (LC) separation of enantiomers [1]. These chiral stationary phases (CSPs) seemed to resemble more closely the functionalized cellulosic stationary phases (produced in Japan) than the original native CD bonded-phase packings [2–4]. They were able to resolve a variety of racemates using either hexane–isopropanol mobile phases or (for more strongly retained polar analytes) mobile phases of 100% alcohol or acetonitrile. Inclusion complexation did not appear to play an important role in these separations. However, unlike the cellulosic stationary phases that are adsorbed onto large pore silica gel, the functionalized CD phases are covalently bonded. This makes them more stable and allows them to be used in a greater variety of mobile phase conditions.

The naphhylethylcarbamate (NEC)-functionalized β-CD stationary phase seemed to be the most widely applicable of all the various derivatives (Fig. 1). Also, the NEC moiety contains a stereogenic center and can be produced in *R*, *S* or racemic forms [1]. In fact the (*R*)- and (*S*)-NEC-β-CDs often had different enantioselectivities

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in normal-phase LC. The number of NEC substituents on the CD affected both the selectivity and efficiency [1,5]. In general, enantioselectivity (*i.e.*, α values) tended to increase with increasing degree of substitution. However, very high degrees of substitution tended to produce stationary phases of poor efficiency (presumably due to mass transfer problems). As a compromise, a degree of substitution of five NEC groups per CD molecule was used. This product seemed to give good selectivity and efficiency for the largest number of racemates [1].

Originally it was thought that the bulky substituents at the mouth of the CD cavity (Fig. 1) would interfere with inclusion complexation, which is very important in the reversed-phase mode. Early, studies with the original native CD bonded phases seemed to indicate that inclusion complexation was necessary for chiral recognition in the reversed-phase mode [4]. Thus, it is no surprise that the derivatized CD columns (operated in the normal-phase mode) and native CD columns (operated in the reversed-phase mode) separate different types of racemates. Thus far no reversed-phase separations have been attempted on the NEC-functionalized β-CD supports. In this work we evaluate the use of these columns in the reversed-phase mode. Particular attention is paid to their enantioselectivity and whether it is similar or different from other cyclodextrin bonded stationary phases in any mode.

EXPERIMENTAL

Materials and methods

Cyclobond I stationary phase was obtained from Advanced Separation Technologies (Whippany, NJ, U.S.A.). (*R*)- and (*S*)-1-(naphthyl)ethyl isocyanate were obtained from Aldrich (Milwaukee, WI, U.S.A.). Pyridine was used as the solvent for the isocyanate-derivatized phases. The mixture was refluxed until all the water was removed (as an azeotrope into a Dean-Stark trap). The derivatizing agent was added (neat) and the mixture was refluxed for about 4 h. The isocyanate-derivatized β-CD bonded phases were collected on a fritted glass filter and washed with approximately

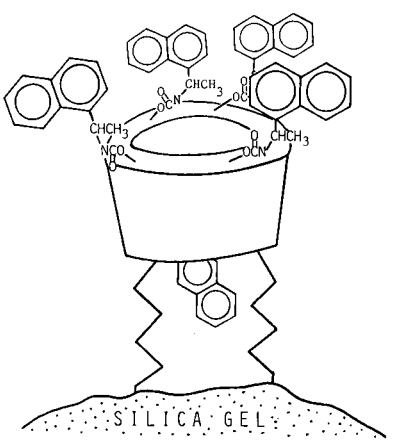


Fig. 1. A simplified model of the NEC-functionalized β-CD bonded stationary phase. In this model the NEC degree of substitution is five and there are two linkage chains from the CD to the silica gel support.

100 ml of pyridine followed by 200 ml of methanol and then air-dried.

The bonded sorbents were submitted for carbon analysis. The surface concentration was calculated according to the equation below:

$$(\mu\text{mol/m}^2) = \frac{\%\text{C} \cdot 10^6}{S[1200N_c - \%\text{C}(M - 1)]}$$

where N_c is the number of carbons in the ligand, M is the molecular weight of the ligand, and S is the surface area of the substrate, which, according to the manufacturer, is 170 m^2/g. For β-CD ($N_c = 42$, $M = 1135$), the coverage was calculated to be 0.20 $\mu mol/m^2$. To determine the degree of substitution on the β-CD of the derivatized β-CD phases, the % C from the CD + linkage chain was subtracted from the total % C and $M - 1$ was substituted by M in the denominator. The degrees of substitution for each of the phases was calculated. The sorbents were all packed into 250 × 4.6 mm I.D. stainless steel columns.

The structures of all resolved racemates are given in Table I. All compounds were obtained from Sigma or Aldrich except for Terodiline (N-*tert.*-butyl-1-1-methyl-3,3-diphenylpropylamine) which was the generous gift of Dr. Marit Olsson (Kabi Pharma, Stockholm, Sweden). Compounds **29** and **30** were the generous gifts of Dr. D. Rothley and Professor Oelschlager (University of Frankfurt). Ancymidol, Ruelene and Dyfonate were obtained from Chem. Service (West Chester, PA, U.S.A.). The 5,6-dihydro-4-[(2-methylpropyl)amino]4H-thieno{2,3-β}thiopyran-2-sulfonamide-7,7-dioxide was obtained from Merck Sharp & Dohme (West Point, PA, U.S.A.).

The chromatographic experiments were done on a Shimadzu LC-6A Chromatograph interfaced with a C-R3A Chromatopac Data System. Detection was accomplished using a variable wavelength detector.

RESULTS AND DISCUSSION

Although the NEC-functionalized β-CD was not expected to be an effective CSP in the reversed-phase mode, the opposite turned out to be true (Fig. 2). Table I lists 30 racemates that were resolved on the (*R*)- or (*S*)-NEC-β-CD bonded phases. It is apparent from this data that the configuration of the NEC group plays an important role in the enantiomeric separation process. Twenty-one of these compounds were resolved best (or exclusively) on the (*S*)-NEC-β-CD column, five resolved best (or exclusively) on the (*R*)-NEC-β-CD, and four of the racemates seemed to resolve equally well on either column. Also the data indicate that pH plays a major role in both the retention and chiral recognition of most of these analytes. This is to be expected for ionizable compounds when using hydro–organic mobile phases. Often pH was shown to be a critical parameter for other reversed-phase CSP-based separations, including the original, native β-CD bonded phases and protein-based stationary phases [6–8]. Optimization of pH improved the resolution of essentially all of the racemates in this study (Table I). Over one third of the compounds could be resolved at one pH but not another (Table I).

The most interesting feature of these reversed-phase separations involves the types of compounds resolved on the (*R*)- and (*S*)-NEC-β-CD columns. In no case were the compounds in Table I effectively resolved by the same columns in the

TABLE I

SEPARATION DATA AND STRUCTURES OF RACEMIC ANALYTES SEPARATED IN THE REVERSED-PHASE MODE ON THE (*R*)- AND (*S*)-NEC-β-CD STATIONARY PHASES

k' = Capacity factor of the first eluted enantiomer; α = separation factor; buffer = 1% triethylammonium acetate.

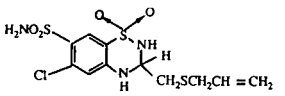
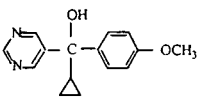
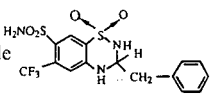
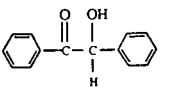
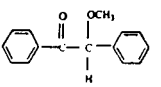
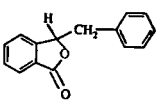
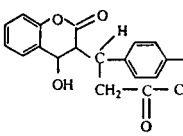
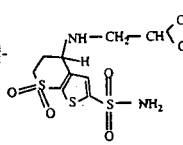
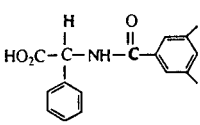
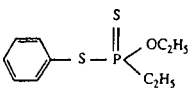
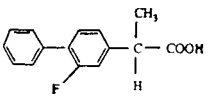
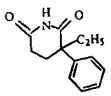
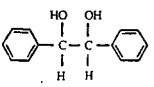
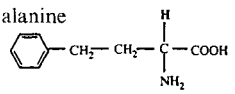
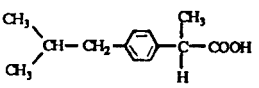
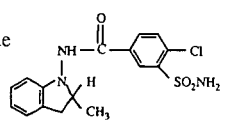
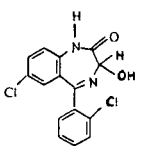
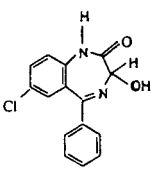
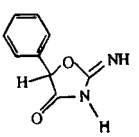
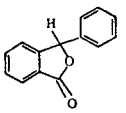
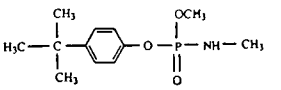
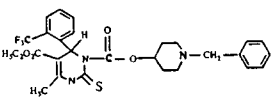
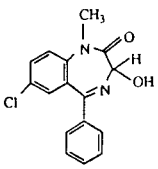
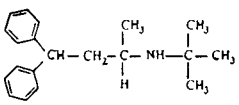
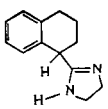
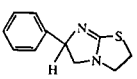
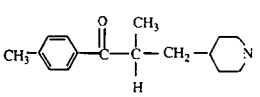
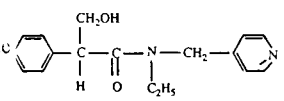
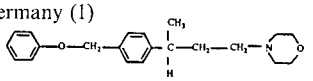
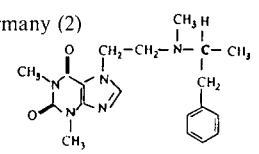
Compound	Structure	Configuration of the NEC group	Mobile phase acetonitrile–buffer	pH of buffer	k'	α
1 Althiazide		*R*	20:80	4.5	8.9	1.03
		S	20:80	7.1	6.8	1.02
		S	20:80	4.5	3.0	1.03
2 Ancymidol		*S*	20:80	7.1	2.9	1.16
		R	20:80	7.1	5.4	1.08
		R	30:70	4.5	2.2	1.06
3 Bendroflumethiazide		*R*	20:80	4.5	19.0	1.02
		S	30:70	7.1	3.0	1.10
		S	30:70	4.5	2.0	1.10
4 Benzoin		*S*	20:80	4.5	3.4	1.04
5 Benzoin methylether		*S*	20:80	4.5	5.6	1.05
6 3-Benzylphthalide		*R*	35:65	7.1	4.4	1.07
		R	40:60	4.5	3.2	1.04
		S	30:70	7.1	4.1	1.06
		S	30:70	4.5	2.7	1.07
7 Coumachlor		*R*	35:65	7.1	6.6	1.04
		~~*R*~~	~~20:80~~	~~7.1~~	~~8.9~~	~~1.10~~
		~~*S*~~	~~20:80~~	~~7.1~~	~~5.4~~	~~1.09~~
8 5,6-Dihydro-4[(2-methylpropyl)amino]-4H-thieno{2,3-β}-thiopyran-2-sulfonamide-7, 7-dioxide		corrected 26 DEC 34				
		R	20:80	7.1	8.9	1.10
		S	20:80	7.1	5.4	1.09
9 3,5-Dinitrobenzoyl phenylglycine		*S*	50:50	4.5	2.1	1.50

TABLE I *(continued)*

Compound	Structure	Configuration of the NEC group	Mobile phase acetonitrile–buffer	pH of buffer	k'	α
10 Dyfonate		*R*	25:75	7.1	22.0	1.02
		R	30:70	4.5	12.0	1.02
11 Flurbiprofen		*S*	20:80	4.5	25.0	1.04
12 Glutethimide		*S*	30:70	7.1	4.1	1.10
13 Hydrobenzoin		*S*	20:80	4.5	1.6	1.08
14 Homophenylalanine		*S*	5:95	5.5	3.1	1.22
15 Ibuprofen		*R*	35:65	7.1	3.4	1.13
		R	40:60	4.5	5.7	1.07
		S	40:60	7.1	2.8	1.11
		S	30:70	4.5	9.5	1.07
16 Indapamide		*R*	20:80	4.5	9.4	1.04
		S	20:80	7.1	6.0	1.18
		S	20:80	4.5	3.0	1.15
17 Lorazepam		*S*	20:80	4.5	5.0	1.04
18 Oxazepam		*S*	30:70	7.1	1.7	1.15
		S	30:70	4.5	1.2	1.16
19 Pemoline		*S*	20:80	7.1	0.5	1.09
		S	20:80	4.5	0.7	1.12

(Continued on p. 88)

TABLE I *(continued)*

Compound	Structure	Configuration of the NEC group	Mobile phase acetonitrile–buffer	pH of buffer	k'	α
20 3-Phenylphthalide		*R*	35:65	7.1	4.0	1.03
		R	30:70	4.5	6.6	1.03
		S	30:70	7.1	3.6	1.06
		S	30:70	4.5	2.6	1.05
21 Ruelene		*R*	25:75	7.1	14.0	1.03
		R	30:70	4.5	7.3	1.03
22 SQ 31 579		*R*	40:60	4.5	2.1	1.05
		S	20:80	7.1	1.7	1.08
23 Temazepam		*S*	20:80	4.5	5.2	1.03
24 Terodiline		*R*	27:73	7.1	11.0	1.05
		S	27:73	7.1	6.9	1.08
25 Tetrahydrozoline		*S*	30:70	7.1	1.3	1.08
		S	20:80	4.5	0.5	1.13
26 Tetramisole		*R*	20:80	4.5	0.8	1.07
		S	30:70	7.1	2.0	1.10
		S	30:70	4.5	0.3	1.20
27 Tolperisone		*R*	10:90	4.5	6.6	1.09
		S	20:80	7.1	5.8	1.09
		S	20:80	4.5	1.3	1.11
28 Tropicamide		*S*	30:70	7.1	1.2	1.20
		S	30:70	4.5	0.9	1.20
29 West Germany (1)		*R*	20:80	4.5	12.0	1.02
30 West Germany (2)		*R*	20:80	4.5	4.4	1.09
		S	20:80	7.1	8.0	1.06

normal-phase mode. Likewise, most of the racemates previously reported resolved on the (*R*)- and (*S*)-NEC-β-CD columns in the normal-phase mode [1,5], were not resolved in this study. Thus, the NEC-β-CD column acts like two different chiral stationary phases in that it resolves two different sets of enantiomers depending on whether it is used in the normal-phase mode or the reversed-phase mode. This is logical as the separation mechanism is thought to be different in the two modes.

The question arises as to whether the selectivity of the NEC-β-CD columns, in the reversed-phase mode, is similar to that of the original native β-CD bonded phase, which also is used in the reversed-phase mode. Of the thirty compounds in Table I only one (ibuprofen) can be easily resolved on the native β-CD phase. Furthermore, compounds that are easily resolved on the native β-CD column, such dansyl-amino acids, crown ethers and a variety of other biologically active compounds [9], are generally not resolved on the NEC-β-CD column.

From these results it appears that the NEC-β-CD CSP may be the first effective multimodal chiral stationary phase for liquid chromatography. We believe that to be an effective multimodel CSP, several criteria must be met. It is not enough to simply use a normal-phase CSP briefly in the reversed-phase mode thereby achieving a poorer separation of compounds already effectively resolved in the normal-phase mode, or *vice versa*. It is necessary, but not sufficient that a multimodel CSP be stable in both normal-phase and reversed-phase solvents. On the whole, significantly different racemates should be resolved in each mode. Most likely, this would be due to differences in the retention and chiral recognition mechanisms in each mode. Also, it would be useful if there was a logical, systematic approach for the selection and optimization of the various modes. This will be addressed in a subsequent work.

Many of the current chiral stationary phases (*i.e.*, protein, adsorbed derivatized cellulose, etc.) cannot be considered multimodel because of stability problems that prevent their use with either normal- or reversed-phase solvents. Other CSPs such as the π-complex/hydrogen bonding types (Pirkle) and native CD bonded phases have

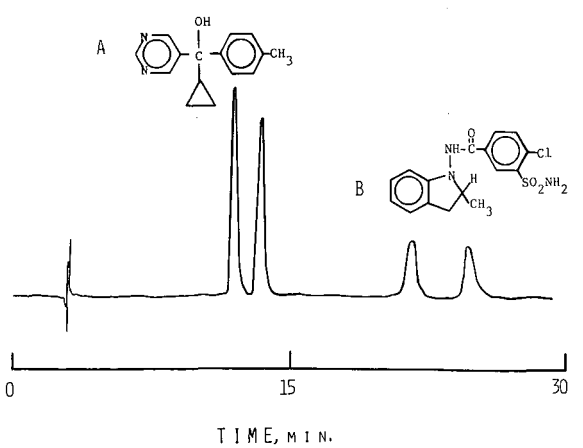


Fig. 2. Reversed-phase chromatogram showing the enantiomeric resolution of (A) the herbicide Ancymidol and (B) the diuretic Indapamide. The separation was done on a (*S*)-NEC-β-CD column. The mobile phase consisted of 1% triethylammonium acetate buffer (pH 7.1)–acetonitrile (80:20, v/v). The flow-rate was 1.0 ml/min.

been used in more than one mode [10,11]. However, thus far they are exceedingly limited in one or the other modes. For example, the CD bonded phase is stable in normal- and reversed-phase solvents. However, >95% of all enantiomers separated with this stationary phase have been in the reversed-phase mode. As far as the resolution of enantiomers is concerned it should not yet be considered an effective multimodal column. Likewise, the π-complex/hydrogen bonded phases are used almost exclusively in the normal-phase mode. Although a reversed-phase separation has been reported [10], these stationary phases often are not as stable in these solvents. More importantly, the enantioselectivity seems to be a diminished version of what is observed in the normal-phase mode.

Because of the plethora of available chiral stationary phases (>50) and their high cost, some consolidation and simplification will occur sooner or later. The development of true, effective multimodal CSPs may be the best way to reduce the total number of CSPs needed and eliminate duplicate phases. This could result in a net savings to those who are involved in enantiomeric separations.

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Determination of pituitary and recombinant human growth hormone molecular weights by modern high-performance liquid chromatography with low angle laser light scattering detection

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ABSTRACT

The determination of molecular weight for pituitary and recombinant human growth hormone (p-hGH/Crescormon and r-hGH/Protropin) has been performed. This has involved on-line coupling of size-exclusion chromatography (SEC) and gradient elution, reversed-phase high-performance liquid chromatography (RP-HPLC) with low-angle laser light scattering (LALLS) detection. A 5-μm, 300 Å, Delta-bond octyl column was used. Traditional specific refractive index increment (dn/dc) and refractive index (n) measurements have been performed in order to derive absolute weight-average molecular weight (M_w) information for p-hGH and r-hGH. Known concentrations of each protein have been separated using reversed-phase gradients utilizing acetonitrile with on-line LALLS determination of excess Rayleigh scattering factors. Accurate M_w data has been obtained for both proteins under conventional RP-HPLC gradient elution conditions.

SEC data of both hGHs were found to be concentration, mobile phase, and column dependent for the particular analyses. Both medium- and high-resolution SEC–LALLS studies were performed, and all of these determinations further confirmed our RP-HPLC results. On-line LALLS provides certain advantages in identifying aggregates that may be present, even in medium-resolution SEC, where incomplete resolution occurs. The on-line coupling of modern RP-HPLC for biopolymers with LALLS detection represents a major step forward in the ability of bioanalytical chemists to determine the nature (monomer *versus* higher-order aggregate) of such materials. Other classes of biopolymers should prove suitable for studies with the same RP-HPLC–LALLS–UV approaches.

INTRODUCTION

The separation and determination of biopolymers by modern high-performance liquid chromatography (HPLC) has become an area of intense interest within the past few years [1–5]. Separation approaches for biopolymers have advanced rapidly; however, methods of detection have lagged somewhat behind these advances [6,7].

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Most of the routine detection systems for HPLC, such as ultraviolet–visible (UV–VIS), fluorescence (FL), differential refractive index (DRI), and electrochemistry (ED), offer little in the way of biopolymer information or characterization. Most biopolymers show little or no response under conventional ED conditions, in the absence of some type of derivatization [8,9]. Though UV–FL detectors may provide some information, they rarely provide biopolymer identification or characterization. When different prosthetic groups are present, UV–FL may provide characteristic spectra for certain biopolymers. This is because in UV–FL detection, most biopolymers have very similar absorbance and emission spectra [10,11]. Recent studies using derivative optical spectroscopy illustrate how small differences in protein structure can be used to identify certain materials [12,13]. In the past, chromatographic performance *via* capacity factor determination, or *via* the use of an internal standard, have been the most widely used identification parameters.

The development of recombinant DNA technology has made available substantial amounts of proteins that were formerly available in very limited amounts. These recombinant proteins must be obtained in a state that assures full biological activity. In some cases, the activity is dependent on stoichiometry, mainly that the pure monomer is present, while in others, it may be the dimer or even higher-order oligomer(s). Clearly, it is of practical interest to understand the possible self-association of proteins under a variety of conditions. Association and aggregation have both been used to indicate higher order species greater than monomer that exist(s) on the chromatographic timescale. However, association may be a better word, since it can include both short- and long-lived, higher order species (dimer, trimer, etc.). These higher order species may, or may not, be reversible once they have left the chromatographic system. It is quite possible that associates can form on dissolution of the solid protein, prior to HPLC injection, as a function of the solvents/buffers used and concentrations.

In the HPLC separation of biopolymers, an issue that can arise is the stoichiometry of the eluting species. Many recombinant materials are hydrophobic and can form higher order aggregates either in solution/clean-up prior to chromatography or within the chromatographic column itself [14]. These aggregates are frequently immunogenic and from a regulatory point of view, they require evidence of absence in the final product. Moreover, the formation of aggregates can lead to multiple peaks in a chromatogram, requiring identification of individual peaks. Such behavior has been shown in the hydrophobic interaction chromatography (HIC) of alkaline phosphatase [10] and β-lactoglublin A [15].

In addition, it is usually recommended to inject high concentrations of biopolymer into an HPLC column in preparative-scale chromatography. Such concentration conditions are often conducive to biopolymer–biopolymer interaction, leading to association. Such association can occur without precipitation taking place. Here again, assessment of eluting fractions in terms of molecular weight could be most helpful in deciding on conditions for high sample capacity within the column.

Human growth hormone (hGH) is a single chain polypeptide of 191 amino acids, containing two intramolecular disulfide bonds, where the molecular weight of the monomer is 22 125 dalton [16,17]. The observation of a lower-molecular-weight variant of approximately 20 000 dalton and higher-order aggregates/associates have been reported in the literature [18,19].

In the polymer field, low-angle laser light scattering (LALLS) has been used as a standard technique for measuring the molecular weights in the bulk solution state or in eluting fractions on-line with size-exclusion chromatography [20–29]. Recently, this laboratory has demonstrated that LALLS can be coupled to gradient HIC and reversed-phase (RP) HPLC to yield molecular weight on-line [14,30,31]. An added benefit of LALLS is that it provides an "absolute" measure of molecular weight, since the method of calibration is based on simple geometry, rather than referenced to a different solvent of known Rayleigh scattering characteristics, or to some external standard.

Our previous efforts employed the use of standard biopolymers (proteins) that are commonly available for purposes of thorough system validation [10,14,29–32]. This current research was designed, in part, to further test and extend the usefulness of the gradient LALLS technique to a formulated product made by recombinant DNA technology.

EXPERIMENTAL

Equipment

The RP-HPLC–LALLS and SEC–LALLS systems consisted of two modular arrangements. System I, used primarily for SEC–LALLS, consisted of an LDC ConstaMetric III analytical metering pump (1/3 speed), a Rheodyne Model 7125 injection valve equipped with a 100-μl loop, a Chromatix (LDC Analytical/Thermo Instruments, Riviera Beach, FL, U.S.A.) KMX-6 LALLS photometer configured for flow analyses, an LDC SpectroMonitor-D variable UV–VIS and a modified RefractoMonitor IV DRI detector. The modifications were performed by the manufacturer within their facilities at LDC Analytical. The DRI modification consisted of replacing the heat blocking filter with an in-line filter (650 nm, $\Delta\lambda = 40$ nm, Corion, part No. S40-650-A-M125, Holliston, MA, U.S.A.), while increasing the light source available from 3.3 to 5.0 V (6.0 V maximum), to compensate for the decreased light (wavelength range) throughput. Detector outputs were all linked to Soltec Model 1242 strip chart recorders (Soltec, Sun Valley, CA, U.S.A.), LDC CMX-10A (analog-to-digital, A/D) converters and to a IBM-AT compatible compute r (Dell Computer, Austin, TX, U.S.A.). The LDC A/D converters were further linked to a DEC (Digital Equipment, Boston, MA, U.S.A.) Micro PDP-11/23+ computer system for digitization of instrumental analog outputs and further graphics/data manipulation. Standard software packages for calculating LALLS molecular weights were from LDC Analytical, versions MOLWT3 (DEC computer) and PCLALLS (IBM-AT compatible computer).

System II, used for RP-HPLC–LALLS studies performed at ambient temperature (25°C), was composed of an LDC Model CM4000 multiple solvent delivery system, a Rheodyne Model 7125 syringe loading injector equipped with either a 20-μl or 100-μl loop, a Chromatix KMX-6 LALLS photometer configured for flow analyses, and an LDC SM4000 programmable UV–VIS detector. Detector outputs were linked to both a Soltec Model 1242 strip chart recorder, and to an IBM PC-AT compatible computer using PCLALLS software.

Mobile phase refractive index (RI) measurements were performed on an Abbe refractometer (Analytical Products Division/Milton Roy, Rochester, NY, U.S.A.)

with the appropriate temperature control. In order to obtain the proper RI of the solvents at the wavelength in use throughout these experiments, the addition of 632.8 ± 0.2 nm narrow band pass filter (Melles Griot, Rochester, NY, U.S.A.) was installed between the traditional light source and the receiving optics.

The specific RI increment measurements, dn/dc values, were performed off-line using a Chromatix Model KMX-16 laser differential refractometer connected to a low-temperature (20°C) water bath (Fisher Scientific, Boston, MA, U.S.A.).

Chemicals and supplies

Pituitary (p-hGH/Crescormon) and recombinant (r-hGH/Protropin) human growth hormones were the kind gift of Genentech. All samples were shipped from Genentech to Northeastern University in dry-ice and were immediately transferred to the freezer (−15°C), for storage. All solutions were prepared daily, without further sample purification, and kept in the refrigerator (5°C) in-between analyses/injections. Before chromatography taking place, samples were allowed to equilibrate to room temperature.

Acetonitrile, methanol, and water were HPLC Omnisolv grade, trifluoroacetic acid (TFA) and ammonium acetate were reagent grade, all from EM Science (Cherry Hill, NJ, U.S.A.). The following reagents and chemicals were used without further purification: TFA, highest purity available, and imidazole, 99% pure, Aldrich Chemical (Milwaukee, WI, U.S.A.); ammonium hydroxide, 30%, Baker Analyzed, J. T. Baker (Phillipsburg, NJ, U.S.A.); N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), Sigma (St. Louis, MO, U.S.A.); EDTA, monobasic phosphate, dibasic phosphate, sodium chloride (all Certified ACS reagent grade); sodium acetate (HPLC buffer salt grade), Fisher Scientific (Springfield, NJ, U.S.A.); sodium azide, Eastman Kodak (Rochester, NY, U.S.A.); non-ionic surfactant, octa(ethylene glycol) mono-N-dodecyl ether, Nikko Chemical (Tokyo, Japan).

Chromatographic columns used were: (RP-HPLC) Deltabond octyl, 5 μm, 300 Å pore size, 10 cm × 4 mm I.D. (Keystone Scientific, State College, PA, U.S.A.); (SEC) TSK-SW-2000 or SWXL-2000, 30 cm × 7.5 mm I.D. (Supelco/Division Rohm & Haas, Bellefonte, PA, U.S.A.), where specified.

Mobile phases

Two mobile phases were prepared for gradient elution RP-HPLC–LALLS–UV studies. The first consisted of 0.15% TFA in water (A), and the second consisted of 0.15% TFA in acetonitrile–water (95:5) (B). Both solvents were adjusted to pH 3.0 with ammonium hydroxide. The column was equilibrated overnight, when not in use, with 90% B at a flow-rate of 0.2 ml/min. The phosphate-buffered saline (PBS) used for the SEC studies consisted of: 25 m*M* of monobasic phosphate, 25 m*M* dibasic phosphate, and 150 m*M* sodium chloride, pH 7.2. The modified protein buffer (MPB) used for the SEC studies consisted of: 18 m*M* HEPES, 7 m*M* imidazole, 1 m*M* EDTA, 3 m*M* sodium azide, 200 m*M* sodium acetate, 0.5 m*M* non-ionic surfactant, octa(ethylene glycol) mono-N-dodecyl ether, pH 7.0.

All mobile phases used throughout this research were first thoroughly degassed with helium, then filtered under vacuum using a 0.22-μm hydrophilic Durapore membrane (Millipore, Bedford, MA, U.S.A.) via standard laboratory vacuum filtration apparatus.

Procedures

One important point that must be considered before performing LALLS analyses is accurate injected mass. Each new loop was carefully custom fitted onto a particular injector and then that loop was dedicated to the injector. Subsequently the loop was removed, and its volume was gravimetrically determined. Injection conditions were performed at least 5 times ($n = 5$, or more).

SEC utilized a bonded diol phase, TSK-SW-2000 (medium-resolution) or SWXL-2000 (high-resolution) SEC column at a constant flow-rate of 0.775 ml/min, using PBS or MPB as the mobile phase, with a fixed injection volume of 100 μl. As is customary in SEC analyses, all protein solutions were prepared in the same buffer as used in the SEC experiment and analyzed the same day. All data are given as the mean (ave) $\pm$ S.D. in kilodalton.

The RP-HPLC gradients consisted of 80% to 20% A, linear, over 30 min (pH 3.0, 25°C), flow-rate 1.13 ml/min (calibrated), injected masses were 20–100 μl of approximately 8 mg/ml using the Deltabond Octyl column. All protein solutions were prepared by dissolving a weighed amount of each protein in mobile phase A at the appropriate pH and allowing to equilibrate for about one hour in the refrigerator at 5°C.

The KMX-6 LALLS detector was configured as for SEC with the 5-mm flow-through cell, 6–7° annulus, 0.2 mm field stop; G_0 (incident intensity) was in the range of 200–600 mV, with 2,3,4 attenuation, transmittance of the attenuators used in measuring the incident illuminating light beam (D) = approximately $4.2 \cdot 10^{-9}$; $G_\emptyset$ (scattered light intensity at initial conditions) ranged from 150–450 mV, and a 0.3-s time constant. Axial dispersion, or band broadening corrections were not performed; however, extra-column effects were minimized by removing the "zero dead volume" filter body, and allowing the eluent to be directly transferred from the column into the light scattering flow cell. This was only possible by using a column that had been excessively flushed with mobile phase in order to minimize particulate (fines) contamination.

All RP-HPLC and SEC–UV measurements were performed at 280 nm. Mobile phase RI (n) determinations were conducted using a modified Abbe refractometer incorporating a 633-nm narrow bandpass filter between the light source and the receiving optics. Experimental values of n were in close agreement with interpolated literature values. RI measurements, with varying percentages of B, were performed in an off-line study over the gradient range of B used in the RP-HPLC separations. Solutions of 0–100% B were prepared volumetrically in final volumes of 10 ml, with the %B increasing successively by 10% in each solution. Refractive indices of those solutions were determined in triplicate.

The dn/dc values for each protein were determined with the Chromatix KMX-16 using concentrations in the range of 3–5 mg/ml. Blanks were prepared identically, with matching supporting buffer (*i.e.*, glycine for p-hGH and mannitol for r-hGH) as in the formulation, side-by-side, to avoid the need for dialysis. Protein dn/dc values were determined by plotting $(n_i - n)/c_i$ *versus* c_i and extrapolating to zero concentration, where n_i is the RI at concentration c_i, and n is the RI of the mobile phase.

Protein solutions for dn/dc determinations used in reversed phase were prepared in the calculated mobile phase composition resulting in peak elution. The weighed portion was first placed in a 20-ml scintillation vial, dissolved in mobile phase A, which

consisted of 0.15% TFA in water (pH adjusted to 3.0 with ammonium hydroxide) and capped. Once the solutions were homogeneous and allowed to equilibrate, roughly one hour later, appropriate amounts of solvent B [0.15% TFA in acetonitrile–water (95:5), pH adjusted to 3.0 with ammonium hydroxide] were added slowly and capped again. These solutions were mildly swirled for about 10 min, then refrigerated at 5°C overnight, and analyzed the very next morning. Blanks were prepared side-by-side. All HPLC–LALLS solutions were allowed to re-equilibrate at room temperature prior to analysis and were filtered through a 0.45-μm, low protein binding, hydrophilic Durapore membrane, just prior to HPLC introduction.

Traditional optical alignment procedures, as are used for isocratic HPLC/SEC–LALLS, were not satisfactory when used for gradient RP-HPLC–LALLS, due to changes in mobile phase RI and solid angle, resulting in actual scattering volume changes [33]. A modification of the alignment procedure was developed, where optical realignment was performed once during an initial blank gradient. This procedure produced consistently stable LALLS baselines and avoided any extra-scattering effects from within the cell that could result in experimental artifacts.

RESULTS AND DISCUSSION

Theory

The basic principles of LALLS pertaining to the simplification of multi-component theory used in this research has been previously described elsewhere [14,29–32]. When the individual solvents and their mixtures employed in the light scattering experiments are considered isorefractive (*i.e.*, their difference is <0.025 RI units), one is able to use the solvent combinations without the need to perform exhaustive dialysis [26]. Ideal solvents do not interact with proteins in solution to produce newer species that scatter light differently than the free biopolymer. This concept allows for a three- (or greater)-component system to be artificially reduced to that of a two-component system. Careful choice of experimental conditions allows one to obtain true weight-average molecular weights, M_w (*vs.* apparent M_w values) under gradient conditions.

Background and operational principles

As with all light scattering methodologies, the specific RI increment (dn/dc) determination is a required quantity for the deduction of molecular weight data. It is one of the most important values that must be determined with utmost precision and accuracy. Even though HPLC–LALLS measurements were made under dynamic conditions, and dn/dc values were measured in an off-line manner, it is clear that different protein stoichiometries can be present in each measurement technique. Accumulated data generally suggests that dn/dc attains a constant value above a certain molecular weight, which is in the range of 10–20 k dalton [24–26]. Therefore, when studying these biopolymers, errors associated with measuring the dn/dc of monomers, dimers, or even higher-order associates in an off-line condition (via the KMX-16), should not significantly affect the measurements made under dynamic conditions (HPLC–LALLS) where higher-order aggregates may be present. All measurements were made at 25°C. The uncertainty of this measurement was approximately $\pm 3\%$, and was our major source of experimental error. This was determined by repetitive measurements under identical conditions.

Experimentally, another consideration that had to be taken into account was constant LALLS baselines, especially for gradient elution HPLC–LALLS. Baselines had to be both highly reproducible and linear for valid quantitation purposes. Factors have been described with regard to solvent combination RI limitations [14,15,26,27]. In addition, the HPLC pumping system must offer excellent on-line, pre-injector solvent mixing in order to minimize local concentration fluctuations in the combined mobile phase RI. For this purpose, we incorporated an analytical HPLC silica column having the following characteristics: 5-μm particle size, 10 cm $\times$ 4.6 mm I.D., into the HPLC–LALLS system just prior to the injector. This system produced consistent LALLS chromatograms throughout this work, in addition to its benefit by serving as a saturator column for protection of the analytical column.

A minimum of five individual chromatograms were generated per protein and condition, with blank gradients in-between, in order to ensure chromatographic and data reproducibility. Proper quantitation of individual peaks was not straightforward, since the commercial software package (PCLALLS) was intended for isocratic HPLC operation, where (in theory) all polymer variables remain constant. This is not the case in gradient HPLC–LALLS operation. Peak molecular weights were performed off-line by measuring the corresponding values for each peak's elution condition at its apex (*i.e.*, RI, scattering volume, and the specific RI increment). The second virial coefficient (a term used to indicate solution non-ideality), A_2, was not determined due to the considerable effect of on-column dilution, and a nominal value of $5 \cdot 10^{-4}$ ml mol/g^2 was used throughout these experiments [10]. Individual peak concentrations via relative peak area and known injected mass were determined on-line using the UV detector. These values were then incorporated into the software, and each peak was quantitated individually. The peak apex M_w was reported and several important M_w values deduced, without the need for computer deconvolution software. Taking into account all sources of error associated with these techniques, we have measured an overall experimental error of about $\pm 5\%$ [34].

Aim and purpose of current studies

This study has been designed to understand various phenomena regarding protein separations under normal (<2% solvent change per minute) gradient elution RP-HPLC, and under isocratic SEC conditions. This has involved: (1) determination of M_w for each hGH peak as a function of injected sample mass: (2) determination of association, if any, as a function of the injected concentration; (3) biopolymer solution–mobile phase effects; (4) column choice differences; and (5) overall reproducibility, accuracy, and precision of on-line LALLS determinations under certain gradient RP-HPLC and isocratic SEC conditions for hGH.

Our first efforts were in using gradient RP-HPLC–LALLS to deduce dynamic biopolymer M_w values, since the reported and experimentally measured refractive indices for water and acetonitrile, at 25°C and 633 nm, were 1.332 and 1.341, respectively (their difference is 0.009, well below the isorefractive criteria of 0.025). It is well known that when RI *versus* %(w/w) is plotted for acetonitrile in water, the resultant plot is not linear. When the resultant plot of acetonitrile was compared to that of methanol or isopropanol, acetonitrile showed less deviation from linearity when viewing the 20–80% acetonitrile in water range [14]. Reversed-phase gradients using acetonitrile can be used with LALLS detection because of the small change in RI

TABLE I

SUMMARY OF CONCENTRATION DEPENDENT RP-HPLC–LALLS MOLECULAR WEIGHT DETERMINATIONS FOR PITUITARY AND RECOMBINANT HUMAN GROWTH HORMONE

Form/storage (mg/ml) (μl)	Injected mass 10^{-4} (g)	M_w[a] overall	Peak 1		Peak 2	
			M_w[a]	% area[b]	M_w[a]	% area[b]
p-hGH/new (8.35) (20)	1.67	24.1 ± 1.2	22.3 ± 0.4	93.4 ± 0.4	[c]	6.4 ± 0.3
p-hGH/new (4.17) (100)	4.17	24.2 ± 0.8	22.9 ± 0.7	91.6 ± 0,5	[d]	8.3 ± 0.3
p-hGH/new (8.35) (100)	8.35	23.8 ± 0.4	22.0 ± 0.4	91.2 ± 1.0	43.1 ± 1.9[e]	8.9 ± 1.1
r-hGH/5°C (7.52) (100)	7.52	22.3 ± 0.8	22.1 ± 0.8	98.9 ± 0.1	[f]	1.1 ± 0.1
r-hGH/45°C (7.52) (100)	7.52	21.7 ± 0.8	21.6 ± 0.8	98.0 ± 0.0	[f]	2.0 ± 0.0

[a] Expressed as kdalton ± S.D., where $n = 5$.
[b] % determined by UV peak area at 277 nm ± S.D.
[c] LALLS response is below the minimum signal-to-noise (S/N) ratio, M_w is invalid.
[d] LALLS response is just below the minimum S/N ratio, M_w is invalid.
[e] LALLS response is above minimum S/N ratio, M_w is valid.
[f] Insufficient LALLS signal for M_w determination.

with change in weight percent. The LALLS signals were obtained free of any baseline anomalies, and background correction procedures were not necessary.

Reversed-phase chromatography

Pituitary human growth hormone (p-hGH/Crescormon). Table I contains the RP-HPLC–LALLS data for both p-hGH and r-hGH samples stored under different conditions. Chromatography was performed under identical conditions that have been previously shown to deter on-column association of several proteins, that is, a slow solvent changeover, while varying injected mass [14]. It is more than likely that under these particular RP-HPLC conditions, there is little or no on-column association of p-hGH occurring. For all RP-HPLC–LALLS molecular weight determinations, the following constants were determined and used: mobile phase $n = 1.349$ RI units, solid angle $= 646.6$, and $dn/dc = 0.149$ ml/g; resulting in a polymer optical constant, K, of $1.648 \cdot 10^{-7}$ mol cm^2/g^2. The refractive indices for these particular mobile phase combinations over the entire range of chromatographic elution have been previously determined [14]. The dn/dc values of the protein as a function of the water–acetonitrile composition were experimentally determined as described before, and plotted, though not shown here, for at least three individual concentrations of p-hGH.

Initial analysis was performed on p-hGH. Injected mass was determined, based on previous similar analyses, and 20 μl of 8.35 mg/ml (167 μg mass) was used with efforts addressed at characterizing the main protein peak [10,14]. A second peak eluting just after the main peak was obscured, and was proposed to be the p-hGH dimer. In addition, a third peak appearing as a lower retention volume shoulder of the main peak was observed. Quantitation of the minor peaks was impossible, due to the low concentrations present. Injection of 417 μg of p-hGH under the same RP-HPLC–LALLS conditions also did not allow for M_w determinations of the minor peaks. Injection of 835 μg total mass (Fig. 1a) produced adequate LALLS responses for valid integration of the first p-hGH monomer and second, assumed dimer peaks. Based on

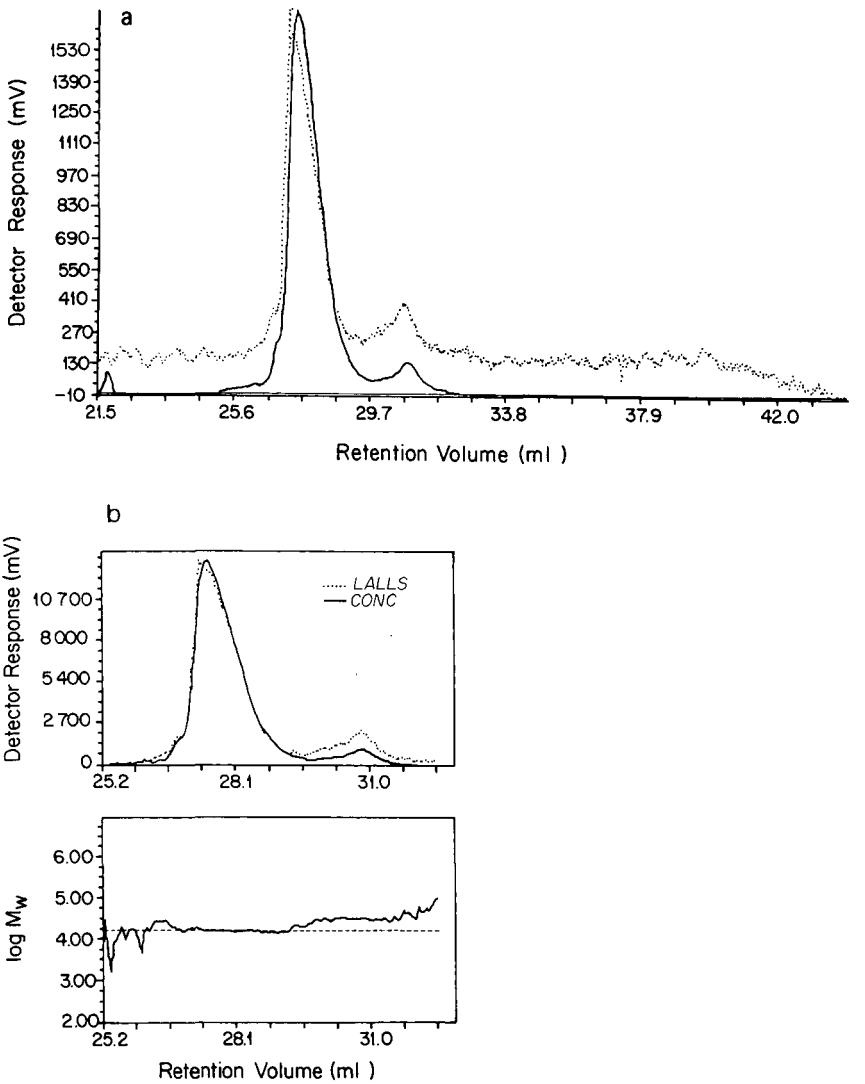

Fig. 1. (a) RP-HPLC–LALLS–UV of p-hGH, 835 μg injected mass, Keystone Deltabond Octyl column. Solid line = UV (280 nm/0.2 a.u.f.s.), dotted line = LALLS. x-Axis = retention volume (ml); y-axis = detector response (mV). (b) Integrated normalized chromatograms (top) and on-line plot of LALLS determined log M_w *versus* retention volume (bottom), showing two distinct molecular weight species for the two peaks, monomer and dimer, respectively.

previous studies under similar RP conditions, it was assumed here that this second peak was the dimer of p-hGH [12]. Integration of both peaks yielded an average M_w = 23.8 $\pm$ 0.4 kdalton, with the first peak M_w = 22.0 $\pm$ 0.4 kdalton (monomer) with a % relative UV area = 91.2 $\pm$ 1.0%, and peak 2 M_w = 43.1 $\pm$ 1.9 (dimer) at a % relative UV area = 8.9 $\pm$ 1.1%. The lower M_w peak was even more pronounced, but still of too low concentration for adequate LALLS signal integration (<1%).

Previous studies had suggested that this peak was a variant of p-hGH [12]. Since RP-HPLC baseline fluctuations (noise) were higher than in isocratic SEC, *i.e.* ± 30 mV as compared to ± 10 mV, three times the usual minimum peak height is required for M_w integration. Fig. 1b shows the integrated, normalized chromatograms (top: solid line = UV, dotted line = LALLS) and an on-line plot of LALLS determined log M_w *versus* retention volume (bottom), showing two distinct molecular weight species. From our calculations, it is suggested that in order to properly quantitate the p-hGH dimer, a minimum quantity of 75 μg of this dimer would be required in this RP-HPLC system. We also noticed an increased % relative UV area for the dimer as a function of higher, injected mass/concentration. This suggested, in the absence of on-column association at higher concentrations, increased association on dissolution in the injection medium/solvent. Further results using SEC-LALLS, as below, tended to support these initial conclusions via RP-HPLC–LALLS studies. We do not, at present, believe that association occurred under these particular SEC conditions.

Recombinant human growth hormone (r-hGH/Protropin). When Protropin, stored at 5 and 45°C, was chromatographed under similar conditions (752 μg injected mass) as for p-hGH (835 μg injected mass), some interesting observations were made. For Protropin stored at 5°C (Fig. 2a), the overall M_w was 22.3 ± 0.8 kdalton, with the principle peak having a M_w = 22.1 ± 0.8 kdalton (monomer) and percent relative UV area = 98.9 ± 0.1%. M_w could not be determined for the second peak due to its low percent relative UV area = 1.1 ± 0.1%, Fig. 2b. Based on our previous work with p-hGH, as above, when comparing retention characteristics, we can only presume that this second peak is a dimer. However, without adequate LALLS response due to the low mass of this peak, this can still be in question. Identical chromatographic analysis with Protropin stored at 45°C yielded similar data, with a slight increase in the percent relative UV area of the second peak to 2.0 ± 0.0%.

Size-exclusion chromatography

Much of the literature on the SEC of proteins involves derivatized silica packings, and the TSK-SW series are the most popular [35–37]. In 0.2 *M* ionic strength and neutral pH, numerous globular proteins display ideal chromatographic behavior on SW columns. The primary requirement of the SEC support is that the gel porosity be such that all of the species are well separated. Since resolution affects the accuracy of the results (especially the M_w distribution), by providing a larger change in retention volume, a column should be chosen that offers optimal separation of all components.

Three molecular moments, *viz.*, M_z, M_w, and M_n (z-average, weight-average, and number-average molecular weight) were determined as well as the polydispersity ratio, M_w/M_n, which is an indicator of homogeneity for polymer molecular weight distributions (Table II). A polydispersity value of 1.00 is expected for a monodisperse protein, while using chromatographic band broadening corrections. Previous experiments using this particular SEC–LALLS system have determined that with a pure protein, standard (*e.g.*, ribonuclease A) having a true polydispersity ratio of 1.00, experimentally determined polydispersity was 1.03 [14]. For this reason, band broadening corrections were not applied.

Medium-resolution SEC. Pituitary human growth hormone (p-hGH/Crescormon). In order to define more fully the exact nature of the protein species present on receipt, we initially performed conventional SEC using a TSK-SW-2000 column (medium

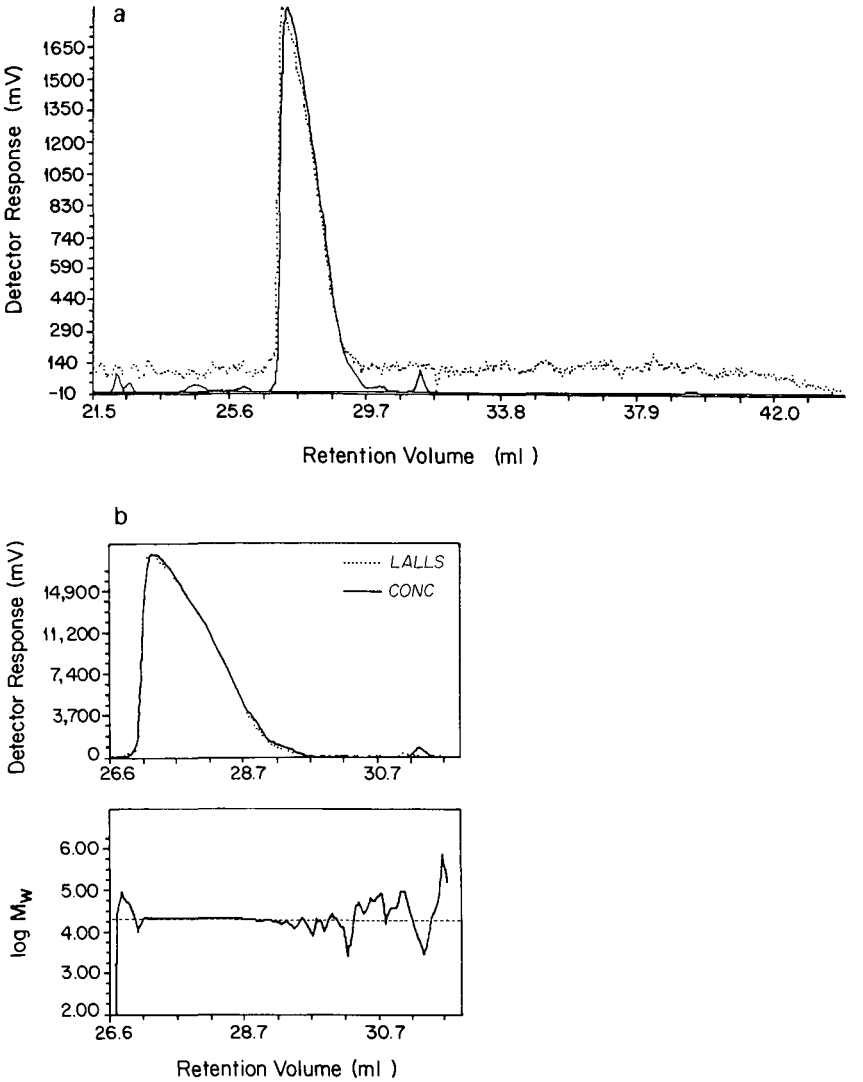

Fig. 2. (a) RP-HPLC–LALLS–UV or r-hGH stored at 5°C for two months, 752 μg injected mass, Keystone Deltabond Octyl column. Solid line = UV (280 nm/0.2 a.u.f.s.), dotted line = LALLS. *x*-Axis = retention volume (ml); *y*-axis = detector response (mV). (b) Integrated normalized chromatograms (top) and on-line plot of LALLS determined log M_w *versus* retention volume (bottom), identifying the major peak as the monomer. Similar conditions as in Fig. 1 where monomer and dimer were identified, but insufficient LALLS signal for proposed dimer characterization.

resolution with LALLS–UV–DRI detection in series for M_w determination in a special buffer (MPB), in addition to PBS. The MPB special buffer has been described and was used to prevent associate formation and dissociation through minimizing ionic and hydrophobic interactions between the protein itself and the mobile or stationary phase [14,29–32]. With many different proteins, we have never found association or

TABLE II

SUMMARY OF MEDIUM RESOLUTION SEC-LALLS MOLECULAR WEIGHT DETERMINATIONS FOR PITUITARY AND RECOMBINANT HUMAN GROWTH HORMONE STORED UNDER DIFFERENT CONDITIONS

Experimental conditions using the TSK-SW-2000 column.

Form/buffer/storage (mg/ml) (μl)	Injected mass (10^{-4} g)	Molecular weight moment[a]			
		M_z	M_w	M_n	M_w/M_n
p-hGH/MPB/new (1.67) (100)	1.67	24.9 ± 1.1	22.2 ± 0.6	18.0 ± 0.6	1.23 ± 0.03
p-hGH/PBS/new (1.67) (100)	1.67	26.2 ± 1.5	23.3 ± 0.4	19.8 ± 0.2	1.18 ± 0.02
p-hGH/PBS/old (1.83) (100)	1.83	427.0 ± 9.2	63.3 ± 1.1	44.6 ± 1.1	1.42 ± 0.01
r-hGH/PBS/5°C (2.20) (100)	2.20	25.8 ± 0.6	22.9 ± 0.6	19.7 ± 0.9	1.16 ± 0.03
r-hGH/PBS/45°C (2.20) (100)	2.20	26.9 ± 0.4	23.1 ± 0.2	20.3 ± 0.5	1.14 ± 0.03

[a] Expressed as kdalton ± S.D., where $n = 5$. M_n = number-average molecular weight; M_w = weight-average molecular weight; M_z = z-average molecular weight.

dissociation of once-formed aggregates to occur in this MPB medium. Under these particular medium resolution SEC conditions, M_w values were determined for p-hGH. The LALLS parameters for integration using MPB as the mobile phase were: mobile phase n was 1.336 RI units and subsequent solid angle = 639.3, dn/dc was 0.160 ml/g, resulting in a polymer optical constant, K, of $1.864 \cdot 10^{-7}$ moles cm^2/g^2. All medium resolution SEC results are summarized in Table II.

With an injected mass of 167 μg, the principle biopolymer peak, M_w = 22.2 ± 0.6 kdalton (monomer), and M_n = 18.0 ± 0.6 kdalton (Fig. 3), showed a leading shoulder which resulted in a non-Gaussian molecular weight distribution (M_w/M_n = 1.23 ± 0.03), incorporating both the monomer and higher M_w species. This can be

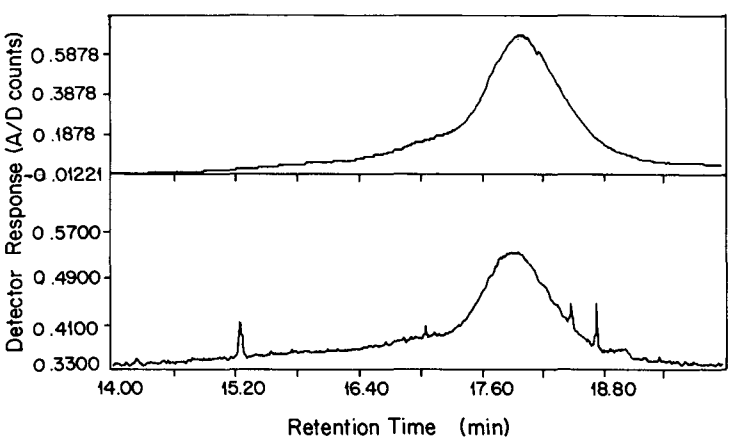

Fig. 3. Medium resolution SEC–LALLS–UV of p-hGH in MPB, new sample, 167 μg injected mass, TSK-SW-2000 column. Top = UV (280 nm/0.1 a.u.f.s.), bottom = LALLS. x-Axis = retention time (min); y-axis = detector response (A/D counts).

seen, both qualitatively and quantitatively, by comparing the normalized peak heights to one another, and by inspection of the processed data, yielding M_{w_i} for each data point at the corresponding elution volume ($M_{w_i} = M_w$ at elution volume i). Both procedures suggested that this peak, though incompletely resolved from the main peak, was the dimer having a percent relative UV area of about 2%, even though at small concentrations relative to the monomer (approximately percent relative UV of 98%). The low M_w (20 kdalton) variant of p-hGH was not readily apparent, under any SEC conditions herein described, most probably due to the inherent lower resolution capability of SEC with respect to RP-HPLC and the small amounts present.

We also investigated the use of the common physiological SEC buffer, PBS. The mobile phase n was similar to MPB having a value of 1.338, solid angle = 640.8. The specific dn/dc was identical to MPB, 0.160 ml/g, using three concentrations for its determination. With an identical injected mass of 167 μg, the M_w was 23.3 $\pm$ 0.4 kdalton and M_n = 19.8 $\pm$ 1.18 kdalton (M_w/M_n = 1.18 $\pm$ 0.02), again indicative of a mixture of species, including a small amount of higher M_w material (co-eluting shoulder on the high M_w side of the peak). These species were identified as primarily monomer (approximately 95% relative UV area) and dimer (approximately 5% relative UV area). When both the PBS and MPB chromatograms were overlaid, they were nearly superimposable.

When a solution of p-hGH was prepared in PBS (183 μg injected mass) and stored in the refrigerator at 5°C of 24 h, it was chromatographically noticed that the biopolymer appeared to randomly "self-associate" producing an increase in the overall M_w and showing a more pronounced high M_w shoulder close to the column exclusion volume, between 13.7–15.1 min (Fig. 4). The elevated LALLS baselines, with respect to the UV response, over the retention times of 13.7–19.3 min suggest that there are small amounts of very high M_w species showing signs of protein mobile/stationary phase competition, resulting in mild, yet reversible adsorption. If this were not the case,

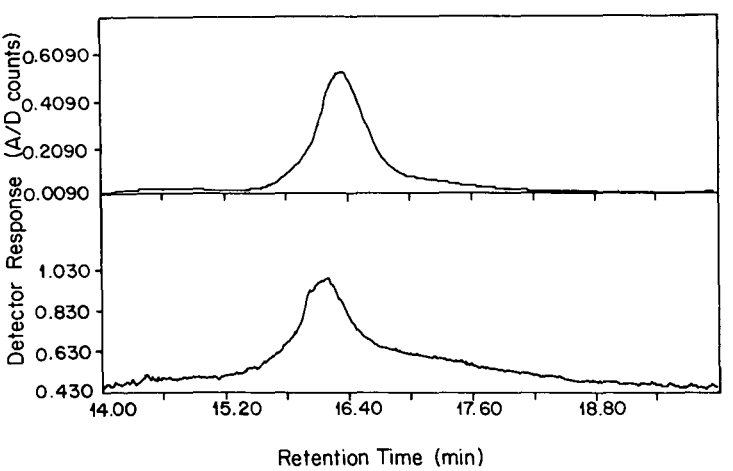

Fig. 4. Medium resolution SEC–LALLS–DRI of p-hGH in PBS, old sample, 183 μg injected mass, TSK-SW-2000 column. Top = DRI (1 V f.s. = $2 \cdot 10^{-4}$ RI units), bottom = LALLS. x-Axis = retention time (min); y-axis = detector response (A/D counts).

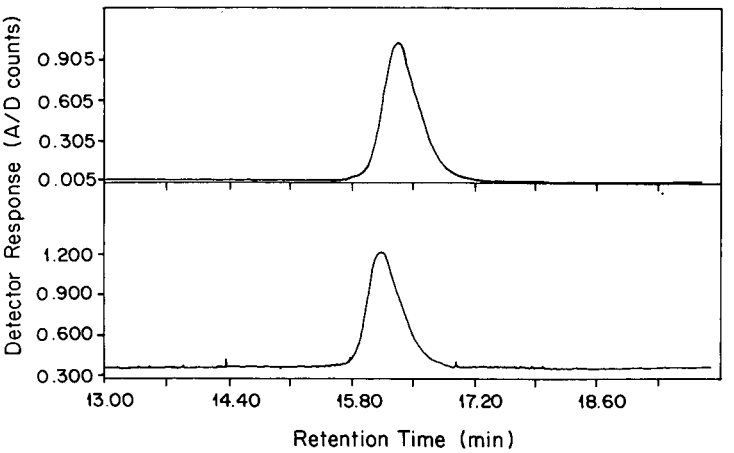

Fig. 5. Medium resolution SEC–LALLS–DRI of r-hGH in PBS, storage at 5°C for 2 months, 220 μg injected mass, TSK-SW-2000 column. Top = DRI (1 V f.s. = 2 × 10^{-4} RI units), bottom = LALLS. x-Axis = retention time (min); y-axis = detector response (A/D counts).

and the separation was based on purely entropic considerations as SEC should be performed, then one would see similar LALLS and UV responses, but with a large LALLS peak at the column exclusion volume. Molecular weight moments for the major peak(s) were elevated here, with an overall M_w = 63.3 ± 1.1 kdalton and M_n = 44.6 ± 1.1 kdalton (M_w/M_n = 1.42 ± 0.01).

Medium-resolution SEC. Recombinant human growth hormone (r-hGH/Protropin). Similar analyses to the above were performed on two Protropins, stored at 5°C and 45°C for two months, under identical chromatographic conditions. M_w determination procedures were very similar to p-hGH. Fig. 5 illustrates Protropin stored at 5°C, having a M_w = 22.9 ± 0.6 kdalton and M_n = 19.7 ± 0.9 kdalton with a polydispersity = 1.16 ± 0.03. Protropin, stored at 45°C, had a M_w = 23.1 ± 1.2 kdalton and M_n = 20.3 ± 0.5 kdalton with a polydispersity = 1.14 ± 0.03. Both Protropins were chromatographically superimposable. When Protropin was compared to p-hGH in PBS, peak shapes were Gaussian, showing no contribution of the higher M_w dimer. SEC showed only a slight difference between Protropin stored at 5 and 45°C, and when statistically treated, was within experimental error.

We have presented the medium-resolution SEC–LALLS results in order to show that even in the absence of baseline resolution of monomer from dimer hGH species, it is still possible to denote the presence of a higher order species at much lower relative concentrations. Though UV does not easily reveal higher molecular weight species in such situations, the LALLS responses very clearly can and do. Such results should then suggest the use of higher-resolution SEC–LALLS, under identical or similar mobile phase conditions, in order to more fully resolve monomer from dimer or other aggregates that may be present.

High-resolution SEC. Pituitary human growth hormone (p-hGH/Crescormon). High-resolution SEC using a TSK-SWXL-2000 column was utilized in order to separate monomer/dimer. Fig. 6 clearly shows that for p-hGH stored at −15°C for

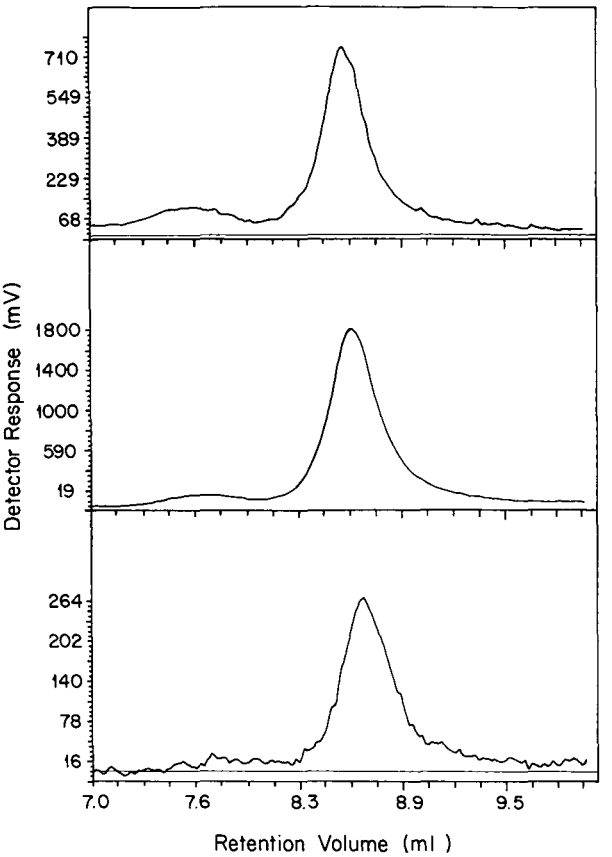

Fig. 6. High-resolution SEC–LALLS–UV–DRI of p-hGH in PBS stored at −15°C for 3 months, 334 μg injected mass, using the TSK-SWXL-2000 column. Top: LALLS; middle: UV (0.2 AUFS = 1000 mV); bottom: DRI ($2.0 \cdot 10^{-4}$ RI units full scale = 1000 mV).

3 months in this laboratory and chromatographed in PBS under similar conditions to the above, the proposed dimer was almost fully resolved from the monomer. Since the RP-HPLC–LALLS experiments suggested that dimer production was concentration related, pre-injection, we again varied concentration in SEC, while keeping the injection volume fixed, for each set of data in Table III ($n = 5$).

Overall M_w values remained constant (*ca.* 25.8 ± 0.5 kdalton) while reducing injected masses from 334–111 μg/injection. However, the percent relative UV area of the monomer increased from 90.3 to 91.8 ± 0.4%, while for the dimer, it decreased from 9.7 to 8.2%. These changes were real, beyond experimental error and fully reproducible. In these SEC experiments, we observed a similar percentage of monomer/dimer as in the RP-HPLC–LALLS experiments for p-hGH (Table I).

We then changed the mobile phase to MPB in order to see if the hGH experienced any solution effects. It was very interesting to note that the M_w values deduced in MPB were identical to those in PBS, but the percent relative UV areas for

TABLE III

SUMMARY OF HIGH RESOLUTION SEC-LALLS DETERMINATIONS FOR PITUITARY AND RECOMBINANT HUMAN GROWTH HORMONE: MOBILE PHASE EFFECTS

Experimental conditions using the TSK-SWXL-2000 column.

Protein[a]/Buffer (mg/ml) (μl)	Injected mass (10^{-4} g)	M_w(overall)[b]	% Monomer[c]	% Dimer[c]
p-hGH/PBS (3.34) (100)	3.34	25.6 ± 0.4	90.3 ± 0.3	9.7 ± 0.1
p-hGH/PBS (2.23) (100)	2.23	25.8 ± 0.4	91.1 ± 0.3	8.9 ± 0.1
p-hGH/PBS (1.11) (100)	1.11	25.8 ± 0.5	91.8 ± 0.4	8.2 ± 0.2
p-hGH/MPB (3.34) (100)	3.34	25.8 ± 0.5	90.1 ± 0.2	9.9 ± 0.1
p-hGH/MPB (2.23) (100)	2.23	25.7 ± 0.5	90.2 ± 0.2	9.8 ± 0.1
p-hGH/MPB (1.11) (100)	1.11	25.7 ± 0.6	90.3 ± 0.2	9.7 ± 0.1
r-hGH/PBS (3.33) (100)	3.33	22.7 ± 0.4	98.0 ± 0.3	2.0 ± 0.1
r-hGH/PBS (2.22) (100)	2.22	22.7 ± 0.4	98.2 ± 0.3	1.8 ± 0.1
r-hGH/PBS (1.11) (100)	1.11	22.7 ± 0.5	98.4 ± 0.3	1.6 ± 0.2
r-hGH/MPB (3.33) (100)	3.33	23.0 ± 0.4	98.3 ± 0.1	1.7 ± 0.1
r-hGH/MPB (2.22) (100)	2.22	23.1 ± 0.4	98.4 ± 0.1	1.6 ± 0.1
r-hGH/MPB (1.11) (100)	1.11	23.0 ± 0.6	98.5 ± 0.1	1.5 ± 0.2

[a] Storage at −15°C for three months.
[b] Expressed as kdalton ± S.D., where $n = 5$.
[c] Determined by % relative UV peak area at 277 nm ± S.D.

the monomer and dimer were more constant in MPB than in PBS when concentration was varied. This again suggested that MPB does not allow association or dissociation to occur, or to a lesser extent, with varying concentrations, in comparison to other buffers or mobile phases (*e.g.*, RP-HPLC). These MPB results also suggested that the monomer/dimer ratio observed at the lowest concentration studied was probably most representative of the original sample composition before dissolution into any solvent(s). Extrapolation to lower concentrations, if possible under SEC–LALLS requirements, would be expected to show the same monomer/dimer ratios already observed at higher concentrations.

High-resolution SEC. Recombinant human growth hormone (r-hGH/Protropin). A similar workup as for p-hGH was performed on r-hGH. Protropin in PBS (Fig. 7, x and y-coordinates expanded for visual purposes of highlighting the dimer peak) yielded an overall M_w of approximately 22.7 ± 0.5 kdalton with a percent relative UV area for the monomer and dimer of 98.0 ± 0.3% and 2.0 ± 0.1%, respectively. Protropin was not as concentration dependent as previous determinations with p-hGH in the same buffer. The percent monomer/dimer for r-hGH in MPB did not change much with respect to that in PBS when varying concentration. A plot of the ratios of percent relative UV area *versus* injected concentration is shown in order to better visualize the hGH concentration dependence of dimer production in both PBS and MPB (Fig. 8). These percentages of monomer and dimer coincided with previously determined RP-HPLC–LALLS values, again confirming that the RP-HPLC–LALLS dimer peaks were not an artifact of the overall technique. The agreement, not coincidence, also suggested that changes in the monomer/dimer ratios occur prior to injection, as a function of the mobile phase or buffer, rather than on-column under RP-HPLC or SEC [12,13,16,19].

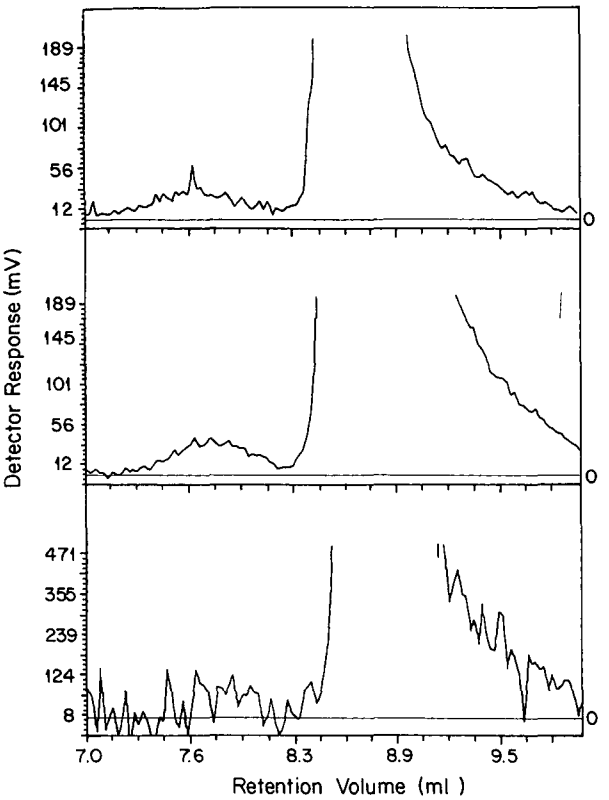

Fig. 7. High-resolution SEC–LALLS–UV–DRI of r-hGH in PBS stored at −15°C for 3 months, 333 μg injected mass, using the TSK-SWXL-2000 column. Chromatographic coordinate expansion. Top: LALLS; middle: UV (0.2 AUFS = 1000 mV); bottom: DRI ($2.0 \cdot 10^{-4}$ RI units full scale = 1000 mV).

CONCLUSIONS

From this study one may conclude that the higher M_w propagation was due, in major part, to pre-column aggregation showing concentration dependence, which may be protein and buffer specific. Pituitary hGH tended to aggregate more readily, since higher order species (*i.e.*, dimer) were already present in the original sample and at higher concentrations than for r-hGH. Recombinant hGH showed signs of similar association, buffer dependent, but of lesser magnitude than the pituitary form.

Proteins, either naturally derived or from recombinant DNA techniques, may form associates during various HPLC assays which LALLS may be able to characterize. These associates may, or may not, be immunogenic and regulatory agencies require evidence of absence in the final product. The results obtained with routine RP-HPLC gradients (solvent change of less than 2%/min), do indicate that it is possible to separate and characterize these proteins in a reasonable amount of time using the RP-HPLC–LALLS technique.

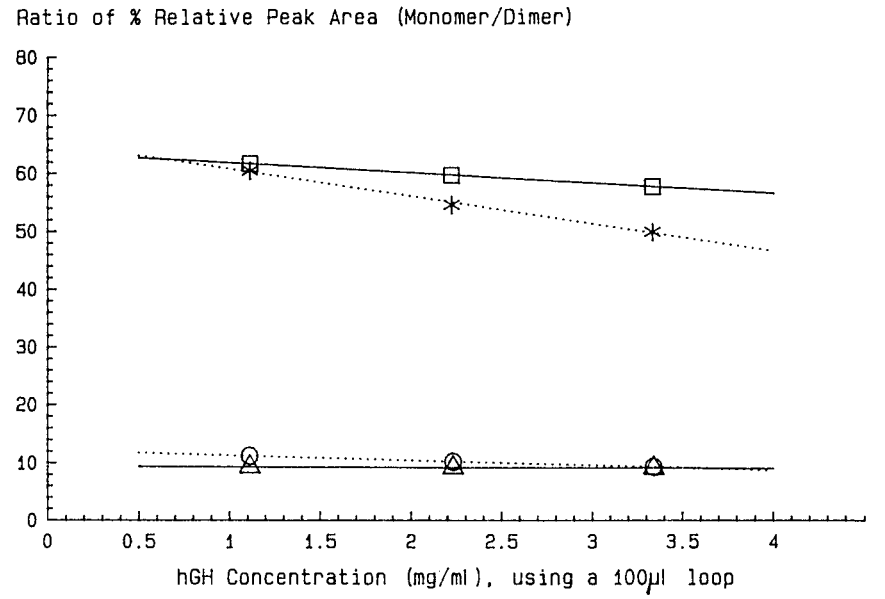

Fig. 8. Ratio of SEC–UV peak area (monomer/dimer) at 277 nm *versus* injected concentration. ∗···∗ = Protropin in PBS; □--□ = protropin in MPB; ○···○ = crescormon in PBS; △—△ = crescormon in MPB. TSK-SWXL-2000 column. SEC–LALLS–UV (277 nm)–DRI (633 nm) system.

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Isoelectric focusing field-flow fractionation

Experimental study of the generation of pH gradient[a]

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ABSTRACT

Isoelectric focusing field-flow fractionation is a method for the separation of ampholytes using, in addition to the electric field and pH gradient, the flow of the carrier liquid through the fractionation channel as the third active separating factor. Flow action permits a decrease in the channel dimension in the direction of the electric field. It makes it possible to decrease the absolute value of the voltage while maintaining a high field strength and results in a lower Joule heat production. Moreover, the laminar flow of the carrier liquid stabilizes the pH gradient against convection. A channel was designed from which ampholyte samples can be taken at twelve different positions. pH value of the samples taken were measured with the aid of a capillary pH electrode. The pH dependence of the applied voltage, flow-rate of ampholyte solutions and concentrations of the solutions of ampholyte and electrode electrolytes were measured at different channel positions. It follows from the measurements performed so far that the pH gradient generation is sufficiently fast and reproducible for use in isoelectric focusing field-flow fractionation.

INTRODUCTION

Isoelectric focusing (IEF), an electromigration method for the separation and characterization of amphoteric compounds [1], has become an important method for protein studies. Gel IEF, a technique with very high resolution [2], has been widely used, but a number of works have recently appeared that assume the initial idea of IEF in solutions [1]. This makes it possible to extend the field of applications to particles and cells which cannot pass through gels, to create continuous and discontinuous preparative systems and to reduce the time required for analyses [3–8].

Despite considerable progress in instrumentation, some problems have remained unsolved. The first is the time required for the analysis which, with rare exceptions, reaches several hours, also the application of high voltages, leading to intense Joule heat production and requiring the use of efficient cooling systems and finally the difficult detection and characterization of the substances being separated.

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The application of focusing field-flow fractionation is a possible solution to these problems [9]. The method, utilizing for the separation, in addition to the electric field and pH gradient, the flow of a liquid through the separation channel as the third active separating factor, was named isoelectric focusing field-flow fractionation (IEF_4) [10]. The shape of the flow velocity profile of the carrier liquid is influenced by the geometry of the fractionation channel [9]. The method was put into practical use by Chmelík *et al.* [10] in a trapezoidal cross-section channel and by Thormann *et al.* [11] in a rectangular cross-section channel. The latter group named this technique electrical hyperlayer field-flow fractionation (EHF_3).

The zone of an amphoteric solute is established owing to the action of the electric field and pH gradient at that position in the channel where the intensity of forces is zero. After dynamic equilibrium between the focusing and dispersive processes has been reached, the narrow focused solute zones, having an approximately Gaussian distribution of concentrations, are located at different positions of the channel depending on their isoelectric points. The direction of the liquid flow inside the fractionation channel is perpendicular to the direction of the effective electric field. The velocity profile formed in the liquid flow causes the migration of focused zones along the channel at different velocities, so that the solutes are longitudinally separated.

The flow acting as the separation factor makes it possible to reduce the channel dimension in the direction of the electric field, which enables absolute voltage values to be decreased with the maintenance of a high field strength. This results in a decrease in the Joule heat production. The reduction in the channel dimensions also decreases the time required for focusing. Further, the laminar flow of the carrier liquid stabilizes the pH gradient against convection [10]. Another advantage is provided by the detection mode permitted by the elution character of the method [11]. Steady-state solute zones need not be mobilized in order to pass them across a sensor, but detectors used in liquid chromatography can be used.

Sufficient speed of the establishment of the pH gradient and its stability are fundamental assumptions for effective separations by IEF_4. A number of theoretical and experimental studies characterizing pH gradients in different techniques have been published (for reviews, see refs. 12 and 13). However, no study has been published concerning the generation of pH gradients in IEF_4. This work was aimed at investigating the dynamics of the pH gradient in the IEF_4 channel and studying the influence of various experimental parameters on the pH gradient.

EXPERIMENTAL

The IEF_4 channel used in the study of pH gradient generation is illustrated schematically in Fig. 1. The channel is composed of three Perspex parts clamped together with brass bolts. The core is the block in which is the channel proper with dimensions of length 250 mm, height 12 mm and width 1 mm. This block also includes inlet and outlet capillaries which are used for the introduction of ampholyte solution (the capillary situated at the channel head at its half-height). Injection of the sample (the capillary situated in the distance of 2 cm from the channel head at its half-height) and taking of ampholyte samples for pH measurements (four triplets of capillaries situated at one quarter, half and three quarters of the channel length and at its end with individual capillaries of one triplet situated at one quarter, half and three quarters of

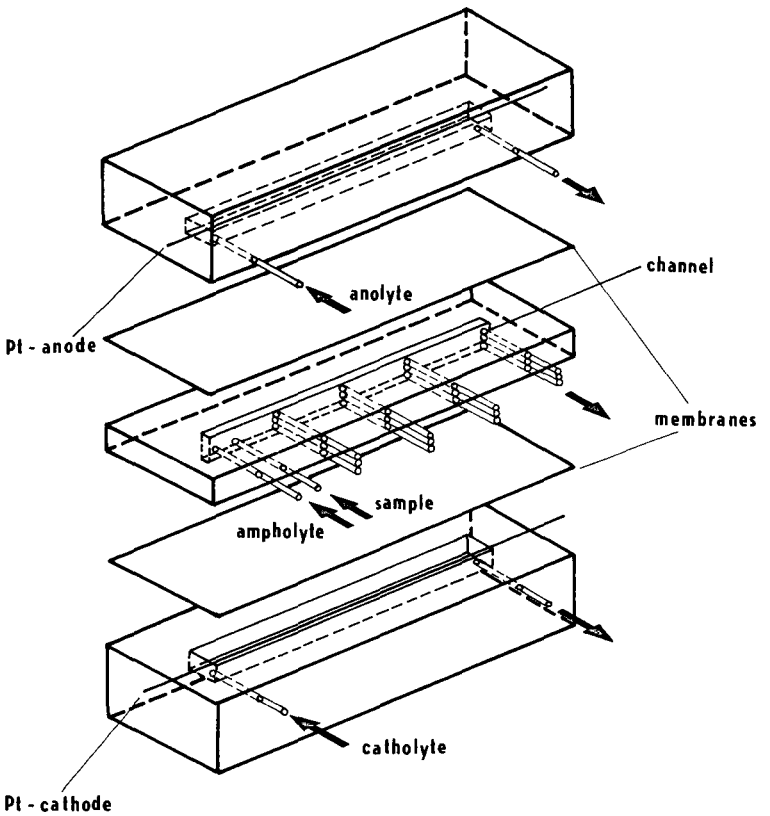

Fig. 1. Schematic illustration of the IEF_4 channel used for the study of pH gradient generation.

the channel height). The remaining two blocks, used for fixing platinum wire capillaries and comprising electrode reservoirs, are separated from the channel interior space with PGCL ultrafiltration membranes (Millipore, Bedford, MA, U.S.A.) with a nominal molecular weight cut-off of 10 000.

The scheme of the experimental arrangement for the measurement of pH gradients is shown in Fig. 2. An M 122 doser (Mikrotechna, Prague, Czechoslovakia) with two injection syringes was used to pump solutions of electrode electrolytes, *i.e.*, solutions of sodium hydroxide and acetic acid, into electrode reservoirs. The flow-rate was 500 μl/min in all instances. An LD 2 linear feeder (Development Workshops of the Czechoslovak Academy of Sciences, Prague, Czechoslovakia) was used to pump ampholytes into the channel. Another LD 2 device with three identical syringes was used to suck ampholyte samples from the channel. These linear feeders were adjusted in such a way that the volumes of the ampholyte injected were identical with the total volume taken by all three syringes. The syringe denoted a was used to take ampholyte samples from the anodic side of the channel (*i.e.*, in the acidic range of the pH gradient), syringe b was used to take samples from the alkaline range of the pH gradient in the cathodic side of the channel and syringe c served for taking samples

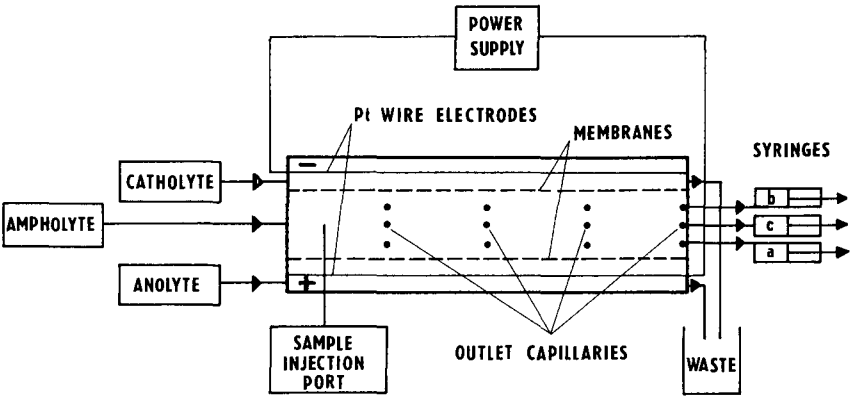

Fig. 2. Scheme of the experimental arrangement for the measurement of pH gradients in the IEF_4 channel.

from the middle of the channel. This arrangement ensured good reproducibility of the measurements. Another LD 2 feeder was used for the continuous injection of amphoteric dyestuffs.

The pH values of ampholyte samples taken with syringes a, b and c were measured with a capillary pH microelectrode (Radelkis, Budapest, Hungary).

The synthetic carrier ampholyte Servalyt 4-9T was a product of Serva (Heidelberg, F.R.G.). Other chemicals were obtained from Lachema (Brno, Czechoslovakia).

RESULTS AND DISCUSSION

Generation of pH gradient in the IEF_4 channel was studied as a function of various experimental parameters. Sampling from twelve different positions in the channel at three flow-rates of ampholyte solution (50, 150 and 500 μl/min), at three different concentrations (0.25, 0.5 and 1%, w/v) and at three applied voltages (25, 50 and 100 V) made it possible to obtain numerous experimental data characterizing the generation of pH gradients. In view of the large number of the data, it is not possible to present all of them here and only some examples have been selected. In spite of the fact that the height of the channel used exceeds several-fold the height of the channels used in earlier work [10,11], it is probable that, with certain restrictions, the results obtained could also be applied to channels of smaller height that are used for analytical purposes.

To illustrate the results, with the following four dependences the data obtained from the first triplet of sampling capillaries situated at one quarter of the channel length and those from the triplet of the sampling capillaries situated at the channel end only are presented in the figures. In the former instances this means from the location where pH gradient is just being generated and in the latter from the location where the steady-state pH gradient has already been reached, *i.e.*, a pH gradient which corresponds to the pH gradient that would be generated in a flowless arrangement at the given voltage and at the given compositions of ampholyte solutions and electrode electrolytes in the course of a sufficiently long time period. pH values from the

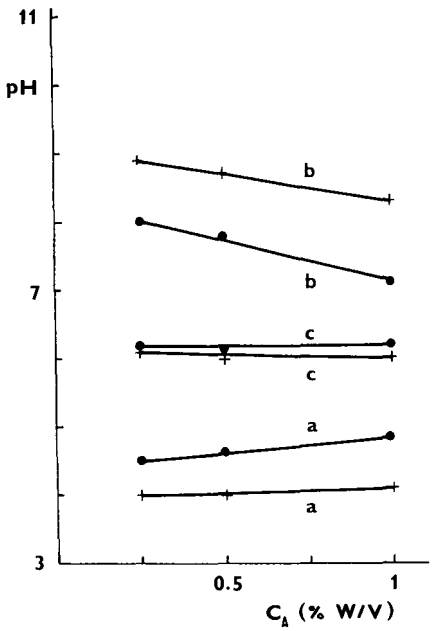

Fig. 3. Dependence of pH gradient on the concentration of ampholyte, c_A. Conditions: applied voltage, E = 100 V; flow-rate of ampholyte solution, v = 150 μl/min; concentration of electrode electrolytes, c_E = 0.05 M. Letters a, b and c denote syringes sucking ampholyte samples from the anodic and cathodic sides and the middle of the channel, respectively. (●) pH values obtained from the capillaries situated at one quarter of the channel length; (+) data from the capillaries situated at the channel end.

capillaries situated at half and three quarters of the channel length lie between these boundary values.

It is obvious from Fig. 3 that under the given experimental conditions the difference between the pH values in the anodic and cathodic sides of the channel decreases with increasing ampholyte concentration. The influence of the increase in the concentration of H^+ ions in the anolyte and of OH^- ions in catholyte appears as a decrease or increase in the pH of the anodic and cathodic sides of pH gradients (see Fig. 4). An increase in the applied voltage results in an acceleration of the migration of compounds that show electrophoretic mobility and a faster generation of the pH gradient, which under the experimental conditions used (Fig. 5) also appeared as an increase in the difference between the pH values in the cathodic and anodic sides of the channel. It follows from the results obtained that the steady-state pH gradient is not generated in the channel at lower values of the applied voltage and at higher flow-rates.

The influence of the flow-rate of the ampholytes on the generation of the pH gradient is illustrated in Fig. 6. This influence is evident in those parts of the channel where, under the given conditions, the steady-state gradient is not generated. This is illustrated in Fig. 6 by the pH values obtained from the capillaries situated in the first quarter of the channel. As long as under the given conditions (applied voltage and composition of solutions) the steady-state pH value is generated at a certain position in the channel at the highest flow-rate used, it is evident that the pH at this position in the

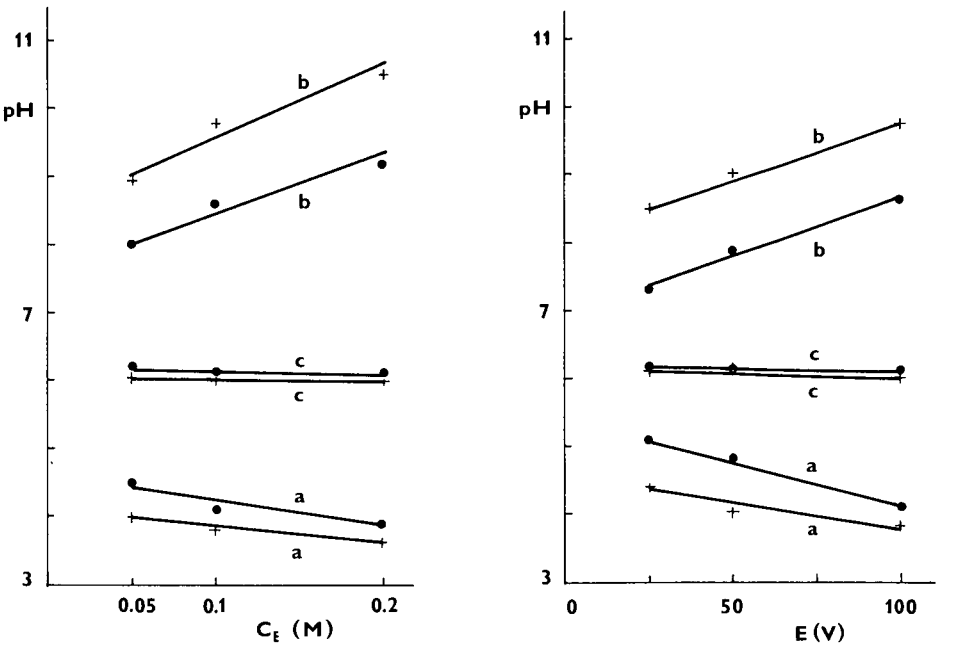

Fig. 4. Dependence of pH gradient on the concentration of electrode electrolytes, c_E. Conditions: E = 100 V; v = 150 μl/min; c_A = 0.25%. Other symbols as in Fig. 3.

Fig. 5. Dependence of pH gradient on the applied voltage E. Conditions: v = 150 μl/min; c_A = 0.25%; c_E = 0.1 M. Other symbols as in Fig. 3.

channel will not be dependent on the flow-rate at lower values. This situation is illustrated in Fig. 6 by the pH values obtained from the capillaries situated at the channel half-height and by those obtained from the capillary situated at the end of the channel in the anodic side of the channel. On the other hand, the situation in the cathodic side of the channel is more complicated, as certain changes in pH values appear, depending on the flow-rate.

It follows from the results of the measurements that at an applied voltage of 100 V the pH gradient corresponding to the steady-state pH gradient at a flow-rate of 50 μl/min is reached between a quarter and a third of the channel length. It is also evident that the steady-state pH values are reached faster in the acidic than the alkaline region of the pH gradients. At a flow-rate of 150 μl/min, pH values corresponding to the steady-state values are reached in the last quarter of the channel. At a flow-rate of 500 μl/min the pH values corresponding to the steady-state values in acidic and neutral regions of the pH gradient only are reached.

Measurements of the long-term stability of the pH values of the samples taken from a certain triplet of capillaries were also performed. The highest stability was obtained in the case of the capillaries situated at the end of the channel and at three quarters of the channel length, where no changes were observed at a flow-rate of 150 μl/min. The greatest changes were observed with the capillaries situated at one quarter

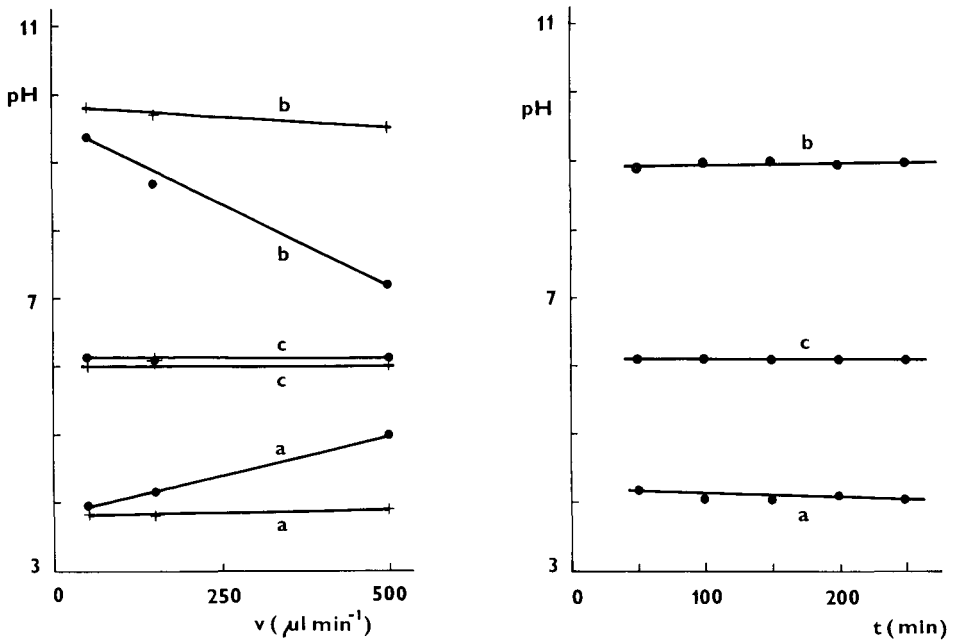

Fig. 6. Dependence of pH gradient on the flow-rate of the solution of ampholytes, v. Conditions: E = 100 V; c_A = 0.25%; c_E = 0.1 M. Other symbols as in Fig. 3.

Fig. 7. Stability of pH gradient studied with the aid of ampholyte samples taken from the channel half-length. Conditions: E = 100 V; v = 150 μl/min; c_A = 0.25%; c_E = 0.1 M. Letters a, b and c as in Fig. 3.

of the channel length in the cathodic side of the channel (±0.2 pH unit) and smaller changes in the anodic side of the channel (±0.1 pH unit). In the case of the capillaries situated at the channel half-length, no changes were observed if the samples were taken from the middle of the channel, whereas certain changes were observed if the samples were taken from the anodic and cathodic sides of the channel (±0.05 pH unit). As follows from Fig. 7, the stability of the pH gradient studied at the channel half-length for 250 min is very good. This is supported by the findings that the laminar flow of the carrier liquid favourably affects the stability of the pH gradient [14].

The dynamics of the generation of the pH gradient in the IEF_4 channel can be deduced from the results illustrated in Fig. 8, showing the pH values measured at different positions in the channel. The pH values measured at a flow-rate of 50 μl/min are plotted at relative positions corresponding to the positions of the sampling capillaries in the channel. In order to characterize pH gradient at the beginning of the channel, *i.e.*, before the sampling capillaries situated at one quarter of the channel length, the data from the sampling capillaries obtained at a flow-rate of 150 and 500 μl/min, respectively, were transformed.

The transformation was performed in such a way that the position of the given triplet of sampling capillaries was divided by the ratio of the flow-rate used to a flow-rate of 50 μl/min. This means that, *e.g.*, at a flow-rate used of 150 μl/min, the positions of the sampling capillaries were divided by three and the measured pH values

were plotted for these calculated positions. This transformation substantially means plotting pH values for those positions of the channel which would be reached by ampholyte solutions at a flow-rate of 50 μl/min within the same time during which the sampling capillaries are reached at a higher flow-rate. The data obtained at a flow-rate of 50 μl/min can easily be transformed into the state occurring at higher flow-rates and thereby an idea of the course of the pH gradient under these conditions can be acquired. Although the agreement of the data obtained in this way is not perfect (see Fig. 8), these results give an idea of the speed of the generation of the pH gradient under the conditions of the flow along the IEF_4 channel, for the scale of relative distances can be transformed into the time scale by dividing the absolute channel lengths by the linear flow-rate.

The results confirm the finding that the generation of pH gradients is relatively fast [15], but it is nevertheless necessary to be aware that the generation of a pH gradient in solutions of carrier ampholytes is a much faster process than reaching the steady-state distribution of various components of a mixture of carrier ampholytes and conductivity [13]. The generation of a pH gradient is thus one of the conditions required for successful separation. Reaching the steady-state distribution of carrier ampholytes gives rise to an increase in buffering capacity, *i.e.*, an increase in the stability of the pH gradient and thereby the ability of carrier ampholytes to dictate the pH characteristics of the gradient even in the presence of further ampholytes whose zones in such a pH gradient are narrower than those in a pH gradient with low buffering capacity.

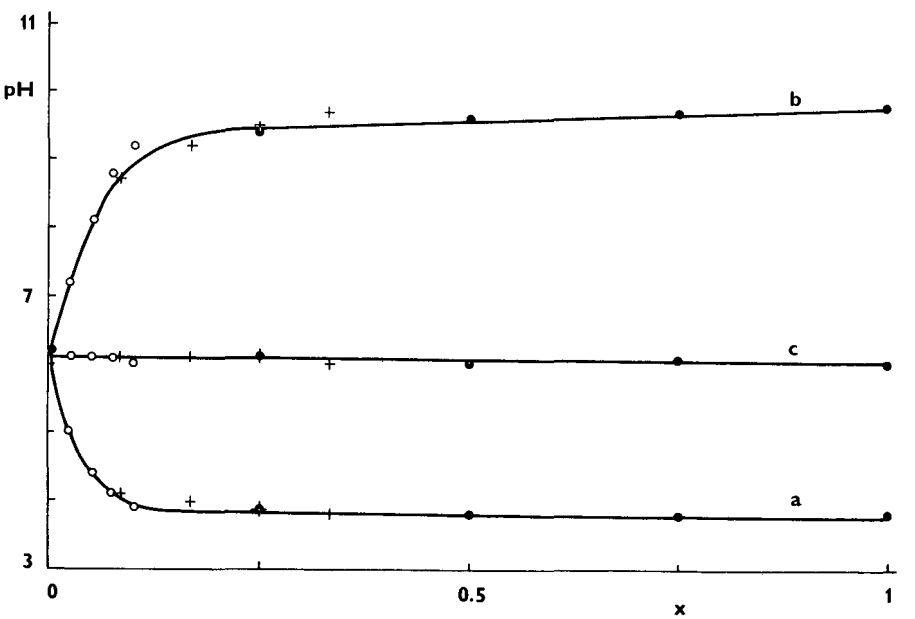

Fig. 8. Dependence of pH gradient on the relative position in the IEF_4 channel. Conditions: E = 100 V; c_A = 0.25%; c_E = 0.1 M. Relative positions x = 0 and x = 1 correspond to the beginning and end of the channel, respectively. Letters a, b and c as in Fig. 3. (●) pH values at a flow-rate of 50 μl/min; (+) and (○) pH values obtained at flow-rates of 150 and 500 μl/min, respectively, plotted on transformed positions.

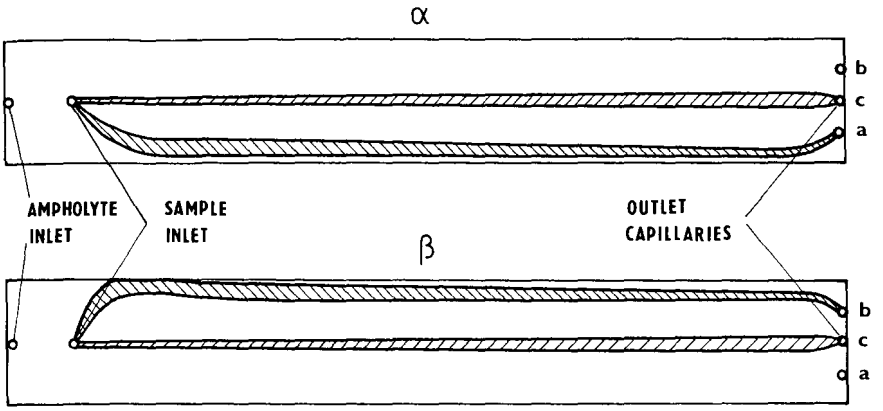

Fig. 9. Schematic illustration of the behaviour of continuously injected amphoteric dyestuffs in the IEF_4 channel. Conditions: flow-rate of the solution of dyestuffs, $v_D = 30$ µl/min; flow-rate of ampholyte, $v = 150$ µl/min; $c_A = 0.25\%$; concentration of dyestuffs, $c_D = 0.1$ *M*. The hatched regions in the middle of both panels denote the flow of the dyestuff without the action of the applied electric field. The other hatched regions denote the flow of the dyestuff at an applied electric field of 100 V. Letters a, b and c as in Fig. 3. (α) Methyl red, electrode electrolytes used at a concentration $c_E = 0.1$ *M*; (β) 4-(4′-hydroxyphenylazo)-1-naphthylethylenediamine, electrode electrolyte used at a concentration $c_E = 0.2$ *M*.

The behaviour of low-molecular-weight ampholytes was studied with the aid of continuous injection of amphoteric dyestuffs into the channel. With both of the dyestuffs selected, methyl red for the acidic region and 4-(4′-hydroxyphenylazo)-1-naphthylethylenediamine for the alkaline region, the transformation from yellow to red occurs in the pH range between the neutral medium of the solution of ampholytes (pH 6.2) and their isoelectric points. This change in colour aids the visual investigation of the processes in the channel. As is obvious from the schematic illustration in Fig. 9, only a small part of the channel is required for transferring dyestuffs from the site of their injection to the positions corresponding to their isoelectric points. In the next part of the channel the zone of dyestuffs becomes narrower, which corresponds to gradual focusing of the dyestuff and obviously also to an increase in the buffering capacity of the pH gradient.

In addition to this visual investigation of the focusing of low-molecular-weight amphoteric dyestuffs, a more detailed study of the focusing of methyl red was performed by using Hewlett-Packard HP 1040 A diode-array detector. The detector was situated between a sampling capillary and a suction syringe. It was found that without the applied electric field, methyl red was only detected in samples taken from the middle of the channel (capillaries c). With an applied electric field of 100 V methyl red was only detected in samples taken from the anodic side of the channel (capillaries a).

The spectra of methyl red recorded both without and with the applied electric field are shown in Fig. 10. Spectrum a (without the applied electric field) corresponds to the yellow form of methyl red in the neutral pH range; the pH of the ampholyte solution was determined to be 6.2. Spectrum b (with an applied electric field of 100 V) corresponds to the red form of methyl red in the acidic pH range. The determined pH

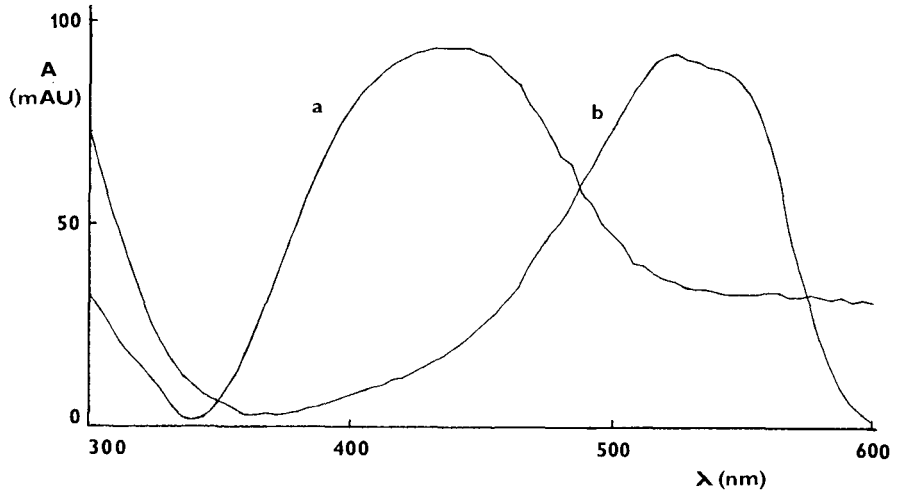

Fig. 10. Spectra of methyl red obtained in the investigation of the behaviour of continuously injected dyestuffs in the IEF_4 channel. Conditions: flow-rate of solution of methyl red, v_D = 30 μl/min; concentration of methyl red, c_D = 0.01 *M*; flow-rate of ampholyte solution, v = 150 μl/min; concentration of ampholytes, c_A = 0.25%; concentration of electrode solutions, c_E = 0.1 *M*. Capillaries a and c as in Fig. 3. (a) Spectrum of methyl red obtained from capillary c without the applied electric field; (b) spectrum of methyl red obtained from capillary a at an applied electric field of 100 V.

values of ampholyte solutions from capillaries a ranged from 3.9 to 3.8, which corresponds to the isoelectric point of methyl red.

Methyl red was not detected in any sample taken from each of the twelve capillaries if the pH of anolyte was increased from 3.0 to 5.0, *i.e.*, the pH of the anolyte was higher than the isoelectric point of methyl red. In this instance methyl red did not focus inside the channel but passed through the membrane into the electrode (anode) reservoir. These results confirm that the zone of methyl red is formed by isoelectric focusing if the pH gradient generated inside the channel contains the pH value corresponding to the isoelectric point of methyl red.

The results obtained only represent the first step in characterizing pH gradients in IEF_4 channels. With respect to the procedure used for taking samples for the pH measurements, these are of only a qualitative character. It will be possible to obtain more detailed information by placing the pH electrodes (*e.g.*, antimony or bismuth) directly in the channel or by studying changes in the potential gradient at different channel positions with the aid of an electrode array detector.

On the basis of the experience obtained so far, the conditions can be found under which a pH gradient is generated in the IEF_4 channel such that amphoteric compounds can be separated successfully. This is confirmed by results published recently on the fractionation of proteins [10,11]. Further research aimed at using IEF_4 must, in addition to a search for suitable experimental conditions, also include optimization of the separation channel with respect to both its design and the materials used [11,16]. The solution to these problems could lead to a reduction in the time required for the analysis to a few minutes for channels of rectangular cross-section [11] or to tens of

minutes for channels of trapezoidal cross-section [10], which would make IEF_4 suitable for various applications.

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Multi-component principal component regression and partial least-squares analyses of overlapped chromatographic peaks

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ABSTRACT

Principal component and partial least-squares in latent variable regression methods were applied to the multivariate calibration of overlapping chromatographic peaks for toluene, isooctane and ethanol mixtures. The degree of peak overlap was varied using column temperatures of 105, 120 and 130°C. Even using the most severely overlapped peaks (130°C), the analysis errors obtained for validation set samples using both regression techniques were of the same size as those encountered using simple linear regression for individual determination of the three constituents. Truncation of the overlapped peak chromatograms appeared to lower the noise level without a significant loss of statistical information about the constituent concentrations.

INTRODUCTION

Quantitative chromatographic analysis of complex samples is often complicated by the occurrence of overlapping peaks of the mixture constituents. Expensive investment in sophisticated chromatographic equipment capable of separating overlapped peaks can be made, although more modest outlays involved in computer software and hardware can lead to accurate quantitative determintations of the constituents of mixtures. In some instances optimization techniques can be used to eliminate overlap, but longer elution times often result [1]. In others, direct numerical treatment of the overlapped band system is applied. Chemometric techniques, such as the partial least-squares in latent variables (PLS) [2,3] and the principal component regression [4] (PCR) methods, have been applied to complex multi-component analysis using a variety of chemical instrumentation [5,6], including high-performance liquid chromatography [7]. The theoretical basis and mathematical formulations of these multivariate methods have also been described [8–10].

In this work, the quantitative gas chromatographic analysis of toluene, isooctane and ethanol mixtures using the multivariate PLS and PCR methods is reported. Although diode-array detectors permit more sophisticated multivariate applications, the chromatographic system employed here has a simple thermal conductivity detector. Different degrees of peak overlap were investigated using chromatograms obtained at different column temperatures. The results of the

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multi-component analysis compare favourably with those obtained for the individual analyses of the three constituents. Preprocessing of the raw chromatographic data, eliminating analytical signal for the wing portions of the band system, reduces the number of principal components or latent variables needed to perform the analysis, indicating that truncation reduces the noise level without a significant loss of statistical information about the concentrations of the constituents of the mixture.

EXPERIMENTAL

Fourteen calibration samples of toluene, isooctane and ethanol mixtures with mass ranges of 0.300–1.014, 0.201–0.787 and 0.308–0.964 mg, respectively, were prepared by direct weighing so that the concentration interval between these limits was more or less evenly covered. Six validation set mixtures were prepared in the same manner as the calibration samples. Analytical-reagent grade reagents from Merck (ethanol) and Carlo Erba (toluene and isooctane) were used. Ethanol was first treated with a molecular sieve to eliminate water.

All chromatographic work was done using a Varian Model 920 chromatograph with a thermal conductivity detector and a 2 m × 1/8 in. I.D. stainless-steel column packed with 5% SE-30 on Chromossorb W (80–100 mesh). The measurements were performed at an injector temperature of 160°C and a detector temperature of 180°C with hydrogen as the carrier gas at a flow-rate of 30 ml min^{-1}. In Fig. 1 representative chromatograms obtained with column temperatures of 150, 120 and 130°C for a toluene–isooctane–ethanol mixture (0.613, 0.608 and 0.311 mg, respectively) are shown.

PLS calculations were performed on an 8-bit CPM DICON microcomputer using the SIMCA-3B program acquired from Principal Data Components. [11]. PCR

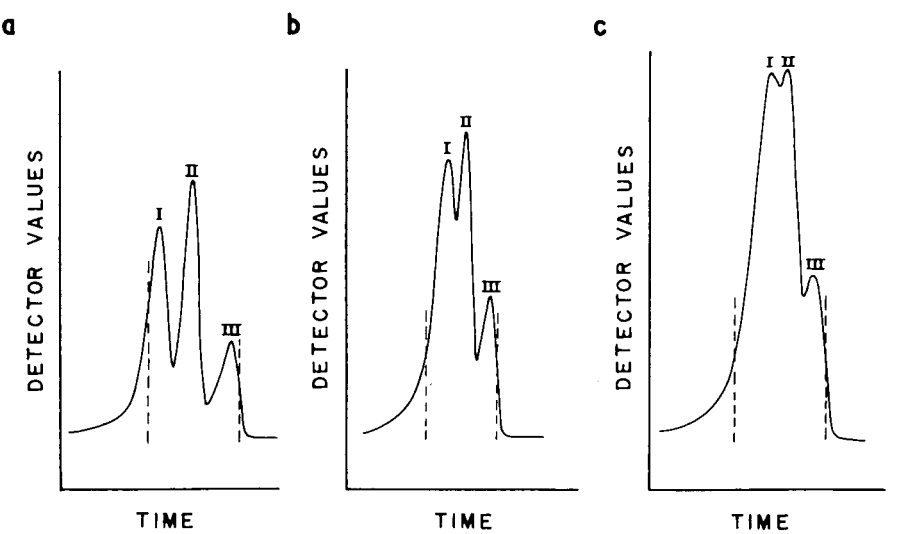

Fig. 1. Representative chromatogram obtained with column temperatures of (a) 105, (b) 120 and (c) 130°C for (I) Toluene (0.613 mg), (II) isooctane (0.608 mg) and (III) ethanol (0.311 mg).

was carried out using the KARLOV subroutine of the ARTHUR/75 computer program [12] adapted for the PC-XT microcomputer [13] and a standard multiple regression program.

PREPROCESSING

The calibration concentration matrix is 14 × 3, each row containing the masses of the three constituents of the mixtures. Each row of the 14 × 41 chromatographic matrix contains the chromatographic responses of 41 evenly spaced intervals chosen to cover completely the overlapping peak system. Each row of both the concentration and the chromatographic matrix were normalized so that the sum of their elements was equal to one. The validation set chromatographic matrix (6 × 41) was formed in the same way as that used in the calibration step. Visual inspection of the overlapped peak chromatograms showed that the wings of the chromatogram superimpose for the calibration and validation set samples. As these portions of the peak system probably contribute little information about the constituent concentrations and also introduce noise into the PCR and PLS calculations the effect of peak truncation was tested using only the 26 central detector responses falling between the limits illustrated in Fig. 1. Normalization of the rows in the (14 × 26) calibration and (6 × 26) validation matrices of the truncated data set was also carried out.

RESULTS AND DISCUSSION

Individual analysis

Separate chromatographic quantitative analyses of each of the three mixture constituents were performed to estimate the errors in the measurement procedures. The chromatographic conditions were identical with those employed for the mixture analyses, except that the column temperature was held constant at 120°C and benzyl alcohol was used as a mixture component. Eight calibration set samples with masses in approximately the same ranges as those used in the mixture analyses were used. Validation set standard prediction errors for three samples were calculated using

$$SEP = \sqrt{\sum (y_{calc} - y_{exp})^2/n} \quad (1)$$

where y_{calc} and y_{exp} are the percentage constituent masses calculated from the calibration graph and by direct weighing, respectively. The number of validation set samples, n, was three for the individual analyses. Standard prediction errors (*SEP*), expressed as percentages, for toluene, isooctane and ethanol of 0.95, 0.82 and 1.22%, respectively, were estimated by this procedure.

Principal component regression

The number of principal components appropriate for use in a multi-component analysis is not known *a priori*. Table I contains information relevant for the determination of these numbers for analyses performed using the three column temperatures. *F*-values calculated using standard statistical equations for regressions, fluctuate over wide ranges for all the constituents at all column temperatures as the number of principal components included in the regression is increased. However,

TABLE I

CALIBRATION SET *F*-TEST AND CORRELATION COEFFICIENT VALUES AND VALIDATION SET STANDARD ERROR VALUES FOR TOLUENE, ISOOCTANE AND ETHANOL CONCENTRATIONS FOR 41 VARIABLE EXPERIMENTS WITH COLUMN TEMPERATURES OF 105, 120 AND 130°C

Column temperature (°C)	Compound	Parameter	Number of PC[a]				
			1	2	3	4	5
105	Toluene	*F*-value[b]	186	131	217	492	352
		Corr. coef.[c]	0.969	0.980	0.992	0.998	0.998
		SEP[d] (%)	3.35	3.28	1.84	1.45	1.41
	Isooctane	*F*-value	1.47	153	201	646	663
		Corr. coef.	0.331	0.983	0.992	0.998	0.999
		SEP (%)	12.7	2.55	2.25	2.24	2.02
	Ethanol	*F*-value	9.99	1510	1270	862	823
		Corr. coef.	0.674	0.998	0.999	0.999	0.999
		SEP (%)	11.3	1.50	1.07	1.09	1.01
120	Toluene	*F*-value	107	492	202	143	195
		Corr. coef.	0.948	0.948	0.992	0.992	0.996
		SEP (%)	6.21	6.23	2.63	2.43	2.77
	Isooctane	*F*-value	2.21	20.7	171	141	156
		Corr. coef.	0.394	0.889	0.990	0.992	0.995
		SEP (%)	13.8	11.5	3.17	2.73	1.10
	Ethanol	*F*-value	6.47	156	167	177	128
		Corr. coef.	0.592	0.983	0.990	0.994	0.994
		SEP (%)	9.17	5.85	3.47	2.30	2.64
130	Toluene	*F*-value	67.1	31.7	93.1	84.3	392
		Corr. coef.	0.921	0.923	0.983	0.987	0.998
		SEP (%)	2.45	1.85	1.58	1.39	0.78
	Isooctane	*F*-value	1.58	46.5	106	73.1	183
		Corr. coef.	0.341	0.946	0.985	0.985	0.996
		SEP (%)	12.7	2.22	1.61	1.60	2.27
	Ethanol	*F*-value	7.26	342	357	580	428
		Corr. coef.	0.614	0.992	0.995	0.998	0.998
		SEP (%)	10.8	2.02	2.14	2.05	1.97

[a] Number of principal components included in regression calculations.
[b] Standard *F*-value calculated for the sums of squares due to regression and to residuals.
[c] Correlation coefficient, *r*, for experimental and calculated concentrations.
[d] Calculated using eqn. 1.

once the *F*-values remain essentially constant or decrease, correlation coefficients for the experimental and analysed values of the constituent concentrations of the calibration set samples are larger than 0.99. For the analyses at column temperatures of 105 and 120°C, three principal components included in the regression result in correlation coefficients of 0.99 or higher for all constituents. However, for the more severely overlapped groups of peaks, obtained with a column temperature of 130°C, four or five principal components are necessary to obtain correlation coefficients this high.

In principle, the standard errors for the six validation set samples could also be

used to estimate the appropriate number of principal components by comparing their values for the overlapped band systems with the standard errors obtained for the individual analyses of toluene, isooctane and ethanol. The standard error values in Table I are almost always much larger than those obtained for the individual analyses. Even when five principal components are included in the regression, errors in the PCR results of about twice the size of the errors for the individual analyses can be encountered. The larger errors in the mixture analyses can be the result of several error contributions that do not exist in the individual analyses. As shown later, some validation set samples have constituent concentrations which are not in the concentration domain of the calibration set samples. This is not a problem for the individual analyses as the validation set sample concentrations were always obtain by interpolation procedures applied to the calibration graphs. Also, it is reasonable to expect that errors propagated in the mixture results may be larger than those for the individual results owing to more severe detector non-linearity problems and possibly matrix effects.

For some constituents, analysis with certain column temperatures can be carried out using less than three principal components. For example, the determination of ethanol in the extremely overlapped peak system obtained with a column temperature of 130°C provides acceptable results using only two principal components. In Fig. 2 the ethanol concentrations of both the calibration and validation set samples as a function of the scores of the first two principal components are shown. These points form a well defined plane in this space, illustrating the usefulness of a two-variable regression in the analysis of the overlapped ethanol peak.

Partial least-squares regression

The PLS technique is especially convenient for estimating the number of components or latent variables to be included in the regression and for studying the effects of the peak truncation suggested above. Total standard prediction errors obtained from results of two block PLS treatments, for the validation set samples were calculated using eqn. 1. The y_{calc} and y_{exp} quantities are the weight percentages (normalized constituent masses) determined by the analyses and those obtained from the masses of the mixture preparations. The sum is taken over all the validation set

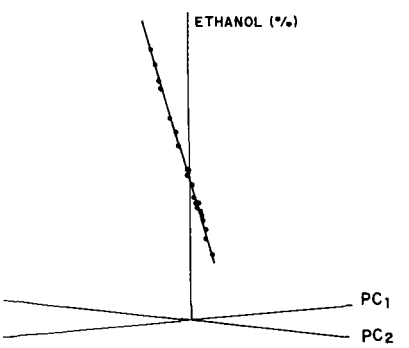

Fig. 2. Ethanol concentrations of both the calibration and validation set samples as a function of the scores of the first two principal components, obtained with a column temperature of 130°C.

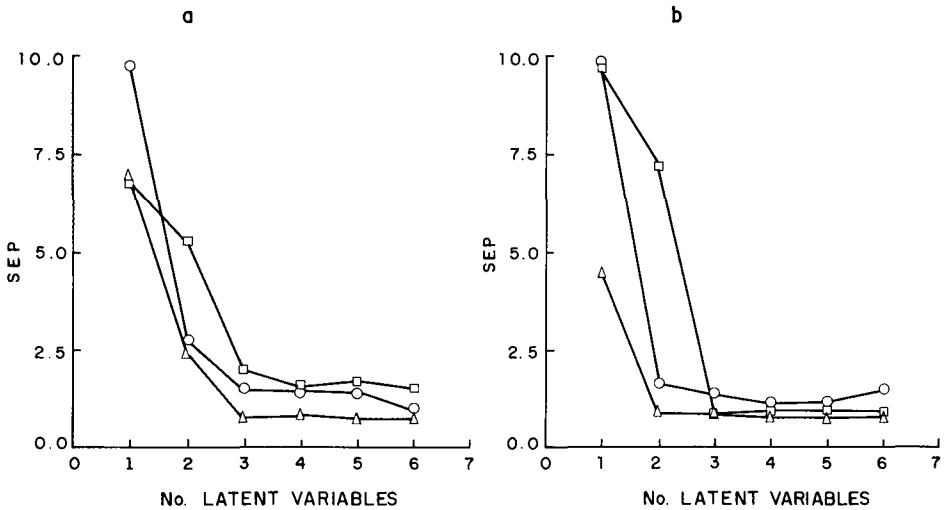

Fig. 3. *SEP* values as a function of the number of components used in the PLS analysis for (a) the complete chromatograms (41 detector responses) and (b) the truncated peak systems (26 responses). Column temperature: △ = 105; □ = 120; ○ = 130°C.

samples and all three constituents. Hence n is equal to the number of validation set samples times the number of constituents. Fig. 3 shows the graphs of the SEP values as a function of the number of components used in the PLS analysis for both the complete chromatograms (41 detector responses) and the truncated peak systems (26 responses). It can be observed that the SEP values become almost constant for three components or more for the complete 41 response data set. For the truncated 26 detector response set, values of SEP below 2.0% can be obtained using only two components for the 105 and 130°C column temperature data sets. As the results of cross-validation were not clear for determining the number of components, two components were used to describe the 105 and 130°C truncated systems and three components for the others.

Truncation of chromatograms leads to a reduction in the number of components necessary to perform the analysis for two reasons. First, the discarded data belong to regions where the chromatograms superimpose and apparently do not depend on the concentrations of the constituents. The data corrsponding to these regions of the chromatograms probably contain more noise than useful information about the constituent concentrations. Second, as the truncated regions correspond to low concentration values, the effects of non-linearity of the detector responses are also minimized.

The 120°C data set represents a special case for which the intermediate resolution of the chromatograms results in the appearance or not of a third peak depending on the constituent concentrations. A graph of the scores of the first two principal components, shown in Fig. 4, confirms the existence of sub-classes with two or three partially resolved peaks. In spite of this, a one-class model was used in all our calculations.

PLS-predicted weight percentages for the three constituents are compared with the experimental values for the six validation set samples in Table II. Results are

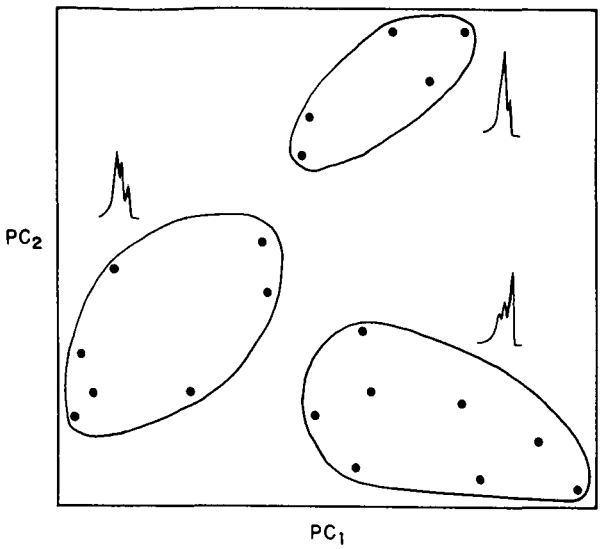

Fig. 4. Score plot of the first two principal components for the 120°C data set containing 87% of the total data variance. Clustering of samples for different types of peak profiles is clearly evident.

included for the three column temperatures studied. The residual standard deviations of the calibration set samples,

$$S_0 = \sum_i^n \sum_k^p e_{ki}^2/[(p-A)\ (n-A-1)]^{1/2} \tag{2}$$

and the individual residual standard deviations of the validation set samples,

$$S_t = \sum_k^p e_k^2/(p-A)^{1/2} \tag{3}$$

are also presented in Table II. In these equations the e_{ki} and e_k are the differences between chromatographic detector values and those predicted by the PLS model. The sums are taken over the independent variables ($p = 26$ for the truncated system and 41 for the complete peak system) and the number of samples in the calibration set ($n = 14$). The number of components (latent variables), A, is two for the 105 and 130°C column temperature analyses and three for those at 120°C. Only those validation set samples fitting the PLS calibration model within about $2S_0$ can be expected to have reliable PLS prediction values if a 95% confidence envelope for the calibration model is used as the classification criterion. For this reason, the sample numbers labelled *b* in Table II were not included in the calculation of the standard prediction errors of the validation set samples using eqn. 1. These error values (see Table II) are 1% or less for the three constituents analysed using column temperatures of 105 and 120°C for which overlap is less severe. For a column temperature of 130°C the errors are larger, but always less than 2%.

TABLE II

SAMPLE COMPOSITION AND PLS-PREDICTED VALUES (%, w/w) FOR TWO- AND THREE-COMPONENT PLS MODELS FOR 26-VARIABLE CHROMATOGRAPHIC ANALYSIS WITH COLUMN TEMPERATURES OF 105, 120 AND 130°C

Column temperature (°C)	S_0	Sample No.	Toluene		Isooctane		Ethanol		S_T
			Exp.	PLS	Exp.	PLS	Exp.	PLS	
105	0.254	1	40.4	39.9	25.8	25.9	33.8	34.1	0.215
		2[b]	13.9	16.4	61.5	57.2	24.7	26.4	0.752
		3	26.2	26.3	40.7	38.9	33.1	34.8	0.328
		4	26.7	26.6	19.5	18.8	53.9	54.7	0.145
		5	40.0	39.8	39.7	39.3	20.3	20.9	0.179
		6	33.8	33.0	32.2	33.1	34.0	33.9	0.246
		SEP (%)		0.4		1.0		0.9	
120	0.284	1	40.4	40.9	25.8	27.0	33.8	32.1	0.151
		2[b]	13.9	16.2	61.5	65.9	24.7	17.9	1.302
		3	26.2	27.4	40.7	40.3	33.1	32.3	0.389
		4	26.7	26.5	19.5	19.5	53.9	53.9	0.202
		5	40.0	39.3	39.7	40.5	20.3	20.2	0.409
		6	33.8	33.7	32.2	32.6	34.0	33.7	0.212
		SEP (%)		0.7		0.7		0.9	
130	0.247	1[b]	40.4	45.4	25.8	25.3	33.8	29.3	0.571
		2	13.9	14.3	61.5	61.8	24.7	23.9	0.130
		3	26.2	25.6	40.7	39.9	33.1	34.5	0.121
		4	26.7	26.4	19.5	19.3	53.9	54.3	0.128
		5	40.0	37.7	39.7	43.5	20.3	18.8	0.174
		6	33.8	32.7	32.2	33.4	34.0	33.9	0.123
		SEP (%)		1.2		1.8		1.0	

[a] S_T and S_0 values calculated using eqns. 2 and 3. *SEP* values calculated using eqn. 1. PLS results are from two block calculations. For all calculations PLS_2 and PLS_1 calculations gave identical results within experimental error.

[b] Results for these samples were not included the calculation of *SEP* as they do not fit the PLS calibration models, *i.e.*, $S_T > 2.0S_0$.

The importance of excluding samples that do not fit the calibration model should be emphasized. Their constituent concentrations were not included in the calculation of the SEP values shown in Fig. 3 or in the determination of the appropriate number of latent variables to be included in the PLS analysis. Also, the PCR validation set sample errors in Table I are grossly inflated by errors from the constituent concentrations of these same samples which are not accurately described by the PCR model. Even though this three-analyte system is not complicated and the detector might be expected to provide linear additivity, it is usually risky to extrapolate with PLS or PCR.

In Table III, standard prediction errors for the validation set samples for analyses made with the three column temperatures are presented for calculations using both the PCR and PLS methods. The number of principal components or latent variables for the calculations at each column temperature are indicated. The PLS and PCR prediction errors are very similar.

TABLE III

STANDARD PREDICTION ERRORS (%) FOR VALIDATION SET SAMPLES USING THE PLS AND PCR METHODS FOR COMPLETE AND TRUNCATED CHROMATOGRAMS

Method	Column temperature (°C)	A^a	Toluene	Isooctane	Ethanol
PLS, complete	105	3	0.4	0.9	1.0
	120	3	2.6	1.3	1.7
	130	3	0.7	1.5	1.6
PLS, truncated	105	2	0.4	1.0	0.9
	120	3	0.7	0.7	0.9
	130	2	1.2	1.8	1.0
PCR, complete	105	4	0.3	1.0	1.0
	120	4	2.1	1.8	1.9
	130	4	0.7	1.7	1.5
PCR, truncated	105	3	0.8	1.2	0.9
	120	4	0.8	1.1	0.8
	130	3	0.7	1.7	1.1
Individual analyses			1.0	0.8	1.2

[a] A = number of latent variables (PLS) or principal components (PCR) used in the regressions.

The effect of eliminating detector readings for the wings of the overlapped peak systems on the standard prediction errors can also be seen in Table III. For either the PLS or PCR regression techniques the peak truncation performed here does not appear to have decreased the accuracies of the analyses. Some standard prediction errors decreased dramatically with peak truncation (*e.g.*, PLS calculations for analysis with a column temperature of 120°C) whereas others increased slightly.

CONCLUSIONS

Both the PCR and PLS regression techniques result in similar standard prediction errors for the toluene–isooctane–ethanol mixtures studied here. The PCR method required one more regression variable (or component) than PLS. This has already been observed for multi-component analysis using fluorescence spectra [14]. Increasing overlap of the chromatographic peaks did not result in large increases in the prediction errors. This is especially interesting because the calibration and validation set samples are identical for the three column temperatures used to control the degree of peak overlap. Peak truncation effected by eliminating the detector values of the wing portions of the overlapped peak system resulted in prediction errors slightly smaller than those obtained using the entire overlapped peak system.

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Effect of noise on peak heights calculated using an exponentially modified gaussian peak shape model

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ABSTRACT

Most computer-based methods for finding chromatographic peak heights are relatively crude, relying on finding an appropriate baseline, then measuring a maximum signal height relative to the baseline. The error in finding a precise signal height of a weak signal can be increased by noise spikes. In this article, data are presented to show that the use of the exponentially modified gaussian peak shape model can effectively increase the quality of height measurements of peaks deliberately degraded to near undiscernability by dilution.

INTRODUCTION

The measurement by computer-based data systems of peak heights generated by chromatographic techniques is generally carried out by automating manual methods, *i.e.*, finding the appropriate baselines on either side of a peak and assigning the peak height by measuring the signal maximum relative to the baseline. This method can lead to imprecise data, if the signal is noisy because the real peak height maximum can be misassigned by noise spikes.

Although peak area measurements can be used, peak height measurements are generally preferred because of their better accuracy and precision [1]. Problems associated with peak area measurements include interferences with compounds eluting close to the analyte peak and uncertainty in assigning the beginning and end of a peak.

Several years ago, our automated data acquistion and processing system, QSIMPS (quantitative selected-ion monitoring processing system) was developed to quantitate drug concentrations in plasma and/or urine samples from pharmacokinetic experiments [2–4]. Because of the high sensitivity requirement of drug assays, peaks are generally fairly noisy at the lower limit of quantitation. For this reason, the exponentially modified gaussian (EMG) peak shape model [5–15] was incorporated into QSIMPS because it is widely regarded as giving the most accurate description of chromatographic peaks. Additionally, the EMG model has been shown to give results more precise than manual measurements [16].

This paper describes a study of the effect of noise on peak heights calculated using the EMG model.

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EXPERIMENTAL

The data was obtained from a gas chromatographic–mass spectrometric (GC–MS) plasma assay for rimantadine, an antiviral agent [17]. Only calibration standards and quality assurance (QA) samples were used. The calibration standards contained either 500, 200, 50, 20 or 5 ng/ml of rimantadine and 100 ng/ml of [$^2H_4$]rimantadine. The QA samples contained either 64 ng/ml (QA high) or 25 ng/ml (QA low) of rimantadine and 100 ng/ml of [$^2H_4$]rimantadine. All samples were diluted by a factor of 1000, 5000, 10 000 or 20 000 and were analyzed in duplicate. These dilution factors were chosen in order to obtain both high quality and noisy peaks at the same electron multiplier setting. All four diluted sets of calibration and QA samples were analyzed together on four separate days.

A Finnigan Model 9500 gas chromatograph was equipped with a 4 ft. × 2 mm I.D. glass column packed with 3% OV-1 on 100–120 mesh Gas-Chrom Q (Alltech). Methane (Liquid Carbonic, 99%) at 14 kg/m^2 was used as the GC carrier gas and negative chemical ionization (NCI) reagent gas. The injector, column, interface oven and transfer line were operated at 300, 250, 250 and 240°C, respectively. Prior to use, the column was conditioned with a 3-μl injection of Silyl-8® (Pierce). The GC system was equipped with an air-actuated divert valve which allowed the diversion of the solvent front from the mass spectrometer. An aliquot of 2 μl of the sample (50 μl total volume) was injected onto the column using a modified Hewlett-Packard autosampler (Model 7672A; Palo Alto, CA, U.S.A.).

A Finnigan 3200 quadrupole mass spectrometer was tuned to give the maximum response consistent with reasonable ion peak shape and near unit resolution. The voltage across the continuous dynode electron multiplier was −800 V and the voltage on the conversion dynode was +3.0 kV. The unlabelled and deuterium-labelled ions were monitored using a Finnigan Promim® (programmable multiple ion monitor, Model 015-80) unit. Each channel was set up to monitor either the $[M-HF]^{-\cdot}$ ion (m/z 353) of the unlabelled analyte or the $[M-{}^2HF]^{-\cdot}$ ion (m/z 356) of the tetradeuterated reference standard. QSIMPS [18] was used to control the autosampler, the divert valve, and to collect and process the SIM data.

The calibration curves were fit using weighted ($1/y^2$) non-linear regression, to the following equation:

$$R = \frac{P1 + x}{P2(x) + P3}$$

where R is the ion ratio [(m/z 353)/(m/z 356)], x is the analyte concentration, and $P1$, $P2$ and $P3$ are parameters adjusted to give the best fit to the calibration data.

For the signal-to-noise (S/N) calculation, noise was defined as the detector signal range between parallel lines that enclose random fluctuations for 25 scans starting with scan 125 [19], and the signal was defined as the maximum intensity at the apex of the peak minus the baseline response.

RESULTS

Fig. 1 shows SIM current profiles of the $[M-HF]^{-\cdot}$ ion (m/z 353) from rimantadine and the $[M-{}^{2}HF]^{-\cdot}$ ion (m/z 356) from $[^{2}H_4]$rimantadine from the 5 ng/ml standard diluted factors of 1000, 5000, 10 000 and 20 000. The raw data is represented by the solid line, actually made up to 512 data is represented by the solid line, actually made up to 512 data points over the retention time window shown. The crosses represent the EMG fit using the top 80% of the peak. The baseline was chosen by extrapolation between the average voltage from scans 90–100 and the average voltage from 450–460 scans. Note the good correspondence of the calculated and raw data. This figure also demonstrates the excellent sensitivity of the assay; the most dilute sample respresents 4.2 ag injected on column.

The results of all the data for the diluted calibration standards are compiled in Table I. The overall accuracy (amount found *versus* amount added) summarized in Table I can be seen to be surprisingly good. In no case is the mean found value more than 10% different than the added value. The relative standard deviations are acceptable except at the two lowest concentrations at the 1:20 000 dilution. However, for this amount of analyzed material, the noise is so high and the signal is so small, that a peak often cannot be visually discerned and no calculation of signal to noise could be calculated. The overall mean inter-assay precision can be seen to diminish more than three times, while the overall mean intra-assay precision diminished over six times, over the twenty-fold dilution range. The correlation coefficients for the fit of the calibration data to the equation used were all greater than 0.99.

Data for the high and low QA samples at the various dilutions are shown in Table II. The analyte response for all of these samples gave a discernible peak. Student's t-test analysis of the measured concentrations for each standard showed no difference among the mesured values at $p<0.01$. In spite of an approximately 25–45 fold decrease in the S/N ratio over the 20-fold dilution range, the relative standard deviations for the analyses only increased approximately 2-fold. A plot of relative

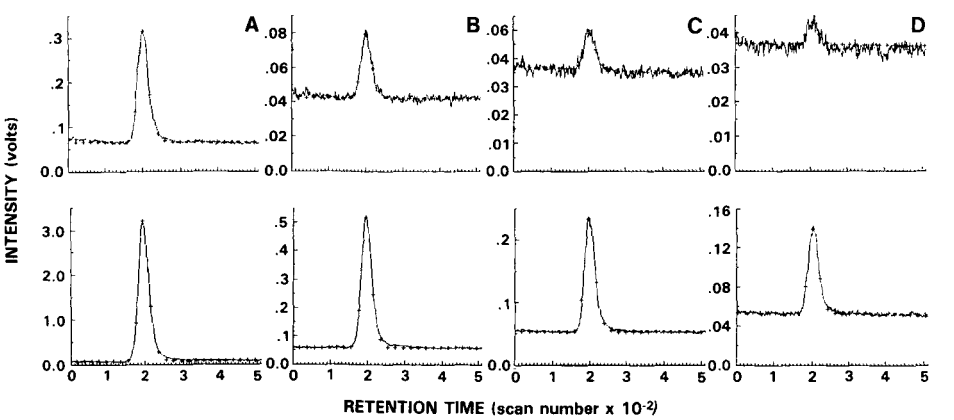

Fig. 1. SIM current profiles of m/z 353 and m/z 356 from the 5 ng/ml calibration standard diluted (A) 1:1000, (B) 1:5000, (C) 1:10 000 and (D) 1:20 000. The profile of the 1:20 000 dilution is one of the better ones at this dilution. The solid line represents the raw data and the crosses denote the EMG fit to the data.

TABLE I

CALIBRATION CURVE DATA FOR STANDARD DILUTED 1:1000; 1:5000; 1:10 000 or 1:20 000 ($n=4$).

Dilution	5 ng/ml	20 ng/ml	50 ng/ml	200 ng/ml	500 ng/ml	Inter[b]	Intra[c]
1:1000	5.0 ± 0.15 (3.0%)	20 ± 1.10 (5,4%)	52 ± 0.75 (1.5%)	196 ± 5.9 (3.0%)	505 ± 22 (4.4%)	2.8 ± 0.9%	3.0 ± 0.48%
1:5000	5.1 ± 0.40 (7.9%)	20 ± 0.64 (3.2%)	51 ± 0.64 (1.3%)	198 ± 6.6 (3.3%)	502 ± 21 (4.2%)	3.2 ± 1.3%	2.8 ± 2.4%
1:10 000	5.0 ± 0.43 (8.6%)	20 ± 1.40 (6.8%)	51 ± 0.99 (1.9%)	196 ± 3.9 (2.0%)	505 ± 13 (2.6%)	3.7 ± 1.3%	5.2 ± 3.6%
1:20 000	5.4 ± 1.30 (24%)	20 ± 2.70 (10%)	51 ± 3.00 (5.8%)	203 ± 15 (7.2%)	499 ± 16 (3.2%)	9.6 ± 2.5%	19 ± 8.0%

[a] Values between parentheses are relative standard deviations (R.S.D.).

[b] Mean R.S.D. ± S.D. for added and back-calculated concentration values.

[c] Mean R.S.D. for ratio of duplicate back-calculated concentrations (first value of duplicate pair divided y the value of the second). Calibration standards were analyzed in duplicate.

TABLE II

RESULT FOR HIGH[a] AND LOW[b] QA SAMPLES AT VARIOUS DILUTIONS

QA	Dilution	S/N[c] ± S.D.	Mean concentration[d] ± S.D. (R.S.D.)[e]
High	1:1000	272 ± 144	64 ± 2.6 (4.1%)
High	1:5000	24 ± 11	63 ± 1.9 (3.0%)
High	1:10 000	11 ± 3.2	63 ± 3.5 (5.6%)
High	1:20 000	6.4 ± 2.3	65 ± 4.3 (6.7%)
Low	1:1000	81 ± 21	25 ± 0.8 (3.4%)
Low	1:5000	11 ± 5.0	25 ± 0.7 (3.0%)
Low	1:10 000	6.0 ± 3.4	25 ± 1.4 (5.6%)
Low	1:20 000	3.0 ± 1.1	25 ± 1.8 (7.0%)

[a] QA sample containing 64 ng/ml of rimantadine from pooled patient samples.
[b] QA sample fortified to contain 25 ng/ml of rimantadine.
[c] S/N = Signal-to-noise ratio.
[d] ng/ml.
[e] R.S.D. = Relative standard deviation.

standard deviation (y) *versus* S/N (x) for the data in Table II would give a plot similar to the previously reported data of Vanderwal and Snyder [20] for high-performance liquid chromatographic drug assays. From the 1:1000 calibration data (Table I), the intrinsic precision of the GC–MS assay can be estimated to be 3%. The relative standard deviation for the high and low QA samples from the 1:5000 dilution is not too different than this intrinsic sensitivity. The relative standard deviation at a dilution of 1:10 000 is somewhat over this intrinsic precision and the relative standard deviation of the 1:20 000 dilution is considerably over the intrinsic precision.

A plot of the relative difference from global means *versus* S/N, for the high and low QA samples is shown in Fig. 2. There is a trend towards increasing error with decreasing S/N, although comparing the error in Group 8 ($S/N > 100$) to that of Group 1 ($S/N > 3$), there is only a 3-fold increase in error in spite of the >30-fold decrease in S/N.

DISCUSSION

Previously the EMG chromatographic peak model was shown to give more accurate peak height determinations than manual methods. In this study, data are presented showing that the use of the EMG model can effectively increase the quality of height measurements of peaks deliberately degraded to almost undiscernability relative to noise by dilution.

Signals from mass spectral assays are subject to all forms of noise, *e.g*, thermal, shot and exogeneous noise. Like any analytical measurement, increased sensitivity in mass spectral assays ultimately depends on incrasing the signal-to-noise ratio of the corrected response from a given amount of analyte, and decreasing the noise is a potentially effective way to accomplish this. Varous digital [21–24] methods are available to minimize noise. Analog, *i.e.*, hardware methods, are available, but can cause phase and amplitude distortion [21]. Each of the digital filtering techniques essentially uses a software algorithm to smooth and filter stored data. Some of the

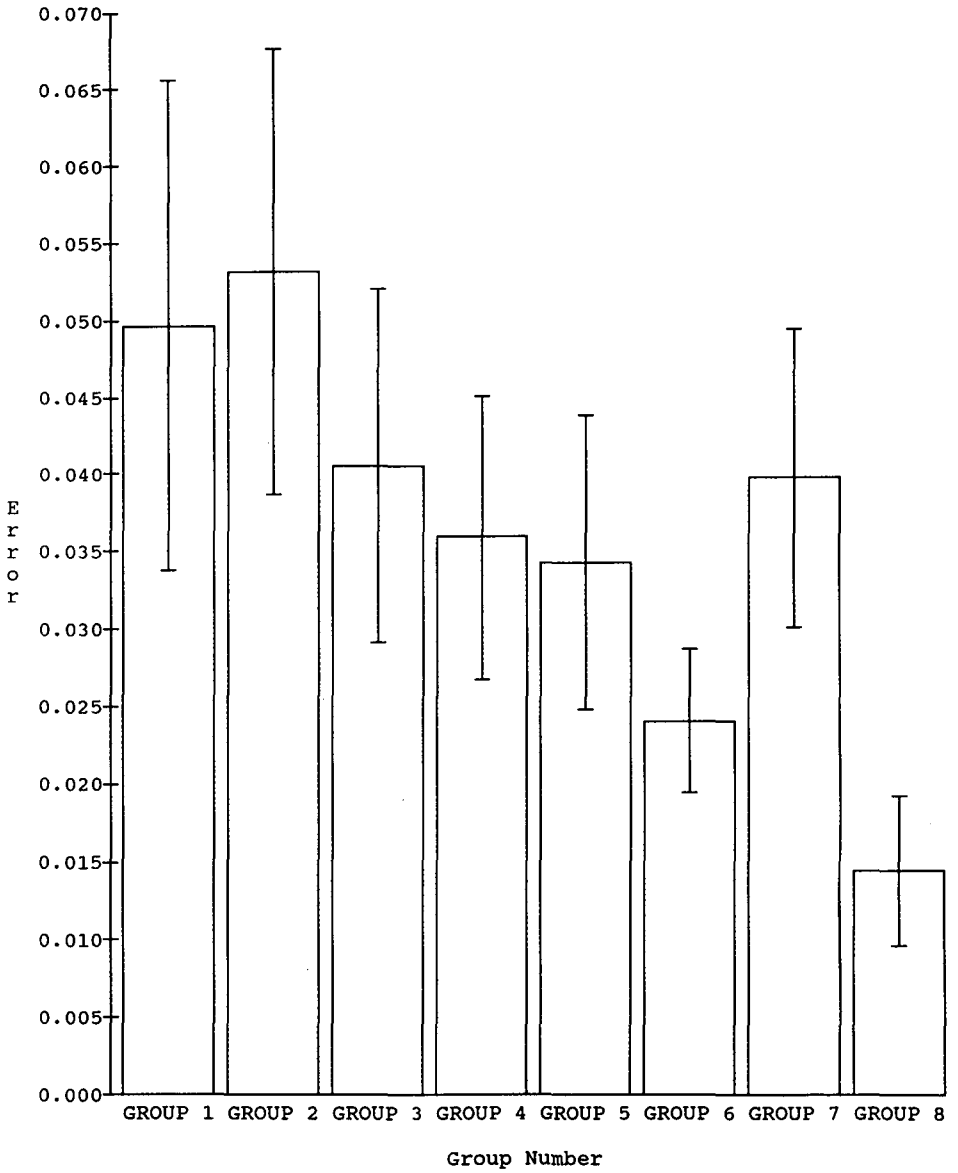

Fig. 2. Plot of relative difference (error) from global means for high and low QA samples (ordinate) *versus* *S/N* for the individual assay. Group 1 = *S/N* of 1–3 ($n=7$); Group 2 = *S/N* of 4 and 5 ($n=10$); Group 10 = *S/N* of 6–8 ($n=9$); Group 4 = *S/N* of 9–11 ($n=8$); Group 5 = *S/N* of 13–18 ($n=8$); Group 6 = *S/N* of 20–49 ($n=7$); Group 7 = *S/N* of 56–95 ($n=7$); Group 8 = *S/N* of 106–272 ($n=8$). All groups are not of equal size in order that no particular *S/N* ratio be in more than one group. Error bars are standard errors.

more common techniques are boxcar averaging [21], ensemble averaging [21], weighted or unweighted polynomial smooting [21,22] and Fourier transformation [25]. With modern microprocessors the previous disadvantage of slow processing times with complex algorithm is typically not significant.

Because no systemic direct comparison between the various smoothing routine has been done, it is difficult to evaluate whether the aproach described in this study is superior or even equal to any or all of the other methods. However, it is fundamentally different than from the others because the data are fit to a model, and not smoothed. Noise rejection in this approach is based on observed inconsistency with the model which is typically accepted as being the most accurate representation of chromatographic peaks, and not on datapoints inconsistencies with adjacent data points. The approach described in this study offers a method for rejecting noise which is based on a chromatographic, and not an electronic perspective. This is in addition to its ability to generate accurate chromatographic features of merit such as a value for theoretical plates [12,14].

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Determination of primary and secondary amines in foodstuffs using gas chromatography and chemiluminescence detection with a modified thermal energy analyser

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ABSTRACT

A simple method is described for the determination of primary and secondary amines in foodstuffs by gas chromatography with a modified thermal energy analyser, operated in the nitrogen mode. Food samples were subjected to mineral oil vacuum distillation and the isolated amines were derivatized with benzenesulphonyl chloride to form the corresponding sulphonamides, which were fractionated to yield primary and secondary amine derivatives using a modified Hinsberg procedure. The detection limit for individual amines using a 10-g food sample was 10 μg/kg (ppb) and recoveries were in excess of 80%.

INTRODUCTION

Amino compounds are important in the human diet and environment. Aliphatic primary and secondary amines are widely distributed in the environment as they are endogeneously synthesized and excreted by living organisms and synthesized in bulk as raw materials for both chemical and manufacturing industries. In foodstuffs, aliphatic amines have seldom been investigated [1–4], despite the fact that they can act as precursors to carcinogenic N-nitroso compounds [5], which occur in the human diet [6] and the environment [7]. N-Nitroso compounds can also be formed endogeneously from ingested amines and nitrate/nitrite [8]. The dietary exposure to nitrate is well documented [9]. However, until the dietary exposure to nitrosatable amines in foods is determined, it remains impossible to evaluate the potential risk of endogeneous formation of N-nitroso compounds.

Quantitative methods for the determination of amines involving either high-performance liquid chromatography (HPLC) or gas chromatography (GC) have several inherent problems related both to the difficulty in handling low-molecular-weight amines owing to their volatility and high water solubility, and to the necessity to form derivatives to enhance both the detection limit and chromatographic separation. Derivatization is required in order to increase the detection sensitivity for HPLC analysis using ultraviolet (UV) [10–13], fluorescence [14,15], electrochemical [16] and chemiluminescence excitation detection [17]. However, despite the increased sensi-

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tivity of detection, amine derivative selectivity cannot be guaranteed during the analysis of complex matrices, limiting the practical use of HPLC for the determination of amines in foodstuffs and environmental and biological samples. The determination of underivatized amines by GC [18–20] results in amine adsorption (tailing) and possible decomposition on the column. These problems can be effectively solved by derivatization of amines to either benzenesulphonamide [21,22], dimethylthiophosphinicamide [23], *p*-toluenesulphonamide [4,24], trifluoroacetamide [1] or 2,4-dinitrofluorobenzene [25] derivatives in which the volatility of the amine is reduced and the hydrophobic derivative is readily extractable into organic solvents. Most GC methods using these derivatives employ flame photometric detection (FPD) [21–23], flame ionization detection (FID) [1,18–20] or GC–mass spectrometry with selected ion monitoring (GC–MS/SIM) [4,24] in which amines, or their derivatives, are determined in the presence of other potentially interfering analytes.

The thermal energy analyser (TEA) was originally designed for the specific detection of N-nitroso compounds [26], but after modification it can also be used in the nitrogen mode for the detection of nitrogen-containing organic compounds [27]. Despite the obvious advantages of this highly sensitive nitrogen-specific detection method, TEA detection has never been used for the routine determination of amines. We report here a simple method for the routine determination of amines in foodstuffs using mineral oil vacuum distillation and derivatization with benzenesulphonyl chloride (BSC) to form benzenesulphonamides, which are separated using a modified Hinsberg procedure prior to determination by GC with a modified TEA detector.

EXPERIMENTAL

Reagents

All solvent and reagents were of analytical-reagent grade and, with the exception of amines, were used without further purification. Amines were purified by fractional distillation. The internal amine standards (ethylpropylamine and *tert.*-butylamine) were prepared in 0.1 *M* hydrochloric acid at a concentration of 150 μg/ml.

Standard benzenesulphonamines were prepared by condensation of BSC with the amine under alkaline conditions prior to acidification and extraction with hexane. The crude benzenesulphonamides were purified by double recrystallization or fractional distillation. Purity was confirmed by GC with FID, TEA and MS detection, ^{1}H NMR and determination of melting or boiling points. Standard mixtures of representative primary and secondary amine benzenesulphonamides (10 μg/ml) were prepared in isooctane.

Sample distillation and derivatization

The food sample was homogenized and a representative sample (10–20 g) was removed and placed in a 100-ml distillation flask together with glycerine (10 ml), 2 *M* sodium hydroxide solution (2 ml) and distilled water (5–15 ml, depending on the water content of the sample). The sample was spiked with two internal standards (150 μg each of *tert.*-butylamine and ethylpropylamine in 1 ml of 0.1 *M* hydrochloric acid). The distillation flask was fitted with a short distillation bridge and connected to a 100-ml distillation receiver fitted with a side-arm which was connected to a vacuum pump. The distillation receiver was charged with 1 *M* hydrochloric acid (5 ml) and

cooled to −80°C in a Dewar flask containing acetone–solid carbon dioxide. The food sample was distilled under vacuum (8 mmHg) for 20 min at ambient temperature and then heated at 100°C for 40 min. On completion of the distillation, the heating mantle was switched off and the system left to cool for 20 min before venting and removal of the distillation receiver. The acidic distillate, containing the amine hydrochloride salts, was removed and corrected to a total volume of 30 ml with distilled water. Aliquots (2 ml) were removed and placed in a 15-ml screw-topped Pyrex glass reaction vial containing 10 *M* sodium hydroxide solution (1 ml) and BSC (0.4 ml). The reaction vial was then heated at 105°C for 60 min with occasional shaking.

The benzenesulphonamides were isolated using a modified Hinsberg separation as follows. The alkaline reaction mixture was extracted with hexane (4 × 4 ml), the combined hexane fractions were washed by back-extraction with 1 *M* sodium hydroxide solution (2 ml) and concentrated under a stream of nitrogen to yield a final concentrate of 1 ml of hexane containing the secondary amine fraction derivatized as benzenesulphonamides.

The aqueous alkaline wash fraction was added to the original derivatization mixture and made acidic by careful addition of 10 *M* hydrochloric acid (1 ml). The acidic mixture containing primary amine derivatives was extracted with diethyl ether (3 × 3 ml) and the ether fractions were dried by filtration through a short column containing anhydrous sodium sulphate prior to concentration to dryness under a stream of nitrogen. The residue was taken up in 10% dichloromethane in hexane (1 ml) for analysis.

Sample analysis by gas chromatography using a thermal energy analyser in the nitrogen mode

Sample aliquots (10 μl) were analysed on a gas chromatograph (Hewlett-Packard Model 5880A) using a 3 m × 2 mm I.D. glass column packed with 10% OV-101 on Volaspher A2 (120–140 mesh). Helium was used as the carrier gas at a flow-rate of 18 ml/min and the injection port temperature was maintained at 250°C. For the primary amine derivatives the oven was programmed directly from 210 to 224°C at 0.5°C/min and then to 280°C at 2°C/min and maintained at 280°C for 10 min. The retention times for standard primary amine benzenesulphonamides were methylamine 9.7, ethylamine 10.9, *tert.*-butylamine (internal standard) 12.9, propylamine 14.0, isobutylamine 15.9, *n*-butylamine 18.4, isopentylamine 21.5, pentylamine 24.2, hexylamine 31.3 and phenethylamine 49.6 min.

For the secondary amine derivatives the oven was programmed directly from 210 to 222°C at 0.5°C/min and then to 250°C at 5°C/min and maintained at 250°C for 10 min. The retention times for standard secondary amine benzenesulphonamides were dimethylamine 9.4, methylethylamine 11.3, diethylamine 13.3, ethylpropylamine (internal standard) 16.2, dipropylamine 20.3, pyrrolidine 21.3, morpholine 23.3, piperidine 25.4, dibutylamine 29.7 and methylbenzylamine 37.9 min. The limit of detection for both primary and secondary amines was 10 μg/kg foodstuff.

For detection, a Model 502 thermal energy analyser (Thermo Electron, Waltham, MA, U.S.A.) was modified for use in the nitrogen mode as shown in Fig. 1. The column outlet from the gas chromatograph was connected to the pyrolyser tube with a metal T-piece through which oxygen, at a flow-rate of 5 ml/min, was mixed with the carrier gas prior to passing through the pyrolyser tube. The pyrolyser tube

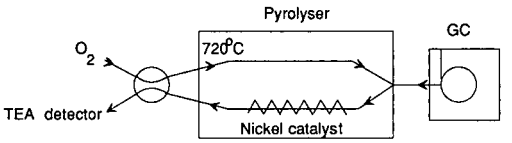

Fig. 1. GC with thermal energy analyser modified for use in the nitrogen mode.

contained a tightly packed nickel gauze which acted as a catalyst for pyrolysis of nitrogen-containing compounds to NO radicals at a pyrolyser temperature of 720°C. The pyrolyser was connected to the reaction chamber of the TEA detector via a short PTFE tube and an on-line CTR gas stream filter (Thermo Electron). Thermal pyrolysis of the N–NO bond in N-nitroso compounds at 450°C or pyrolysis of amines at 720°C with a nickel catalyst produces NO radicals which react with ozone in the reaction chamber of the TEA detector to produce NO_2^* in an electronically excited state. Decay of NO_2^* back to the ground state results in photon emission which is detected and quantified using a photomultiplier tube (chemiluminescence detection). Quantification was effected by peak-area integration using a Trilab 3000 integrator (Trivector Computer Systems).

Amine confirmation using capillary gas chromatography–mass spectrometry

Sample aliquots (3 μl) were analysed on a gas chromatograph (Hewlett-Packard Model 5890A) using a 12 m × 0.2 mm I.D. U-1 fused-silica capillary column (Hewlett-Packard). Helium was used as the carrier gas (2 ml/min) and sample injection was made in the splitless mode with an automatic sample injector (Hewlett-Packard Model 7673). For both primary and secondary amine derivatives, the oven was programmed directly from 70 to 300°C at 10°C/min and maintained at 300°C for 10 min. The capillary column was interfaced with a mass-selective detector (Hewlett-Packard Model 5970) operated either in the scanning mode (range 40–400 u) or programmed for selective ion monitoring (SIM). The ionizing voltage was 70 eV. The retention times for standard primary amine benzenesulphonamides were methylamine 7.1, ethylamine 7.5, *tert.*-butylamine (internal standard) 8.0, propylamine 8.4, isobutylamine 8.8, *n*-butylamine 9.3, isopentylamine 9.9, pentylamine 10.3 and hexylamine 11.2 min. The retention times for standard secondary amine benzenesulphonamides were dimethylamine 7.8, methylethylamine 8.6, diethylamine 9.3, ethylpropylamine (internal standard) 10.3, dipropylamine 11.0, pyrrolidine 11.2, morpholine 11.6, piperidine 12.9 and dibutylamine 14.4 min.

RESULTS AND DISCUSSION

Derivatization of amines as benzenesulphonamides proved to be a reliable and versatile method for the GC determination of amines using the TEA detector. Using a reagent blank, recovery experiments (determined five times) at a spiking concentration of 10 and 100 μg amine/kg (ppb) gave recoveries of secondary amine benzenesulphonamides of the order of 80–90% with little cross-contamination from primary amine benzenesulphonamides; traces of longer chain ($>C_5$) primary amines occurred occasionally (at less than 5% carryover) in the secondary amine fraction.

Almost quantitative recovery (90–100%) of primary amine benzenesulphonamides was achieved. In the presence of a food matrix, no change in the recoveries was apparent and other food components did not interfere with the analytical procedure.

The standard mixtures were based on a representative selection of primary and secondary amines known to occur in foodstuffs [1]. Similarly, the two internal standards (ethylpropylamine and *tert.*-butylamine) were selected for their known absence from foodstuffs. We have so far analysed over 200 food samples during which we regularly checked for the presence of these two amines in samples analysed without addition of the internal standards. We have never detected their presence, or the presence of other amines with identical retention times. Representative chromatograms obtained using TEA detection for the primary and secondary amine benzenesulphonamide fractions of standard compounds and a typical food sample (chocolate powder) are shown in Figs. 2 and 3, respectively.

In previous studies using BSC derivatives of secondary amines, Hamano and co-workers [21,22] reported the use of 2% OV-1, 3% OV-1 and 3.5% SE-30 on Chromosorb W as column packing materials. However, in our studies these packing materials resulted in unacceptable peak tailing of primary amine derivatives. To permit the separation of both primary and secondary amine benzenesulphonamides on the same column, 10% OV-101 on Volasphere A2 (120–140 mesh) was used, which resulted in good resolution of secondary amine derivatives and greatly reduced the tailing of primary amine benzenesulphonamides.

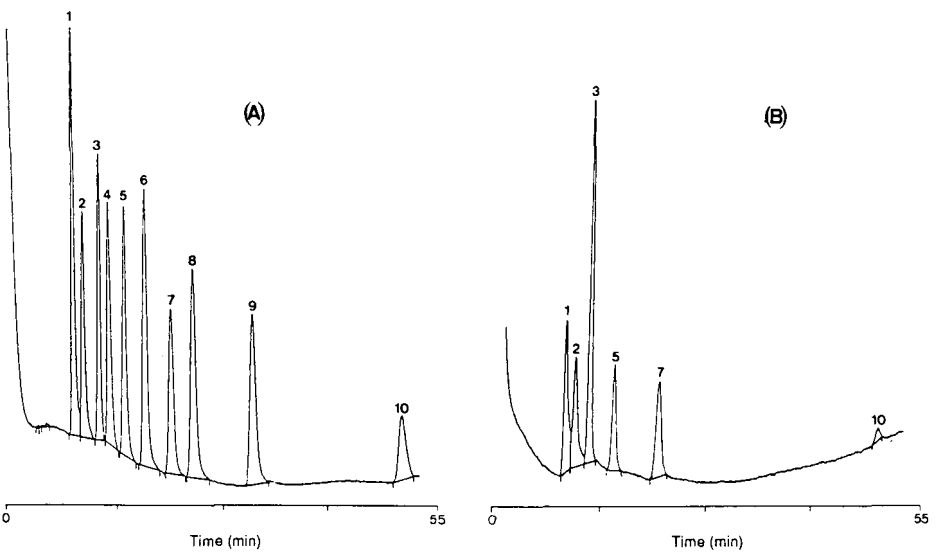

Fig. 2. Gas chromatograms of primary amine BSC derivatives on a 3 m × 2 mm I.D. glass column packed with 10% OV-101 on Volasphere A2, carrier gas helium (18 ml/min), injection port temperature 250°C, oven programmed from 210 to 224°C at 0.5°C/min and then to 280°C at 2°C/min with TEA detection. Amount injected, 5 μl, corresponding to 50 ng of each standard compound. (A) Standard mixture; (B) chocolate powder. Peaks: 1 = methylamine; 2 = ethylamine; 3 = *tert.*-butylamine (internal standard); 4 = propylamine; 5 = isobutylamine; 6 = *n*-butylamine; 7 = isopentylamine; 8 = pentylamine; 9 = hexylamine; 10 = phenethylamine.

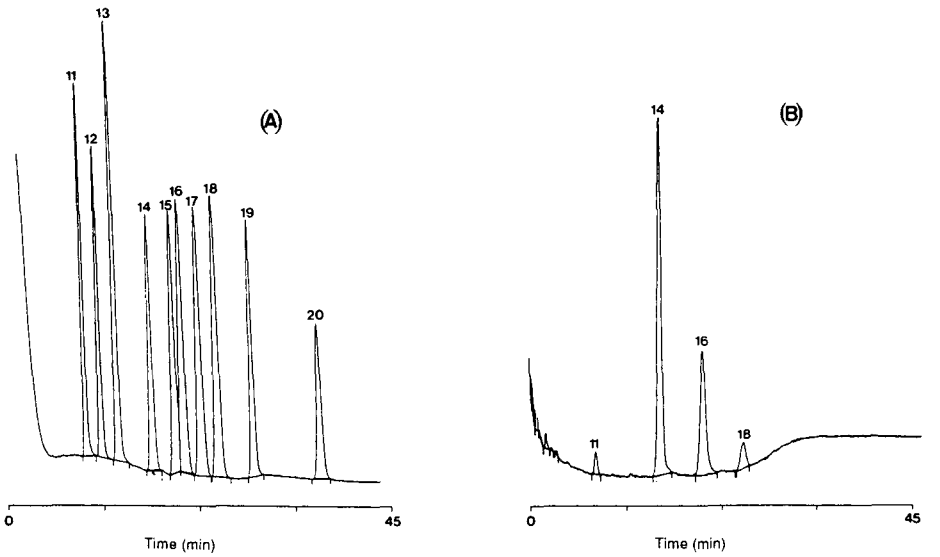

Fig. 3. Gas chromatograms of secondary amine BSC derivatives on a 3 m × 2 mm I.D. glass column packed with 10% OV-101 on Volasphere A2, carrier gas helium (18 ml/min), injection port temperature 250°C, oven programmed from 210 to 222°C at 0.5°C/min and then to 280°C at 5°C/min with TEA detection. Amount injected, 5 μl, corresponding to 50 ng of each standard compound. (A) Standard mixture; (B) chocolate powder. Peaks: 11 = dimethylamine; 12 = methylethylamine; 13 = diethylamine; 14 = ethylpropylamine (internal standard); 15 = dipropylamine; 16 = pyrrolidine; 17 = morpholine; 18 = piperidine; 19 = dibutylamine; 20 = methylbenzylamine.

The choice of BSC derivatives for amine analysis by GC has two major advantages over other commonly used derivatization techniques. The identification of unknown compounds is simplified, as fractionation using a modified Hinsberg method immediately allows the distinction between primary and secondary amines. Further, benzenesulphonamides have very characteristic mass spectral fragmentation patterns at ion masses of m/z 77 and 141 relating to the structure of the derivatization reagent, which can be easily located using GC–MS/SIM for gaining structural information on the suspected amine parent molecule.

Changing from packed to capillary columns resulted in an even better separation of secondary amine derivatives while tailing of primary amine benzenesulphonamides still occurred, as shown in Fig. 4. However, for routine analysis of a large number of food samples the advantages gained from superior peak resolution using capillary columns are outweighed by the disadvantages of reduced column life and the problems incurred on interfacing capillary columns with the TEA pyrolyser [28,29].

The advantages of the TEA detector for the determination of nitrogen-containing compounds are immediately apparent on comparison of the detection limit for other reported methods for amines. Using the reported method, analysis of a 10-g food sample allows detection down to 10 μg amine/kg (ppb) foodstuff (signal-to-noise ratio = 3). Other methods give detection limits of 4 μg amine/kg foodstuff (100 g foodstuff, as *p*-toluenesulphonamides, with FID) [4] and 5–50 μg/kg (50–500 g foodstuff, as trifluoroacetamides, with FID) [1].

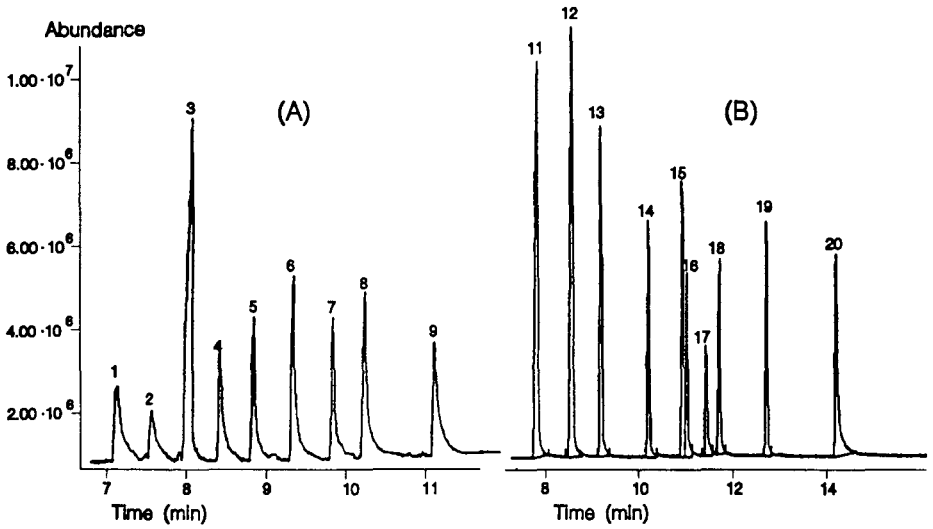

Fig. 4. Gas chromatograms of (A) primary amine and (B) secondary amine BSC derivatives on a 12 m × 0.2 mm I.D. U-1 fused-silica column, carrier gas helium (2 ml/min), oven programmed from 70 to 300°C at 10°C/min with MS detection (scanning mode 40–400 u). Splitless 2-μl injection corresponding to 20 ng of each standard compound. Peaks as in Figs. 3 and 4.

The use of BSC derivatives for the determination of secondary amines in foods using FPD [23] and mixtures of primary and secondary amines in water and sediments using GC–MS/SIM [24] has been reported to give similar detection limits to the present method.

The use of sulphur-containing BSC derivatives results in a gradual partial poisoning of the nickel catalyst in the pyrolysis tube of the modified TEA detector. Under normal operating conditions, the nickel gauze catalyst requires replacement after about 200 sample injections if the detection limit is to be maintained.

We have recently conducted a food survey using the above methodology for the determination of primary and secondary amines (results to be reported). The proposed method has proved robust and reliable for routine analysis of foodstuffs and with little modification can be used for the determination of amines in biological samples (*e.g.*, urine, faeces, gastric juice and plasma) and environmental samples (*e.g.*, tobacco smoke).

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Separation of polychlorinated terphenyls from lipoidal material by preparative gel permeation chromatography and gas chromatography

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ABSTRACT

The potential of preparative gel permeation chromatography to separate polychlorinated terphenyls (PCTs) from lipoidal material derived from fish was investigated. When applied to styrene–divinylbenzene gel permeation chromatography columns, PCTs eluted prior to commonly examined environmental contaminants, such as polychlorinated biphenyls (PCBs). Coelution of PCTs with lipoidal material was observed with some columns tested, attributable to the large molecular size of the chlorinated terphenyls. Capillary gas chromatography coupled with electrolytic conductivity detection provided adequate characterization of two PCT formulations, Aroclor 5432 and 5460. Some lowly chlorinated PCTs eluted in the same retention window as highly chlorinated PCBs.

INTRODUCTION

Polychlorinated terphenyls (PCTs) are structurally similar to the comparatively well-studied polychlorinated biphenyls (PCBs). Substantial volumes of the former compounds were produced, amounting to approximately 15% that of PCBs [1]. PCTs were synthesized as complex mixtures, the components of which possessed varying degrees of chlorination. Two common formulations marketed by Monsanto were Aroclor 5432 and 5460, which possessed 32% and 60% chlorine by weight, respectively. A third formulation, Aroclor 5442, has been observed to consist of a mixture of the two previously described products [1]. Manufacture of PCTs apparently ceased prior to 1980 [2]. Chemical and physical properties of PCTs and PCBs are similar. As a consequence, the two groups of chemicals were employed commercially for related purposes. This similarity also raises concerns about their environmental distribution, fate and toxicity.

Reports of the analysis of PCTs are rare in the literature, particularly when compared with those concerning PCBs. The majority of papers were published in the 1970s and utilized gas chromatography (GC) [3–6]. The packed-column techniques used in those investigations provided extremely poor resolution of the constituents of

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these complex mixtures. The presence of a third phenyl ring in PCTs not only allows additional positions for chlorination, but also permits the terphenyl molecule to assume *ortho, meta* and *para* orientations. This greatly increases the number of possible PCT congeners, compared with PCBs, posing a complex task for the chromatographer.

Discovery of significant concentrations of PCTs in sediment and shellfish samples during an exploratory monitoring program in the Commonwealth of Virginia, U.S.A. [7], prompted an examination of analytical methods for the accurate determination of these compounds. Gel permeation chromatography (GPC) has received increasing interest for the separation of organic pollutants from naturally occurring material [8,9]. This latter material typically interferes with subsequent GC analyses, which are ultimately used for identification and quantification purposes. The behavior of PCTs on several preparative GPC columns and with two solvent systems is described. In addition, the application of capillary GC with electrolytic conductivity detection (ELCD) for the analysis of PCTs is introduced.

EXPERIMENTAL

Chemicals and test materials

High-purity-grade dichloromethane (DCM) and cyclohexane (CCH) were obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). Aroclor standards and 2,2′,4,4′,5,5′-hexabromobiphenyl were obtained from Chem Service (West Chester, PA, U.S.A.). Decachlorobiphenyl was purchased from Aldrich (Milwaukee, WI, U.S.A.).

Non-polar material, operationally defined as lipid, was extracted from catfish (*Ictalurus catus*). The organisms were collected from the James River, VA, U.S.A. The entire organism was initially homogenized in a tissue grinder and the resulting material dried in an FTS Systems lyophilizer for 24–48 h. Approximately 20 g of lyophilized tissue were then extracted with DCM for 48 h in Soxhlet apparatuses. The resulting lipid extracts were reduced in volume with a rotary evaporator.

Gel permeation chromatography

An automated gel permeation chromatograph (ABC Labs., Autoprep 1002A) was used to separate the PCT formulations, Aroclor 5432 and 5460, from lipoidal material. GPC columns (60 cm × 2.5 cm) equipped with PTFE fittings were obtained from the same source. One column was prepacked with 60 g of 200–400 mesh Bio-Beads S-X3 resin by the manufacturer. Additional columns were slurry packed in the laboratory with 70 g S-X3 or 60, 80 or 100 g of 200–400 mesh S-X8 (Bio-Rad Labs.). This resin was sieved over a 250-mesh screen to remove fines and allowed to swell for 24 h in the packing solvent prior to use. The 60-g S-X3 column was packed in a mixture of DCM–CCH (1:1, v/v). The remaining columns were packed in 100% DCM. S-X3 and S-X8 resins have manufacturer-specified molecular-size-exclusion limits of approximately 2000 and 1000 dalton, respectively. The flow-rate through the columns was 5.0 ml min^{-1}. Extracts, representing less than 1 g of total lipid, and solutions containing Aroclor 5432, Aroclor 5460 and decachlorobiphenyl standards were injected onto the columns. Decachlorobiphenyl was proposed as a surrogate standard for the analysis of PCTs in environmental matrices. Fractions, 5 or 10 ml in

volume, were then collected, evaporated to dryness at room temperature and the lipid residue determined gravimetrically. PCTs were determined as described below. The PCT solutions were augmented with PCBs consisting of Aroclor 1242, 1254 and 1260 for tests conducted on the 100-g S-X8, and the 60- and 70-g S-X3 columns to examine the relative elution characteristics of these compounds.

Gas chromatography

The presence of PCTs, as well as PCBs, in the various GPC fractions was determined by capillary GC, employing a Varian Model 3300 gas chromatograph. The instrument was equipped with an OI Analytical Model 4420 ELCD system. The detector was operated in the halogen-specific mode with *n*-propanol as the conductivity solvent. Helium at a linear velocity of 30 cm s^{-1} was used as the carrier gas. The injector was maintained at 300°C and the reactor tube of the ELCD system at 950°C. The chromatograph was equipped with a DB-5 fused-silica column (J & W Scientific, 30 m long × 0.32 mm I.D., 0.25 μm film thickness). Following exchange of the original GPC solvent to hexane, injections onto the chromatograph were made in the splitless mode. The column effluent was purged for 3 min after injection, to eliminate the bulk of the solvent prior to its entrance into the detector. Column temperature was initially held at 90°C for 2 min. It was then programmed at 4°C min^{-1} to 300°C. Final column temperature was held at 300°C for 10 min.

RESULTS AND DISCUSSION

The major constituents of Aroclor 5460 contain seven to ten chlorines [1]. Its presence in the environment has been reported sporadically in the literature, generally at low concentrations. However, Aroclor 5460 was detected recently in sediments collected near the mouth of the James River at concentrations as high as 26 mg kg^{-1} [7].

Aroclor 5432 is composed of terphenyls with a lower degree of chlorination than 5460, 2–5 chlorines per molecule [1]. Our laboratory has identified Aroclor 5432 in sediments and shellfish from a tributary of the Chesapeake Bay at concentrations as high as 250 and 35 mg kg^{-1}, respectively [10]. The only other report uncovered, detailing the environmental occurrence of this formulation was published in 1978 [11]. Those authors separated Aroclor 5432 from 5460 by thin-layer chromatography. The resulting residue was then perchlorinated and analyzed by packed-column GC with electron-capture detection.

In an attempt to improve the methodology for the detection of PCTs, the ability of various GPC packing and solvent systems to resolve PCTs from lipoidal material was examined. The technique with S-X8 and 100% DCM as the eluting solvent has been employed in this laboratory to purify biota and sediment extracts since 1978. It was found to be effective in separating lipids from a range of anthropogenic compounds, such as PCBs, polynuclear aromatic hydrocarbons and various heterocyclic xenobiotics [12,13]. However, difficulties were initially encountered when PCTs were detected in the Chesapeake Bay samples mentioned above. Some of the terphenyls, particularly the highly chlorinated components of Aroclor 5460, eluted in the early lipid fraction. This was attributed to the relatively large molecular size of the PCTs. As a consequence, a more detailed examination of the separation characteristics of various GPC columns was conducted.

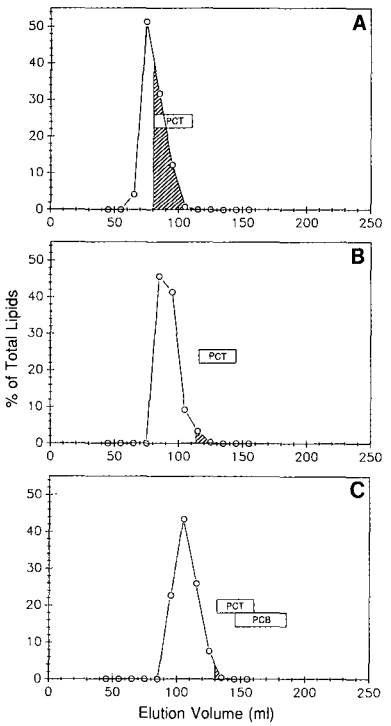

Fig. 1. GPC elution profiles of lipoidal material and windows for PCTs on columns packed with: (A) 60 g S-X8; (B) 80 g S-X8; and (C) 100 g S-X8. The elution window for PCBs on the 100-g column is also shown. All columns were eluted with 100% DCM. Shaded areas of the lipid curves denote overlap with PCTs.

Elution profiles of lipoidal material from tissue extracts on 60-, 80- and 100-g S-X8 columns are provided in Fig. 1; column bed lengths were 30, 40 and 50 cm, respectively. The elution volumes of the PCTs, in relation to extracted fish lipids, are also presented. Considerable overlap between PCTs and lipids occurred, particularly with the two shorter columns. Approximately 45 and 3.7% of the total lipid injected coeluted with the PCTs in the case of the 60- and 80-g columns, respectively. Coelution of more than 0.5% of the lipids with PCTs was observed on the 100-g S-X8 column. The majority of the PCTs eluted from 131 to 160 ml on this column, with the higher-molecular-weight Aroclor 5460 components eluting first. PCBs eluted from 146 to 185 ml. The inability of a 50-g S-X8 column, eluted with DCM, toluene or CCH, to adequately resolve fish lipids and organochlorine pesticides has been described previously [14].

The 60-g S-X3 column employed a solvent regime of CCH–DCM (1:1). This column had a bed length of 45 cm. The PCTs were well resolved from the bulk of the lipids, although the latter peak tailed (Fig. 2A). This suggests the presence of modest amounts of relatively low-molecular-size lipoidal material. No such tail was observed for the S-X8 columns examined. PCTs began to elute at 151 ml and continued to 200 ml. PCBs eluted from 171 to 210 ml.

A column containing 70 g of S-X3, using 100% DCM as the solvent, is recommended by the U.S. Environmental Protection Agency for the elimination of

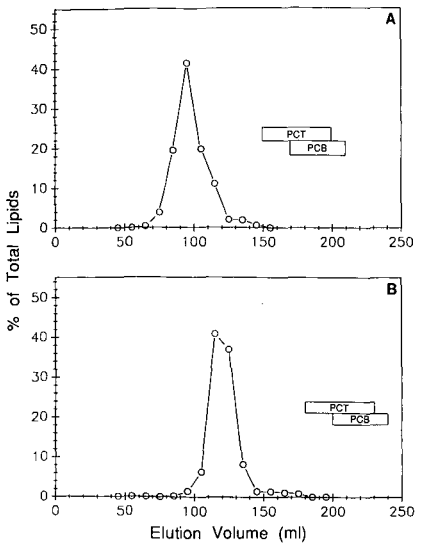

Fig. 2. GPC elution profiles of lipoidal material and windows for PCTs and PCBs on: (A) the 60-g S-X3 column, eluted with DCM–CCH (1:1); and (B) the 70-g S-X3 column, eluted with 100% DCM.

interferents in solid waste extracts [9]. The column tested possessed a bed length of 52 cm. PCTs eluted from 181 to 230 ml. PCBs eluted from 201 to 240 ml. Lipids and PCTs were resolved from each other, although the lipid peak again tailed (Fig. 2B). The observation that tailing occurred with both solvent regimes, with S-X3 but not with SX-8, suggests a simple molecular-size effect, rather than a polarity-based interaction with the resin.

A chromatogram obtained with the GC–ELCD system for Aroclor 5460 and 5432 standards is provided in Fig. 3A. Although the electron-capture detector is widely used to determine the concentration of organochlorine pollutants, ELCD provides greater specificity and response is less sensitive to the structure of the compounds examined [15]. The electron-capture detector does exhibit somewhat higher sensitivity. Aroclor 5460 elutes from relatively non-polar GC columns after decachlorobiphenyl. As many investigators discontinue their chromatographic analyses prior to the emergence of this compound, they may fail to detect the presence of this late-eluting formulation. Decachlorobiphenyl elutes from the chromatographic column between Aroclor 5432 and 5460 (Fig. 3A), demonstrating its potential as a standard in the GC analysis of PCTs. This compound has been used in some investment casting applications [2] and is a minor constituent of Aroclor 1260 [16]. Nonetheless, it typically represents less than 1% of the total PCB burden in environmental samples [17,18]. As shown in Fig. 3A, 2,2′,4,4′,5,5′-hexabromobiphenyl also elutes between the two PCT formulations examined.

Some difficulties have been encountered in distinguishing the components of Aroclor 5432 from highly chlorinated biphenyls, even with high-resolution GC columns. This is apparent when a chromatogram of Aroclor 5432 and that of a mixture of PCB formulations are compared (Fig. 3A and B). Confirmation of the identities of individual chromatographic peaks requires mass spectrometric analysis [10].

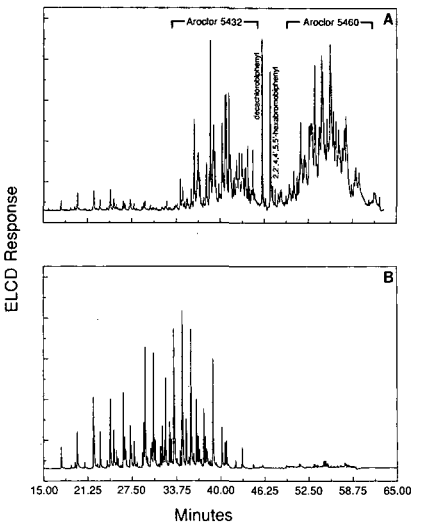

Fig. 3. GC–ELCD of: (A) Aroclors 5432 and 5460; (B) PCB formulation Aroclor 1260. GC retentions of decachlorobiphenyl and 2,2′,4,4′,5,5′-hexabromobiphenyl are also provided.

Alteration or removal of selected components of PCB formulations via processes such as microbial or physical degradation have been reported [19,20]. The structural similarity between PCTs and PCBs suggests that both groups of compounds may be acted upon in analogous manners. To date, residues of Aroclor 5432 detected in the environment have closely resembled the parent formulation [10]. However, some depletion of the lowly chlorinated components of Aroclor 5432 from sediments, at sites several kilometers from the original source of the PCTs, have been observed (Fig. 4). At present, insufficient data are available to establish the mechanism of the alterations.

In summary, the S-X8 columns tested in this study did not adequately separate extracted lipoidal material from PCTs. However, resolution from PCBs was ac-

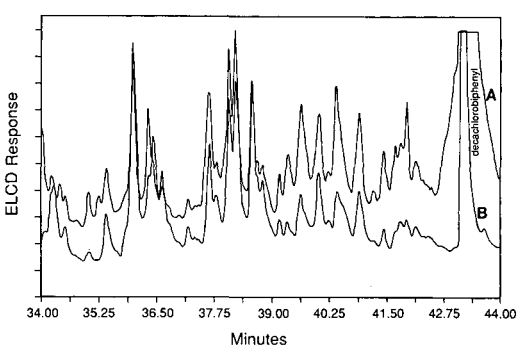

Fig. 4. (A) GC–ELCD of an extract of sediment containing Aroclor 5432. The sample was collected 5 km from the suspected source of the contamination. (B) Chromatogram of an Aroclor 5432 standard. Note the diminution of the lowly chlorinated PCT constituents in the sediment extract, compared with the standard.

ceptable with the 100-g column. Retention times and attendant solvent consumption were lower than with the S-X3 resin. Both S-X3 systems provided satisfactory resolution. The progression in elution from large to small molecular-size compounds, for complex organochlorine mixtures, with GPC is demonstrated in Fig. 5A–C. In the first chromatogram presented (Fig. 5A), representing the 181–190-ml fraction, Aroclor 5460 predominates. This formulation is succeeded by Aroclor 5432 (Fig. 5B) in the 191–200-ml aliquot and finally the PCB mixture in the 201–210-ml fraction (Fig. 5C). Thus, the potential for coelution of lipoidal material with PCTs was most severe for Aroclor 5460. The use of the 100% DCM solvent regime has the advantage of simplicity and is not vulnerable to compositional changes in the eluting solvent over time, as is the binary solvent system. Utilization of DCM as the sole solvent also allows more gentle conditions to be applied during subsequent concentration steps, with attendant minimization of losses of the more volatile sample constituents. The GPC and GC–ELCD methods described are currently being applied to the analysis of PCTs in a variety of aquatic biota and sediments in this laboratory.

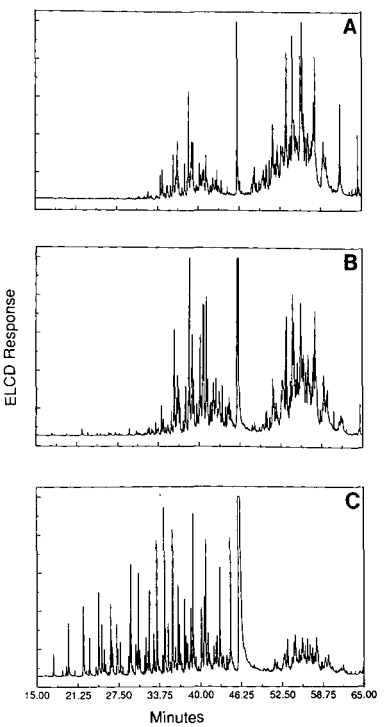

Fig. 5. GC–ELCD of fractions obtained from the 70-g S-X3 column. Fractions analyzed were: (A) 181–190 ml; (B) 191–200 ml; and (C) 201–210 ml. The succession in elution from predominantly highly chlorinated PCTs to lowly chlorinated PCTs and finally PCBs is shown.

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Capillary gas chromatographic and combined gas chromatography–mass spectrometric study of the volatile fraction of a coal tar pitch using OV-1701 stationary phase

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ABSTRACT

The components of the volatile fraction of a coal tar pitch were studied by capillary gas chromatography and combined gas chromatography–mass spectrometry using the OV-1701 stationary phase in both instances. The temperature-programmed retention indices and the molecular weight of each identified compound are given. It is shown that the volatile fraction of the coal tar pitch is composed of a group of homologous series of polycyclic aromatic compounds.

INTRODUCTION

The identification and determination of polycyclic aromatic compounds (PACs) is a fundamental task in the study of pitches, tars and other carbonaceous materials, not only for the optimization of production processes but also for their more satisfactory utilization. This area is also of interest from other points of view, such as environmental pollution and health, owing to the mutagenic and carcinogenic properties of some of these compounds. For these reasons many of analytical studies on PACs have been reported in recent years [1–4].

Coal tar is a by-product obtained in coal carbonization, and coal tar pitch is the primary distillation residue of coal tar. Coal tar pitches are complex mixtures of hundreds of PACs. Many of them (50–70% or more) are volatile substances. The boiling points of these compounds range from 218°C for naphthalene to 525°C for coronene. Unfortunately, most of the PACs present in a coal tar pitch are not available commercially. Some excellent gas chromatographic–mass spectrometric (GC–MS) studies on the composition of coal tars have been published [5–11], but coal tar pitch has not received much attention [12].

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In this paper a capillary GC study of the components of the volatile fraction of a coal tar pitch using OV-1701 (86% methyl, 7% phenyl and 7% cyanopropyl) stationary phase is reported. To the best of our knowledge, this moderately polar stationary phase has not been used before in the study of these complex mixtures. The retention indices of all peaks obtained using temperature programming were determined. Also, a mass spectrometric study of the sample was carried out using the same capillary column and the same separation conditions as in the GC study, in order to identify as many components of this complicated mixture as possible.

EXPERIMENTAL

The sample was the extracted fraction of a coal tar pitch, obtained by extraction in an ultrasonic bath, using toluene as solvent. The characteristics of the coal tar pitch and the extraction procedure are described in detail in another paper [13].

Retention times were determined with a Model 8320 gas chromatograph (Perkin-Elmer, Beaconsfield, U.K.) with flame ionization detection (FID). Separation was carried out on a fused-silica capillary column (25 m × 0.22 mm I.D.) (Chrompack) coated with OV-1701 stationary phase (McReynolds polarity [14] ≈ 789) with hydrogen as the carrier gas at a flow-rate of 1.8 ml/min (measured at the working temperature). The splitting ratio was 1:120. The detector and injector temperatures were 300°C and the column temperature was programmed from 50 to 300°C at 4°C/min.

The retention indices were calculated using Van den Dool and Kratz's method [15]:

$$I = 100\left[n + \frac{t_r(\text{compound}) - t_r(n)}{t_r(n+1) - t_r(n)}\right] \quad (1)$$

where I is the retention index to be calculated, n is the number of rings in the hydrocarbon standard that elutes prior to the substance measured, t_r(compound) is the retention time of the analyte compound and $t_r(n)$ and $t_r(n + 1)$ are the retention times of the PAC standards that elutes just before and after the analyte compound, respectively. The standards used were naphthalene, phenanthrene, chrysene and picene. Some retention indices after picene were calculated from an extension of the chrysene–picene interval. The number of determinations was more than 10.

Mass spectral data were obtained with a Hewlett-Packard combined Model 5880A gas chromatograph–Model 5987A mass spectrometer. The same capillary column as used in GC coated with OV-1701 stationary phase was connected directly to the ion source. The instrument was calibrated with perfluorotributylamine and electron impact mass spectra were recorded at an ionization energy of 70 eV. Peaks in the mass spectra were identified through comparison with other spectra in the literature [16]. Further, for the identification of the compounds, previous knowledge of the chromatographic behaviour (on stationary phases of different polarity) of a large group of compounds belonging to the different families of compounds present in coal tar pitches was used [3,4]. Likewise, the chromatographic behaviour and GC–MS data of the same coal tar pitch extract as studied here on SE-54 stationary phase were also used to help in identification [17].

RESULTS AND DISCUSSION

Fig. 1 shows the chromatogram of the volatile fraction of the coal tar pitch, and Table I gives the retention index, the mass and the compound assigned to each peak in the chromatogram.

It can be seen that high peaks generally belong to one only compound and small peaks sometimes belong to more than one compound. The compounds with the highest concentrations in the mixture are the polycyclic aromatic hydrocarbons (PAHs). The following are present in this carbonaceous material: hydrocarbons with two and three aromatic rings (compounds 1, 39 and 40), hydrocarbons with four aromatic rings *peri-* (compounds 72 and 77) and *cata*-condensed (compounds 103_b, 109, 110, 111 and 112_a), five-ring systems also *peri-* (compounds 144, 145_a, 146, 149_a, 150 and 152) and *cata*-condensed (compounds 170_a, 174, 175_b, 176_a, 177, 178 and 180_b) and six-ring *peri*-condensed hydrocarbons (compounds 175_a, 179 and 181_a).

The *peri*-condensed hydrocarbons always elute before the *cata*-condensed hydrocarbons with the same number of rings, and even *peri*-condensed systems with n aromatic rings elute before other *cata*-condensed hydrocarbons with $n - 1$ aromatic rings (see compounds 104, 175_a and 179). Among the *peri*-condensed hydrocarbons the non-alternant systems always elute before the alternant systems, *e.g.*, fluoranthene elutes before pyrene and benzofluoranthenes elute before benzopyrenes.

It can also be seen that *cata*-condensed hydrocarbons whose aromatic rings form a straight line are more retained than the corresponding standard (see retention indices of anthracene and naphthacene in relation to phenanthrene and chrysene, respectively). Taking into account this observation, compounds 180_b may be either benzo[*a*]naphthacene or pentacene. On the other hand, *cata*-condensed hydrocarbons whose aromatic rings form a curved line are less retained and elute before the corresponding standard (*e.g.*, benzo[*c*]phenanthrene elutes before chrysene). For this reason, compounds 155_c, 158_b and 170_a could be identified as benzo derivatives of benzo[*c*]phenanthrene.

Acenaphthene, fluorene and some partially hydrogenated PAHs, such as 9,10-dihydro- and 1,2,3,4-tetrahydroanthracene (compounds 15, 22, 29 and 37), are also present in this mixture in considerable concentrations. In addition, other partially hydrogenated PAHs have been found in low concentrations. Hydrogenated PAHs always elute before the corresponding aromatic hydrocarbons.

Two different fluorene series have been found: on the one hand, 4*H*-cyclopenta[*def*]phenanthrene (compound 51) and its benzo derivatives 11*H*-benzo[*bc*]aceanthrylene, 4*H*-cyclopenta[*def*]chrysene and 4*H*-cyclopenta[*def*]triphenylene (compounds 125_a, 126_a and 127_a) and on the other benzo[*a*]-, benzo[*b*]- and benzo[*c*]fluorene (compounds 85, 86_a and 87) and some dibenzofluorenes (compounds 157_a and 158_a). It should be noted that compound 51 and its benzo derivatives shows retention indices similar to those of the methyl derivatives of hydrocarbons with the same number of aromatic rings, whereas benzo- and dibenzofluorenes show retention indices similar to those of methyl derivatives of *peri*-condensed hydrocarbons with an additional aromatic ring but with the same molecular weight.

Other compounds detected in considerable amounts in this coal tar pitch contain sulphur. Taking dibenzo[*b*,*d*]thiophene (compound 38) as the starting point, two families have been detected: the family of phenanthro[4,5-*bcd*]thiophene (com-

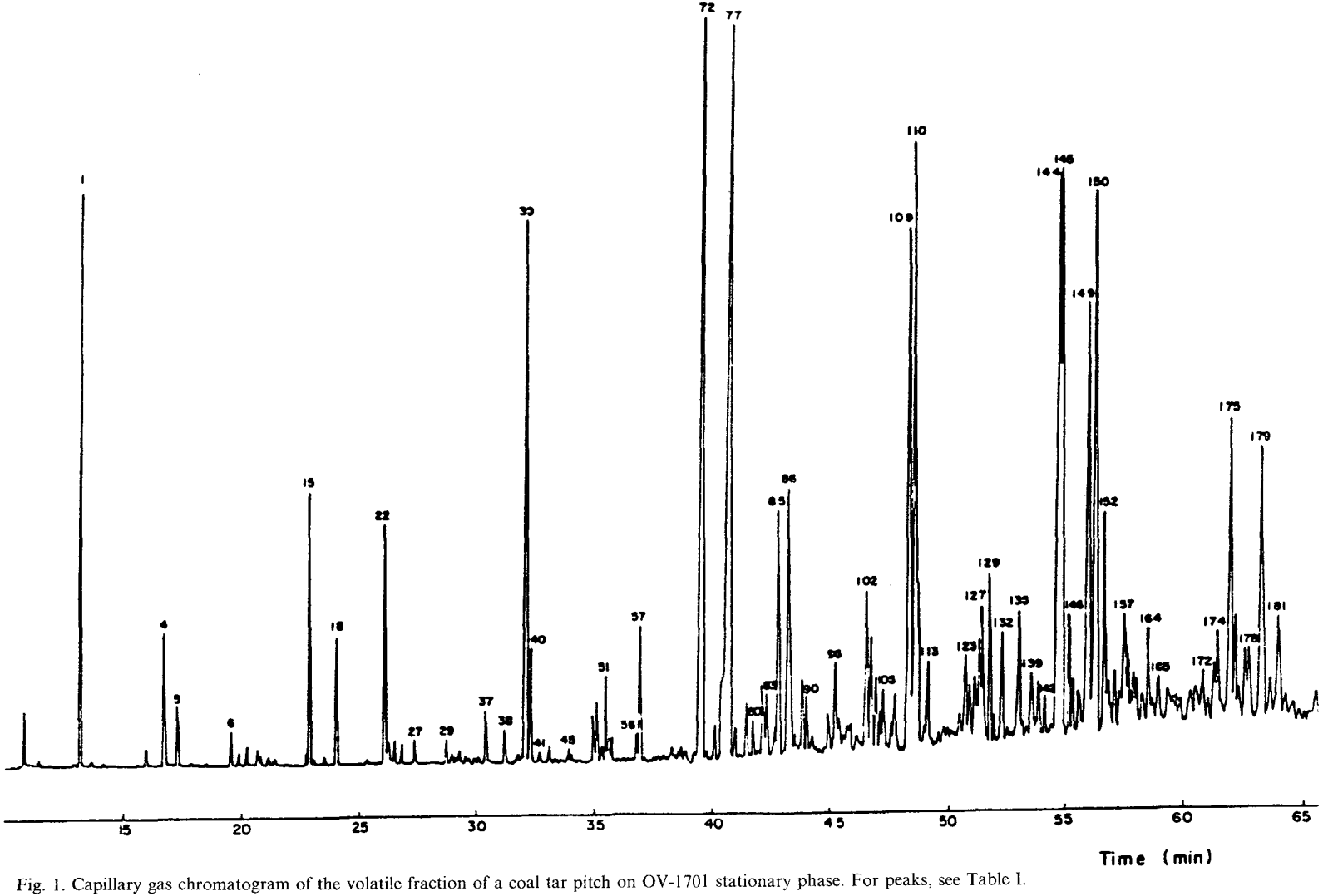

Fig. 1. Capillary gas chromatogram of the volatile fraction of a coal tar pitch on OV-1701 stationary phase. For peaks, see Table I.

TABLE I

RETENTION INDICES (I) ON OV-1701 STATIONARY PHASE AND MOLECULAR WEIGHTS OF THE COMPONENTS IDENTIFIED IN THE VOLATILE FRACTION OF A COAL TAR PITCH

Peak No.	$I^{OV\text{-}1701}$	Molecular weight	Possible compound
1	200.00	128	Naphthalene
2	201.66	134	Benzo[*b*]thiophene
3	213.46	129	Quinoline
4	217.87	142	2-Methylnaphthalene
5	220.81	142	1-Methylnaphthalene
6	232.50	154	Biphenyl
7	234.22	156	2-Ethylnaphthalene
8	236.22	156	Dimethylnaphthalene
9	238.34	156	Dimethylnaphthalene
10	238.93	156	Dimethylnaphthalene
11	241.01	156	Dimethylnaphthalene
12	242.53	156	Dimethylnaphthalene
13	244.99	156	Dimethylnaphthalene
14	249.89	168	Methylbiphenyl
15	250.59	154	Acenaphthene
16	251.54	170	Trimethylnaphthalene
17	253.62	168	Methylbiphenyl
18	256.56	168	Dibenzofuran
19	259.90	170	Trimethylnaphthalene
20	262.30	170	Trimethylnaphthalene
21	263.56	153	Naphthonitrile or azaacenaphthylene
22	266.72	166	Fluorene
23	267.78	168	Methylacenaphthene
24	268.49	168	Methylacenaphthene
25	269.90	168	Methylacenaphthene
26	271.53	182	Methyldibenzofuran
27	274.59	182	Methyldibenzofuran
28	275.42	198	
29	281.56	180	9,10-Dihydroanthracene
30_a	282.55	180	9,10-Dihydrophenanthrene
30_b	282.55	196	Dimethyldibenzofuran
31_a	283.49	180	9-Methylfluorene
31_b	283.49	196	Dimethyldibenzofuran
32	284.38	180	Methylfluorene
33	285.75	180	Methylfluorene
34	286.63	180	Methylfluorene
35	287.74	196	Dimethyldibenzofuran
36	288.81	196	Dimethyldibenzofuran
37	290.56	182	1,2,3,4-Tetrahydroanthracene
38	294.83	184	Dibenzo[*b*,*d*]thiophene
39	300.00	178	Phenanthrene
40	300.72	178	Anthracene
41	302.96	179	Benzoquinoline
42	305.32	179	Benzoquinoline
43	306.79	204	1-Phenylnaphthalene
44	307.32		
45	308.43	179	Benzoquinoline
47	313.48		
48	316.78	192	Methylphenanthrene, -anthracene
49	317.56	192	Methylphenanthrene, -anthracene

(Continued on p. 162)

TABLE I *(continued)*

Peak No.	$I^{OV\text{-}1701}$	Molecular weight	Possible compound
50	318.67	192	Methylphenanthrene, -anthracene
51	319.53	190	4*H*-Cyclopenta[*def*]phenanthrene
52	320.49	192	Methylphenanthrene, -anthracene
53	321.24	192	Methylphenanthrene, -anthracene
54	323.07		
55	324.64		
56	327.63	204	2-Phenylnaphthalene
57	328.19	167	Carbazole
58_a	328.72	206	Dimethylphenanthrene, -anthracene
58_b	328.72	193	Methylbenzoquinoline or isomer
59	330.06	206	Dimethylphenanthrene, -anthracene
60	330.75	206	Dimethylphenanthrene, -anthracene
61	331.39	206	Dimethylphenanthrene, -anthracene
62	332.57	206	Dimethylphenanthrene, -anthracene
63	333.58	206	Dimethylphenanthrene, -anthracene
64_a	334.65	206	Dimethylphenanthrene, -anthracene
64_b	334.65	204	Methyl 4*H*-cyclopenta[*def*]phenanthrene
65	335.49	206	Dimethylphenanthrene, -anthracene
66_a	337.03	206	Dimethylphenanthrene, -anthracene
66_b	337.03	204	Dihydropyrene or isomer
67	338.29	204	Dihydropyrene or isomer
68	338.92	204	Benzacenaphthene or isomer
69	339.68	181	Methylcarbazole
70	340.54		
71	342.52		
72	344.20	202	Fluoranthene
73	345.60	181	Methylcarbazole
74	347.77	208	Phenanthro[4,5-*bcd*]thiophene
75	349.40	203	Azafluoranthene, -pyrene
76	350.43	218	Benzonaphthofuran
77	351.21	202	Pyrene
78	353.13	218	Benzonaphthofuran
79	356.09	218	Benzonaphthofuran
80	357.99	218	Benzo[*kl*]xanthene
81	359.53	220	Trimethylphenanthrene, -anthracene
82_a	360.64	216	Methylfluoranthene
82_b	360.64	203	Azafluoranthene, -pyrene
83	361.58	203	Azafluoranthene, -pyrene
84	363.45	216	Methylfluoranthene, -pyrene
85	364.26	216	Benzo[*a*]fluorene
86_a	367.22	216	Benzo[*b*]fluorene
86_b	367.22	232	Methylbenzonaphthofuran or isomer
87	368.23	216	Benzo[*c*]fluorene or isomer
88	368.90	232	Methylbenzonaphthofuran or isomer
89	370.62	232	Methylbenzonaphthofuran or isomer
90	371.70	216	Methylpyrene
91	373.41	232	Methylbenzonaphthofuran or isomer
92	374.44	232	Methylbenzonaphthofuran or isomer
93_a	375.41	217	Azabenzofluorene
93_b	375.41	230	Dimethylfluoranthene, -pyrene
94	377.51	230	Dimethylfluoranthene, -pyrene
95	379.47	230	Dihydrochrysene or isomer

TABLE I *(continued)*

Peak No.	$I^{OV\text{-}1701}$	Molecular weight	Possible compound
96_a	380.01	191	4*H*-Benzo[*def*]carbazole
96_b	380.01	230	Dimethylfluoranthene, -pyrene
97	381.21	230	Dimethylfluoranthene, -pyrene
98_a	382.36	230	Dimethylfluoranthene, -pyrene
98_b	382.36	246	Dimethylbenzonaphthofuran or isomer
99_a	383.11	230	Dimethylfluoranthene, -pyrene
99_b	383.11	246	Dimethylbenzonaphthofuran
100_a	384.65	230	Dimethylfluoranthene, -pyrene
100_b	384.65	246	Dimethylbenzonaphthofuran
101	385.31	246	Dimethylbenzonaphthofuran
102	387.46	234	Benzo[*b*]naphtho[2,1-*d*]thiophene
103_a	388.58	232	Tetrahydrochrysene or isomer
103_b	388.58	228	Benzo[*c*]phenanthrene
104	389.64	226	Benzo[*ghi*]fluoranthene or isomer
105	390.65	234	Benzo[*b*]naphtho[1,2-*d*]thiophene
106	391.52	229	Dibenzoquinoline or isomer
107	393.60	244	Trimethylfluoranthene, -pyrene
108	394.49	234	Benzo[*a*]naphtho[2,3-*d*]thiophene
109	397.91	228	Benz[*a*]anthracene
110	400.00	228	Chrysene
111	400.33	228	Triphenylene
112_a	402.35	228	Naphthacene
112_b	402.35	248	Methylbenzo[*b*]naphthothiophene
113_a	403.44	242	Methylbenz[*a*]anthracene or isomer
113_b	403.44	248	Methylbenzo[*b*]naphthothiophene
114_a	407.39	248	Methylbenzonaphthothiophene
114_b	407.39	258	Tetramethylfluoranthene or isomer
115	408.73	242	Methylbenz[*a*]anthracene or isomer
116_a	409.68	248	Methylbenzonaphthothiophene or isomer
116_b	409.68	258	Tetramethylfluoranthene or isomer
117	410.49	242	Methylchrysene or isomer
118_a	411.48	241	Methylazabenzo[*ghi*]fluoranthene or isomer
118_b	411.48	258	Tetramethylfluoranthene or isomer
119_a	412.27	242	Methylchrysene or isomer
119_b	412.27	248	Methylbenzonaphthothiophene
120	412.73	242	Methylbenz[*a*]anthracene or isomer
121	413.58	256	Dimethylbenz[*a*]anthracene or isomer
122	414.48	242	Methylbenz[*a*]anthracene or isomer
123	415.35	242	Methylbenz[*a*]anthracene or isomer
124	416.68	242	Methylbenz[*a*]anthracene or isomer
125_a	418.02	240	11*H*-Benz[*bc*]aceanthrylene or isomer
125_b	418.02	254	Binaphthalene or isomer
126_a	419.66	240	4*H*-Cyclopenta[*def*]chrysene or isomer
126_b	419.66	254	Binaphthalene or isomer
127_a	420.68	240	4*H*-Cyclopenta[*def*]triphenylene or isomer
127_b	420.68	256	Dimethylbenz[*a*]anthracene or isomer
128	421.67	254	Phenylphenanthrene or isomer
129	422.92	217	11*H*-Benzo[*a*]carbazole
130	423.94	254	Phenylphenanthrene or isomer
131	425.50	254	Dihydrobenzofluoranthene or isomer
132	426.28	217	7*H*-Benzo[*c*]carbazole
133	428.17	254	Dihydrobenzofluoranthene or isomer

(Continued on p. 164)

TABLE 1 *(continued)*

Peak No.	$I^{OV\text{-}1701}$	Molecular weight	Possible compound
134_a	429.18	241	Methylazabenzo[*ghi*]fluoranthene or isomer
134_b	429.18	256	Dimethylchrysene
135	430.69		
136	431.52	217	5*H*-Benzo[*b*]carbazole
137_a	432.43	241	Methylazabenzo[*ghi*]fluoranthene or isomer
137_b	432.43	256	Dimethylchrysene
137_c	432.43	268	Dibenzonaphthofuran or isomer
138	433.62	256	Dimethylchrysene or isomer
139_a	435.64	256	Dimethylchrysene or isomer
139_b	435.64	268	Dibenzonaphthofuran or isomer
140_a	436.32	256	Dimethylchrysene or isomer
140_b	436.32	268	Dibenzonaphthofuran or isomer
141_a	437.60	219	Dimethylbenzo[*c*,*d*,*f*]carbazole
141_b	437.60	231	Methylbenzocarbazole
142_a	439.45	254	Dihydrobenzofluoranthene or isomer
142_b	439.45	268	Dibenzonaphthofuran or isomer
143	442.25	268	Dibenzonaphthofuran or isomer
144	443.36	252	Benzo[*j*]fluoranthene
145_a	444.29	252	Benzo[*b*]fluoranthene
145_b	444.29	268	Dibenzonaphthofuran
146	446.96	252	Benzo[*k*]fluoranthene
147_a	448.40	268	Dibenzonaphthofuran
147_b	448.40	258	Benzophenanthrothiophene
148_a	450.08	254	Dihydrobenzopyrene or isomer
148_b	450.08	258	Benzophenanthrothiophene
148_c	450.08	268	Dibenzonaphthofuran
149_a	452.90	252	Benzo[*e*]pyrene
149_b	452.90	268	Dibenzonaphthofuran or isomer
150	454.96	252	Benzo[*a*]pyrene
151	455.58	253	Azabenzopyrene
152	458.04	252	Perylene
153	459.31	266	Methylbenzofluoranthene or isomer
154_a	460.67	266	Methylbenzofluoranthene or isomer
154_b	460.67	272	Methylbenzophenanthrothiophene
155_a	461.41	266	Methylbenzofluoranthene or isomer
155_b	461.41	253	Azabenzopyrene or isomer
155_c	461.41	278	Dibenzophenanthrene
156_a	462.59	253	Azabenzopyrene or isomer
156_b	462.59	266	Methylbenzofluoranthene or isomer
157_a	463.47	266	Dibenzofluorene or isomer
157_b	463.47	282	Methyldibenzonaphthofuran
158_a	464.23	266	Dibenzofluorene or isomer
158_b	464.23	278	Dibenzophenanthrene, -anthracene
159_a	465.24	266	Methylbenzofluoranthene or isomer
159_b	465.24	282	Dimethyldibenzonaphthofuran
160	466.01	266	Methylbenzopyrene or isomer
161_a	467.13	266	Methylbenzopyrene or isomer
161_b	467.13	282	Dimethyldibenzonaphthofuran
162	467.86	266	Methylbenzopyrene or isomer
163_a	469.66	266	Methylbenzopyrene or isomer
163_b	469.66	282	Dimethyldibenzonaphthofuran
164_a	471.50	264	11*H*-Cyclopenta[*ghi*]perylene

TABLE I *(continued)*

Peak No.	$I^{OV\text{-}1701}$	Molecular weight	Possible compound
164_b	471.50	266	Methylbenzopyrene
165	474.94	280	Dimethylbenzopyrene or isomer
166_a	478.01	241	4*H*-Naphtho[1,2,3,4-*def*]carbazole or isomer
166_b	478.01	280	Dimethylbenzofluoranthene or isomer
167	481.40		
168_a	483.22	284	Dibenzonaphthothiophene
168_b	483.22	280	Dimethylbenzofluoranthene or isomer
169_a	484.45	280	Dimethylbenzofluoranthene
169_b	484.45	296	Trimethyldibenzonaphthofuran
170_a	488.36	278	Dibenzophenanthrene
170_b	488.36	280	Dimethylbenzopyrene or isomer
171	489.49		
172_a	490.76	284	Dibenzonaphthothiophene
172_b	490.76	279	Dibenzophenanthridine or isomer
172_c	490.76	294	Trimethylbenzofluoranthene or isomer
173	491.37		
174	492.08	278	Dibenz[*a,j*]anthracene or isomer
175_a	494.80	276	Indenopyrene or isomer
175_b	494.80	278	Dibenzo[*a,c*]anthracene
176_a	496.15	278	Dibenzo[*a,h*]anthracene
176_b	496.15	267	
177	498.96	278	Benzo[*b*]chrysene or isomer
178	500.00	278	Picene
179	502.47	276	Benzo[*ghi*]perylene
180_a	504.80	290	Methyl derivative of indenopyrene or isomer
180_b	504.80	278	Benzo[*a*]naphthacene or pentacene
181_a		276	Anthanthrene
181_b		290	Methyl derivative of indenopyrene or isomer

pound 74, detected for the first time in 1977 in tars [18]) and their benzo derivatives (compounds 147_b and 148_b), and the family of benzo and dibenzo derivatives of dibenzo[*b,d*]thiophene (compounds 102, 105, 108, 168_a and 172_a). The significant concentration of benzo derivatives of benzo[*b,d*]thiophene in this complex mixture should be noted. All these compounds with n aromatic rings are retained on the stationary phase in an order similar to that of hydrocarbons which have $n + 1$ aromatic rings and a similar geometrical shape.

In addition to the compounds mentioned above, pyridine and pyrrole derivatives are also present in this mixture. Pyrrole derivatives begin with carbazole (compound 57) and compounds corresponding to its *peri-* and *cata*-condensed series (compounds 96_a and 166_a, and 129, 132 and 136, respectively) have been found. It is interesting that all these compounds are much more retained on OV-1701 than hydrocarbons or sulphur derivatives with the same geometrical shape. All of them elute among other compounds which have a higher molecular weight and a higher boiling point (*e.g.*, boiling point of carbazole, compound 57, 355°C; boiling point of 4*H*-cyclopenta[*def*]phenanthrene, compound 51, 358°C; and boiling point of 2-phenylnaphthalene, compound 56, 359.8°C). This can be attributed to the fact that

the pyrrole derivatives are able to establish hydrogen bonds with the stationary phase. For this reason, they are more retained than the other compounds. Further, the considerable concentration of carbazole and its *cata*-condensed benzo derivatives in the mixture should be pointed out; the elution order of these latter compounds could be first 11*H*-benzo[*a*]carbazole (boiling point 450°C), then 7*H*-benzo[*c*]carbazole (boiling point 452°C) and 5*H*-benzo[*b*]carbazole (boiling point 455°C), if this sequence agrees with the sequence of increasing boiling points.

The group of pyridine derivatives has a very large number of members but all of them at very low concentrations. All these compounds (compounds 3, 41, 42, 45, 75, 82_b, 83, 93_a, 106, 151, 155_b, 156_a and 172_b) elute near to the corresponding hydrocarbons. The chromatographic behaviour of this family of compounds has been previously studied using stationary phases of different polarity [3,19].

Another interesting group to be detected are oxygenated compounds. The first, dibenzofuran (compound 18), is followed by its benzo and dibenzo derivatives (compounds 76, 78, 79, 139_b, 140_b, 142_b, 143, 145_b, 147_a and 148_c), but the *peri*-condensed series previously found for dibenzo[*b*,*d*]thiophene and carbazole have not been detected. Compound 80 has been identified as benzo[*k*]xanthene. According to Lang and Eigen [20], the boiling point of this latter compound is 400°C. However, the boiling points of benzo[*b*]naphtho[2,1-*d*]furan and benzo[*b*]naphtho[2,3-*d*]furan are 387.7 and 394.5°C, respectively.

In addition to all the above-mentioned types of compounds, their methyl derivatives have also been detected. The concentration of monomethyl derivatives is always lower than those of the unmethylated compounds but higher than those of the di- or trimethyl derivatives.

Phenyl and naphthyl derivatives of hydrocarbons (compounds 6, 43, 56, 125_b, 130, 131, etc.) and other compound types in very small proportions have also been detected. These small peaks are more difficult to identify because they generally belong to more than one compound.

All components found in the volatile fraction of coal tar pitch are also present in coal tar, as expected, but the concentrations of most of the compounds are very different. Likewise, most of these compounds are not available commercially and their physico-chemical properties are unknown, but they are present in many carbonaceous and environmental samples.

The volatile fraction of the coal tar pitch covers wide ranges of boiling points (from 218 to *ca.* 525°C) and molecular weights (from *ca.* 128 to 300). Most of the compounds contained in this fraction belong only to the different families mentioned before, and compounds with other special functional groups have not been detected. Because of this, it could be concluded that the involatile fraction of this coal tar pitch is formed by the components of the different series mentioned before with higher molecular weights.

In the light of this, it might be worthwhile looking for relationships between the qualitative and/or quantitative composition of the volatile fraction of coal tar pitches, obtained through a chromatographic study, and the properties and behaviour of the whole coal tar pitches.

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Thin-layer chromatographic behaviour and separation of zirconium(IV) and hafnium(IV) on silica gel in mineral acid–hydrogen peroxide media

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ABSTRACT

The thin-layer chromatographic behaviour of Zr(IV) and Hf(IV) on silica gel in HNO_3, HCl and H_2SO_4 media containing various amounts of H_2O_2 was investigated as a function of the concentration of each component of the respective solvents. In all the systems, Zr(IV) gave much higher R_F values than Hf(IV) because of the stronger complexation of Zr(IV) with H_2O_2, so that the complete separation of the two metals, present in Zr:Hf weight ratios ranging from 20:1 to 1:40, could be accomplished simply and easily without any sample pretreatment.

INTRODUCTION

The complete separation of zirconium(IV) and hafnium(IV) is one of the most difficult tasks in inorganic analysis, because both metals exhibit almost the same chemistries owing to their similar outer electronic configurations and ionic radii, and is particularly troublesome when Zr(IV) is present in much higher proportions than Hf(IV), as in natural zirconium ores. In general, solvent extraction, ion-exchange and column chromatographuc methods have effectively been utilized for this purpose [1]. Good results have also been obtained with several paper chromatographic methods [2]. However, much less information is available on the separation of these ions by thin-layer chromatography (TLC).

The TLC separation of Zr(IV) and Hf(IV) from each other was first achieved [3] using microcrystalline cellulose and diethyl ether–14 mol/dm^3 HNO_3 (1:1, v/v). Shimizu and Muto [4] also accomplished the separation of the two metals by two-dimensional TLC on DEAE-cellulose by development with 0.1 mol/dm^3 H_2SO_4–0.05 mol/dm^3 $(NH_4)_2SO_4$ solution and 0.1 mol/dm^3 H_2SO_4–1.0 mol/dm^3 $(NH_4)_2SO_4$ solution. However, these two methods did not provide a sufficiently large resolution for this pair, having centred on the separation of equimolar amounts of the metals. In

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contrast, Olsina *et al.* [5] quantitatively separated both metals present in a wide range of amounts and ratios on a silica gel layer by development with $HCl–H_3PO_4–H_2O$ (10:1:9, v/v/v), in which both metals have been reported to be inseparable by paper chromatography [6]. This TLC method of separation, however, requires fuming of the sample solution with H_2SO_4 just before its application on the plate and special care in drying the spotted sample solution in order to avoid anomalous results caused by hydrolysed and polymerized species of these metals in aged solutions.

In this work, the TLC behaviour of Zr(IV) and Hf(IV) on silica gel in HNO_3, HCl and H_2SO_4 media containing various amounts of H_2O_2 was studied in order to establish simple and effective methods for the separation of these metals. All the TLC systems investigated allow the complete separation of both metals coexisting in a wide range of amounts (0.2–4 mg) and ratios (Zr:Hf = 20:1–1:40), without any pretreatment of aged sample solutions.

EXPERIMENTAL

Stock solutions

Stock solutions of Zr(IV) and Hf(IV) were prepared from their oxychlorides (99.99% purity), as follows, to give a concentration of 0.05 mol/dm^3 with respect to each metal. The oxychloride solutions were obtained by dissolving suitable amounts of the respective compounds in a small volume of 6 mol/dm^3 HCl, evaporating to dryness and dissolving the residues in the same acid. For oxynitrate and oxysulphate solutions, appropriate amounts of the oxychloride of each metal were dissolved in a small volume of 6 mol/dm^3 HNO_3 or $\frac{3}{2}$ mol/dm^3 H_2SO_4 and evaporated to dryness, followed by dissolution of the residue in the same acid. When necessary, the stock solutions were diluted with the same acid of the same concentration.

Test solutions for the measurement of the R_F values were prepared by diluting the individual stock solutions to give a 1 mg/cm^3 metal solution. Sample solutions were obtained by mixing suitable amounts of the stock solutions or by diluting the resulting mixtures.

Preparation of thin-layer plates

A 10-g portion of silica gel without binder (Wakogel B-O; Wako, Osaka, Japan) was thoroughly blended with 22.5 cm^3 of distilled, deionized water. The slurry was spread to a thickness of 250 μm on glass plates (20 × 20 cm) with an applicator. The plates were allowed to stand for 30 min at room temperature and then dried in an oven at *ca.* 70°C for 30 min.

In order to remove inorganic impurities from the silica gel used, the plate was thoroughly washed by developing overnight up to the upper edge of the plate by the ascending technique with 30 cm^3 of 1 mol/dm^3 HCl–3% (w/v) H_2O_2 solution, using a sandwich type of chamber made of plastic. Subsequently, the plate was dried on a hot-plate at *ca.* 90°C for 20 min. After cooling, they were stored in a desiccator containing saturated sodium chloride solution, until ready for use.

Procedure

A 0.5-μl portion of the test or sample solution was applied to the plate by means of a microcap (Drummond, Broomall, PA, U.S.A.) at a point 2.5 cm from one edge

and the spots were dried in air for 10 min. The plate was immediately placed in a chromatographic chamber (22 × 10 × 22 cm), equipped with a small tank (21 × 5 × 4 cm) containing 30 cm^3 of a developing solvent, and developed up to 10 cm from the starting point by the ascending technique at room temperature. The developing solvents used were HNO_3, HCl and H_2SO_4 containing H_2O_2 at various concentrations.

Detection

After development, the plate was dried on a hot-plate at *ca.* 90°C and the positions of the metals were revealed by spraying with a 0.02% aqueous arsenazo III solution. Both metals were detected as green spots on a pink background.

RESULTS AND DISCUSSION

Adsorption behaviour

HNO_3–H_2O_2 system. The R_F values of Zr(IV) and Hf(IV) on silica gel in HNO_3–H_2O_2 media are summarized as a function of the concentration of each component of the solvents in Table I, where the separation factors (α) and the resolutions (R_s), each defined as follows, are also given to clarify the effectiveness of the system for the separation of the metals:

$$\alpha = \left[R_{F(\mathrm{Hf})}^{-1} - 1 \right] \Big/ \left[R_{F(\mathrm{Zr})}^{-1} - 1 \right]$$

TABLE I

R_F VALUES (× 100) OF Zr(IV) AND Hf(IV) ON SILICA GEL IN HNO_3–H_2O_2 MEDIA

Concentration of HNO_3 (mol/dm^3)	Concentration of H_2O_2 (%, w/v)	R_F × 100		Separation factor (α)	Resolution (R_s)
		Zr(IV)[a]	Hf(IV)[a]		
0.1	0	1–11	1–5	2	0.4
0.1	0.6	75–97	0–4	300	6.5
0.1	1	84–99	2–7	230	8.7
0.1	3	91–100	1–8	450	11
0.1	6	88–100	0–9	330	8.5
0.1	10	92–100	2–25	150	5.3
1	0	0–9	0–7	1	0.1
1	0.3	2–93	2–8	17	0.9
1	0.6	33–95	2–11	26	1.6
1	1	62–98	2–11	58	3.3
1	3	87–100	1–20	120	5.2
1	6	92–100	1–23	180	5.6
1	10	92–100	3–38	93	3.5
0.1	3	91–100	1–8	450	11
0.5	3	90–100	2–11	270	9.3
1	3	87–100	1–20	120	5.2
3	3	91–100	3–35	90	3.7
6	3	92–100	4–44	76	3.0
9	3	91–100	12–33	71	5.0

[a] Oxynitrate used.

where R_F is the average of the R_F values of the rear and the front of each spot; and

$$R_S = 2[D_{(Zr)} - D_{(Hf)}]/[W_{(Zr)} + W_{(Hf)}]$$

where D is the migration distance of the centre of each spot and W is the width of each spot in the longitudinal direction.

When the concentration of HNO_3 was kept constant at 0.1 or 1.0 mol/dm^3, Zr(IV) migrated near the solvent front even in the presence of a small amount of H_2O_2 and gave more compact spots as the concentration of H_2O_2 became higher. In contrast, Hf(IV) remained near the point of application in almost all the solvents tested and showed longer spots at higher concentrations of H_2O_2.

On the other hand, when the concentration of H_2O_2 was kept constant at 3.0% (w/v), Zr(IV) displayed well defined and compact spots in contact with the solvent front, regardless of the concentration of HNO_3, whereas Hf(IV) showed low R_F values and gave longer spots as the concentration of HNO_3 increased.

In addition, without any H_2O_2, the adsorption behaviour of Zr(IV) and Hf(IV) were very similar; they adsorbed very tightly on silica gel in all the HNO_3 solutions within the concentration range 0.1–6.0 mol/dm^3, and showed a slightly increasing tendency in their R_F values with further increase in HNO_3 concentration, although all the data obtained are not quoted in Table I.

Hence in this system Zr(IV) gives extremely high R_F values even in the presence of small amounts of H_2O_2, but Hf(IV) remains near the point of application, although

TABLE II

R_F VALUES (× 100) OF Zr(IV) AND Hf(IV) ON SILICA GEL IN HCl–H_2O_2 MEDIA

Concentration of HCl (mol/dm^3)	Concentration of H_2O_2 (%, w/v)	R_F × 100		Separation factor (α)	Resolution (R_s)
		Zr(IV)[a]	Hf(IV)[a]		
0.1	0	1–11	1–5	2	0.4
0.1	0.6	44–97	2–6	57	2.3
0.1	1	91–100	0–6	690	12
0.1	3	95–100	0–6	1300	17
0.1	6	94–100	1–11	510	12
0.1	10	96–100	1–15	560	10
1	0	−1–8	−1–6	1	0.1
1	0.3	0–55	0–12	6	0.6
1	0.6	41–99	0–17	25	1.6
1	1	86–100	0–16	150	5.6
1	3	90–100	0–16	220	6.7
1	6	94–100	2–26	200	5.5
1	10	95–100	0–26	260	5.5
0.1	3	95–100	0–6	1300	17
0.6	3	92–100	1–16	260	7.6
1	3	90–100	0–16	220	6.7
3	3	83–100	0–46	36	2.2
6	3	88–100	55–76	8	1.7

[a] Oxynitrate used.

both metals adsorb very strongly on silica gel in pure HNO_3 without H_2O_2. Therefore, the complete separation of the two metals can be achieved over a wide range of concentrations of HNO_3 and H_2O_2.

HCl–H_2O_2 system. The R_F values, the separation factors and the resolutions for Zr(IV) and Hf(IV) on silica gel in HCl–H_2O_2 media are listed as a function of the concentration of each component of the solvents in Table II.

In general, the TLC behaviour of the two metals in this system was very similar to that in the HNO_3–H_2O_2 system, although the former offered larger separation factors and resolutions than the latter. However, when the stock solution of Zr(IV) oxychloride, aged for at least 4 h, was applied to the silica gel layer, another very weak spot was observed, just at the point of application. On the other hand, when that of the oxynitrate, aged for at least 6 months, was used, Zr(IV) did not show any abnormal adsorption. Fortunately, the abnormal adsorption of Zr(IV) oxychloride could easily be eliminated by adding a few drops of 3% (w/v) H_2O_2 and allowing the solution to stand for at least 4 h or by pretreating the sample solution three times with small amounts of 6 mol/dm^3 HCl–3% (w/v) H_2O_2 solution or aqua regia. These observations suggest that the oxychloride solutions can produce more hydrolysed and polymerized species for a long aging period than the oxynitrate solutions and that it is very difficult to dissociate rapidly the resulting species to less hydrolysed and

TABLE III

R_F VALUES (×100) OF Zr(IV) AND Hf(IV) ON SILICA GEL IN H_2SO_4–H_2O_2 MEDIA

Concentration of H_2SO_4 (mol/dm^3)	Concentration of H_2O_2 (%, w/v)	R_F × 100		Separation factor (α)	Resolution (R_s)
		Zr(IV)[a]	Hf(IV)[a]		
0.05	0	0–78	0–13	9	0.7
0.5	0	55–99	0–44	12	1.3
1	0	83–98	58–82	4	1.1
2	0	93–100	87–98	2	0.4
3	0	95–100	89–96	3	0.8
0.05	3	91–100	0–34	100	3.7
0.5	3	92–100	26–59	33	2.6
1	3	90–100	69–90	5	1.0
2	3	91–100	78–95	3	0.7
3	3	90–100	89–100	1	<0.1
0.05	0.6	72–98	4–29	29	2.7
0.05	1	92–100	3–33	110	4.1
0.05	3	91–100	0–34	100	3.7
0.05	6	92–100	0–39	99	3.2
0.05	10	92–100	0–52	68	2.3
0.5	0.3	88–100	10–42	45	3.1
0.5	0.6	89–100	12–50	38	2.6
0.5	1	88–100	19–53	28	2.5
0.5	3	92–100	26–59	33	2.6
0.5	6	90–100	26–76	18	1.5
0.5	10	90–100	42–83	11	1.3

[a] Oxysulphate used.

polymerized species during chromatography. In addition, Hf(IV) always remained at or near the point of application with or without any treatment. Therefore, all the data in Table II are given with respect to the oxynitrates.

In conclusion, this system also allows the complete separation of Zr(IV) and Hf(IV) to be achieved simply and effectively, as with the HNO_3–H_2O_2 system, if the oxynitrates of the metals are used as the starting material.

H_2SO_4–H_2O_2 system. The R_F values, the separation factors and the resolutions for Zr(IV) and Hf(IV) are given as a function of the concentration of each component of the solvents in Table III, and those in pure H_2SO_4 without H_2O_2 are also listed for comparison.

In H_2SO_4 without H_2O_2, the TLC behaviour of these metals was notably different from those in pure HNO_3 and pure HCl; Zr(IV) showed weaker adsorption than Hf(IV), as with the cation-exchange resin–dilute H_2SO_4 system [7]. With lower concentrations of H_2SO_4, these two metals could be separated on silica gel only with difficulty, because the resolutions were not satisfactory owing to long tailing of the peaks of the metals. On the other hand, with higher concentrations of H_2SO_4, both metals were hardly adsorbed, because they occurred as less hydrolysed and polymerized species produced by the complexation with sulphate ions.

In contrast, in the H_2SO_4 media with H_2O_2, Zr(IV) showed a compact spot in contact with the solvent front, irrespective of the change in the concentration of H_2SO_4, whereas Hf(IV) gradually moved upwards as the concentrations of both H_2O_2 and H_2SO_4 increased. Thus, the addition of suitable amounts of H_2O_2 to H_2SO_4 solutions was very effective for the separation of Zr(IV) and Hf(IV), particularly when the concentration of H_2SO_4 was kept constant at 0.5 mol/dm^3.

Hence, in all of the present systems the R_F values of Zr(IV) increase significantly with increasing concentration of H_2O_2, but those of Hf(IV) are hardly affected, so that Zr(IV) always provides much higher R_F values than Hf(IV). This observation can be explained by the fact that Zr(IV) forms much more stable peroxo complexes than Hf(IV) [8]. Another particularly interesting characteristic common to the present systems is that the sequence of adsorption is contrary to those in systems consisting of silica gel and HCl–H_3PO_4–H_2O (10:1:9, v/v/v) [5] and of a strongly basic anion-exchange resin and 2.5 mol/dm^3 H_2SO_4–0.1% H_2O_2 solution [9]. These facts and the present results suggest that in the present systems cation-exchange adsorption of hydrolysed and polymerized species of both metals and desorption of Zr(IV) based on the formation of peroxo complexes participate in determining the R_F values of the metals. Consequently, it is clear that the presence of appropriate amounts of H_2O_2 is essential to obtain better resolutions and separation factors for this pair of metals.

Separation

The most outstanding characteristic of the present TLC systems is that the separation factors and the resolutions for Zr(IV) and Hf(IV) are large enough to separate them clearly. The R_F data in Tables I and II suggest that all of the HNO_3 and HCl media, containing H_2O_2 in concentrations of 0.6% (w/v) or more, allow the complete separation of both metals present in a wide range of amounts and ratios to be accomplished simply and easily. The maximum separation factor (α = 1300) and the best resolution (R_s = 17) can be obtained in 0.1 mol/dm^3 HCl–3% (w/v) H_2O_2 solution, whereas in 0.1 mol/dm^3 HNO_3–3% (w/v) H_2O_2 solution the values are α = 450 and R_s = 11.

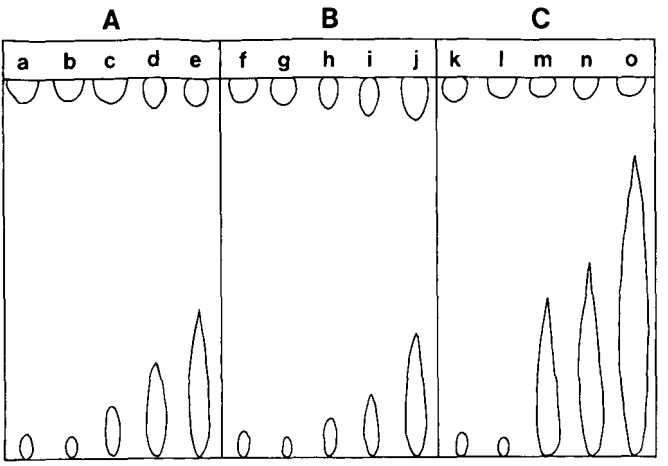

Fig. 1. Separation of Zr(IV) (upper spots) and Hf(IV) (lower spots). Solvent: (A) 0.1 mol/dm^3 HNO_3–3% (w/v) H_2O_2; (B) 0.1 mol/dm^3 HCl–3% (w/v) H_2O_2; (C) 0.05 mol/dm^3 H_2SO_4–3% (w/v) H_2O_2. Amounts of metals taken (mg): a, f and k, Zr 4, Hf 0.2; b, g and l, Zr 2, Hf 0.2; c, h and m, Zr 0.2, Hf 2; d, i and n, Zr 0.2, Hf 4; e, j and o, Zr 0.2, Hf 8. Metal: (A) and (B) oxynitrate used; (C) oxysulphate used.

On the other hand, in H_2SO_4–H_2O_2 media the resolution and the separation factors are smaller, *viz.*, $\alpha = 110$ and $R_s = 4.1$ with 0.05 mol/dm^3 H_2SO_4–1% (w/v) H_2O_2 solution.

Typical thin-layer chromatograms for the separation of Zr(IV) and Hf(IV), coexisting in a wide range of amounts (0.2–4 mg) and ratios (Zr:Hf = 20:1–1:40), are given in Fig. 1. The validity of the separation of each mixture was confirmed by visual comparison with the same amounts of standards chromatographed alongside the sample on the same plate. The R_F values of the metals separated were in good agreement with those obtained individually.

In conclusion, it can be expected that the present systems, consisting of silica gel and acidic media containing H_2O_2, will be applicable not only to the TLC separation of Zr(IV) and Hf(IV) coexisting in a more extended range of amounts and ratios, but also to the column separation of these two metals.

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Separation of antibody–antigen complexes by capillary zone electrophoresis, isoelectric focusing and high-performance size-exclusion chromatography

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ABSTRACT

The separation of antibody–antigen complexes by free-solution capillary zone electrophoresis (CZE) has been demonstrated. The antigen, a monoclonal antibody specific for the antigen, and the complex were well resolved. The entire separation was achieved in less than 10 min using on-column UV detection. The p*I* values for the three species were estimated separately by isoelectric focusing (IEF) experiments on polyacrylamide gels. Reasonably good agreement was found between the relative migration times measured by CZE and the p*I* values. Both IEF and high-performance size-exclusion chromatography of the antibody–antigen mixtures confirmed the formation of the complexes observed by CZE. This study demonstrates the utility of CZE as a new and complementary technique for the characterization of antibody–antigen complexes.

INTRODUCTION

Capillary zone electrophoresis (CZE) in free solution has gained widespread recognition as a powerful separation tool that is complementary to gel-electrophoretic and chromatographic techniques. Recent literature describes several applications, along with information on basic principles, instrumentation and methodology [1–11].

This paper describes the separation of antibody–antigen complexes formed by an immunochemical reaction by free-solution CZE; the antigen is a recombinant human growth hormone (hGH) that reacts with a monoclonal antibody [MoAb, immunoglobulin G (IgG) class] specific to hGH. The complexes were well resolved from the unreacted (or free) antibody and antigen in solution. Recently, Chen *et al.* [12] also reported a successful separation of antibody–antigen complexes by CZE. However, they employed an isoelectric focusing experiment that requires a stabilizing medium and ampholytes in capillary tubes. In our work, the separation was achieved in a free-flowing buffer solution without ampholytes or stabilizing media. Preliminary results of this work were reported elsewhere [6]. We also present data comparing CZE to conventional isoelectric focusing (IEF) and high-performance size-exclusion chromatography (HPSEC).

The monoclonal preparation employed in this study, at least in theory, contains

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a single, homogeneous population of antibody molecules specific to a well-defined epitope on the antigen. Thus, only two types of complexes [IgG–hGH and IgG–$(hGH)_2$] will be formed, that correspond to reaction at the two antigen binding sites of each antibody molecule:

$$IgG + hGH \rightleftarrows IgG\text{–}hGH \quad (1)$$

$$IgG\text{–}hGH + hGH \rightleftarrows IgG\text{–}(hGH)_2 \quad (2)$$

We will refer to these complexes in general as IgG–$(hGH)_n$, where n is the valency of the antibody. These complexes are non-covalent in nature, tightly held together by various combinations of electrostatic, hydrophobic, dipole–dipole, hydrogen bonding and Van der Waals interactions [13].

EXPERIMENTAL

Chemicals and reagents

Highly purified recombinant DNA-derived hGH was obtained from Lilly Research Labs. (Indianapolis, IN, U.S.A.). The anti-hGH monoclonal antibody (part No. 20065) was purchased from Hybritech (San Diego, CA, U.S.A.). This preparation was a murine IgG made by ammonium sulphate fractionation and subsequent purification by DEAE ion-exchange chromatography. Calibration standards (p*I* 3–9) for IEF and the precast polyacrylamide gels were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ, U.S.A.). Tricine was purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were analytical-reagent grade, and the solutions were made in distilled water, deionized using the Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Apparatus

CZE experiments were performed using an Applied Biosystems (Foster City, CA, U.S.A.) Model 270A instrument. The fused-silica capillary was 100 cm long (80 cm to the detector) with 50 μm I.D. and 360 μm O.D. (Polymicro Technologies, Phoenix, AZ, U.S.A.). The capillary was rinsed with the 0.10 *M* tricine buffer (pH 8) between injections. New capillaries were rinsed with 0.10 *M* NaOH, water and 0.10 *M* HCl prior to use. In all experiments, the detector end of the capillary was negatively polarized relative to the injector end. The IEF experiments were carried out on the Pharmacia PhastSystem (Pharmacia LKB Biotechnology). HPSEC was performed using a high-performance liquid chromatographic system consisting of a Hewlett-Packard Model 1050 series gradient pump (Waldbron Analytical Division, F.R.G.), ISS-100 sampling system (Perkin-Elmer Norwalk, CT, U.S.A.) and a Spectroflow Model 757 absorbance detector (Kratos, Ramsey, NJ, U.S.A.).

Electrophoretic conditions

CZE separations were performed in 0.1 *M* tricine buffer, pH 8, using an applied voltage of 30 kV, with a current of about 19 μA. Injection volumes were *ca.* 9 nl in all cases (3-s injection using a 127-mmHg vacuum), and the separated proteins were detected at 200 nm. Operating temperature was maintained at 30.0 ± 0.1°C.

In the IEF experiments, 1-μl volumes of solutions containing individual proteins or protein mixtures (prepared as described below) were directly applied to the precast polyacrylamide gels (5%) using special sample application combs provided for the Pharmacia PhastSystem. The gels were pre-focused for 75 V · h at 15°C, 2000 V, prior to sample application for 15 V · h (200 V, 2.5 mA, 3.5 W). Focusing was carried out at 15°C for 410 V · h during which time the voltage rose from 200 to 2000 V (2.5 mA, 3.5 W). Gels were fixed in 20% trichloroacetic acid and stained with Coomassie Blue R-350, following standard procedures described in the PhastSystem manual.

Chromatographic conditions

Separations were achieved with a GF-250 (25 cm × 9.4 mm) size-exclusion column (DuPont, Wilmington, DE, U.S.A.) at ambient temperature using a 0.20 *M* Na_2HPO_4 (pH 7.6) mobile phase flowing at 0.60 ml/min. Eluted peaks were detected at 214 nm. Sample injection was carried out using a 20-μl loop.

Sample preparation

Both hGH and the anti-hGH MoAb (IgG) were dissolved directly into or dialyzed against 0.1 *M* tricine buffer (pH 8). The final concentration of IgG was 3.03 mg/ml ($2.35 \cdot 10^{-5}$ *M*), and that of hGH was 0.508 mg/ml ($2.29 \cdot 10^{-5}$ *M*), based on molecular weights of 150 000 dalton for IgG and 22 124 dalton for hGH. These concentrations were determined by UV absorbance at 276 nm for hGH (a = 0.74 ml/mg · cm) and IgG (a = 1.40 ml/mg · cm), where a is the absorbance of a 1 mg/ml solution in a 1 cm path length cell. Appropriate volumes of hGH and IgG solutions were mixed to initiate the reaction. The approximate molar ratios of IgG:hGH in the mixtures used for CZE and HPSEC were 1:4, 1:2, 1:1, 2:1 and 3:1.

For the IEF experiments, hGH and the monoclonal IgG were dissolved in 0.05 *M* potassium phosphate buffer, pH 7.6. The final concentration of IgG was 2.2 mg/ml, and that of hGH was 1.0 mg/ml. Two solutions containing IgG and hGH were made by mixing appropriate volumes of the above protein solutions. The approximate molar ratios of IgG:hGH in the above mixtures were calculated to be 1:1.5 and 1:3.1.

RESULTS

The model antigen (hGH) and the monoclonal anti-hGH antibody (IgG) were readily available in highly purified preparations. They were well characterized in-house to establish purity, identity and binding properties. Results [14] showed that both preparations contained a single protein component of appropriate molecular mass by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (hGH *ca.* 22 124 dalton, IgG *ca.* 150 000 dalton). No significant protein impurities were detected when stained with Coomassie Blue R-350. The apparent affinity constant for the reaction (K_f) was *ca.* $1.0 \cdot 10^9$ l/mol by cold competition radioimmunoassay [14]. This strong binding affinity of the antibody for hGH results in strong, stable antibody–antigen complexes that form very rapidly in solution. Mixtures containing various molar ratios of IgG:hGH were analyzed by CZE, IEF and HPSEC to study the properties of the complexes formed. These techniques separate species based on differences in electrophoretic mobility, isoelectric point and size, respectively.

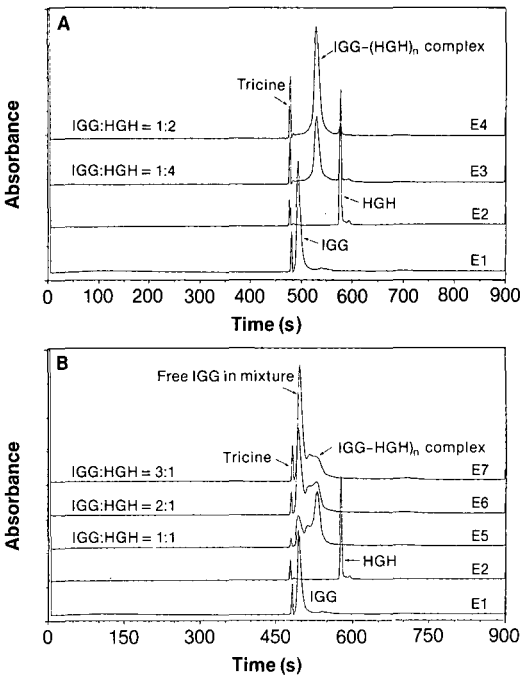

Fig. 1. Separation of IgG, hGH and IgG–(hGH)$_n$ complexes by CZE. (A) Electropherograms of IgG, hGH and mixtures containing an excess of hGH. (B) Electropherograms of IgG, hGH and mixtures containing an excess of IgG. Experimental conditions are described in the text.

CZE experiments

Fig. 1 shows seven electropherograms (E1–E7) representing separate injections. Electropherograms E1 and E2 are for individual injections of IgG and hGH, respectively. They show that these species have different migration times and are well resolved under the experimental conditions employed. Note that IgG migrates much faster than hGH, due to the fact that the p*I* for hGH is 5.2 and that for the monoclonal IgG is in the range 7.2–8.0 (multiple bands in Fig. 2). Therefore, hGH molecules have much greater negative charge than IgG molecules in the CZE separation buffer (0.1 *M* tricine, pH 8). This results in faster migration of the IgG molecules towards the negatively polarized detector end of the capillary. The first peak in all electropherograms is due to a slightly higher tricine concentration in the sample buffer compared to that of the separation buffer.

Electropherograms E3 and E4 show the results for 1:4 and 1:2 molar ratios of IgG:hGH, respectively (Fig. 1A). Based on migration-time comparison, the peak at *ca.* 531 s is a new molecular entity, most probably corresponding to one or more IgG–(hGH)$_n$ complexes. The peak at *ca.* 576 s corresponds to free hGH present in excess in both mixtures. As the IgG:hGH molar ratios decrease in the mixtures, one observes distinct changes in the peak profiles (Fig. 1B, electropherograms E5, E6 and E7). In electropherogram E5, the free IgG peak was observed at *ca.* 495 s along with two distinct peaks for IgG–(hGH)$_n$ complexes at *ca.* 513 and *ca.* 531 s, respectively.

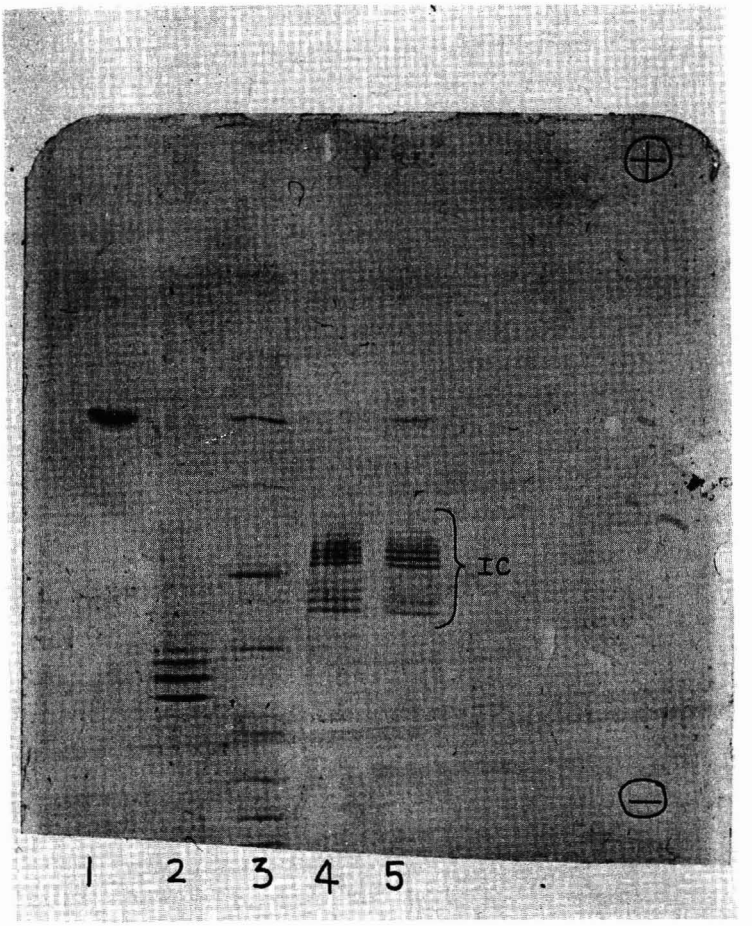

Fig. 2. Separation of IgG, hGH and IgG–$(hGH)_n$ (IC) complexes by IEF. Lanes: 1 = hGH; 2 = IgG; 3 = p*I* markers (p*I* 3–9); 4 = IgG:hGH mixture, molar ratio = 1:1.5; 5 = IgG:hGH mixture, molar ratio = 1:3.1. Experimental conditions are described in the text.

Note that the peak at *ca.* 513 s remains the same, but the late migrating peak for the second IgG–$(hGH)_n$ complex decreases rapidly as the hGH concentration is decreased (E6, E7). No excess hGH was detected in electropherograms E5, E6 and E7. IgG, hGH and various mixtures of IgG–hGH were analyzed on IEF gels (p*I* 3–9) and by HPSEC to further explore the nature of IgG–$(hGH)_n$ complexes observed in CZE experiments.

IEF experiments

IEF results in Fig. 2 show that the IgG–$(hGH)_n$ complexes were well resolved from free hGH, and appear not to dissociate significantly during the experiment. In addition, hGH shows a single band on the gel (p*I* 5.2), while IgG and the complexes show multiple bands with a p*I* range of 7.2–8.0 and 6.1–7.0, respectively. The acidic

shift associated with the IgG–$(hGH)_n$ bands (lanes 4 and 5) is probably due to the presence of hGH molecule(s) in the complex. This shift in p*I* is consistent with slower migration of the complexes relative to IgG, but faster than hGH, in CZE experiments, and correlates well with the migration order observed in CZE. These observations are consistent with results described in a previous report [4].

In lanes 4 and 5, the IgG–$(hGH)_n$ complexes are present in two distinct groups of bands, whereas IgG in lane 2 was resolved into only one group of bands with fewer components (Fig. 2). The more acidic group of bands appears to be present in higher amounts, since they stain more intensely than the lower, relatively more basic, group. These two groups may correspond to the two peaks observed in the CZE experiments in Fig. 1 (E5, E6 and E7). Note also that the excess hGH in the second mixture (IgG:hGH = 1:3.10) is clearly visible in lane 5.

It is not clear if the multiplicity of bands (or micro-heterogeneity) seen *within* these two groups on IEF gels does correspond to different IgG–$(hGH)_n$ complexes. Previous work [15,16] shows that artifactual micro-heterogeneity can be induced by proteins binding to carrier ampholytes in IEF gels, but other studies dispute such observations [17]. This phenomenon is being investigated by CZE in the presence and absence of carrier ampholytes.

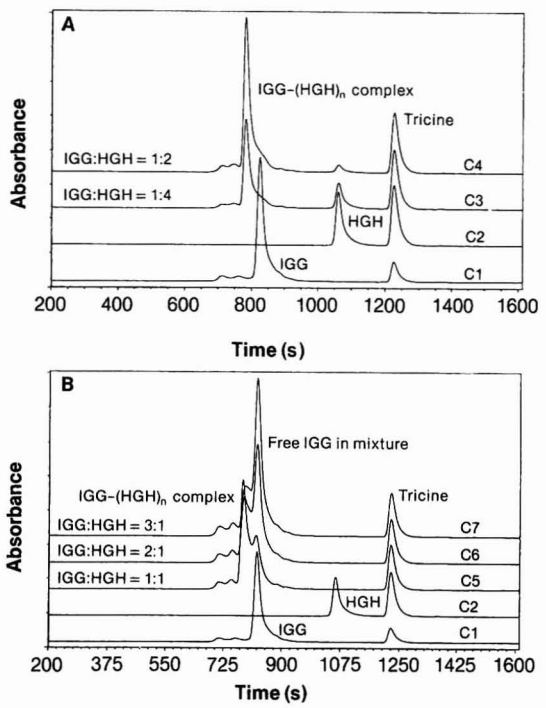

Fig. 3. Separation of IgG, hGH and IgG–$(hGH)_n$ complexes by HPSEC. (A) Chromatograms of IgG, hGH and mixtures containing an excess of hGH. (B) Chromatograms of IgG, hGH and mixtures containing an excess of IgG. Experimental conditions are described in the text.

HPSEC experiments

The hGH, IgG and mixtures were analyzed with a GF-250 HPSEC column to compare resolution and/or formation of high-molecular-weight complexes (Fig. 3). The peak at *ca.* 1220 s in all chromatograms is due to tricine present in the samples (buffer for CZE). The IgG peak elutes at *ca.* 820 s (chromatogram C1) while hGH is well resolved at *ca.* 1050 s (chromatogram C2). Chromatograms C3–C7 in Fig. 3A and B represent various molar ratios of the IgG:hGH mixtures that show varying amounts of free IgG, free hGH and the complexes. These were the same mixtures analyzd by CZE and shown in Fig. 1A and B.

Chromatograms C3 and C4 show free hGH and probably a single IgG–$(hGH)_n$ complex at *ca.* 770 s; note that the amount of free hGH decreases in C4 since the mixture contains less hGH. As the hGH:IgG molar ratio decreases, one observes an increased peak area for free IgG (chromatograms C5, C6 and C7 in Fig. 3B); the amount of IgG–$(hGH)_n$ complex decreases accordingly. Note that HPSEC resolves one major peak corresponding to IgG–$(hGH)_n$, whereas CZE clearly shows the formation of two distinct complexes. Ionic strength and buffer composition changes did not sufficiently improve the efficiency of the HPSEC separation so that the second complex observed by CZE or multiple complexes seen by IEF could be resolved. GF-450 (DuPont) columns with higher size-exclusion limits (25 000–900 000 dalton) were also unsuccessful in improving the resolution of the multiple complexes.

DISCUSSION

Assuming that the antibody was a pure monoclonal preparation, we expected two types of complexes to be formed as predicted by eqns. 1 and 2. Indeed, the CZE results demonstrate two peaks that may correspond to the stoichiometry assumed in these equations. Furthermore, as the hGH:IgG ratios change, the relative peak ratios also change in a manner qualitatively consistent with the above equations. The peak at *ca.* 513 s migrates slower than the IgG peak, suggesting the presence of hGH in the complex, probably as IgG–hGH. By similar reasoning, the peak at *ca.* 531 s should contain more hGH and, therefore, corresponds to IgG–$(hGH)_2$. The IEF results support the formation of two types of complexes, but does not reveal the stoichiometry. Analysis by HPSEC demonstrates the formation of a major complex peak, larger than the antibody itself, but does not conclusively demonstrate the formation of multiple complexes observed in CZE and IEF experiments. Attempts to estimate the molecular weight of the IgG–$(hGH)_n$ complex uisng high-molecular-weight protein calibrators were unsuccessful because the optimum separation conditions for the calibrators were not the same as that for the antibody–antigen complexes. Thus, we were unable to verify the molecular size or stoichiometry of the complexes observed in CZE, IEF and HPSEC.

In addition, we found that the total peak area by CZE for all peaks containing IgG is proportional to the amount of added IgG. Since the absorption at 200 nm is essentially independent of complex formation, this implies that (1) no material is lost by adsorption or other processes, and (2) all IgG is distributed between the three species observed. It is also apparent that the CZE peak at *ca.* 513 s is never a predominant species. These observations indicate that the complex at *ca.* 513 s may have a lower equilibrium constant (*i.e.*, lower stability) than the complex at *ca.* 531 s.

Note that the peaks for IgG and the IgG–$(hGH)_n$ were relatively broad in CZE. Multiply charged IgG species with different electrophoretic mobilities or analyte interactions with the wall may cause such broadening. It is worth noting, however, that the complexes (*ca.* 172 000–194 000 dalton) and IgG (*ca.* 150 000 dalton) were much better resolved by CZE than by HPSEC, and that the CZE separations were much faster (Figs. 1 and 3). Also, to our knowledge, these are the largest protein moieties separated by free solution CZE in less than 10 min with reasonable resolution.

CONCLUSIONS

CZE has been successfully employed to separate antibody–antigen complexes. Formation of stable IgG–$(hGH)_n$ complex(es) were confirmed by IEF and HPSEC experiments, but CZE clearly supports the formation of two types of complexes as predicted by theory. The IEF results show that the p*I* values for the complexes were more acidic than for free IgG, but more basic than hGH. The relative migration times for the three species in CZE experiments are consistent with the p*I* values observed. The CZE experiments were simple, fast, and can be readily automated with instruments that are commercially available [18].

In the long run, CZE may be a useful tool to study not only the formation of antibody–antigen complexes, but also the dynamics of other macromolecular interactions where biologically significant complexes of varying "stability" are formed. The advantage of free-solution CZE is that no supporting media such as chromatographic packings or gels are employed, which may enhance the dissociation of weak, non-covalent complexes during separation [19–21].

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Short Communication

Determination of impurities in dextropropoxyphene hydrochloride by high-performance liquid chromatography on dynamically modified silica

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ABSTRACT

A high-performance liquid chromatographic method was developed for the separation and determination of small amounts of impurities in dextropropoxyphene hydrochloride. The separation was achieved on a column of bare silica (LiChrosorb Si 100), using as the eluent methanol–tetrahydrofuran–water–0.2 *M* potassium phosphate buffer (pH 7.5) (350:84:516:50) containing 2.5 m*M* cetyltrimethylammonium bromide. The selectivity of the system towards a mixture of dextropropoxyphene hydrochloride and two possible impurities was investigated using different brands of silica. Only minor variations were found relative to those of a chromatographic system based on chemically bonded ODS-silica as the column material. The method is well suited for pharmacopoeial purposes, especially as the utilization of reference substances is not necessary.

INTRODUCTION

Dextropropoxyphene hydrochloride is a narcotic analgesic and monographs fixing the quality of the substance can be found in several modern pharmacopoeias. In the monographs in both the U.S. Pharmacopeia (USP XXII) [1] and the British Pharmacopoeia (B.P. 88) [2] a test for related substances is prescribed by means of gas chromatography (GC) using authentic samples of two known impurities as the reference substances. The two substances are an acetoxy compound, which is a possible impurity originating from the route of synthesis, and a carbinol compound, which may originate both from the route of synthesis and from degradation. The structures of dextropropoxyphene and the two impurities are given in Table I. As the drug substance itself and probably also the related substances are considered to be narcotics, the exchange of reference substances between countries may be difficult or even impossible owing to national and international restrictions. Hence it would be useful to have at the disposal of the authorities a method that allows the investigation of the content of related substances without the use of reference substances.

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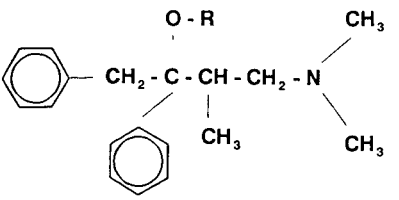
TABLE I

STRUCTURES OF DEXTROPROPOXYPHENE AND TWO POSSIBLE IMPURITIES



No.	Substance	R
I	Dextropropoxyphene	$-COCH_2CH_3$
II	Acetoxy compound	$-COCH_3$
III	Carbinol compound	-H

A separation method based on high-performance liquid chromatography (HPLC) with chemically bonded ODS-silica as the column material is an obvious possibility for a basic drug substance such as dextropropoxyphene. Previously published methods using bonded-phase materials as the column packing has not been specifically intended for purity testing of the raw material [3–5]. Further, the selectivity of such chromatographic systems has previously been shown to depend strongly on the brand of column material [*e.g.*, 6, 7] and, therefore, a suitability test will most often be needed involving the use of one or more reference substances.

This present investigation was performed with a view to developing an HPLC method suitable for separating and determining possible impurities in dextropropoxyphene hydrochloride for pharmacopoeial purposes and without the need for reference substances. For this purpose, the dynamically modified silica approach was chosen. This technique has been shown to be independent of the brand of column material [7].

EXPERIMENTAL

Chemicals

Samples of dextropropoxyphene hydrochloride were of pharmacopoeial quality. Dextropropoxyphene impurities were B.P. Chemical Reference Substances. All reagents were of analytical-reagent grade from E. Merck (Darmstadt, F.R.G.).

Chromatography

A liquid chromatograph consisting of a Model 410 LC pump (Kontron, Zürich, Switzerland), a Model 2012 spectrophotometer detector (Cecil, Cambridge, U.K.) operated at 220 nm and a Model 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.) equipped with a 20-μl loop was used. Chromatograms were recorded on a Model BD 8 recorder (Kipp and Zonen, Delft, The Netherlands) and retention data were measured and processed by means of a Model 3353A laboratory data system (Hewlett-Packard, Cuperline, CA, U.S.A.).

All experiments were performed on columns of 120 × 4.6 mm I.D. (Knauer, Oberursl, F.R.G.) packed by the dilute slurry technique with silica or chemically

bonded octadecylsilyl (ODS) silica of 5 μm particle size. For chromatography on dynamically modified silica the eluents were mixtures of methanol, acetonitrile or tetrahydrofuran with water and a fixed amount (5%) of 0.2 *M* potassium phosphate buffer (pH 7.5), and with the addition of 2.5 m*M* cetyltrimethyl ammonium (CTMA) bromide. The columns were equilibrated by eluting overnight. During chromatography, the analytical column was guarded by a silica precolumn situated between the pump and the injection valve. Following each series of experiments the column was brought to its initial state by rinsing with methanol–0.05 *M* nitric acid (1:1) and finally with methanol. For bonded-phase chromatography the eluent was acetonitrile–0.2 *M* sodium perchlorate (adjusted to pH 2.0 with hydrochloric acid) (40:60).

Test and standard solutions

For impurity testing, 0.5% solutions of the individual samples of dextropropoxyphene hydrochloride in the eluent were used. A 0.0025% solution of dextropropoxyphene hydrochloride in the eluent was used as an external standard. Volumes of 20 μl of each solution were injected.

For system suitability testing, 50 mg of dextropropoxyphene hydrochloride were boiled for 30 min in 5.0 ml of 1 *M* alcoholic potassium hydroxide, after cooling 2.5 ml of 2 *M* hydrochloric acid were added and the mixture was diluted to 50 ml with eluent.

RESULTS AND DISCUSSION

The HPLC method was elaborated using a sample of dextropropoxyphene hydrochloride (I) containing two potential impurities (II and III). Several parameters will influence the retention and selectivity in a chromatographic system based on the dynamically modified silica approach [7–9]. The starting point in choosing the actual eluent was a previously used mixture, methanol–water–0.2 *M* potassium phosphate buffer (pH 7.5) (50:45:5) containing 2.5 m*M* CTMA [8]. The separation of III and dextropropoxyphene hydrochloride using this eluent was insufficient. On replacing 50% of methanol by 30% of acetonitrile, resulting in an isoeluotropic eluent, the shape of the dextropropoxyphene peak became unacceptably poor. Also, the use of tetrahydrofuran as the organic modifier proved unsuccessful. It was not possible to achieve a sufficient separation unless a percentage of tetrahydrofuran was used that resulted in an unacceptably long retention time. Furthermore, problems were encountered in dissolving the drug substance in such eluents.

Optimization of eluent compositions by using ternary mixtures of solvents has been described previously in chromatography on chemically bonded phases by Schoenmakers *et al.* [10] and also in reversed-phase chromatography on dynamically modified silica by Hansen and Helboe [9]. When performing optimization experiments using methanol at concentrations between 0 and 50% and tetrahydrofuran at concentrations between 32 and 0%, the optimum mixture turned out to be methanol–tetrahydrofuran–water–0.2 *M* potassium phosphate buffer (pH 7.5) (350:84:516:50).

In order to compare possible variations in selectivity using different brands of column materials to those of a chromatographic system based on the use of chemically bonded ODS-silica, a separation method utilizing such column materials was required. The method used is a proposal from the BP Laboratory discussed during the elaboration of a draft monograph on dextropropoxyphene hydrochloride for the

TABLE II

SEPARATION FACTORS (α) BETWEEN DEXTROPROPOXYPHENE AND TWO POSSIBLE IMPURITIES MEASURED ON SIX DIFFERENT SILICA COLUMNS AND FIVE DIFFERENT ODS-SILICA COLUMNS

Column material	Separation factor	
	II	III
Bare silica		
LiChrosorb Si 100	1.69	1.23
Spherisorb S 5 W	1.63	1.27
Partisil 5	1.64	1.32
Zorbax SIL	1.66	1.29
Nucleosil 50-5	1.63	1.28
Nucleosil 100-5	1.62	1.20
ODS-silica		
LiChrosorb RP-18	1.22	1.34
Hypersil ODS	1.30	1.42
Spherisorb ODS-1	1.35	1.44
Spherisorb ODS-2	1.49	1.49
Partisil ODS-3	1.49	1.70

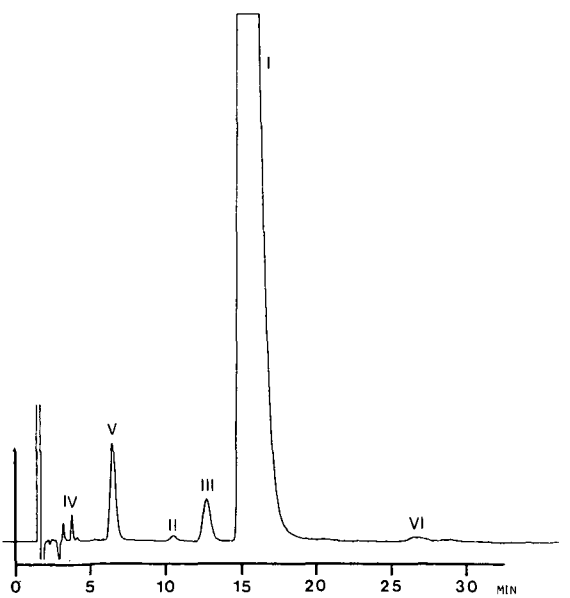


Fig. 1. Chromatogram of a sample of dextropropoxyphene hydrochloride solution that had been stored for several days. Column, Partisil 5 (120 × 4.6 mm I.D.); eluent, methanol–tetrahydrofuran–water–0.2 *M* potassium phosphate buffer (350:84:516:50) containing 2.5 m*M* CTMA; detection wavelength, 220 nm (0.1 a.u.f.s.); flow-rate, 1 ml/min. Peaks I–III as in Table I; IV–VI = unknown impurities.

European Pharmacopoeia [11]. The eluent for this chromatographic system was acetonitrile–0.2 *M* sodium perchlorate (pH 2.0) (40:60).

The two separation methods were tested using six different brands of bare silica and five different brands of ODS-silica. The selectivities of the individual chromatographic systems towards a mixture of dextropropoxyphene hydrochloride and impurities II and III as expressed by the separation factor of each compound from the dextropropoxyphene peak are given in Table II. It can be seen that the variations in selectivity of the system based on bonded-phase materials are considerably larger than those of the dynamically modified silica system, and in one instance the two impurity peaks coincide. This is in accordance with experience from previous investigations on the determination of impurities in propranolol [12] and on separations of corticosteroids [13]. The possible lack of separation of the two impurities in the bonded-phase system might constitute a serious drawback with regard to the requirement for the content of related substances to be expressed as is often done in pharmacopoeial monographs, *e.g.*, "not more than 0.5% of each individual impurity". A batch containing the allowed amount of each of II and III would appear in the test as containing 1% of a single impurity, hence the batch would be rejected, Another drawback to the bonded-phase system is that the order of elution of the two possible impurities has reversed in comparison with that in the dynamically modified silica system (see below).

In Fig. 1 shows a chromatogram of a sample of dextropropoxyphene hydrochloride that had been stored in solution at room temperature for several days. Apart from the known impurities (II and III), several unknowns are seen. Unknown V was found in detectable amounts only in stored solutions and, further the peak corresponding to carbinol impurity III increases on standing. Therefore, sample solutions should be prepared fresh daily. It appears that the peak corresponding to impurity III is closer to the main peak than that corresponding to II. As impurity III is a degradation product, it may be prepared directly from the sample to be investigated (see Experimental) and used for a suitability test, as the order of elution in a chromatographic system based on dynamically modified silica is independent of the brand of column material used. This possibility is not available when using the chromatographic system based on bonded-phase material, as impurity II, the one originating solely from the route of synthesis, is that which elutes most closely to the main peak.

Using the chromatographic system based on dynamically modified silica as described above, a series of samples of dextropropoxyphene hydrochloride were analysed. For quantification, external standardization relative to a dilute solution of the sample to be investigated was used. Linearity of the detector response for the external standard was ensured up to a content corresponding to 2% of the amount in the main peak in the chromatogram of the test solution, injecting amounts ranging between 0.5 and 10 μg ($n = 5$), $y = 1.05x + 0.05$ ($r = 1.0000$). The limit of quantification was *ca.* 0.2 μg (corresponding to 0.05% of the amount in the main peak). The response factor of impurities II and III was shown to be comparable to that of dextropropoxyphene hydrochloride in the range 215–220 nm. Hence the amount of impurity can be taken as that determined relative to the external standard, which is a dilution of dextropropoxyphene hydrochloride. The absorption maximum for compounds I–III is *ca.* 215 nm. However, the detection wavelength used was 220 nm in

TABLE III

RESULTS OF THE ANALYSIS OF EIGHT SAMPLES OF DEXTROPROPOXYPHENE HYDROCHLORIDE

Sample	Amount of impurity (%)[a]	
	II	III
A	0.15	0.05
B	0.18	0.05
C	–	–
D	0.12	0.05
E	0.17	0.05
F	–	–
G	–	0.05
H	–	–

[a] Each determination was performed in triplicate.

order to improve the signal-to-noise ratio, as 215 nm is close to the UV cut-off limit of the eluent.

The precision of the method was investigated by analyzing separately ten portions of a 0.0025% solution of dextropropoxyphene hydrochloride (corresponding to a 0.5% impurity level). The relative standard deviation was 0.9%. The results of the analyses are given in Table III.

CONCLUSION

An HPLC method based on dynamically modified silica has been developed and shown to be suitable for the separation and determination of possible impurities in dextropropoxyphene hydrochloride. The efficiency of the column can be ensured by means of a system suitability test without using any reference substances. The method implies a requirement on the minimum resolution between the peak corresponding to the substance to be examined and a degradation product which can be prepared *in situ*. Hence the method has been shown to be superior to an alternative HPLC method based on chemically bonded ODS-silica, as the latter exhibited large brand-to-brand variations in selectivity towards a test mixture containing dextropropoxyphene hydrochloride and two possible impurities. The method is, therefore, deemed well suited for pharmacopoeial purposes.

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Short Communication

High-performance liquid chromatography of biogenic amines in the corpus cardiacum of the American cockroach, *Periplaneta americana*

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ABSTRACT

The simultaneous determination of biogenic amines in the corpus cardiacum of the American cockroach, *Periplaneta americana*, was carried out using high-performance liquid chromatography with a Neurochem neurochemical analyser. Vanillic acid, dopamine, octopamine and tyramine were detected. Tyrosine and tryptophan were also detected at high levels. Octopamine levels in the corpus cardiacum were increased on injection of an acetone solution. The biological function of the biogenic amines detected is discussed.

INTRODUCTION

In insects, the corpora cardiaca are the principal neurohemal organs. These glands both store and secrete neurosecretory products synthesized in the brain and contain intrinsic cells which produce their own neurosecretory material [1]. In the corpus cardiacum of locusts, biogenic amines have been detected by a radiochemical enzymatic assay [2,3] and by high-performance liquid chromatography (HPLC) [4]. However, there are only two reports on the measurement of biogenic amines in the corpora cardiaca of the cockroach [5,6]. Recently, HPLC with electrochemical detection (ED) using coulometric electrodes has been used successfully to determine catecholamines in some samples from discrete regions of the insect nervous system [7–13].

In this work, we investigated amines in the corpus cardiacum of the American cockroach, *Periplaneta americana*.

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EXPERIMENTAL

Insects

Adult male cockroaches were taken from a colony of *Periplaneta americana* maintained under a 13 h light–11 h dark photoperiod at 26°C and a relative humidity of 65%. They were reared on a pelleted diet and water. Insects were taken between 1 and 5 months after the final moult and divided into two groups of ten 24 h prior to dissection. One group was in the normal state and the other group was subjected to chemical stress.

Injection

As a chemical stressor, 2 μl of acetone were injected into the abdominal haemocoel through the abdominal intersegmental integument between the second and third segments using a microlitre syringe. Thirty minutes after injection, the corpora cardiaca were dissected.

Preparation of corpora cardiaca

The corpora cardiaca of ten male adults were carefully dissected under ice-cold physiological saline [240 m*M* NaCl–2.7 m*M* KCl–3.6 m*M* $CaCl_2$–2.38 m*M* N-2-hydroxyethylpiperazine-N′-2-ethansulphonic acid (HEPES)]. Ten glands from ten animals were placed in 200 μl of ice-cold 0.1 *M* HCl and then homogenized with hand microhomogenizer (glass). Following centrifugation at 10 000 *g* for 10 min, the supernatant was filtered and dried using a rotary evaporator under vacuum. These samples were dissolved in 0.4 *M* perchloric acid (PCA) and aliquots were injected onto the HPLC column. The recovery efficiency was determined by extraction of test samples and the addition of an internal standard followed by analysis. It was found that the recovery was higher than 95% through the extraction, centrifugal filtration (UFC3 OHV, Millipore) and drying process.

HPLC with electrochemical detection (ECD)

The HPLC system used was a Neurochem neurochemical analyser (ESA, Bedford, MA, U.S.A.) consisting of a gradient HPLC system and sixteen high-sensitivity coulometric electrochemical detectors coupled with a compatible computer. The concept and inherent advantages of multi-electrode HPLC systems have been described elsewhere [14,15]. The Neurochem analyser was set to run a mixed linear and step gradient with a reversed-phase C_{18} column, allowing the separation of 24 compounds.

Mobile phase A was 0.1 *M* sodium phosphate containing 10 mg/ml of sodium dodecyl sulphate at pH 3.35 and mobile phase B was methanol–water (1:1, v/v) containing 50 mg/l of sodium dodecyl sulphate at pH 3.45. The sixteen serial electrodes were set in an incremental 60-mV array from 0 to 900 mV. The column and electrodes were maintained at 34°C throughout the run. Data from each electrode were collected on the computer and stored to hard disk for post-run analysis. Each compound would typically be detected on three electrodes, with an average ratio of peak heights between the electrodes of 1:6:1. However, the exact ratio was specific for each compound and could be used to establish the purity of the compound in unknown peaks in the sample eluting from the column at the same time as a known standard.

Special compound recognition algorithms are used with the Neurochem analyser which could match standards with unknowns using both retention time and ratios across the electrodes on which the compound was detected. Final concentration data were calculated based on a comparison of the peak height on the dominant electrode of a known standard with that of dominant unknown peaks in the sample.

RESULTS AND DISCUSSION

Dopamine (DA), tyramine (TYRA), vanillic acid (VA) and octopamine (OA) were detected in the corpus cardiacum (Fig. 1A and B). As amino acids, high levels of tyrosine (TYR-4) and tryptophan (TRP) were also detected. Table I gives the biogenic amine levels in the carpus cardiacum. The levels of DA are considerably lower than those reported for the *Locusta* corpus cardiacum [4] (this level is also considerably higher than that reported by Lafon-Cazal [16]). The OA levels in *P. americana* are similar to those in *Locusta* reported by other workers [16,17]. OA appears to be the neurotransmitter mediating the release of the hyperlipaemic hormone from these glandular cells of the locust [17]. Downer *et al.* [5] suggested that DA is present in the corpus cardiacum of the American cockroach, *Periplaneta americana.*

On treatment with acetone, the levels of DA, TYRA and OA were dramatically increased, whereas the VA level decreased (Fig. 1C; Table I). There is no report on the measurement of the biogenic amine levels in the corpus cardiacum following injection of a chemical stressor. OA, one of these amines, has a neurohormonal role in insects. In *Schistocerca gregaria* the concentration of octopamine in the heamolymph increased drastically during the first 10 min of flight [18] and in response to stress

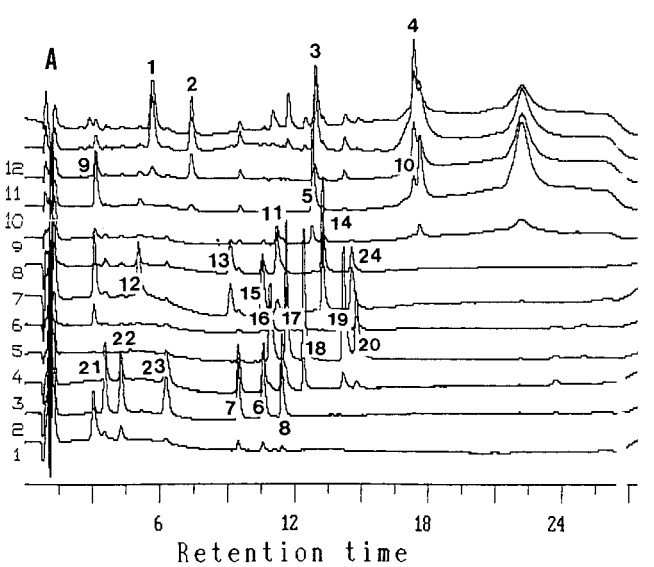

Fig. 1.

(Continued on p. 196)

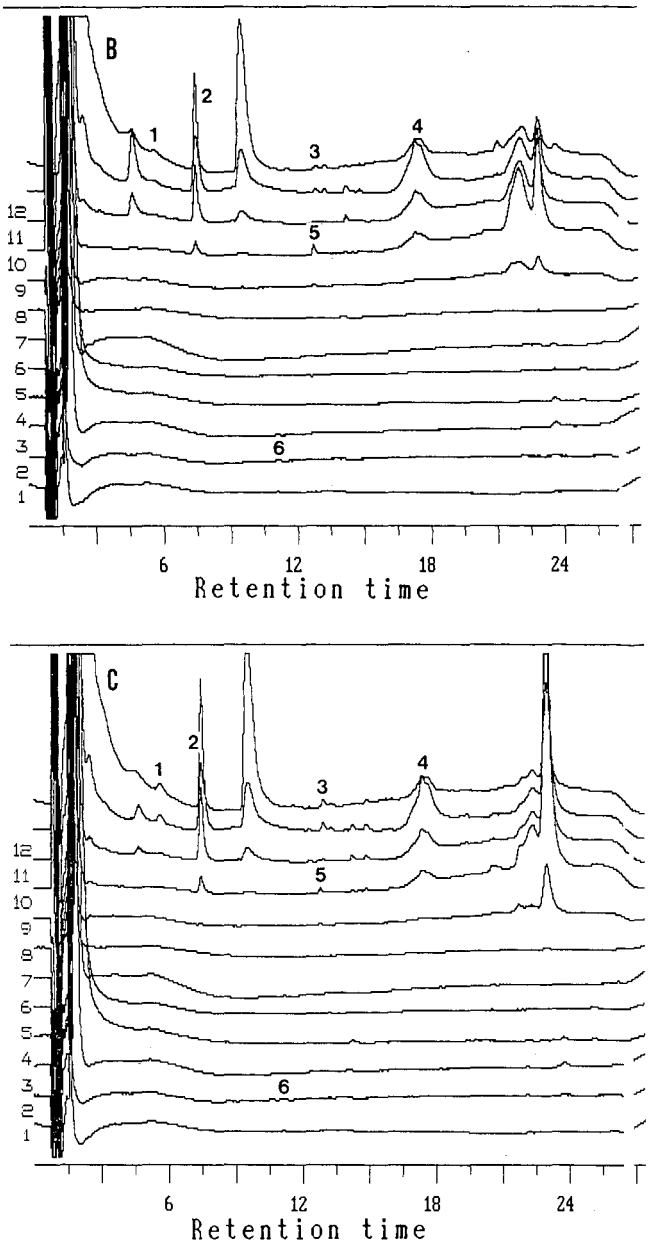

Fig. 1. Chromatograms of (A) standard, (B) sample of the normal carpora cardiaca and (C) sample of carpora cardiaca following injection of 2 μl of acetone solution. Peaks: 1 = octopamine (OA); 2 = tyrosine (TYR-4); 3 = tyramine (TYRA); 4 = tryptophan (TRP); 5 = vanillic acid (VA); 6 = dopamine (DA); 7 = 3,4-dihydroxyphenylacetic acid (DOPAC); 8 = epinine (EPIN); 9 = 4-hydroxy-3-methoxymandelic acid; 10 = melatonin; 11 = metanephrine; 12 = methoxyhydroxyphenyl glycol; 13 = normetanephrine; 14 = homovanillic acid; 15 = methyldopa; 16 = hydroxytyptophan; 17 = hydroxyindoleacetic acid; 18 = acetylserotonin; 19 = serotonin; 20 = methylserotonin; 21 = norepinephrine; 22 = dopa; 23 = epinephrine; 24 = methoxytyramine. Retention time in min.

TABLE I

BIOGENIC AMINE LEVELS IN THE CORPUS CARDIACUM OF *PERIPLANETA AMERICANA*

Treatment	Amount found in corpus cardiacum (pg)							
	TYR-4	DA	TYRA	DOPAC	VA	TRP	EPIN	OA
Untreated	2031.000 ± 22.20	8.100 ± 1.90	35.700 ± 10.70	N.D.[a]	113.400 ± 0.20	147.600 ± 0.00	N.D.	43.100 ± 15.50
Acetone	2655.300 ± 92.10	25.200 ±0.00	121.800 ± 0.00	N.D.	49.200 ± 1.20	N.D.	N.D.	151.100 ± 0.90

[a] N.D. = Not detected.

[19,20]. Under these conditions, circulating OA may act as a sympathetic or stress hormone, increasing the availability of carbohydrate and lipid and stimulating the oxidation of these substances [21]. However, our finding is the first report that the OA, DA and TYRA contents per single corpus cardiacum increased on treatment with acetone solution in comparison with that of a control. As OA in the ganglion is synthesized via TYRA from TYR-4 in the central nervous system of *Manduca sexta* [22]. OA-involved enzyme activity in the ganglion under the influence of a chemical stressor such as acetone may be high. It would be interesting to investigate the induction of OA-involved enzyme activity by chemical stressors.

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Short Communication

Rapid detection and quantification of peroxidase activity in liquid chromatography

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ABSTRACT

A rapid and simple method for the detection and quantification of peroxidase activity is described. The method is based on the visible development of a brownish colour due the reaction of peroxidase in the presence of guaiacol and hydrogen peroxide. The main feature is the detection and quantification of a considerable number of samples at once, instead the spectrophotometric standard methods usually used for this purpose, especially in fractions obtained after liquid chromatography. The effect of substrate concentration and buffer composition on peroxidase determination by the proposed method was investigated.

INTRODUCTION

Detection of enzyme activity during the isolation and chromatographic purification of peroxidases usually relies on spectrophotometric determinations using cuvettes [1–10]. Although very convenient, such methods are laborious and time consuming for routine analysis, especially when the number of samples is large. An alternative and rapid procedure is described here, based on the two-fold serial dilution procedure commonly applied for haemagglutination in microtitre plates. Thus, qualitative and quantitative assays of peroxidase activity can be done directly with observation with the naked eye and automated expensive instruments are not required. Development of a brownish colour, due to the reaction of guaiacol and hydrogen peroxide with the peroxidases, indicates a positive reaction. The quantification is then expressed as the titre of activity, which is defined as the dilution factor of the fraction showing a capacity to develop the brownish colour in a given time. The test was shown

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to be reliable and reproducible in the range 0.5–100 μg/ml of protein and stable for at least 30 min at room temperature.

EXPERIMENTAL

Materials

Microtitre plates (96 wells, rigid U-bottom), microdiluter (Multi-Microdiluter handle) and pipette droppers were purchased from American Scientific Products (McGaw Park, IL, U.S.A.), Sephacryl S-200 superfine, CM Sepharose Cl-4B, bovine serum albumin and ribonuclease A from Pharmacia (Uppsala, Sweden), guaiacol, horseradish peroxidase (HRP) and other chemicals from Sigma (St. Louis, MO, U.S.A.) and hydrogen peroxide (20 volumes, 1.78 *M*) from a local drugstore.

Chromatography

Size-exclusion chromatography was performed on Sephacryl S-200 superfine with a column (94 × 1.6 cm I.D.) equilibrated with 0.1 *M* acetate buffer (pH 5.0) and a sample size of 6 ml of a protein mixture (horseradish peroxidase, bovine serum albumin and ribonuclease A, 2 mg each) at a flow-rate of 45 ml/h. Fractions of 2 ml were collected.

Ion-exchange chromatography was carried out on a CM Sepharose Cl-4B column (20 × 2.6 cm I.D.) with a bed volume of 50 ml. The equilibrium buffer was 0.02 *M* acetate buffer (pH 5.0) and the sample size was 1 ml of a protein mixture of 2 mg of horseradish peroxidase and 1 mg of lysozyme. After washing with two bed volumes of the equilibrium buffer, the molar concentration was increased linearly from 0.02 to 1.0 *M* in five bed volumes. The flow-rate was constant at 47 ml/h and the fraction size was 10 ml.

Qualitative detection of peroxidase activity

A 25-μl volume of 80 m*M* guaiacol solution [prepared in 0.1 *M* acetate buffer (pH 5.0)] were poured into the wells of a microtitre plate, then 25 μl from each chromatographic fraction and finally 25 μl of hydrogen peroxide solution (22.25 m*M*) were added to the wells so that the final volume per well was 75 μl. The plate was incubated at room temperature for 5 min and the development of colour was observed. Colourless wells indicated the absence of activity and, therefore, were considered to be negative. Blank controls (without enzyme) were processed with acetate buffer.

Quantification of peroxidase activity

On a separate plate containing 25 μl of 80 m*M* guiacol solution in each well, 25 μl from the peroxidase-positive fractions were poured into the first line of wells (A). Next, the two-fold serial dilution procedure was carried out with the aid of a 25-μl microdiluter (Multi-Microdiluter handle) from the first row of wells towards the last row at the end of the plate (H). This procedure allowed a consecurive two-fold dilution process with dilution factors from 1/2 to 1/256 to be obtained in a simple manner. Finally, 25 μl of hydrogen peroxide (22.25 m*M*) were added and the titre of activity was determined after incubation for 5 min at room temperature. The reciprocal of the highest dilution showing colour development was considered to be the activity titre. This number is usually plotted in $\log_2$ form to obtain a linear graph.

Effect of substrate concentration and buffer composition on peroxidase assay

Following the previous method, different solutions of guaiacol (2.5, 5, 10, 20, 40, 80 and 160 m*M*) were used to measure the titre of a stock solution of horseradish peroxidase (*ca.* 15 μg/ml), using a constant final concentration of hydrogen peroxide (11.12 m*M*). In the same way, different concentrations of hydrogen peroxide (2.78, 5.56, 11.12, 22.25, 44.5, 89 and 890 m*M*) with a constant final concentration of guaiacol (40 m*M*) were tested. The buffer composition was also varied using different proportions of calcium and manganese chloride (0, 2.5, 5, 10, 25, 50, 75 and 100 m*M*), and the effects of sodium chloride, ammonium sulphate, methyl α-D-glucopyranoside, sodium sulphate (0, 0.05, 0.1, 0.2, 0.3, 0.5, 0.75 and 1.0 *M*), isopropanol, sodium dodecyl sulphate (SDS), Triton X-100 (0, 0.5, 1, 2, 4, 7.5 and 10%) and ethylene glycol (0, 2.5, 5, 10, 20, 40 and 50%) were also studied by mixing them with the hydrogen peroxide solution, valuating the effect on the titre determination of a stock solution of horseradish peroxidase.

Stability of horseradish peroxidase exposed to different chemicals used in liquid chromatography

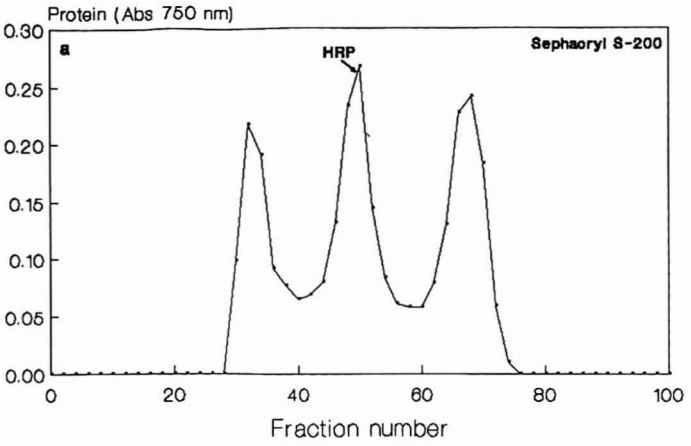
The stability of HRP exposed to different chemicals which could be used during a chromatographic run was studied. One volume of a stock solution of HRP (15 μg/ml) was mixed with one volume of one of the following solutions: calcium chloride (20 m*M*), manganese chloride (200 m*M*); sodium chloride, sodium sulphate, methyl α-D-glucopyranoside, ammonium sulphate (2.0 *M*); isopropanol, SDS, Triton X-100 (20%); ethylene glycol (100%); and acetate (2.0 *M*), phosphate (0.6 m*M*) and citrate (1.0 *M*) buffers (pH 5.0). The mixture was incubated for 5 h at room temperature and then for 16 h at 4°C. The remaining activity was then measured by pouring 50 μl of the corresponding mixture in the empty first line of wells of the assay plate containing 25 μl of 80 m*M* guaiacol. After dilution using the 25-μl microdiluter, 25 μl of hydrogen peroxide solution (22.25 m*M*) were added and the titre was measured as indicated above.

RESULTS AND DISCUSSION

Figs. 1a and 2a, show the distribution of model proteins in size-exclusion chromatography on Sephacryl S-200 superfine and in ion-exchange chromatography on CM Sepharose Cl-4B, respectively.

Peroxidase activity was detected in fractions 35–60 in the size-exclusion experiment (Fig. 1b) and in fractions 2–27 in the ion-exchange chromatographic experiment (Fig. 2b). The higher intensity of the brownish colour also suggested higher peroxidase activity. Obviously this can be evaluated quantitatively using a microplate reader, but the method proposed here can be carried out without such apparatus. Fig. 3a shows the titre of the peroxidase-positive fractions (35–60) from the gel filtration run. The maximum activity corresponded to the HRP peak in Fig. 1a. The corresponding distribution pattern of peroxidase activity was obtained and plotted as the $\log_2$ (titre) against the fraction number (Fig. 3b) in order to obtain a linear plot.

Fig. 4a shows the titre of the peroxidase-positive fractions (2–27) from the ion-exchange chromatographic experiment, where two peaks could be detected. The first (fractions 2–10) corresponded to the unadsorbed material containing peroxidase,



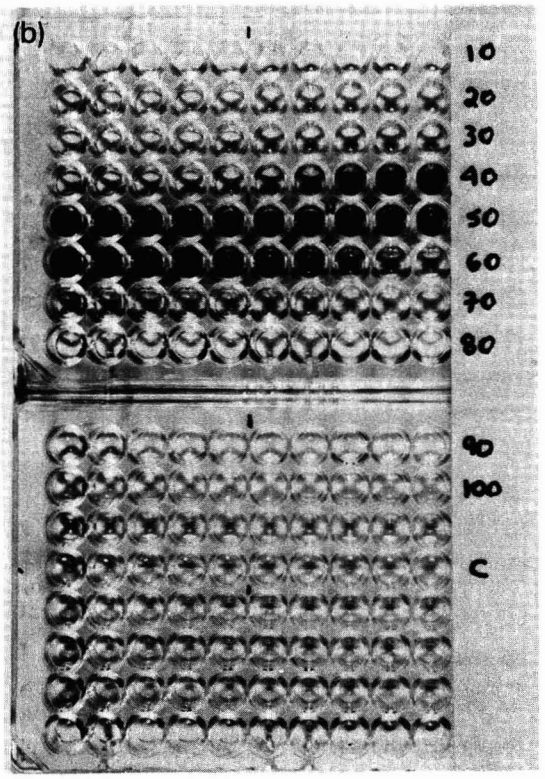


Fig. 1. Size-exclusion chromatography on Sephacryl S-200 of a model protein mixture containing bovine albumin (MW = 65 000), horseradish peroxidase (HRP) (MW = 40 000), and ribonuclease A (MW = 13 700). (a) Protein elution pattern. Protein detection by the Lowry method [11]. (b) Detection of peroxidase-positive fractions. Photograph taken 2 min after the addition of the hydrogen peroxide solution.

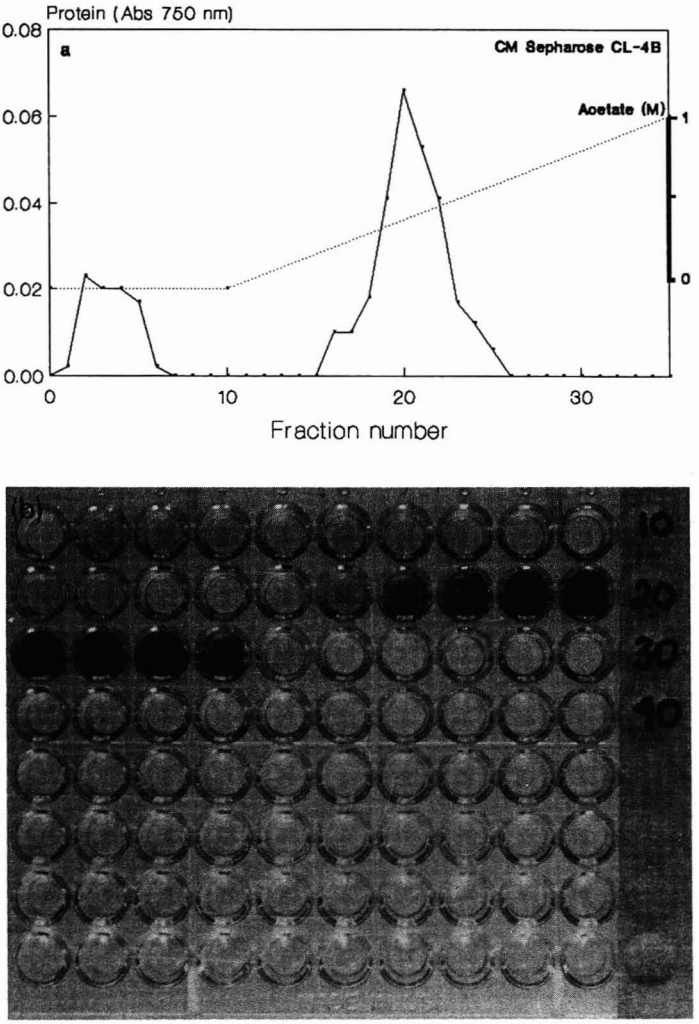


Fig. 2. Ion-exchange chromatography on CM Sepharose CL-4B of a model protein mixture containing horseradish peroxidase (HRP) and lysozyme. (a) Protein elution pattern. Protein detection by the Lowry method [11]. (b) Detection of peroxidase-positive fractions. Photograph taken 1 min after the addition of the hydrogen peroxide solution.

and the second (fractions 14–27) represented the main HRP fraction. The activity distribution also corresponded to the protein bands detected (Fig. 2a). The pattern of elution of the peroxidase activity is shown in Fig. 4b.

The titre of a stock solution of HRP (*ca.* 15 μg/ml) is dependent on guaiacol concentration, being maximum at 40 m*M*. The effect of different concentrations of hydrogen peroxide, on the other hand, is two-fold; it increases colour development up to 22.25 m*M*, above which there is a notable inhibitory effect, reflected in a reduced titre.

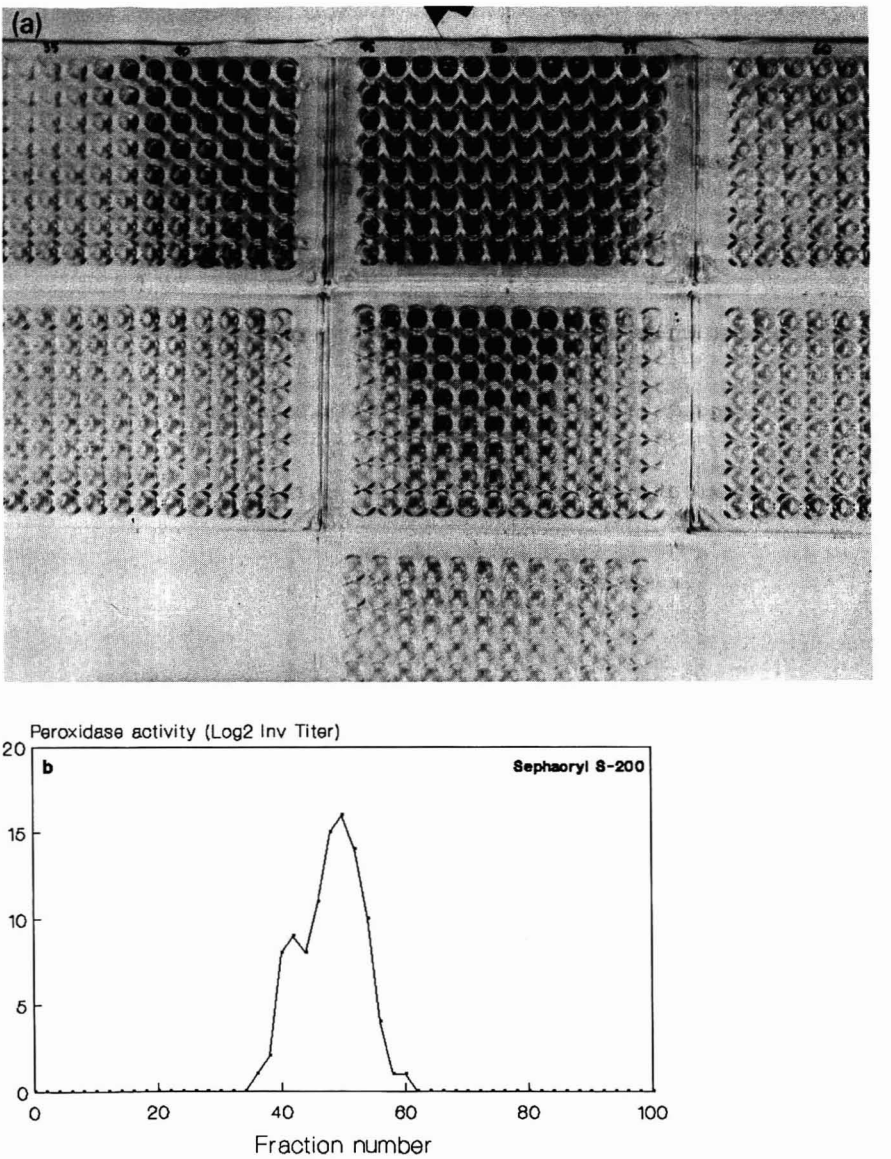
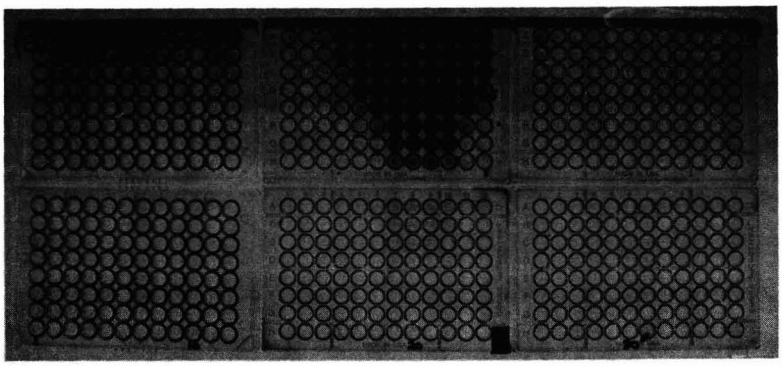


Fig. 3. Quantification of activity of the peroxidase-positive fractions from the size-exclusion run. (a) Titre determination in a microtitre plate. Photograph taken 5 min after the addition of the hydrogen peroxide solution. (b) Elution pattern of the peroxidase activity.

The positive role of calcium in peroxidase activity has been reported previously [12,13]. Therefore, the presence of 25 m*M* of calcium in the reaction mixture induced an increase in the peroxidase titre (2^{10}–2^{12}); however, at higher concentrations the titre was reduced. Interestingly, preincubation of HRP with calcium diminished the requirement for this cation for maximum activity (10 m*M*).



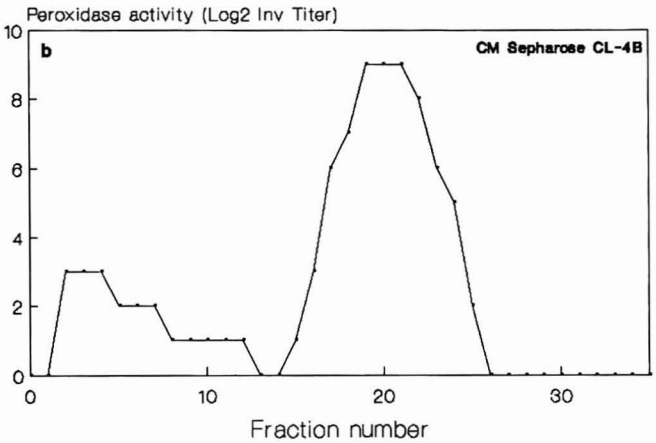


Fig. 4. Quantification of activity of the peroxidase-positive fractions from the ion-exchange run. (a) Titre determination in a microtitre plate. Photograph taken 5 min after the addition of the hydrogen peroxide solution. (b) Elution pattern of the peroxidase activity.

TABLE I

EFFECT OF DIFFERENT CHEMICALS ON THE TITRE DETERMINATION OF PEROXIDASE ACTIVITY

HRP stock solution (*ca.* 15 μg/ml).

Chemical final concentrations	Activity [$\log_2$ (inverse titre)]
Calcium chloride 0, 2.5, 5, 10, 25, 50, 75, 100 m*M*	10, 10, 10, 10, 12, 11, 11, 10
Sodium chloride 0, 0.05, 0.1, 0.2, 0.3, 0.5, 0.75, 1 *M*	10, 10, 10, 10, 10, 9, 9, 8
Ammonium sulphate 0, 0.05, 0.1, 0.2, 0.3, 0.5, 0.75, 1 *M*	10, 10, 10, 9, 9, 9, 9, 8
Sodium sulphate 0, 0.05, 0.1, 0.3, 0.5, 0.75, 1 *M*	10, 10, 10, 10, 10, 10, 10
Isopropanol 0, 1, 2, 4, 5, 7.5, 10%	10, 10, 10, 10, 10, 10, 10
Methyl α-D-glucopyranoside 0, 0.05, 0.1, 0.3, 0.5, 0.75, 1 *M*	10, 10, 10, 10, 10, 10, 10
Manganese chloride 0, 2.5, 5, 25, 50, 75, 100 m*M*	10, 10, 10, 10, 10, 10, 10
Ethylene glycol 0, 2.5, 5, 10, 20, 40, 50%	10, 10, 10, 10, 10, 7, 5
SDS 0, 0.5, 1, 2, 4, 7.5, 10%	10, 10, 8, 7, 7, 6, 6
Triton X-100 0, 0.5, 1, 2, 4, 7.5, 10%	10, 9, 7, 6, 6, 5, 5

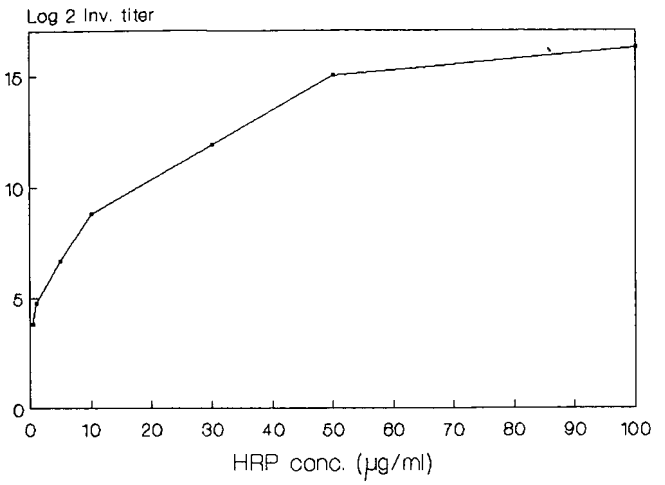


Fig. 5. HRP standard plot. Activity expressed as $\log_2$ (inverse of the titre of HRP solution of different concentration).

The effect on the peroxidase titre of some chemicals frequently used during chromatographic processes was tested (Table I). Sodium chloride, ammonium sulphate and sodium sulphate had little effect on the titre (2^{10}–2^8), even at concentrations as high as 1 *M*. Isopropanol (0–10%), methyl α-D-glucopyranoside (0–1 *M*) and manganese chloride (0–100 m*M*) and also acetate, phosphate and citrate buffers did not show any effect. However, the presence of ethylene glycol at concentrations higher than 20% and of the detergents SDS and Triton X-100 had a negative effect on the titre at concentrations higher than 1%.

The $1/\log_2$ titres of HRP solutions at concentrations of 0.5, 1, 5, 10, 30, 50 and 100 µg/ml were 3.47 ± 0.41, 4.77 ± 0.41, 6.66 ± 0.47, 8.77 ± 0.56, 15.0 ± 0.47 and 16.12 ± 0.60 respectively. The standard plot produced is shown in Fig. 5.

In conclusion, the application of the two-fold serial dilution technique in the chromatographic analysis of peroxidases was found to be convenient and reliable for the rapid detection and quantification of peroxidase activity in a large number of samples, with corresponding savings in time and effort.

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Short Communication

Chiral high-performance liquid chromatography of synthetic pyrethroid insecticides

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ABSTRACT

Fifteen synthetic pyrethroid insecticides were examined by HPLC using a Pirkle column. Complete separation of enantiomer peaks was obtained in almost all cases. Some formulated products were also examined and comments are made about the suitability of the technique for routine analysis.

INTRODUCTION

It is well known that different enantiomers of pesticides and pharmaceuticals can have significantly different biological activity. It therefore seems increasingly likely that national registration procedures will require specification of the enantiomeric content of formulated products [1]. This implies the need for valid analytical methods for enantiomer determination. High-performance liquid chromatography (HPLC) using chiral columns is currently the foremost technique for this purpose though chiral detection, derivatisation with chiral reagents and chiral gas–liquid chromatography all have a role to play.

Cayley and Simpson [2] reported that optical isomers of a variety of synthetic pyrethroid insecticides could be separated by normal-phase HPLC using Pirkle (type 1-A ionic or covalent) columns and the contribution of other workers to this field has been reviewed [3,4]. However, only standard solutions of the pyrethroids have been examined. In the present work the results of Cayley and Simpson have been confirmed, and the range of compounds studied has been extended. Formulated products have also been examined to check the robustness and usefulness of chiral HPLC for these.

EXPERIMENTAL

Pirkle columns (250 × 4.6 mm I.D.) both ionic type 1-A and covalently bonded were obtained from Technicol (Stockport, U.K.). A short guard column of 5-μm

Spherisorb NH_2 (Phase Separations, Deeside, U.K.) was employed. Manual injection was used with a Rheodyne injector, a Pye Unicam PU4010 pump and PU4020 detector set at 230 nm.

Samples of technical pyrethroids and formulated products were obtained as gifts from the manufacturers. Solutions were prepared with about 10 mg of active ingredient in 10–100 ml hexane.

RESULTS

Optimum separation of enantiomers requires careful adjustment of chromatographic parameters. Our preferred experimental conditions and results are summarised in Table I.

Complete separation of the four enantiomers of *d*-allethrin was achieved readily. The racemic *dl*-allethrin could not be fully separated and our findings are in line with earlier work [2]. α-Cypermethrin contains chiefly the two most bioactive *cis* enantiomers of cypermethrin, which are easily separated from each other. This compound is quite a good check-compound to monitor column performance as the colourless crystalline technical material is easy to handle. It was possible to obtain eight peaks from cypermethrin (Fig. 1a) representing an improvement on studies reported in the literature, but the separation was unreliable, and could only be repeated in two consecutive injections before collapsing to seven peaks (Fig. 1b). This may be due to the nature or number of chiral binding sites on the column changing with time. Cyhalothrin and λ-cyhalothrin posed no difficulties yielding four and two peaks respectively. Cyfluthrin yielded eight discernible peaks but overlap of two enantiomer

TABLE I

HPLC SEPARATION OF PYRETHROIDS ON PIRKLE COLUMNS

Compound	Column[a]	Theoretical No. of peaks	No. of peaks observed	Mobile phase[b]	Flow-rate (ml/min)
dl-Allethrin	I	8	7	0.15	0.8
d-Allethrin	I	4	4	0.15	1.5
Cyfluthrin	C	8	8	0.05	1.0
λ-Cyhalothrin	C	2	2	0.15	1.0
Cyhalothrin	C	4	4	0.15	1.0
α-Cypermethrin	C,I	2	2	0.15	1.3
Cypermethrin	I	8	8	0.15	1.3
Fenpropathrin	I	2	2	0.15	0.8
Fenvalerate	C,I	4	4	0.15	2.0
Flucythrinate	C	2	2	0.05	2.0
Flumethrin	C	4	4	0.05	2.0
Permethrin	I	4	4	0.05	0.8
d-Phenothrin	I	2	2	0.05	0.8
Resmethrin	I	4	4	0.05	0.8
Tetramethrin	I	4	4	0.15	2.5

[a] I = Ionic column; C = covalent column. Where both types were used the conditions given are for the ionic column.

[b] Mobile phase is expressed as the percentage of propan-2-ol in hexane.

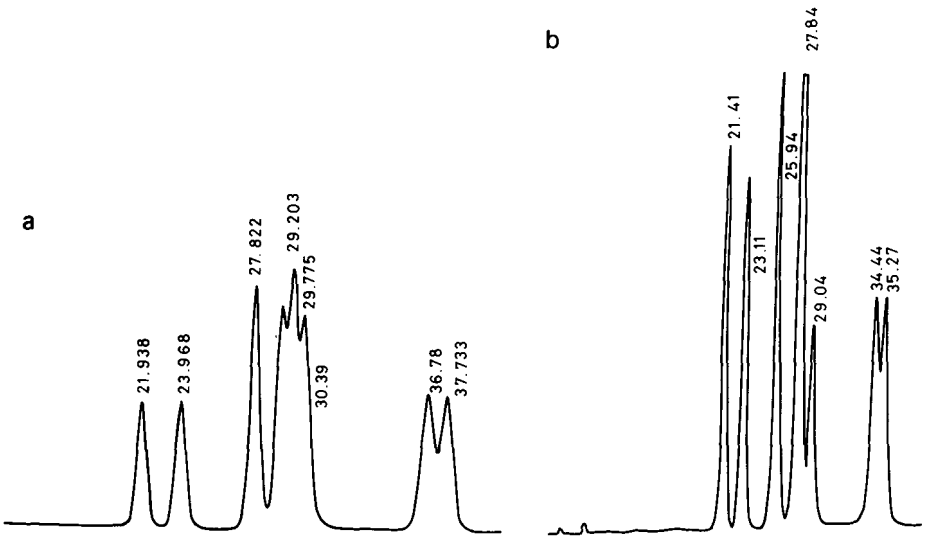

Fig. 1. Chiral separation of cypermethrin enantiomers. (a) 8 peaks; (b) 7 peaks. Pirkle ionic column with Spherisorb NH_2 guard column. Mobile phase: hexane containing 0.15% propan-2-ol, flow-rate 1.3 ml/min. Numbers at peaks indicate retention times in min.

peaks could not be entirely resolved despite long retention times (Fig. 2). Flucythrinate contains two chiral centres but is defined as possessing *S*-stereochemistry in the acid moiety. Two peaks were thus observed. Four peaks were obtained in the analysis of flumethrin, a pyrethroid used in veterinary practice, which contains three chiral centres. It was considered that the sample examined contained only negligible amounts of the four *cis*-isomers obtained. Separation of tetramethrin yielded the theoretical number of peaks.

Formulations of cypermethrin, fenvalerate and permethrin, including wettable powders and emulsifiable concentrates gave similar results to the technical samples,

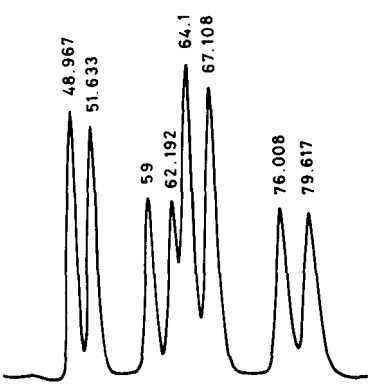

Fig. 2. Chiral separation of cyfluthrin. Pirkle covalent column with Spherisorb NH_2 guard column. Mobile phase: hexane containing 0.05% propan-2-ol, flow-rate 1.0 ml/min. Numbers at peaks indicate retention times in min.

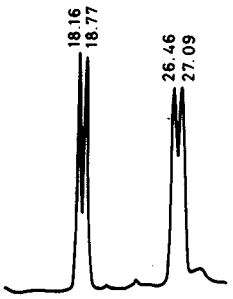

Fig. 3. Separation of enantiomers of formulated permethrin. Pirkle ionic column with Spherisorb NH_2 guard column. Mobile phase: hexane containing 0.05% propan-2-ol, flow-rate 0.8 ml/min. Numbers at peaks indicate retention times in min.

with identical numbers of separated peaks, though the baselines showed more background noise (Fig. 3). In the case of one permethrin formulation, coelution of the synergist piperonyl butoxide with one of the active ingredient enantiomer peaks hindered quantitation.

It was noticed that the commercial ionic column rapidly lost selectivity when exposed to formulations, and required extensive cleaning using hexane–tetrahydrofuran (1:1) and rejuvenation using "Chiral Column Regenerating Solution" (J. T. Baker, Phillipsburg, NJ, U.S.A.) after two weeks of daytime use. It is not known which components of the formulations were responsible for the selectivity loss.

CONCLUSION

Chiral HPLC using Pirkle columns has been shown to effect the separation of a large range of synthetic pyrethroid technical materials and a few formulated products. The columns used may be insufficiently stable for regular analysis of formulated products.

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Short Communication

High-performance liquid chromatographic determination of tentoxin in fermentation of *Alternaria porri* (Ellis) Ciferri

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ABSTRACT

Tentoxin is an interesting non-specific toxin produced by some *Alternaria* species, known as pathogenic organisms. The high-performance liquid chromatographic determination of tentoxin by using a YMCA-312 column, a reversed type of octadecylsilica, with 0.05 *M* ammonium dihydrogenphosphate–acetonitrile (7:3) as the mobile phase is described. The limit of detection is 0.1 μg/ml. One assay can be performed by using culture liquid without any other procedures within 30 min.

INTRODUCTION

In the course of investigations on the bioactive products of *Alternaria porri* (Ellis) Ciferri, the causal fungus of black spot disease in the stone-leek (Japanese name negi) and onion, we have previously reported three reduced anthraquinone derivatives, altersolanol A and B and dactylariol [1], which inhibit elongation of the root in the seeds of lettuce and stone-leek. Altersolanol A also shows antimicrobial activity against some Gram-positive and -negative bacteria [1] and altersolanol B shows high cytotoxicity towards HeLa [2] and Ehrlich ascites carcinoma [3]. Recently, we isolated another bioactive metabolite, tentoxin, a cyclic peptide phytotoxin, from the ethyl acetate-soluble part of the culture liquid of *Alternaria porri* cultured on onion decoction medium [4]. Tentoxin was first isolated by Meyer *et al.* [5] from *A. tenuis*, which is known as a pathogenic organism causing chlorosis in the cotyledons of many

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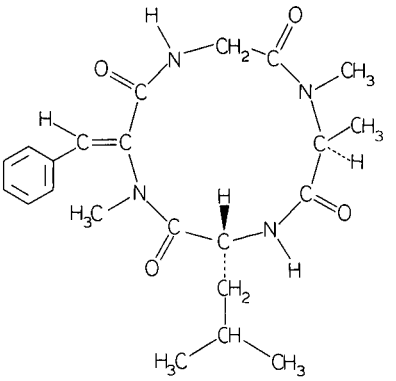

Fig. 1. Structure of tentoxin.

dicotyledonous plant species. The structure (Fig. 1), including the stereochemistry, has also been determined [5,6]. Tentoxin, other than as a metabolite of *A. tenuis*, is also known as a non-specific toxin produced by some *Alternaria* species known as pathogenic organisms, namely *A. mali* [7], *A. citri* [8] and *A. alternata* [9]. From the chemotaxonomic point of view, it is presumed that tentoxin might be found as a metabolite of some *Alternaria* species other than those mentioned above and any fungi related to *Alternaria*. Hence a convenient and accurate method for the determination of tentoxin was required.

Previously, we reported the high-performance liquid chromatographic (HPLC) determination of altersolanol A, macrosporin and alterporriol A, B, C [10] and D and E [11] in the fermentation of *Alternaria porri*. This paper reports the HPLC determination of tentoxin during the period of fermentation when *Alternaria porri* is cultured on onion decoction medium.

EXPERIMENTAL

Material

Tentoxin was isolated as a metabolite of *Alternaria porri* (IFO 9762), which was purchased from the Institute for Fermentation, Osaka, Japan (IFO).

High-performance liquid chromatography

HPLC was performed on a Shimadzu LC-6A liquid chromatograph with a UV detector (Shimadzu SPD-6AV) and integrator (Shimadzu C-R3A) operating at 254 nm in all assays. The solvent system used was 0.05 *M* ammonium dihydrogenphosphate (adjusted to pH 2.5 with phosphoric acid)–acetonitrile (7:3). The column used was a YMC A-312 (Yamamura Chemical Labs., Kyoto, Japan), commercially packed with reversed-phase octadecylsilica (5 μm) (150 mm × 6 mm I.D.). The mobile phase flow-rate was 1.0 ml/min and samples of 10 μl were injected onto the column.

Fermentation and extraction of tentoxin

A 2% (w/v) sucrose solution of onion decoction was used as a culture medium. A number of 500-ml erlenmeyer flasks containing 200 ml of the medium were

sterillized in an autoclave for 20 min at 2.0 bar and 121°C. The fungi, subcultured on an agar medium for 7–10 days, were inoculated into the flasks, which were then kept at 25°C. After fermentation for 2 days, 10 ml of the culture filtrate were extracted with *n*-hexane (4 × 10 ml) to remove lipids. The aqueous layer obtained was called S-2. By a similar procedure, the aqueous layers corresponding to fermentation periods of 5, 7, 14, 21 and 28 days were designated S-3, S-4, S-5, S-6 and S-7, respectively; S-1 was the blank.

RESULTS AND DISCUSSION

Determination of tentoxin during the fermentation period

When the sample of S-6 was used, the chromatograms of tentoxin and the internal standard (I.S.) were as shown in Fig. 2, in which the retention time (t_R) was 17.4 min (tentoxin, capacity factor $k' = 4.3$). We used the internal standard method for quantitation and α-naphthol ($t_R = 21.7$ min, $k' = 9.8$) was used as the internal standard for tentoxin. Methanolic solutions of tentoxin (0.1 mg/ml) (0.4, 0.6, 0.8, 1.0

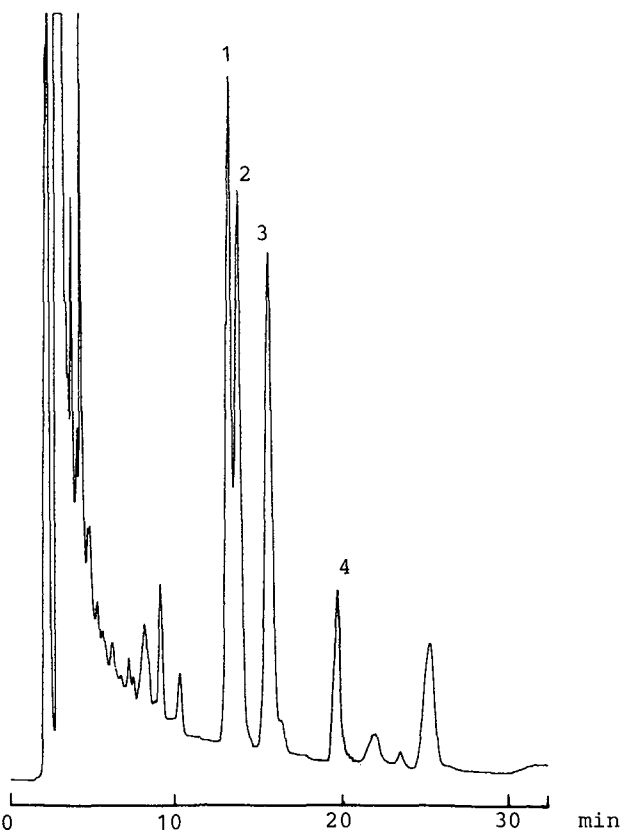

Fig. 2. Chromatograms of tentoxin and I.S. Sample: S-6. Conditions: flow-rate, 1.0 ml/min; detection, UV (254 nm). Peaks: 1 = alterporriol B; 2 = alterporriol A; 3 = tentoxin; 4 = α-naphthol.

and 1.2 ml) were placed in sample vials and 1-ml portions of methanolic solutions of α-naphthol (1 mg/ml) were added. After the volumes had been adjusted to 10 ml with methanol, 10-μl portions of each were subjected to HPLC under the above conditions. By plotting the peak-area ratio against sample weight, a calibration graph for tentoxin was obtained. The limit of detection, based on a signal-to-noise ratio of 10 for tentoxin, was 0.1 μg/ml.

As a practical procedure, α-naphthol (1 mg) was dissolved in each of culture liquids S-1 to S-7 and then 10 μl of each were subjected to HPLC analysis under the above conditions.

The combined results obtained from eight fermentation experiments indicated that tentoxin was detected after fermentation for 5 days and increased continuously for up to 28 days, then levelled off at least for 56 days at 3.3 mg/l.

We conclude that the proposed HPLC method for the determination of tentoxin in fermentation of fungi may be potentially useful, facile and rapid, because it can be carried out by using culture liquid directly without any other procedures and an assay can be completed within 30 min.

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CHROM. 22 854

Short Communication

High-performance liquid chromatographic determination of cephalosporin antibiotics using 0.3 mm I.D. columns

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ABSTRACT

Four cephalosporins, cefazolin, ceftizoxime, cefaloridine and cefaclor, were determined using a novel microbore high-performance liquid chromatographic system designed to be entirely compatible with direct liquid interfacing (DLI) for mass spectrometric analysis. The chromatographic support was a 5-μm C_{18} column of 0.3 mm I.D., compared with the usual microbore column diameters of 1–2 mm. The mobile phase contained no buffers or salts which may have caused column blocking or mobile phase crystallization, and the use of a concentration column allowed the injection of large volumes of analyte (up to 500 μl). The assay was reproducible, the relative standard deviations being less than 20% within-day and between-day for all the drugs. The detection limit for cefaloridine and cefazolin was 1 ng and for cefaclor and ceftizoxime 5 ng.

INTRODUCTION

The occurrence of death due to shock following the use of cephalosporin antibiotics has been encountered in medico-legal practice, hence the development of a high-performance liquid chromatographic–mass spectrometric (HPLC–MS) system for the determination of these drugs in body fluids was necessary. For this purpose, the preferred interface was direct liquid introduction (DLI), which placed various physical and chemical constraints on the properties of the LC system required.

When interfacing mass spectrometers with HPLC systems directly, it is usual to use a splitter valve to reduce the pressure at which the mobile phase enters the spectrometer, resulting in the loss of part of the analyte. An alternative approach is to use super-microbore column chromatography, which allows the analytes to be chromatographed at an extremely low flow-rate, and hence low pressure. In this way, the whole of the mobile phase can be interfaced with the mass spectrometer. This, however, prohibits the use of buffer salts, particularly phosphates, as the width of the tubing linking the various parts of the equipment is so small that blockage caused by

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mobile phase crystallization is a major problem. Further, flow-rates must be kept below a maximum of 10 μl/min to ensure that the pressure does not exceed the acceptable maximum for interfacing with the mass spectrometer.

Conventional HPLC systems for the determination of cephalosporins have been reported. Most of these are limited in the range of drugs which they can determine [1–5], detection by fluorimetry [6] or insufficient detection limits [7–9]. Most of these methods also include the use of phosphate or acetate buffer salts.

One microbore HPLC method for cephalosporins has been reported [10], but this uses phosphate buffer in the mobile phase and no information was given on the UV detection wavelength or detection limits. The advantages of using microbore columns were, however, pointed out; these include a substantial of reduction in the column packing material and solvent consumption (as much as 95%), and a 16-fold increase in sensitivity over conventional columns. In this paper, we report a 10-fold increase in sensitivity over our conventional HPLC procedure [11].

EXPERIMENTAL

Chemicals and reagents

Cefazolin and ceftizoxime were supplied by Fujisawa Pharmaceutical (Japan) and cefaclor and cefaloridine by Shionogi Pharmaceutical (Japan). All solvents were of HPLC grade.

Drug standard solutions

Standard solutions of the pure drugs were made up in distilled water at concentrations of 0.01, 0.05, 0.1, 0.25 and 0.5 μg/ml.

Chromatographic equipment and conditions

The super-microbore HPLC system consisted of two pumps, A and B. Pump A (Milton Roy, U.S.A.) was used to deliver mobile phase A [deionized water–methanol–acetic acid (60:40:0.5)] at a rate of 4 μl/min over the analytical column. Pump B (Jasco, Tokyo, Japan) was used to pass mobile phase B (0.01 *M* ammonium acetate solution adjusted to pH 5 with acetic acid) as the wash solution for the loop and concentration column at a rate of 0.1 ml/min.

The analytical column (15 cm × 0.3 mm I.D.) contained 5-μm ODS C_{18} packing and the concentration column (3 cm × 0.3 mm I.D.) contained 5-μm ODS C_{18} packing. Both columns were supplied by Nomura Chemical (Seto, Japan).

The injection system (Rheodyne) incorporated a 500 -μl fixed loop with a tubing dead volume of 50 μl.

The variable-wavelength UV detector (Jasco) monitored the eluent at 262 nm, and a computer integrator (Shimadzu) was used to collect the chromatographic data.

The tubing used to connect the sections of the system was of 0.05 mm I.D.

Analytical procedure

Accurate volumes of analyte (100 μl) were injected into the loop followed by deionized water (at least 50 μl) to fill the dead volume. The system was such that, in the first instance, mobile phase B was allowed to pass through the loop onto the concentration column, where the analyte was trapped. After 3 min, a switching valve

allowed mobile phase A to pass over the concentration column, causing back-flushing of the analyte from the precolumn onto the main column for determination. The capacity of the loop was 500 μl (maximum injection volume).

Optimization

Mobile phase B was tested at pH 3, 5, 7 and 9. The slightly acidic pH 5 was found to give the best recovery from the concentration column.

The waiting time between delivery of the analyte onto the concentration column and its removal by mobile phase A onto the analytical column was optimized at 3 min (flow-rate 0.1 ml/min). A wait of 5 min resulted in the analyte being completely washed from the column; a wait of 2 min did not allow the system sufficient time to deposit the analyte on the column.

RESULTS AND DISCUSSION

Main analytical column

A typical chromatogram of the four cephalosporins is shown in Fig. 1. The peak shape was excellent for all drugs. The detector response (peak height) was linear for all drugs over the range 0.05–0.5 μg/ml and all graphs passed through the origin (Fig. 2). The retention times were ceftizoxime 5.75, cefaclor 6.71, cefaloridine 7.03 and cefazolin 8.50 min.

The relative standard deviations, both within-day ($n=3$) and between-day ($n=3$), were less than 20% in all instances, and generally the reproducibility increased with increasing concentration, suggesting that the cephalosporins are more unstable at low than at higher concentrations.

The detection limits were 1 ng for cefazolin and cefaloridine on-column (2 ng/ml) and 5 ng for cefaclor and ceftizoxime (10 ng/ml). This is about a 10-fold increase in sensitivity compared with the previously reported conventional HPLC method [11].

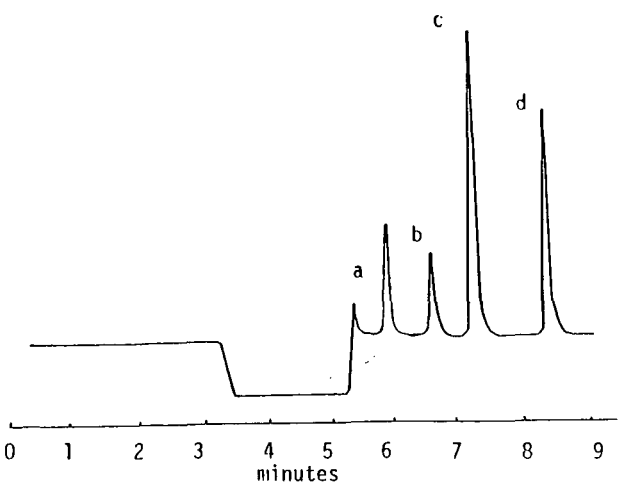

Fig. 1. Cephalosporin standards (0.1 μg/ml; 100 μl). Mobile phase, deionized water–methanol–acetic acid (60:40:0.5); flow-rate, 4 μl/min; detection wavelength, 262 nm; C_{18} column, 15 cm × 0.3 mm I.D. Samples: (a) ceftizoxime; (b) cefaclor; (c) cefaloridine; (d) cefazolin.

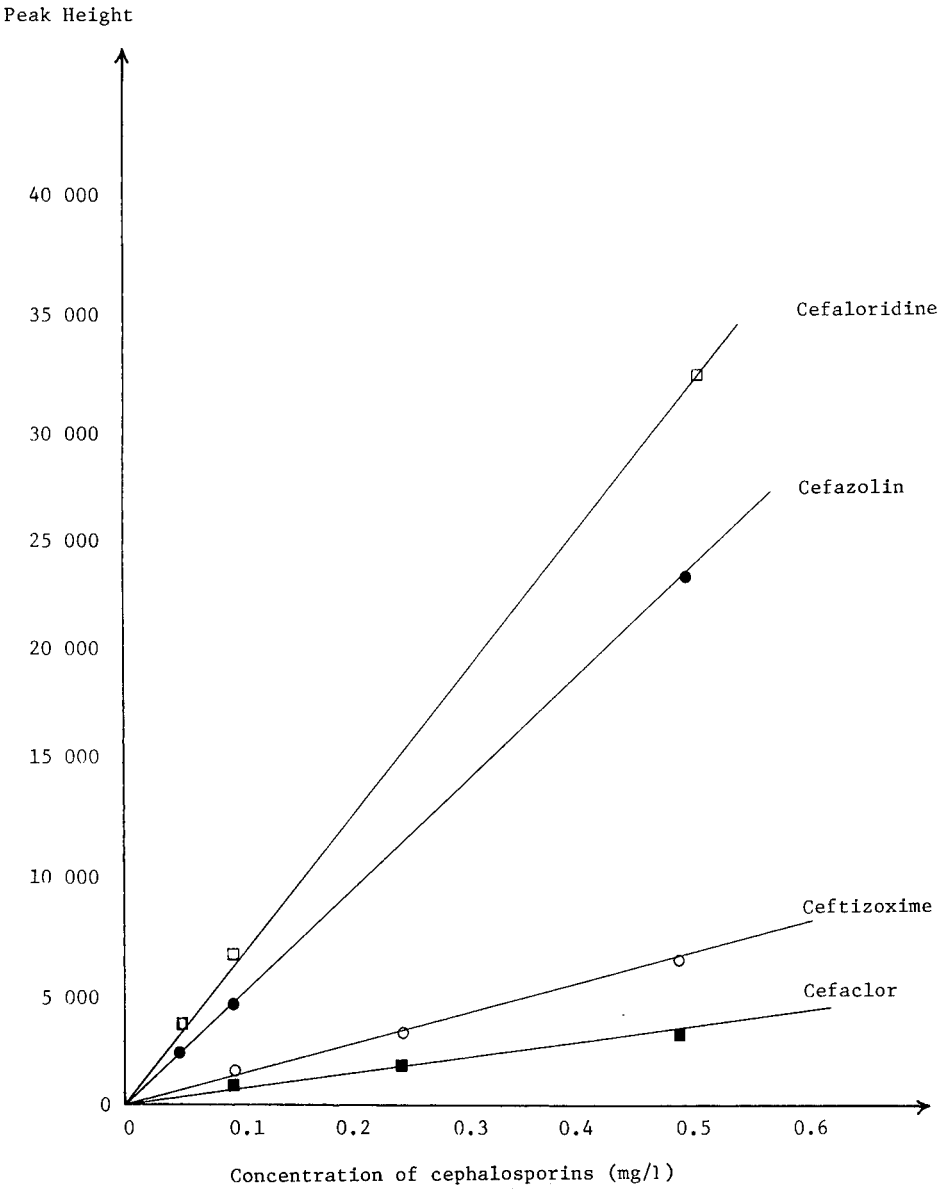

Fig. 2. Linearity of detector response to various drug concentrations. Mean values are shown.

Concentration column

The interactions between the acidic cephalosporin drugs (Fig. 3) and the non-polar C_{18} packing of the concentration column are mostly hydrophobic. Hence the acidic aqueous mobile phase (B) aids the retention of the analytes on the concentration column.

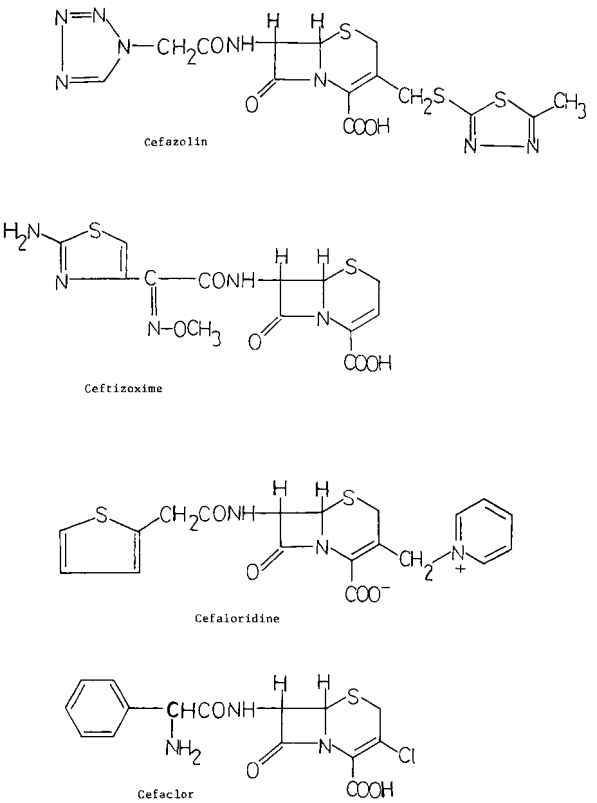

Fig. 3. Structures of the cephalosporins investigated.

The less polar mobile phase (A) which contains 40% methanol removes the drugs from the concentration column and carries them to the main analytical column for determination.

The use of a concentration column circumvents the most common problem associated with the use of microbore columns, *i.e.*, low injection volumes. Using this highly efficient retention/elution system, up to 500 μl of isolate matrix can be analysed.

The purpose of this work was to develop a reproducible HPLC–MS procedure for the determination of cephalosporins. The super-microbore method described is, in itself, suitable for the determination of such antibiotics, as each of the drugs could easily be used as an internal standard for the others. However, the interfacing with a mass spectrometer gives an additional identification procedure and, as we wished to use fast atom bombardment as the fragmentation process, the incorporation of a fluid matrix in the mobile phase was necessary. The addition of glycerol (1%) to the mobile phase shortened the retention times and reduced the peak heights of the cephalosporins.

CONCLUSION

The reproducibility and efficiency of this super-microbore liquid chromatographic system make it ideal for interfacing with a mass spectrometer by direct liquid introduction. Buffer salts and high flow-rates are not necessary. The use of the concentration column allows the injection of up to 500 μl of analyte, so the detection limits, even when glycerol is incorporated in the mobile phase, are easily sufficient to detect therapeutic levels of cephalosporins. The observed decrease in retention time when glycerol was added to the mobile phase caused the cephalosporins to elute closer together, but when mass spectrometry is applied this is not a problem, as separation and resolution using mass chromatograms is possible.

The possibilities of mass spectrometer interfacing are now under investigation.

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CHROM. 22 928

Short Communication

Electrodialysis pretreatment system for ion chromatography of strongly acidic samples and its application to the determination of magnesium and calcium

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ABSTRACT

A newly developed electrodialysis pretreatment system in combination with a new dual anion-selective membrane tubes was employed to reduce the acidity of a strongly acidic sample solution prior to the ion chromatographic determination of magnesium(II) and calcium(II). By using this system, anions such as sulphate or nitrate were also removed from the sample solution through the membrane. The detection limits were 7.8 and 25 ng/ml and the upper limits of the linear response were 2 μg/ml and 5 μg/ml for magnesium(II) and calcium(II), respectively. Results of the analyses of some strongly acidic solutions are given.

INTRODUCTION

Trace ions in a strongly acidic or basic solution cannot be determined by direct injection into an ion chromatograph equipped with a cation- or anion-exchange separation column and conductivity detector, owing to the high background conductance of the sample in addition to the low capacity of the column. Therefore, the solution should be diluted to alleviate the load of excess ions on the column. However, such a dilution makes analysis difficult if the concentrations of the ions in the original sample are close to the detection limits. Pettersen *et al.* [1] reported a method for reducing high concentrations of sodium and hydroxide ions without dilution for the determination of sulphate and nitrate ions. Their method was based on electrolysis in combination with a sheet of cation-selective membrane. In this preliminary work, a new dual anion-selective membrane tube electrodialysis system

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was developed and applied, as an example, to the determination of magnesium and calcium ions in strongly acidic solutions by non-suppressed or single-column ion chromatography.

EXPERIMENTAL

The ion chromatograph consisted of a Tosoh (Tokyo, Japan) Model CCPD double-plunger pump, a Tosoh Model IC-Cation column, a Rheodyne (Cotati, CA, U.S.A.) Model 7125 injector with a 100-μl sample loop and a Tosoh Model CM-8 conductivity detector. The sensitivity of the detector was 0.5 μS/cm. The mobile phase (pH 5.3) consisted of ethylenediamine (0.25 mmol/l) and tartaric acid (0.25 mmol/l). The flow-rate was 1.0 ml/min.

The electrodialysis system consisted of an electrodialysis cell, a constant-current d.c. electric source (0.9 A), a relative conductivity monitor, a Tokyo Rikakikai (Tokyo, Japan) Model MP-3 two-channel peristaltic pump and an M & S Instruments (Osaka, Japan) Model JR magnetic stirrer. For the electric source and the monitor, the components of a Tosoh Model IE-Labo system were used. The design of the electrodialysis cell, shown in Fig. 1, utilized a Tosoh TASN-85 anion-selective membrane tube (40 cm × 1.00 mm I.D. × 2.25 mm O.D.), containing a platinum wire (50 cm × 0.30 mm O.D.), inserted inside a Tosoh TASN-80 anion-selective membrane tube (30 cm × 2.75 mm I.D. × 3.13 mm O.D.). A stainless-steel wire (2 m × 0.50 mm O.D.) was coiled around the outer membrane tube and held negative with respect to the central conductor. Inlet and outlet liquid connections were made with silicone-rubber tees. Sample solution flowed in the outer channel, whereas anode electrolyte solution (0.1 mol/l sulphuric acid) flowed in the inner channel. Sodium hydroxide solution (0.1 mol/l) was used as the cathode electrolyte solution. Other parts of the system were connected with polytetrafluoroethylene tubing (1.0 mm I.D.).

The electrodialysis procedure was as follows. The sample channel was filled with an aliquot of a strongly acidic sample solution (*ca.* 2.5 ml). The solution was then circulated at a flow-rate of 3.7 ml/min and the anode electrolyte solution was also pumped at the same flow-rate. Subsequently, platinum and stainless-steel wire

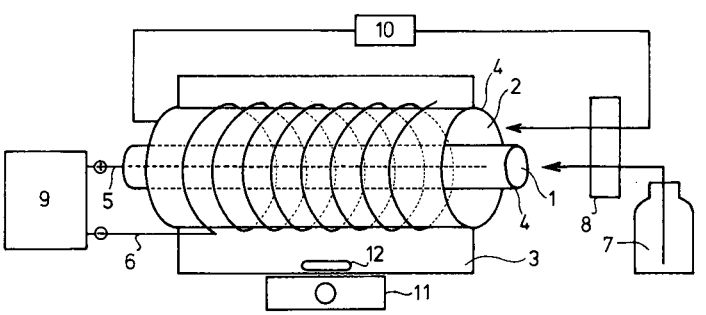

Fig. 1. Schematic diagram of electrodialysis system. 1 = Anode electrolyte solution channel; 2 = sample channel; 3 = cathode electrolyte solution; 4 = anion-selective membrane tube; 5 = anode; 6 = cathode; 7 = anode electrolyte solution; 8 = two-channel peristaltic pump; 9 = electric source; 10 = relative conductivity monitor; 11 = magnetic stirrer; 12 = stirring bar.

electrodes were connected to the source to start the electrodialysis. The relative conductivity was monitored throughout. After the dialysis, the neutralized sample was removed from the apparatus for analysis.

RESULTS AND DISCUSSION

The electrodialysis conditions were optimized using a 0.5 mol/l sulphuric acid test solution spiked with 200 ng/ml of magnesium(II) and 200 ng/ml of calcium(II). During the dialysis, sulphate ions move from the sample solution through the anion-selective membrane into the anode electrolyte solution. At the same time, hydroxide ions move from the cathode electrolyte solution into the sample solution and neutralize the sample. The outer membrane prevents the analyte metal ions from penetrating into the cathode electrolyte solution. The electrodialysis was stopped when the relative conductivity decreased to the level of 10–12%. The time required was at least *ca.* 10 min. Further dialysis should be avoided, becasue gradual penetration of the analyte ions from the sample to the cathode electrolyte solution was apt to occur.

Using this procedure, the determination of magnesium(II) and calcium(II) in strongly acidic solutions by ion chromatography was achieved without any loss of the analyte ions (Table I). The detection limits were 7.8 and 25 ng/ml and the upper limits of the linear response were 2 and 5 μg/ml for magnesium(II) and calcium(II), respectively.

TABLE I

DETERMINATION OF MAGNESIUM AND CALCIUM IN STRONGLY ACIDIC SOLUTIONS

Sample[a]	Magnesium			Calcium		
	Added (ng/ml)	Found[b,c] (ng/ml)	Recovery (%)	Added (ng/ml)	Found[b,c] (ng/ml)	Recovery (%)
0.5 mol/l H_2SO_4	0	N.D.[d]	–	0	28 ± 4	–
	100	98 ± 3	98.0	100	132 ± 3	104
	200	200 ± 2	100	200	242 ± 8	107
1.0 mol/l HNO_3	0	N.D.[d]	–	0	55 ± 2	–
	100	102 ± 2	102	100	146 ± 3	91.0
	200	190 ± 3	95.0	200	244 ± 6	94.5

[a] Commercially available acid diluted with deionized water.
[b] Retention times: magnesium 6.83 min, calcium 12.9 min.
[c] Mean ± average deviation, 3 results.
[d] N.D. = Not detected.

The proposed method is applicable to the determination of trace cations in strongly acidic solutions such as an acid-digested sample. Moreover, it can readily be extended to on-line instrumentation and also to the determination of anions in strongly basic solutions by using a similar system with cation-selective membrane tubes, on which our further work is now focusing in progress.

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Short Communication

Simultaneous determination of molybdenum, vanadium, gallium, copper, iron and indium as 8-quinolinolate complexes by high-performance liquid chromatography

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(First received July 9th, 1990; revised manuscript received October 17th, 1990)

ABSTRACT

8-Quinolinol (HQ) was used as a precolumn chelating reagent for the reversed-phase high-performance liquid chromatographic (HPLC) determination of Mo(VI), V(V), Ga(III), Cu(II), Fe(III) and In(III). The metal–HQ complexes were separated on a C_{18} column using a mobile phase of methanol–water (67:33, v/v) containing $1.25 \cdot 10^{-3}$ *M* HQ and 0.02 *M* chloroacetate (pH 3.5). The metal–HQ complexes were preconcentrated by solvent extraction, evaporation of the solvent and dissolution of the residue in methanol. After preconcentration, $\mu g\ l^{-1}$ levels of the metals can be determined by HPLC with satisfactory precision. The proposed method was applied to the simultaneous determination of Mo(VI), V(V), Cu(II) and Fe(III) in sea water.

INTRODUCTION

High-performance liquid chromatography (HPLC) of metal complexes is an attractive method for the simultaneous determination of trace metal ions. In particular, reversed-phase HPLC seems to offer excellent potential for the determination of many metals owing to its high efficiency and simplicity of operation. Several revies on the HPLC separation of metal complexes have been published [1–5].

8-Quinolinol (HQ) has been extensively used for the separation of metal ions by HPLC. HQ is an suitable reagent for this purpose as it forms stable complexes with many metal ions. A number of papers concerning the determination of metal ions as HQ complexes by HPLC have been published [6–19]. A variety of separation conditions, detectors and derivatization techniques have been used. Separations have been reported with the use of both normal-phase [6–11] and reversed-phase [10–19] HPLC. UV–VIS detection is the most frequently employed detection method in the HPLC of HQ complexes. Fluorescence detection [16] and electrochemical detection

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[14] have been evaluated. In addition, precolumn complexation, on-column complexation [9,14,15,18] and postcolumn complexation [16] have been used. However, their application to real samples is seldom found. A few applications [15] have included the analysis of bovine liver, waste water and river water.

This work involved the low-level determination of Mo(VI), V(V), Ga(III), Cu(II), Fe(III) and In(III) by reversed-phase HPLC of their HQ complexes. For the trace enrichment of metal ions, the complexes have typically been extracted into an organic solvent such as chloroform prior to the chromatographic separation. However, the direct injection of more than 10 μl of an organic extract onto the separation column is inadequate for reversed-phase HPLC. Therefore, after extraction of the HQ complexes into carbon tetrachloride, the solvent was evaporated and the residue was dissolved in methanol prior to injection. Trace levels of the metals can be determined by HPLC after this enrichment, and the simultaneous determination of Mo(VI), V(V), Cu(II), and Fe(III) in sea water by this method is possible.

EXPERIMENTAL

Instrumentation and reagents

The analytical column was a Tosoh (Tokyo, Japan) TSK-GEL ODS-80TM (25 cm $\times$ 4 mm I.D.) with a 5-μm particle size. A Rheodyne Model 7125 injector was used. The detector was a Japan Spectroscopic (Tokyo, Japan) Model 870 variable-wavelength UV detector, together with a Shimazu (Kyoto, Japan) Model U-125 MU recorder. The HPLC pump was a Nihon Seimitsu Kagaku (Tokyo, Japan) NP-DX pump with a hexane damper. The mobile phase was methanol–water (67:33, v/v) containing $1.25 \cdot 10^{-3}$ *M* HQ and 0.02 *M* chloroacetate (pH 3.5). The mobile phase flow-rate was 1.0 ml min^{-1} and the detector was operated at 370 nm.

HQ was obtained from Wako (Osaka, Japan) and used without further purification. Methanol and carbon tetrachloride were purified by distillation. Distilled, deionized water was purified with a Millipore Milli-Q system and was used for all solutions and dilutions. All solvents were filtered through a 0.45-μm filters before use.

Stock solutions (1000 mg l^{-1}) of metal ions were prepared from salts and metals purchased from the following sources: $CuSO_4 \cdot 5H_2O$, Ga, NH_4VO_3, $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ and In from Wako and $Na_2Mo_0O_4 \cdot 2H_2O$ from Kanto Chemicals (Tokyo, Japan). All reagents were of analytical-reagent grade.

Procedure

To 90 ml of sample solution containing Mo(VI), V(V), Ga(III), Cu(II), Fe(III) and In(III), 1.5 ml of 0.05 *M* HQ (0.05 *M* H_2SO_4) solution was added and the pH was adjusted to 4.0 by addition of 2 *M* sodium acetate solution. Then 2 ml of butanol were added and the mixture was heated at 60°C for 10 min. After cooling, the mixture was transferred to a separating funnel and the metal–HQ complexes were extracted into 10 ml of carbon tetrachloride by shaking for 10 min. The extracted organic phase was centrifuged for 2 min and 8-ml aliquots were transferred to eight test-tubes attached to a rotary evaporator. The solvent was evaporated and the residue was dissolved in 1 ml of methanol–water (4:1, pH 3.5) after cooling. Aliquots of 100 μl of this solution were then injected into the chromatograph.

RESULTS AND DISCUSSION

Chromatography and solvent extraction of the metal–HQ complexes

For the study of chromatographic behaviour, the HQ complexes of Mo(VI), V(V), Ga(III), Cu(II), Fe(III) and In(III) were prepared without a preconcentration step in the procedures described above as follows: to a sample solution containing 10 μg of each metal ion, 3 ml of methanol, 1 ml of 10^{-2} *M* HQ solution and 0.5 ml of 1 *M* ammonium acetate buffer solution were added. The mixture was diluted with water to 5 ml, then an aliquot of the solution was injected into the HPLC system.

The proportion of methanol in the methanol–water mobile phase was varied from 60 to 72.5 (v/v). The retention time of each complex increased with decreasing methanol content. Well resolved peaks suitable for the simultaneous determination of the six metal ions were obtained with methanol–water (67:33, v/v).

The effect of the mobile phase pH on the retention behaviour of the metal–HQ complexe was investigated in the pH range 2.5–4.0 and the results are shown in Fig. 1. The retention times of all the complexes were unchanged in the pH range 3–4, except for that time of the In(III) complex, which increased with increasing pH. It is known that In(III) is completely extracted into chloroform to form $In(HQ)_3$ with HQ above pH 4.0. The retention behaviour of the In(III) complex is considered to be due to an increase of the formation of $In(HQ)_3$ with increase in pH. The optimum chromato-

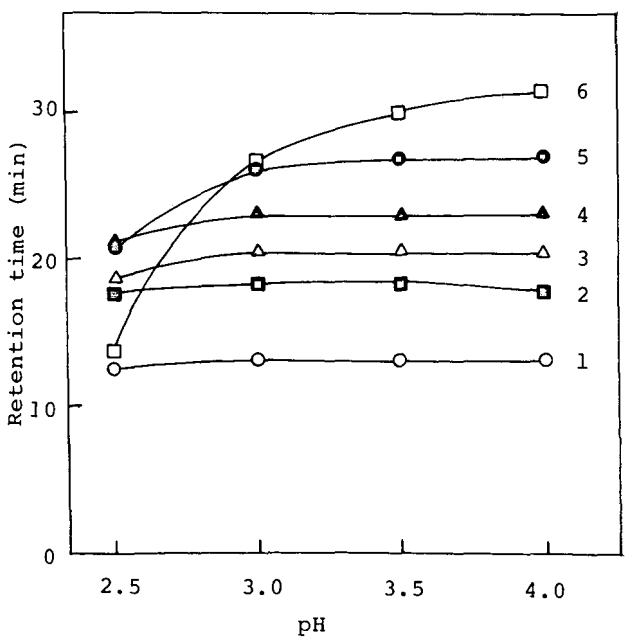

Fig. 1. Variation of retention time with pH of the mobile phase. Column, TSK-GEL ODS-80TM (25 cm × 4 mm I.D.; 5 μm); mobile phase, methanol–water (67:33, v/v) containing $1.25 \cdot 10^{-3}$ *M* HQ and $2 \cdot 10^{-2}$ *M* acetate buffer; flow-rate, 0.65 ml/min; detector wavelength, 370 nm; detector sensitivity, 0.02 a.u.f.s.; injection volume, 20 μl; metal, 20 ng. 1 = Mo(VI); 2 = V(V); 3 = Ga(III); 4 = Cu(II); 5 = Fe(III); 6 = In(III).

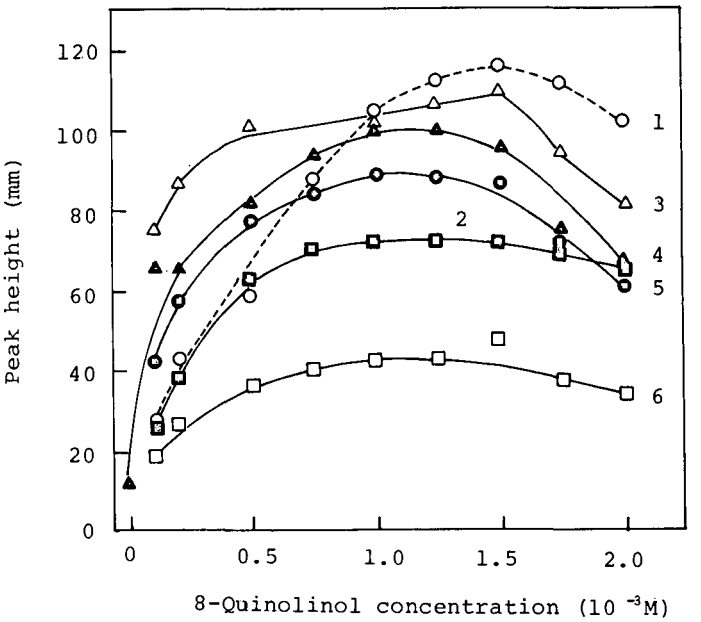

Fig. 2. Variation of peak height with HQ concentration in the mobile phase. Mobile phase, methanol–water (67:33, v/v). Other conditions as in Fig. 1. 1 = Mo(VI); 2 = V(V); 3 = Ga(III); 4 = Cu(II); 5 = Fe(III); 6 = In(III).

gram for the determination of six metal ions was obtained with a mobile phase pH of 3.5.

The peak height of the six complexes increased and the peak symmetry was improved with addition of HQ to the mobile phase. Fig. 2 shows the effect of HQ concentration in the mobile phase on the peak heights of the six complexes. The largest peak heights of the complexes were obtained at an HQ concentration of *ca.* $1.25 \cdot 10^{-3}$ *M*. The peak heights of the complexes decreased at HQ concentrations above $1.5 \cdot 10^{-3}$ *M* because the peaks became broadened. The retention times of the complexes, however, were approximately constant in the HQ concentration range $0.5 \cdot 10^{-3}$–$2.0 \cdot 10^{-3}$ *M*.

A typical chromatogram for the separation of the HQ complexes of Mo(VI), V(V), Ga(III), Cu(II), Fe(III) and In(III) is shown in Fig. 3. The six peaks are well resolved and separated from the peak of HQ.

Solvent extraction of metal complexes is an effective preconcentration method for the low-level determination of metal ions by HPLC. The solvent extraction of HQ complexes has been widely investigated. It has been reported [20] that the HQ complexes of Mo(VI), V(V), Ga(III), Cu(II), Fe(III) and In(III) can be extracted from acidic–neutral or acidic–basic media with various solvents. In this work, the effects of pH, HQ concentration and addition of butanol on the extraction of these complexes from 100 ml of aqueous phase into 10 ml of carbon tetrachloride were investigated according to the procedures previously described. The addition of an alcohol such as butanol is required for complete extraction of V(V) [21]. The aqueous phase was

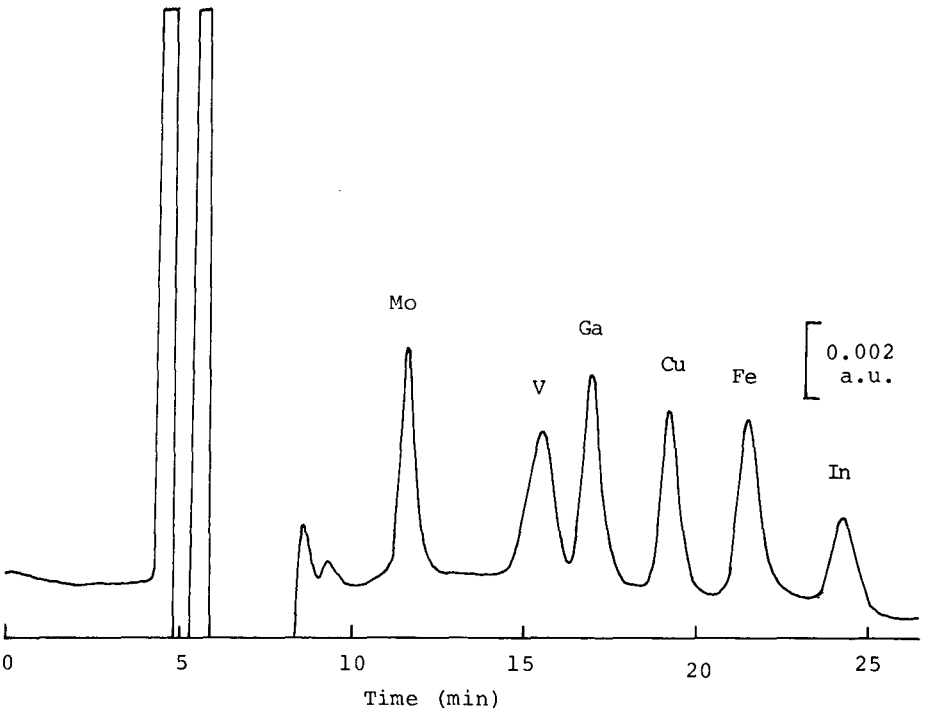

Fig. 3. HPLC separation of metal–HQ complexes. Mobile phase, methanol–water (67:33, v/v). Other conditions as in Fig. 1.

heated at 60°C in order to accelerate the complexation prior to solvent extraction. From the results, an HQ concentration of $7.5 \cdot 10^{-4}$ *M*, 2.5% (v/v) butanol and pH 4 were chosen for subsequent work. All complexes were completely extracted under these conditions. The extracted organic phase was evaporated to dryness and then the residue was dissolved in methanol prior to injection, because the direct injection of more than 10 μl of the organic extract is inadequate for reversed-phase HPLC.

Detection limits and precision

Aqueous solutions containing Mo(VI), V(V), Ga(III). Cu(II), Fe(III) and In(III) were analysed according to the above procedures. The calibration graphs obtained from 100-μl injections were linear for 0.5–50 $\mu g\ l^{-1}$ Mo(VI), V(V) and Ga(III), 1–50 $\mu g\ l^{-1}$ Cu(II), 4–60 $\mu g\ l^{-1}$ Fe(III) and 0.5–60 $\mu g\ l^{-1}$ In(III) when the peak heights (370 nm) were measured. The detection limits, defined as the metal ion concentration which gave a peak height three times larger than the background noise, were 0.04 $\mu g\ l^{-1}$ for Mo(VI), 0.06 $\mu g\ l^{-1}$ for V(V), 0.05 $\mu g\ l^{-1}$ for Ga(III), 0.2 $\mu g\ l^{-1}$ for Cu(II), 1.1 $\mu g\ l^{-1}$ for Fe(III) and 0.1 $\mu g\ l^{-1}$ for In(III). The detection limits could be significantly lowered through the use of large volume sample solution in the solvent extraction step. The relative standard deviations for the peak heights were 2.7, 7.0, 1.6, 2.7, 4.0 and 5.8% for 1.5 $\mu g\ l^{-1}$ Mo(VI), 2.0 $\mu g\ l^{-1}$ V(V), 1.5 $\mu g\ l^{-1}$ Ga(III), 7.0 $\mu g\ l^{-1}$ Cu(II), 15 $\mu g\ l^{-1}$ Fe(III) and 3.5 $\mu g\ l^{-1}$ In(III), respectively.

TABLE I

ANALYTICAL RESULTS FOR Mo(VI), V(V), Cu(II) AND Fe(III) IN SEA WATER

Injection volume, 100 μl; other conditions as in Fig. 1.

Location	Amount found (μg l^{-1})[c]			
	Mo(VI)	V(V)	Cu(II)	Fe(III)
Isozaki coast[a]	7.8 ± 0.5	1.4 ± 0.09	1.4 ± 0.13	16.9 ± 1.4
Hitachi harbour[b]	7.9 ± 0.2	1.6 ± 0.06	0.72 ± 0.08	66.6 ± 4.3

[a] 36°22′40″N, 140°37′56″E.
[b] 36°28′27″N, 140°37′50″E.
[c] Mean ± standard deviation (n = 4).

Determination of Mo(VI), V(V), Cu(II), and Fe(III) in sea water

The possibility of interferences from other metals was investigated for the determination of Mo(IV), V(V), Cu(II) and Fe(III) in sea water. To an artificial sea water containing Al(III), Cr(VI), Mn(II), Ni(II), Sn(IV), Ti(IV), U(VI) and Zn(II) at the natural levels in real sea water, the four metals were added and determined by the above procedures. The chromatogram of the resulting solution showed no interferences from these metals with the peak of Mo(VI), V(V), Cu(II) and Fe(III).

The concentrations of Mo(VI), V(V), Cu(II) and Fe(III) in surface sea water from the Isozaki coast and Hitachi harbour of Ibaraki (Pacific coast of Japan) were determined using the above procedures and the results are given in Table I. All the values, except for Fe(III) in the Hitachi harbour sample, are similar to those reported by Kimura *et al.* [22] for sea water from the Pacific coast of Japan. The high level of Fe(III) in the Hitachi harbour sample is believed to be due to the dissolution of iron from ships' hulls. No Ga(III) or In(III) was detectable because their concentrations in sea water are very low.

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Short Communication

Gas chromatographic analysis of tropic, benzoic and cinnamic acids, biosynthetic tropane alkaloid precursors

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ABSTRACT

A capillary gas chromatographic (GC) method was developed for the determination of tropic, benzoic and cinnamic acids, which are established precursors of many important naturally occurring tropane alkaloids. The acids were derivatized (esterified) by extractive alkylation with pentafluorobenzyl bromide with catalysis by tetrabutylammonium ions. With mandelic acid as internal standard, a direct GC analysis of the methylene chloride extract provides a simple and reliable assay which is applicable to complex sample matrices.

INTRODUCTION

Simple and reliable capillary gas chromatographic (GC) assays for tropic, benzoic and cinnamic acids should prove useful in biochemical research on the medicinally important anticholinergic tropane alkaloids hyoscyamine and scopolamine (tropic acid esters contained, *e.g.*, in *Atropa belladonna* L.) and cocaine (a benzoic acid ester occurring as the main tropane alkaloid constituent of *Erythroxylon coca* Lam.) [1–4]. The acids in question have been assayed both by high-performance liquid chromatography (HPLC) and by GC [5–8]. A promising method for the assay of carboxylic acids seems to be that described by Greving *et al.* [9]. A reliable GC method has been described for the quantification of some of the most important tropane alkaloid precursor amines potentially occurring in *A. belladonna* cell cultures [10].

This investigation was aimed first at the development of a reliable tropic acid assay on the basis of phase-transfer catalysis ("extractive alkylation") and capillary GC [9]. Combination of extraction and derivatization steps is advantageous for the determination of many compounds with exchangeable protons, procedural simplicity providing improved analytical reliability [9,11,12]. From an analytical point of view, the primary alcoholic hydroxyl group of tropic acid renders it more difficult to determine in comparison with benzoic and cinnamic acids (see Fig. 1 for structures),

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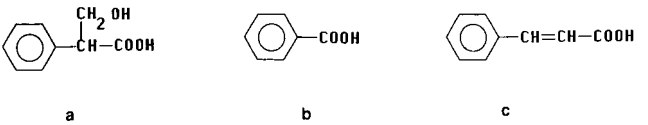

a b c

Fig. 1. Structures of (a) tropic, (b) benzoic and (c) cinnamic acid.

the last two compounds being included here as analytes for potential future applications.

EXPERIMENTAL

A stock solution containing 1 mg of each of tropic acid (EGA-Chemie), benzoic acid (Merck) and cinnamic acid (Merck) in 1 ml of methanol was used for the preparation of standard samples, which were obtained by mixing aliquots of the above solution with lyophilized cells (50 mg) from an *A. belladonna* cell suspension culture devoid of the acids in question. The tetrabutylammonium hydroxide (TBA-OH) counter-ion solution (0.1 *M*, pH 8) for phase-transfer catalysis was obtained from TBA-HSO_4 (Fluka) by neutralization with 2 *M* NaOH solution and dilution with phosphate buffer solution (pH 8) (0.050 *M* KH_2PO_4–0.045 *M* NaOH). The alkylating agent, pentafluorobenzyl bromide (PFB-Br) (EGA-Chemie), was used as a 0.5% solution in methylene chloride.

Lyophilized cell samples (50–100 mg) were shaken in test-tubes with 1 ml of buffer solution (pH 8). Following the addition of 1 ml of the counter-ion solution, 2 ml of PFB-Br solution and 75 μl of an internal standard solution containing mandelic acid (Merck) in methanol (1 mg/ml), the tubes were first shaken for 30 min at room temperature and then placed in an ultrasonic bath (45°C) for 30 min. After centrifugation, the aqueous upper layer was withdrawn by suction. The dried (addition of Na_2SO_4 followed by centrifugation) methylene chloride solution (1 μl) was used for GC analysis.

The alkylation procedure was tested with tropic acid at five pH values (6, 7, 8, 9 and 10; phosphate buffer) plotting the resulting PFB ester peak areas against pH.

To test the hydrolytic stability of hyoscyamine and scopolamine in the alkylation process, 1 m*M* solutions were added to *A. belladonna* cell suspension samples. Even for 4 h shaking/ultrasonic treatments (see above), the chromatograms confirmed the total absence of tropic acid PFB ester peaks.

The GC analysis was carried out with a 25 m × 0.32 mm I.D. OV-1701 fused-silica capillary column with a 0.25-μm coating (Nordibond, Nordion) in a Dani HR 3800 gas chromatograph. The instrument was equipped with a flame ionization detector and a programmed-temperature vaporizer (PTV). Hydrogen was used as the carrier gas at a flow-rate of 3 ml/min and the temperature was programmed from 80 to 255°C at 12°C/min. The PTV temperature range was 70–250°C (splitless operation).

RESULTS AND DISCUSSION

Phase-transfer catalysed alkylation provides an excellent analytical approach for many polar active hydrogen compounds. A wide range of commercially available

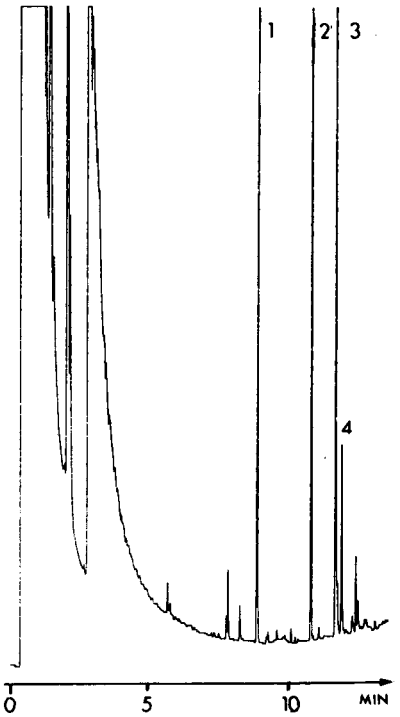

Fig. 2. Gas chromatogram of a standard sample. Peaks: 1 = benzoic acid; 2 = mandelic acid (internal standard); 3 = cinnamic acid; 4 = tropic acid (as PFB esters).

alkylating agents allows the attainment of a sufficient degree of lipophilicity to yield rapid and quantitative extractions with relatively non-polar organic solvents, the derivatization of phenolic compounds, carboxylic acids, barbiturates, etc., thus being easily accomplished. Extraction is based on ion-pair formation, the anionic component of the ion pair being highly susceptible towards derivatization by a suitable carbocation source in organic solution. Even moderately polar compounds are rapidly and quantitatively derivatized, their anions being continuously removed from the aqueous phase by the alkylation process. The aqueous phase pH must be adjusted to allow for the presence of a reasonable ion-pair fraction in the organic phase. The high alkylating power of PFB-Br stems from a good relative stability of the carbocation arising from carbon–bromine bond cleavage. In many GC applications this alkylating reagent has been successfully used for direct drug alkylations within biological sample matrices [9,11,12].

Currently the most advanced tropic acid assays seem to be those based on HPLC [5–7], although in comparison with HPLC methods the present GC assay should provide better separations. In most GC applications an extraction procedure is followed by silylation [13–15]. At least one extraction step can be avoided and the extractant volume can be essentially reduced in the present method, which allows the simultaneous separation and determination of all acids of interest, as demonstrated by the chromatogram of a standard sample in Fig. 2.

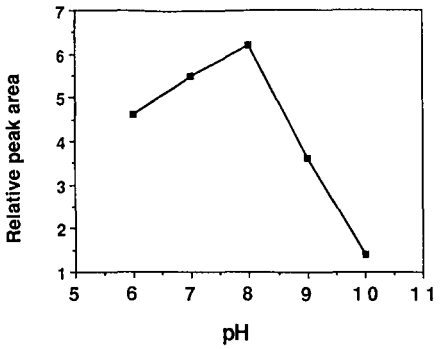

Fig. 3. pH dependence of tropic acid PFB ester peak area in the chromatograms.

Relative simplicity of sample treatment is the outstanding feature of the method, the reaction conditions also being sufficiently mild to leave ester bonds intact (*cf.*, Experimental). It is noteworthy that hydrolytic cleavage of ester alkaloid structures could theoretically provide a source of analytical error through potential "PFB transesterification". pH 8 was found to be optimum for the PFB alkylation of tropic acid (Fig. 3).

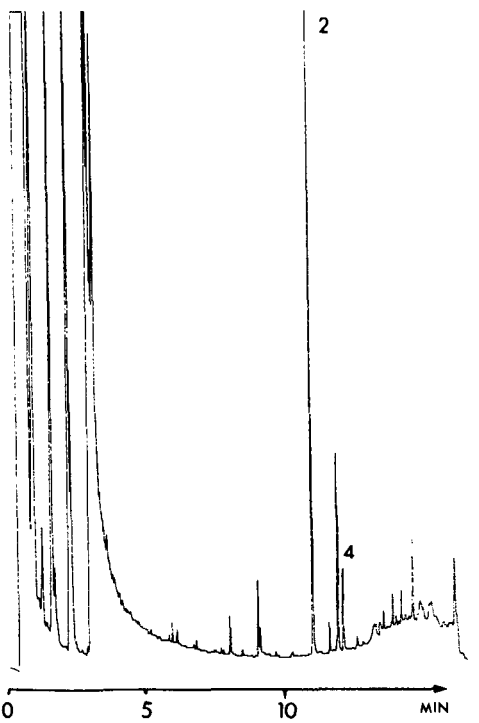

Fig. 4. Gas chromatogram of a cell suspension sample of *Atropa belladonna* [16]. Peaks: 2 = mandelic acid (internal standard); 4 = tropic acid (as PFB esters).

TABLE I

ANALYTICAL PARAMETERS OF THE ASSAY FOR TROPIC, BENZOIC AND CINNAMIC ACIDS

Parameter	Tropic acid	Benzoic acid	Cinnamic acid
Equation of calibration graph[a]	$y = 0.94x - 0.018$	$y = 5.71x - 0.099$	$y = 2.62x - 0.003$
Correlation coefficient (R)	0.995	0.998	0.999
Relative standard deviation ($N = 9$, 30 μg/ml)	5.4	3.5	3.6
Detection limit (ng injected)	5	2	2

[a] y = Peak area ratio; x = concentration (μg/ml) × 10^{-2}.

Table I lists the analytical parameters obtained for the compounds of interest. Of course, a considerable increase in sensitivity can be obtained in GC, if necessary, by the use of an electron-capture detector. The separation of tropic acid PFB ester from various co-analytes present in a suspension culture sample of *A. belladonna* is shown in Fig. 4. The average tropic acid recovery was 113.1% and 97.5% at 10.0 and 40.0 μg, respectively, from spiked cell suspension samples (100 mg, $n = 5$).

The method has been successfully applied in alkaloid and alkaloid precursor feeding experiments involving *A. belladonna* cell suspension cultures [16].

ACKNOWLEDGEMENTS

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Short Communication

Gas chromatographic determination of flumetralin in tobacco

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ABSTRACT

A method for the determination of flumetralin (Prime) residues in tobacco is presented. The procedure is adapted from that of Reif and Moser, which was proposed for the determination of organochlorine pesticide residues in tobacco. Improvements were made by the use of an overflow in the Soxhlet extractor and by replacing the packed column with a fused-silica capillary column. The suggested method is rapid and simple to use.

INTRODUCTION

Flumetralin [N-(2-chloro-6-fluorobenzyl)-N-ethyl-α,α,α-trifluoro-2,6-dinitro-*p*-toluidine], a substance with plant growth regulating activity [1], was introduced under the trade-mark Prime by Ciba-Geigy (Basle, Switzerland). It is applied as a topical treatment for the control of sucker growth on various types of tobacco. The product can provide full season control if applied within 24 h after topping. It has a local systemic effect, and therefore rain occurring later than 2 h after spraying does not reduce its effectiveness, in contrast to Off Shoot T (fatty alcohols). Further, as it is not translocated throughout the plant, as occurs with maleic hydrazide, less residues can be expected in tobacco leaves [2]. A comparison of sucker-control agents performed on Maryland tobacco showed that flumetralin was the most effective local systemic suckercide agent, with a persistent effect [3].

Flumetralin was registered in the U.S.A. for use on tobacco in 1983 and incorporated into the recommended sucker control program [4]. A maximum residue level of 20 ppm of flumetralin in the finished product was set in the German

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Hoechstmengenverordnung [5]. As no rapid and simple method for the determination of flumetralin residues on tobacco appears to have been published, the gas chromatographic (GC) method for organochlorine pesticides described by Reif and Moser [6] was tested for flumetralin and was found to be applicable.

EXPERIMENTAL

Chemicals

n-Hexane for pesticide analysis, used as an extraction solvent, was obtained from Fluka (Buchs, Switzerland) and Florisil (60–100 mesh) from Siber Hegner Rohstoff (Zürich, Switzerland). Flumetralin and mirex pure pesticide were supplied by Dr. Ehrenstorfer (Augsburg, F.R.G.).

Florisil conditioning

The Florisil was heated overnight in a muffle furnace at 500–550°C, then cooled in a desiccator without a desiccant. For deactivation, the Florisil was placed in a round-bottomed flask on a rotary evaporator. With constant agitation, 5 wt.-% of doubly distilled water was added and the flask rotated for 1 h. Before use, the deactivated Florisil was left to equilibrate for at least 48 h in a rightly closed glass container. After each use, the glass container must be tightly reclosed in order to prevent modification of the water content deactivated Florisil.

Gas chromatography

A Hewlett-Packard HP 5890 gas chromatograph equipped with a nickel-63 electron-capture detector, a Model 7673A automatic sampler and a Model 3393A integrator was used. A DB-5 fused-silica capillary column (30 m × 0.32 mm I.D.), film thickness 0.25 μm, was obtained from J & W Scientific (Folsom, CA, U.S.A.). The temperature programme was as follows: the initial temperature (70°C) was held for 1 min, then raised to 150°C at 20°C/min and to 270°C at 3°C/min, and then held at 270°C for 15 min. The carrier gas was helium at a flow-rate of 4 ml/min and the make-up gas was nitrogen at a flow-rate of 30 ml/min. Splitless injection was used. Under these conditions, the retention times of flumetralin and mirex were 23.5 and 34.2 min, respectively.

Extraction/clean-up

The Soxhlet extractor shown in Fig. 1 was filled with 5 g of deactivated Florisil as the lower layer and 5 g of deactivated Florisil mixed with 5 g of ground tobacco as the upper layer.

A 60-ml volume of *n*-hexane and 3 ml of a 4 μg/ml mirex internal standard solution in *n*-hexane were placed in a 250-ml round-bottomed flask. The flask was connected to the Soxhlet extractor and the *n*-hexane was heated with an electrical heater to boil the solution. The Soxhlet extractor was regulated to give a reflux rate of *ca.* 250 ml/h. The level of *n*-hexane above the tobacco had to be kept constant. The overflow prevented drying of the distillation flask. The extraction time was 4.5 h. After extraction, 0.5 μl of the extract was injected directly into the GC system. A typical chromatogram is shown in Fig. 2.

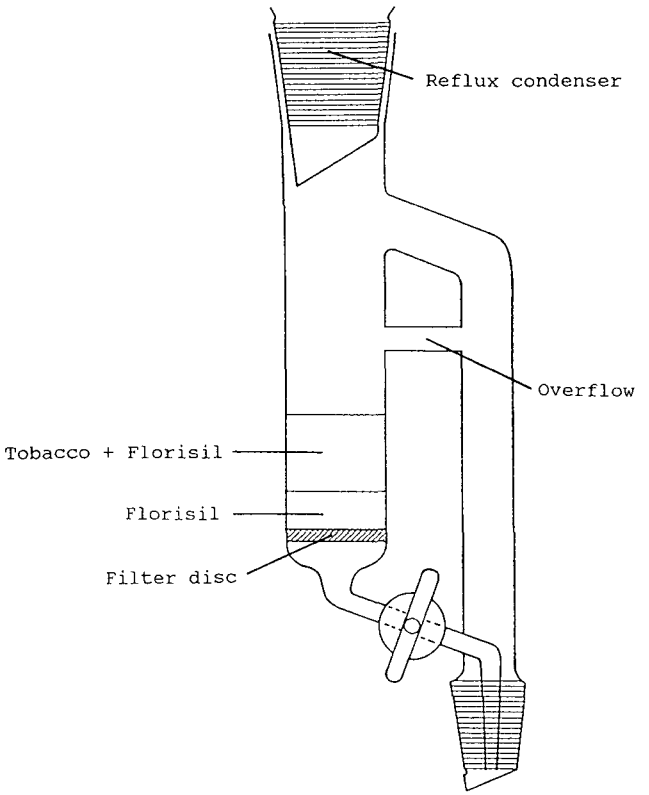

Fig. 1. Diagram of the equipment used for extraction. The filter disc is of porosity 1 and the overflow is at 85 mm. Height without standard taper joint: 170 mm. Inside diameter: 30 mm.

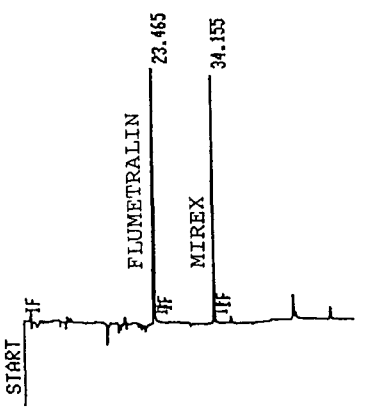

Fig. 2. Example of a chromatogram obtained after Soxhlet extraction. Sample containing 2.9 mg/kg of flumetralin.

TABLE I

FLUMETRALIN RESIDUE VALUES (mg/kg) OBTAINED BY DIFFERENT METHODS (RECOVERY CORRECTED)

The residue values are given at a 95% confidence limit level.

Sample	Coresta mean values (mg/kg)	Soxhlet extractor mean values (mg/kg)
11-L-A	2.6 ± 0.2[a]	2.8 ± 0.1[a]
11-L-B	2.5 ± 0.1	2.9 ± 0.2
11-L-C	2.6 ± 0.2	2.9 ± 0.2
21-L-A	15.0 ± 2.0	15.5 ± 0.4
21-L-B	12.4 ± 1.7	12.1 ± 0.4
21-L-C	26.4 ± 2.9	26.5 ± 0.9
22-L-A	63.1 ± 12.4	59.0 ± 2.1
22-L-B	46.1 ± 7.7	42.1 ± 1.6
22-L-C	103.0 ± 24.1	93.3 ± 4.1

[a] Mean ± standard deviation ($n = 5$).

Calibration

Owing to the non-linear response of the electron-capture detector, a multi-point calibration graph was constructed over the range of interest using standard *n*-hexane solutions containing 0.1–10 μg/ml of flumetralin corresponding to 1.3–126 mg/kg with a constant concentration of 0.2 μg/ml of mirex as an internal standard.

RESULTS

A tobacco sample spiked with 1.3 mg/kg of flumetralin gave a mean recovery of 99 ± 4% at a confidence limit level of 95% (five replicates). The detection limit was 0.1 mg/kg.

Three tobacco cultures grown in Italy and treated with different amounts of flumetralin 7 days after topping were used for the study. For each culture, three tobacco samples (leaves) were collected and the nine resulting samples were analysed for flumetralin. Table I gives the flumetralin residues found in each tobacco sample. These results were obtained by two different methods in a joint experiment of the Coresta pesticide sub-group [7,8] and with the described Soxhlet method. The results were in good agreement with each other.

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Book Review

Troubleshooting LC systems: a comprehensive approach to troubleshooting LC equipment and separations, by J. W. Dolan and L. R. Snyder, Humana Press, Clifton, NJ, 1989, VIII + 515 pp., price £ 63.10, ISBN 0-89603-151-9.

Troubleshooting LC systems is a book organized in three sections with a total of 17 chapters. The first section, "General considerations", contains chapters 2–5 in which the logical approaches to troubleshooting, the basics of separation, the principles of troubleshooting and the prevention of problems are described.

Chapter 2 includes a comprehensive set of flow charts which help in fast problem isolation and corrections of liquid chromatography (LC) problems. Chapters 3–5 are mainly reviews which can be skimmed through by the more experienced chromatographer. However, for the less experienced chromatographer they contain many useful hints and suggestions.

The second section, "Individual LC modules", contains chapters 6–13 and discusses each of the major parts of a typical LC system. Starting with mobile phase reservoirs and degassing, it describe pumps, tubing, fittings, injectors and autosamplers, columns, detectors and recorders and data systems. These chapters not only discuss how to troubleshoot each particular module, but also cover the principles of operation and preventive maintenance. Most readers will probably not take the time to read each chapter, particularly if they are trying to troubleshoot a specific problem, but nevertheless they are a comprehensive source of valuable information.

The third section, "Troubleshooting the separation plus other problems", contains chapters 14–17 and discusses problems related to the separation rather than the equipment. Separation problems, such as band tailing and peak distortion, changes in the appearance of the chromatogram, problems in quantification and the use of gradient elution and sample pretreatment are discussed.

The comprehensive index at the end of this book is a valuable tool for the efficient location of a specific topic. However, I did have some problems in locating cross-referenced information when I was trying to troubleshoot a specific problem, *e.g.*, solvent degassing for gradient elution at low wavelengths.

This book was written to provide chromatographers at all levels of experience with the necessary expertise to isolate and solve an LC problem quickly. The basic rules of troubleshooting, such as make only one change at a time (rule of one), confirm a problem or a solution at least twice (rule of two), substitute a questionable part by a known good part (substitution rule), reinstall the original part for reconfirmation (put it back rule), are common sense but in practice we often do not consider them sufficiently. The book reviews these basic rules very clearly and reminds even the experienced chromatographer to use them more systematically.

I found some other hints, such as preventive recordkeeping (keep track of your

system and the changes you made in your logbook) and preventive maintenance of the system, very useful.

Even in a book of this size it is not possible to cover all subjects of troubleshooting in LC, but I found the wealth of information on topics that most chromatographers have to deal with extremely valuable. In our laboratory, *Troubleshooting LC systems* is now one of the most used handbooks and, since its acquisition 4 months ago, we have successfully solved two LC problems and reduced unnecessary downtime.

Amsterdam (The Netherlands) JEAN PIERRE CHERVET

PUBLICATION SCHEDULE FOR 1991

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Ion Chromatography

Principles and Applications

by **P.R. Haddad,** *University of New South Wales, Kensington, N.S.W., Australia and* **P.E. Jackson,** *Waters Chromatography Division, Milford, MA, USA*

(Journal of Chromatography Library, 46)

Ion chromatography (IC) was first introduced in 1975 for the determination of inorganic anions and cations and water soluble organic acids and bases. Since then, the technique has grown in usage at a phenomenal rate. The growth of IC has been accompanied by a blurring of the original definition of the technique, so that it now embraces a very wide range of separation and detection methods, many of which bear little resemblance to the initial concept of ion-exchange separation coupled with conductivity detection.

Ion Chromatography is the first book to provide a comprehensive treatise on all aspects of ion chromatography. Ion-exchange, ion-interaction, ion-exclusion and other pertinent separation modes are included, whilst the detection methods discussed include conductivity, amperometry, potentiometry, spectroscopic methods (both molecular and atomic) and post-column reactions. The theoretical background and operating principles of each separation and detection mode are discussed in detail. A unique extensive compilation of practical applications of IC (1250 literature citations) is presented in tabular form. All relevant details of each application are given to accommodate reproduction of the method in the laboratory without access to the original publication.

This truly comprehensive text on ion chromatography should prove to be the standard reference work for researchers and those involved in the use of the subject in practical situations.

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