ISSN 0021-9673

VOL. 155 NO. 2 AUGUST 11, 1978

THIS ISSUE COMPLETES VOL. 155

AL OF

HROMATOGRAPHY

NATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

PUBLICATION SCHEDULE FOR 1978

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

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	147 148/1	148/2 149	150/1 150/2	151/1 151/2 151/3	152/1 152/2	153/1 153/2	154/1 154/2	155/1 155/2 156/1	156/2 157	158 160/1	160/2* 161	
Chromatographic Reviews		159/1				159/2				159/3		
Biomedical Applications	145/1		145/2		145/3		146/1		146/2		146/3	

* Cumulative indexes Vols. 141–160.

Scope. The *Journal of Chromatography* publishes papers on all aspects of chromatography, electrophoresis and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental development and their applications. The section *Biomedical Applications*, which is under separate editorship, deals with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis (including the publication of normal values); screening and profiling procedures with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; combinations of chromatographic and electrophoretic methods with other physicochemical techniques such as mass spectrometry. In *Chromatographic Reviews*, reviews on all aspects of chromatography, electrophoresis and related methods are published.

Submission of Papers. Papers in English, French and German may be submitted, if possible in three copies. Manuscripts should be submitted to:
The Editor of *Journal of Chromatography*, P.O. Box 681, 1000 AR Amsterdam, The Netherlands
or to:
The Editor of *Journal of Chromatography, Biomedical Applications*, P.O. Box 681, 1000 AR Amsterdam, The Netherlands.
Reviews are invited or proposed by letter to the Editors and will appear in *Chromatographic Reviews* or *Biomedical Applications*. An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation.

Subscription Orders. Subscription orders should be sent to: Elsevier Scientific Publishing Company, P.O. Box 211, 1000 AE Amsterdam, The Netherlands. The *Journal of Chromatography, Biomedical Applications* can be subscribed to separately.

Publication. The *Journal of Chromatography* (including *Biomedical Applications* and *Chromatographic Reviews*) has 17 volumes in 1978. The subscription price for 1978 (Vols. 145–161) is Dfl. 2006.00 plus Dfl. 255.00 (postage) (total *ca.* US$ 983.00). The subscription price for the *Biomedical Applications* section only (Vols. 145, 146) is Dfl. 244.00 plus Dfl. 30.00 (postage) (total *ca.* US$ 119.00). Journals are automatically sent by air mail to the U.S.A. and Canada at no extra costs, and to Japan, Australia and New Zealand with a small additional postal charge. Back volumes of the *Journal of Chromatography* (Vols. 1 through 144) are available at Dfl. 136.00 (plus postage). Claims for issues not received should be made within three months of publication of the issue. If not, they cannot be honoured free of charge. For customers in the U.S.A. and Canada wishing additional bibliographic information on this and other Elsevier journals, please contact our Journal Information Centre, 52 Vanderbilt Avenue, New York, N.Y. 10017. Tel: (212) 867-9040.

For further information, see page 3 of cover.

Printed in The Netherlands

CONTENTS

(Continued overleaf)

Contents (continued)

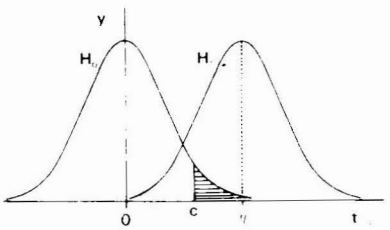
Notes

Statistical Treatment of Experimental Data

by J. R. GREEN, *Lecturer in Computational and Statistical Science, University of Liverpool, and* D. MARGERISON, *Senior Lecturer in Inorganic, Physical and Industrial Chemistry, University of Liverpool.*

Physical Sciences Data, Vol. 2

First published in 1977 and now reprinted with some minor revisions, this book is intended for researchers wishing to analyse experimental data using statistical methods. Statistical concepts and methods which may be employed, are explained, and the ideas and reasoning behind statistical methodology clarified. Formal results are illustrated by many numerical worked examples mainly taken from the laboratory. Concepts, practical methodology, and worked examples are integrated in the text.



Consideration is given in this work to a large number of practical topics which are often omitted from standard texts. These include: obtaining an approximate confidence interval for a function of some unknown parameters; testing for outliers, stabilization of heterogeneous variances, and significant differences between means; estimation of parameters after performing tests; deciding what numbers of significant figures to quote for sample means and variances; straight-line and polynomial regression, through the origin or not, using weighted points, and testing the homogeneity of a set of such lines or curves.

The many examples provided throughout the text will serve as models for the various problems encountered by the readers when employing statistical methods to treat experimental data.

In addition to research workers in universities and industry, the book will be of use for first-year students of statistics, and will be especially suitable as the basis of a graduate course in experimental sciences.

CONTENTS: Chapters: 1. Introduction. 2. Probability. 3. Random Variables and Sampling Distributions. 4. Some Important Probability Distributions. 5. Estimation. 6. Confidence Intervals. 7. Hypothesis Testing. 8. Tests on Means. 9. Tests on Variances. 10. Goodness of Fit Tests. 11. Correlation. 12. The Straight Line Through the Origin or Through Some Other Fixed Point. 13. The Polynomial Through the Origin or Through Some Other Fixed Point. 14. The General Straight Line. 15. The General Polynomial. 16. A Brief Look at Multiple Regression. Appendices: 1. Drawing a Random Sample Using a Table of Random Numbers. 2. Orthogonal Polynomials in x. References. Index.

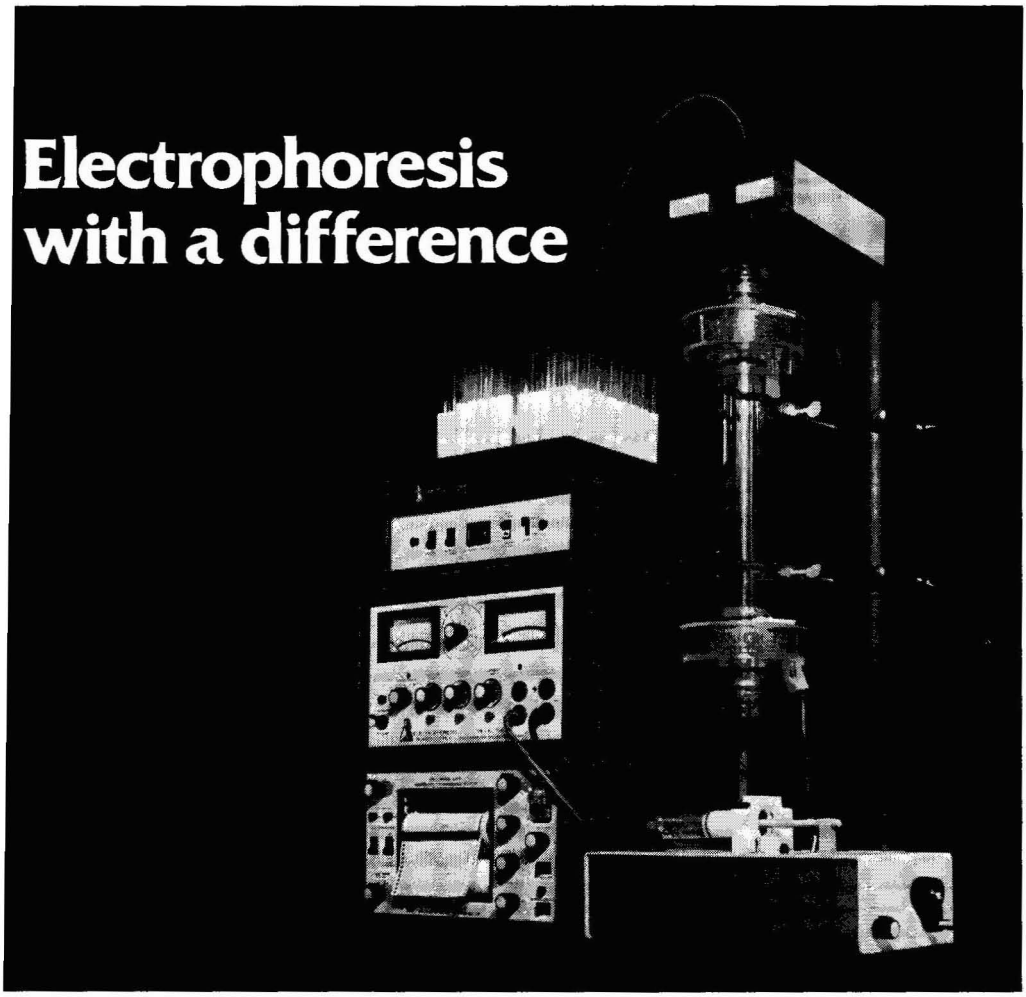
1977 1st revised reprint 1978 xiv + 382 pages US $39.25/Dfl. 90.00
ISBN 0-444-41725-7

ELSEVIER

P.O. Box 211, Amsterdam
The Netherlands
52 Vanderbilt Ave
New York, N.Y. 10017

The Dutch guilder price is definitive. US $ prices are subject to exchange rate fluctuations.

Electrophoresis with a difference



Whether you're involved in special cedures or routine analyses, we can p with our innovative instruments for earch electrophoresis.

Power supplies. ISCO precision power plies deliver reliable constant power, rent, or voltage to your electrophoretic tem. Results: shorter separation time l sharper zones.

Analytical tube gel apparatus. Convenin-line configuration eliminates need to nove or empty tanks to change tubes. Rapid destainer is also available.

Gel scanner. Get high-resolution scanning at UV or visible wavelengths. Dual beam scanning of isoelectrically focused gels eliminates background absorbance.

Density gradient/isoelectric focusing column. Put an end to blind electrophoresis: only an ISCO column lets you record UV absorbance at any time during separation.

For more information, send for your free catalog today. Or dial direct, toll free (800) 228-4250 (continental U.S.A. except NE).

Instrumentation Specialties Company,
P.O. Box 5347, Lincoln, NE 68505.

ISCO

Instruments with a difference

RS Solvents for the analysis of pesticide residues

On the recommendation of international organisations such as FAO and WHO, many nations throughout the world have felt the necessity to regulate the use and the control of pesticides in foodstuffs.
The analysis of pesticide residues presents unusual problems because of the small amounts of substances to be determined and the large number of possible interfering substances which must be first eliminated. In order to determine extremely small quantities, very sensitive analytical methods are required, which however cannot be applied directly to the substances under examination. The general procedure is:

- extraction of the pesticide from the sample;
- concentration of the extract by evaporation of the solvent;
- removal from the extract of naturally-occurring substances which would interfere with the pesticides;
- determination of the extracted pesticide.

In these operations large quantities of polar and non polar solvents are used.
The usual Analytical Grade solvents, when subjected to a more detailed investigation (GLC e.c.d. and Na d.), show peaks due to impurities, whose positions coincide with those of the peaks of pesticides. The use of solvents from which these impurities have been eliminated is therefore indispensable.
Carlo Erba RS solvents for pesticides have been studied and developed in order to satisfy these requirements. Their main characteristic is that of having a greatly reduced quantity of any residue which may interfere with the analytical method. This has been attained by working under special conditions, with small batches which are controlled individually, and by special choice of packing materials, cleaning methods and bottle closure procedures. For chlorinated compounds, a maximum limit of 10^{-9}% as aldrin (GLC e.c.d.) is guaranteed, and for phosphorylated compounds a maximus limit of 10^{-8}% as parathion (GLC Na d.).
All these products are available in bottles of 1000 ml.

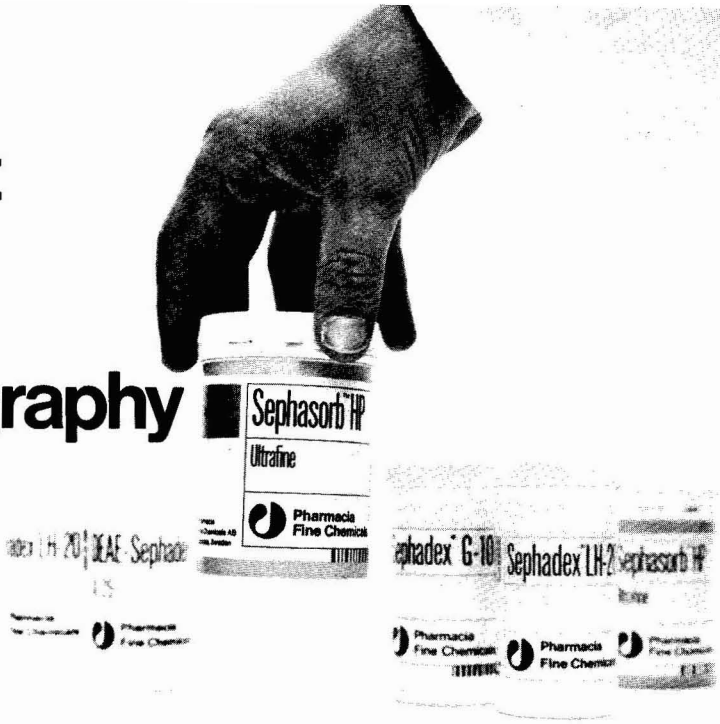
Acetone RS
Acetonitrile RS
Benzene RS
Chloroform RS
Cyclohexane RS
Dioxane RS
Ethyl acetate RS
Ethyl ether RS
n-Hexane RS
Isooctane RS
Isopropyl alcohol RS
Methanol RS
Methylene chloride RS
Petroleum ether 40°-60° RS
n-Pentane RS
Toluene RS

MONTEDISON GROUP
CARLO ERBA

ref.no. 229

CHEMICALS DIVISION
P.O. Box 3996/020159 Milano/Via Imbonati 24 (It
Telex Erba Mi 36314/Tel. 6995

A different selection for liquid chromatography



ake the difference to your results with media from Pharmacia Fine Chemicals. Better elds with excellent reliability over a wider pH range than with conventional supports.

- **First class reproducibility**
- **Catalytically inert**
- **Excellent stability in aqueous and organic solvents**

Select the medium to suit your needs from the following.

ephasorb™ HP Ultrafine

unique new adsorbent for LC and HPLC with novel electivity properties to solve difficult separation roblems. Sephasorb HP Ultrafine has good capacity nd efficiency for adsorption chromatography of ompounds in the MW range 100–2,000.

ephadex® LH-20, LH-60

ephadex LH-20 is suitable for adsorption, partition r gel filtration of a wide variety of synthetic and atural products including hydrocarbons, phenols, eptides, glycosides, vitamins, hormones, steroids, esticides etc. Sephadex LH-60 gives a large stable tationary phase for partition chromatography of pids, peptides, glycosides etc.

Sephadex® Ion Exchangers

Sephadex ion exchangers have excellent stability, high capacity and first class resolution. Strong and weak anion – and cation – exchangers are available for the separation of antibiotics, peptides, nucleotides, hormones, drugs, vitamins and other charged compounds in the range pH 2–12.

Sephadex® G-series

Sephadex has been used extensively for gel filtration. Highly cross-linked types e.g. Sephadex G-10 and G-15 show adsorptive properties which can be utilised for the separation of anions and cations, amino acids, growth substances, vitamins, metabolites etc.

Find out more about the difference from Pharmacia Fine Chemicals.

P.S. All these media are porous and bead-formed.

harmacia Fine Chemicals AB
ox 175
-751 04 Uppsala 1
weden

Pharmacia Fine Chemicals

"Perhaps the most ingenious and effective electrophoretic method for separating proteins is electrofocusing." Lehninger, Biochemistry, 2nd. Ed., Worth, 1975.

Electrofocusing gives you fast, one-step separation of multi-protein mixtures. Superior resolution. More information. A few of the reasons why electrofocusing is the fastest-growing technique in protein separation. And why you should find out more about electrofocusing and what it can do for your separations. The one to ask is the company that started it all. The company with the complete system for electrofocusing. LKB. For our special electrofocusing literature, please contact your nearest LKB office. LKB Produkter AB, S-161 25 Bromma, Sweden.

The resolving power of electrofocusing gives you more information because you can separate — in a single run — components which differ in pI by as little as 0.0025 pH units. It is thus suitable for differentiating closely related molecules and is a rigid test of homogeneity for preparation and analysis. This has spurred the rapid growth of electrofocusing as a major technique in biochemistry. To obtain all that electrofocusing promises LKB offers its complete system: Multiphor, for analytical and preparative electrofocusing; LKB Power Supply, with the constant-power output essential for electrofocusing; Ampholine® carrier ampholytes and Ampholine® PAGplates — your standards for electrofocusing. All supported by Application Notes and *Acta Ampholinae* Literature Reference List.

LKB: the only complete source of experience, knowledge, and equipment for electrofocusing.

LKB

Journal of Chromatography, 155 (1978) 233–240

CHROM. 10,902

MEANINGFUL ERROR ANALYSIS OF THERMODYNAMIC MEASUREMENTS BY GAS–LIQUID CHROMATOGRAPHY

R. J. LAUB, J. H. PURNELL and P. S. WILLIAMS

Department of Chemistry, University College of Swansea, Swansea, Wales SA2 8PP (Great Britain)

and

M. W. P. HARBISON and D. E. MARTIRE

Department of Chemistry, Georgetown University, Washington, D.C. 20057 (U.S.A.)

(First received November 8th, 1977; revised manuscript received January 27th, 1978)

SUMMARY

The usefulness of a simplified error analysis procedure for infinite-dilution partition coefficients (K_R) as measured by gas–liquid chromatography is established by comparison of calculated with experimental relative standard deviations. It is shown that the determination of the column liquid-phase volume, V_L, is the largest source of random error, other error sources, such as that for retention times, being trivial by comparison. As a result, simple apparatus is found to be adequate for the described measurements. An inter-laboratory comparison of partition coefficients shows, further, that the reproducibility of these data can be as good as $\pm 1\%$, these finding, in addition, excellent agreement with those determined by a static technique.

INTRODUCTION

The advantages of gas–liquid chromatography (GLC) for the measurement of thermodynamic properties of solutions, wherein one component is at infinite dilution, have long been recognized[1,2]. The GLC technique requires that the solute partition coefficient, K_R, or the specific retention volume, V_g^0, be determined. These are found, in practice, from the relation,

$$K_R = \frac{t_R' j F_c}{V_L} = \frac{V_g^0 \varrho_L T}{273.15} \tag{1}$$

where t_R' is the solute retention time corrected for dead space, j is the James–Martin[3] gas compressibility correction factor, F_c is the volume flow-rate at the column outlet re-calculated from ambient temperature to the column temperature, T, after correction for the presence of water vapour pressure if a soap-bubble flow meter is employed, and V_L is the volume of liquid (stationary) phase of density, ϱ_L, in the column.

Activity coefficients may be derived from K_R data and fugacity corrections then applied[4] to provide values of γ_1^∞.

The essential simplicity of the GLC approach is so attractive that it is somewhat surprising that it has not been more widely used for thermodynamic studies. It seems to us possible that this reluctance springs from a lack of certainty with regard to the quality of reported data. Indeed, a recently published[5] comprehensive list of GLC-determined activity coefficients indicates that inter-laboratory agreement of γ_f^∞ data is poor, amounting to, at best, $\pm 5\%$, agreement of GLC with static (non-chromatographic) data being no better. Some of the apparent irreproducibility of GLC data from laboratory to laboratory is to be attributed to the use of initial (rather than peak-maximum) retention times in several early studies. Nevertheless, even allowing for this, it is clear that other factors may, in addition, be operative. For example, failure to recognize and to correct for gas–liquid interfacial adsorption is not uncommon while, in converting γ_p^∞ to γ_f^∞ values, inappropriate correction can be identified even in recent work.

At the practical level, since GLC studies often involve the use of high-molecular-weight solvents, it is likely that attainment of consistent levels of liquid-phase purity may also present difficulties. But, in the main, workers in the field have generally attributed discrepancies in K_R and V_g^0 data to instrumental sources and as a result, considerable effort has been expended in developing more reliable apparatus and concomitant error analysis procedures (*e.g.*, refs. 6–9). Our own experience, on the other hand, has led us to question the importance of instrumental factors in comparison with the problems associated with measurement of V_L, as noted by, for example, Wicarova *et al.*[6]. The current study is therefore aimed at defining the various sources of random error and the limits of accuracy of GLC data. To this end, two studies have been concurrently duplicated in laboratories which, where it will be useful, will be herein identified as A and B.

EXPERIMENTAL

The solutes employed were reagent-grade *n*-alkanes (C_5–C_8), cyclohexane, methylcyclohexane, benzene and toluene, the solvents used being dinonyl phthalate (di-3,5,5-trimethylhexyl phthalate, DNP) and squalane (2,6,10,15,19,23-hexamethyltetracosane, SQ). The former was obtained in two lots from BDH (Poole, Great Britain), the latter separately from BDH and Applied Science Labs. (State College, Pa., U.S.A.) and both were used as received. These particular solvents were chosen for this study in order to allow comparison of the chromatographic data with data obtained by extrapolation from finite-concentration of conventionally derived vapour–liquid measurements[10].

The solid support used throughout was Chromosorb G (60–80 mesh, AW DMCS) which was dried overnight, prior to use, in an air oven at 100°C and subsequently stored in a vacuum desiccator over magnesium perchlorate. The material was obtained separately from Applied Science Labs. and Jones Chromatography (Cardiff, Great Britain).

The required amount of liquid phase was dissolved in a volatile solvent (A: methylene chloride; B: acetone + benzene), the support added, and the solvent removed by aspiration. The packing was then finally dried by rotary evaporation.

Coiled stainless-steel columns (0.25 in. O.D.) were packed by applying suction to one end, which had been plugged with silanized glass wool, pouring the packing

into the other end, and gently tapping until no more could be added. Glass wool was then inserted into the filling end. The columns were used as packed, *i.e.*, the filling end was connected to the injector and the suction end to the detector. Liquid loadings of 7–10% (w/w) were used.

Thermo-regulated water-baths were used as column thermostats in both studies. These comprised a Neslab PBC-2 immersion cooling unit and a Vycor 250-W immersion heater coupled to a mercury thermometer-switch (A) and an integrated system based on a Grant, Ltd., 50-l water-bath unit (B). Temperatures were measured with an NBS-calibrated 29.5–30.5°C thermometer (A) or a Hewlett-Packard Model 2802A platinum resistance system (B). The temperature in each system was maintained at 30.00 ± 0.03°C.

Solutes were injected either as individual or as mixed vapours from a 10-μl syringe into a heated Hamilton flash-vaporization unit (A) or a heated injection port constructed from a tube Tee-junction (B), the temperature in either case being maintained at *ca.* 200°C, *i.e.*, above the boiling point of the least volatile solute used.

Both experimental systems employed thermal conductivity detection, the devices used being a Gow-Mac Model 10-952 (A) and a Pye Model 12143 (B). The units and connecting lines were heated to *ca.* 200–250°C with heating tape or Nichrome wire.

The flow of the helium carrier gas was controlled by a conventional cylinder regulator followed in series by one non-lagged (A) or two lagged (B) Negretti-Zambra R-182 NC precision pressure regulators. The column inlet pressure (p_i) was measured with a calibrated U.S. Gauge pressure gauge (A) and a mercury manometer (B), the outlet pressure (p_o) in each case being atmospheric. Column pressure drops ranged from 0.1 to 8 p.s.i. Flow-rates were measured with a water-jacketted 0- to 50-ml soap-bubble flow meter and stopwatch, rates of 20 to 150 ml/min being employed.

Measurement of V_L

The column packing weight was determined by removing the injection-end glass wool plug and displacing the column contents by suction into a single-hole, stoppered, tared vacuum flask, the side-arm of which was fitted with a coarse glass frit. The weight percent of liquid on the support was measured by replicate ashings at red heat (700–1000°C) of tared amounts of packings in porcelain crucibles with a Bunsen burner (A) or a muffle furnace (B). Heating was applied for at least 4 h. Bare support weight losses, due to decomposition of the silanised surface, were determined by the same method and were corrected for when the liquid-phase weight percent was calculated. V_L was calculated from the column packing weight, the weight percent, and the 30°C density of the phases, 0.9638 g/ml (DNP) and 0.8028 g/ml (SQ).

RESULTS

Replicate analysis of the weight loss (mg) per gram of heated support gave, on average, 2.1_2 (A) and 2.7_7 (B) mg/g. These showed some variation when different batches were tested, but the differences were on the order of the errors in weighing milligram amounts. Table I presents the ashing data for all coated packings. Retention data, measured from the air peak, were determined in duplicate with flow-rates being measured before and after all solutes were run, a period of approximately

TABLE I

STATIONARY-PHASE LIQUID LOADINGS (%, w/w) DETERMINED BY ASHING

	Series A				*Series B*			
Run No.	*SQ*		*DNP*		*SQ*		*DNP*	
	Col. 1	*Col. 2*	*Col. 3*	*Col. 4*	*Col. 1*	*Col. 2*	*Col. 3*	*Col. 4*
1	8.45_7	9.73_3	7.93_6	9.85_3	9.79_0	9.95_2	9.80_6	9.86_6
2	8.39_7	9.78_5	7.83_1	9.76_5	9.82_3	10.00_9	10.00_1	9.93_3
3	8.37_6	9.74_7	7.78_8	9.66_0	9.71_0	10.01_2	9.98_4	9.91_3
4	—	—	—	—	9.85_4	9.92_3	9.95_8	9.86_4
5	—	—	—	—	—	10.05_0	9.92_4	9.85_9
Average ($\bar{X}$)	8.41_0	9.75_5	7.85_2	9.75_9	9.79_4	9.98_9	9.93_5	9.88_7
$(\sigma_x/\bar{X}) \cdot 100$	0.50	0.28	0.97	0.99	0.63	0.51	0.78	0.34

TABLE II

COLUMN AND RETENTION DATA FOR LISTED SOLUTES WITH SQUALANE AT 30°C

Solute	*t'_R (min)*			
	Series A		*Series B*	
	Column 1	*Column 2*	*Column 1*	*Column 2*
n-Pentane	1.85 ± 0.00	1.95 ± 0.01	1.86 ± 0.00	1.79 ± 0.01
n-Hexane	5.72 ± 0.00	6.01 ± 0.01	5.79 ± 0.01	5.62 ± 0.00
Cyclohexane	10.84 ± 0.01	11.44 ± 0.00	—	—
n-Heptane	17.36 ± 0.02	18.30 ± 0.01	17.61 ± 0.00	17.09 ± 0.02
Methylcyclohexane	21.79 ± 0.07	23.01 ± 00.1	—	—
n-Octane	52.37 ± 0.09	55.09 ± 0.09	—	—
Benzene	8.09 ± 0.02	8.59 ± 0.01	8.24 ± 0.01	7.99 ± 0.00
Toluene	26.49 ± 0.02	28.33 ± 0.04	27.11 ± 0.02	26.15 ± 0.01
jF_c (ml/min)	69.70 ± 0.05	78.26 ± 0.07	132.33 ± 0.49	144.45 ± 0.08
V_L (ml)	1.3061 ± 0.0065	1.5449 ± 0.0043	2.4945 ± 0.0157	2.6438 ± 0.0135

TABLE III

COLUMN AND RETENTION DATA FOR LISTED SOLUTES WITH DINONYL PHTHALATE AT 30°C

Solute	*t'_R (min)*			
	Series A		*Series B*	
	Column 3	*Column 4*	*Column 3*	*Column 4*
n-Pentane	0.89 ± 0.02	1.07 ± 0.00	1.23 ± 0.01	1.25 ± 0.00
n-Hexane	2.65 ± 0.01	3.20 ± 0.01	3.68 ± 0.00	3.73 ± 0.00
Cyclohexane	5.23 ± 0.02	6.28 ± 0.04	—	—
n-Heptane	7.79 ± 0.01	9.39 ± 0.04	10.80 ± 0.00	10.94 ± 0.02
Methylcyclohexane	10.04 ± 0.01	12.07 ± 0.04	—	—
n-Octane	22.61 ± 0.02	27.40 ± 0.02	—	—
Benzene	8.96 ± 0.00	10.83 ± 0.01	12.40 ± 0.02	12.59 ± 0.02
Toluene	27.77 ± 0.00	33.65 ± 0.01	38.18 ± 0.06	39.16 ± 0.02
jF_c (ml/min)	75.36 ± 0.18	78.54 ± 0.11	114.49 ± 0.60	111.36 ± 0.07
V_L (ml)	1.0138 ± 0.0098	1.2740 ± 0.0126	2.1253 ± 0.0166	2.1180 ± 0.0072

2–4 h. The averaged t'_R and jF_c data are shown in Tables II and III. Reduction of these data according to eqn. 1 yielded the solute partition coefficients shown in Tables IV and V where the data extrapolated from the results of Ashworth[10] using a static system are also presented.

TABLE IV

PARTITION COEFFICIENTS (K_R) OF LISTED SOLUTES WITH SQUALANE SOLVENT AT 30.0°C

Solute	*Series A*			*Series B*			*Static**
	Col. 1	*Col. 2*	*Ave.*	*Col. 1*	*Col. 2*	*Ave.*	
n-Pentane	98.73	98.78	98.76	98.66	97.80	98.23	98.08
n-Hexane	305.3	304.5	304.9	307.1	307.1	307.1	305.5
Cyclohexane	578.5	579.5	579.0	—	—	—	584.4
n-Heptane	926.4	927.0	926.7	934.0	933.8	933.9	927.0
Methylcyclohexane	1163	1166	1164	—	—	—	—
n-Octane	2795	2791	2793	—	—	—	2790
Benzene	431.7	435.1	433.4	437.1	436.6	436.9	434.8
Toluene	1414	1435	1425	1439	1429	1434	—

* Calculated from eqn. 1 and data of ref. 10.

TABLE V

PARTITION COEFFICIENTS (K_R) OF LISTED SOLUTES WITH DINONYL PHTHALATE SOLVENT AT 30°C

Solute	*Series A*			*Series B*			*Static**
	Col. 1	*Col. 2*	*Ave.*	*Col. 1*	*Col. 2*	*Ave.*	
n-Pentane	66.46	65.96	66.21	66.26	65.72	65.99	66.14
n-Hexane	197.0	197.3	197.2	198.2	196.1	197.2	197.4
Cyclohexane	388.8	387.2	388.0	—	—	—	388.2
n-Heptane	579.1	578.9	579.0	581.8	575.2	578.5	572.7
Methylcyclohexane	746.3	744.1	745.2	—	—	—	—
n-Octane	1681	1689	1685	—	—	—	1672
Benzene	666.0	667.7	666.9	668.0	662.0	665.0	670.5
Toluene	2064	2075	2070	2057	2059	2058	—

* Calculated from eqn. 1 and data of ref. 10.

DISCUSSION

For a generalized function, Q, such that

$$Q = a^m b^n c^p \ldots \tag{2}$$

the fractional standard deviation, (σ_Q/Q), is given by[11]:

$$\frac{\sigma_Q}{Q} = \left[m^2\left(\frac{\sigma_a}{a}\right)^2 + n^2\left(\frac{\sigma_b}{b}\right)^2 + p^2\left(\frac{\sigma_c}{c}\right)^2 + \cdots\right]^{\frac{1}{2}} \tag{3}$$

In terms of K_R, eqn. 3 becomes:

$$\frac{\sigma_{K_R}}{K_R} = \left[\left(\frac{\sigma_{t'_R}}{t'_R}\right)^2 + \left(\frac{\sigma_{jF_c}}{jF_c}\right)^2 + \left(\frac{\sigma_{V_L}}{V_L}\right)^2\right]^{\frac{1}{2}} \quad (4)$$

The relative standard deviation to be expected for any set of K_R measurements may therefore be calculated provided reasonable estimates for $\sigma_{t'_R}$, σ_{jF_c}, and σ_{V_L} are available. This treatment differs slightly from that of, for example, Wicarova *et al.*[6], in that sources of random error affecting the parameters, t'_R, jF_c, and V_L, are taken to be reflected adequately by the mean of the parameters themselves, rather than the terms comprising the parameters. For example, calculation of jF_c requires measurement of p_i, p_o, F, T, T_a, p_a, and p_w, for which an expression of the form of eqn. 3 could be written. The relative standard deviation obtained from such a formulation must, however, be very nearly identical to the σ_{jF_c}/jF_c value calculated from the average of measurements of jF_c. Thus, eqn. 4 will provide a reasonable estimate of experimental error in K_R.

Table VI lists the calculated and experimental standard deviations for the K_R data. The legitimacy of eqn. 4 is clearly borne out by this comparison even though

TABLE VI

COMPARISON OF PREDICTED WITH EXPERIMENTAL RELATIVE STANDARD DEVIATIONS FOR K_R DATA

Solute	*Series A* $(\sigma_{K_R}/K_R) \cdot 100$			
	SQ		*DNP*	
	Predicted	*Experimental*	*Predicted*	*Experimental*
n-Pentane	0.45	0.04	1.86	0.53
n-Hexane	0.41	0.19	1.13	0.11
Cyclohexane	0.41	0.12	1.21	0.29
n-Heptane	0.43	0.05	1.08	0.02
Methylcyclohexane	0.47	0.18	1.05	0.21
n-Octane	0.47	0.10	1.02	0.34
Benzene	0.48	0.55	1.02	0.18
Toluene	0.43	1.04	1.02	0.38
Average	0.44	0.28	1.17	0.26
	Series B $(\sigma_{K_R}/K_R) \cdot 100$			
	SQ		*DNP*	
	Predicted	*Experimental*	*Predicted*	*Experimental*
n-Pentane	0.76	0.62	0.91	0.58
n-Hexane	0.65	0.00	0.70	0.75
n-Heptane	0.65	0.02	0.71	0.81
Benzene	0.65	0.08	0.73	0.64
Toluene	0.65	0.49	0.71	0.07
Average	0.67	0.24	0.75	0.57

only two values for each partition coefficient were determined in each study. We note parenthetically that Wicarova *et al.*[6] reported a V_g^0 value for *n*-hexane which, when converted to K_R, gave 291.9 which is in error by 5% from the mean of all K_R data for *n*-hexane with squalane given in Table IV.

The largest contribution to σ_{K_R}/K_R is that from V_L as shown previously by, for example, the comparison of ashing with high-temperature evaporation techniques reported by Petsev *et al.*[12,13]. The former was there found to be more accurate than Soxhlet extraction owing, it was suggested, to the presence of extractable inorganic materials in common supports.

Since squares of relative standard deviations are additive, error sources other than V_L become important only when they approach approximately 10% of the error in this parameter, as shown in Table VII which lists σ_{K_R}/K_R as a function of $\sigma_{t'_R}/t'_R$, σ_{jF_c}/jF_c, and σ_{V_L}/V_L. An increase of from 0.1% to 0.5% in both $\sigma_{t'_R}/t'_R$ and σ_{jF_c}/jF_c increases σ_{K_R}/K_R only from 1.100% to 1.225%. Thus, there is little point in improving control of the pressures and/or flow-rate, or indeed, using automated data acquisition systems if thermodynamic information reliable to $\pm 1\%$ is to be determined by GLC since, in any event, current practice in the measurement of V_L precludes a higher accuracy than this. Relatively simple apparatus is therefore adequate for measurements of this kind, until such time as more reliable techniques for determination of V_L are available.

TABLE VII

EFFECTS OF VARIATION OF σ_i/i ON σ_{K_R}/K_R CALCULATED FROM EQN. 4

Relative standard deviation × *100*

σ_{V_L}/V_L	$\sigma_{t'_R}/t'_R$	σ_{jF_c}/jF_c	σ_{K_R}/K_R
1.0	0.1	0.1	1.1000
1.0	0.5	0.1	1.1225
1.0	0.5	0.5	1.2248
1.0	1.0	0.1	1.4178
1.0	1.0	0.5	1.5000
1.0	1.0	1.0	1.7321

We regard the results given in Tables IV and V as the most accurate data yet reported *for the systems listed*, and suggest their use for the evaluation of accuracy and reproducibility of other studies.

ACKNOWLEDGEMENTS

We thank the National Science Foundation and the Science Research Council for financial support.

REFERENCES

1 A. B. Littlewood, C. S. G. Phillips and D. T. Price, *J. Chem. Soc.*, (1955) 1480.
2 M. R. Hoare and J. H. Purnell, *Trans. Faraday Soc.*, 52 (1956) 222.
3 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.

4 D. H. Everett, *Trans. Faraday Soc.*, 61 (1965) 1637.
5 D. C. Locke, *Adv. Chromatogr.*, 14 (1976) 87.
6 O. Wicarova, J. Novak, and J. Janak, *J. Chromatogr.*, 51 (1970) 3.
7 M. Goedert and G. Guiochon, *Anal. Chem.*, 42 (1970) 962; 45 (1973) 1180, 1188; *J. Chromatogr. Sci.*, 7 (1969) 323.
8 J. A. Jonsson, R. Jonsson and K. Malm, *J. Chromatogr.*, 115 (1975) 57.
9 E. F. Meyer and F. A. Baiocchi, *Anal. Chem.*, 49 (1977) 1029.
10 A. J. Ashworth, *J. Chem. Soc. Faraday Trans. I*, 69 (1973) 459.
11 H. D. Young, *Statistical Treatment of Experimental Data*, McGraw-Hill, New York, 1962, pp. 96–101.
12 N. D. Petsev, R. N. Nikolov and A. Kostova, *J. Chromatogr.*, 93 (1974) 369.
13 N. D. Petsev, V. H. Petkov and Chr. Dimitrov, *J. Chromatogr.*, 114 (1975) 204.

Journal of Chromatography, 155 (1978) 241–247

CHROM. 10,860

SORPTION EFFECTS IN COLUMNS IN THE GAS CHROMATOGRAPHY OF COPPER TRIFLUOROACETYLACETONATE

D. N. SOKOLOV, A. V. DAVYDOV, S. YU. PROKOFYEV, S. S. TRAVNIKOV, E. V. FEDOSEEV and B. F. MYASOEDOV

V. I. Vernadsky Institute of Geochemistry and Analytical Chemistry, U.S.S.R. Academy of Sciences, Moscow, and Section of the Institute of Chemical Physics, U.S.S.R. Academy of Sciences, Chernogolovka, Moscow Province (U.S.S.R.)

(Received December 21st, 1977)

SUMMARY

The distribution of copper trifluoroacetylacetonate during the conditioning of a gas chromatographic column has been studied. It was found that the sorption of the copper chelate in the column depends on the amount of the compound injected, the content of liquid phase in the bed, the nature of the solid support and the temperature. Substantial sorption of the copper chelate by glass- and PTFE-wool plugs was observed. It is shown that the sorption of the copper chelate by the bed is partly a reversible process. The results obtained indicate the necessity for further investigations of inert phases and solid supports and for the suppression of sorption effects in the chromatographic column.

INTRODUCTION

In the gas chromatography of β-diketonates of metals, several effects occur that cause some difficulties in the application of the method for analytical purposes. Of particular importance is the necessity for conditioning the column in order to overcome adsorption of the chelate during the injection of the first portions of the compound being analysed in a column that is new or has not been used for a long period[1,2], displacement of earlier sorbed chelate from the column by other chelates[1,3] or by pure β-diketone[4], difficulties in the determination of small amounts of β-diketonates connected with their losses in the column[5–9], etc. Similar effects have been observed with other compounds[10]. In most papers on the gas chromatography of chelates of metals, sorption effects in columns are rarely considered, except for two papers[1,3].

This paper describes a study of the sorption of bis-(1,1,1-trifluoro-2,4-pentadionato)copper(II) (copper trifluoroacetylacetonate) in columns depending on the conditions used in the gas chromatographic determination. This compound was chosen because the behaviour of copper trifluoroacetylacetonate (CuL_2) in columns is typical of most β-diketonates of metals (an exception are the fluorinated β-diketonates of

beryllium, aluminium and chromium, for which the above effects occur only slightly or not at all). Also, we chose the method of radioactive tracers as the main method of investigation and the isotope copper-64 has nuclear physical characteristics that are convenient for radiometric measurements.

EXPERIMENTAL

Equipment

A gas chromatograph of special construction was used, with which it is possible to detect the compound being chromatographically determined by measuring its γ-radiation at any point along the column and also to record automatically the distribution of γ-radioactive substances during their sorption in the column without cooling and removing it from the chromatograph. In order to measure γ-radiation, a scintillation detector with a NaI(TL) crystal was used that was connected with an intensimeter, the response of which was recorded with an electronic potentiometer. Tsvet-5 and Tsvet-102 gas chromatographs with a katharometer and a flame-ionization detector and an LHM-8 MDP with a detector for γ-radiation of our own construction were used.

For the determination of copper in the liquid phase and separately in the solid carrier a Saturn atomic-absorption spectrophotometer with a Perkin-Elmer NSA-74 graphite furnace atomizer was used.

Columns

Glass or PTFE columns of dimensions 30 $\times$ 0.4 cm (40 $\times$ 0.4 cm in some instances) were used. Various amounts (5–10%) of SE-54 silicone and 5PUE polyphenyl ether on the solid supports Chromaton NAW (0.1–0.125 mm), Chromaton NAW DMCS (0.25–0.315 mm) and Polychrome-1 were used as liquid phases. The beds were fixed in the columns by two plugs of PTFE- or glass-wool. The carrier gas was high-purity helium at a flow-rate of 30 ml/min. The temperature of the columns and the evaporator was varied with the range 120–160°. Before chromatographic operations the columns were purged with helium for 2 h at 180°.

Reagents

Copper trifluoroacetylacetonate was synthesized from trifluoroacetylacetone (Serva, Heidelberg, G.F.R.) according to Berezkin *et al.*[10]. In order to obtain a compound labelled with copper-64, special-purity copper (content of impurities 10^{-4}%), irradiated in a reactor with neutrons, with a specific activity of 5 mCi/mg was used. The copper trifluoroacetylacetonate was purified by sublimation in a current of argon at 200°. The radiochemical purity of the compound obtained was controlled by the γ-spectrometric method.

Methods of investigations

Copper trifluoroacetylacetonate in the form of a 2.5–3.0% solution in benzene or toluene ("pure for analysis") was injected into the columns with a 10-μl microinjector. The volume of the samples injected was 2–10 μl. The amount of the compound sorbed in the column was determined within 5 min of the detection of the last chromatographic peak.

With copper trifluoroacetylacetonate labelled with copper-64, the distribution of γ-radioactivity along the column was recorded and the area of the radiogram obtained was measured in order to determine the amount of the copper compound sorbed in the column. The amount of copper was calculated from the calibration graph of the area of the radiogram *versus* the copper content, which was plotted by measurement of the γ-activity of standard samples. If necessary, a correction for decay of copper-64 (half-life = 12.8 h) was introduced. For the preparation of standards, aliquots of the solution of copper trifluoroacetylacetonate (labelled with copper-64) with a known copper content (12.9–49 μg) were taken, 1 ml of diethyl ether was added and the solutions obtained were applied to strips of filter-paper that were 3 mm wide and of a length equal to that of the chromatographic column (30 cm). The strips were dried and placed in glass tubes analogous to the chromatographic columns. The relative standard deviation of the determination of copper in the column by the given method was 0.05 at $\alpha = 0.95$.

The determination of copper in the liquid phase and separately in the solid carrier was accomplished as follows. The bed was removed from the column, the liquid phase with the sorbed copper trifluoroacetylacetonate was extracted twice with chloroform, the chloroform solutions were combined, brought to a volume of 5 ml and the copper in the solution obtained was determined by atomic-adsorption spectrophotometry with electrothermal atomization. The solid carrier remaining after the removal of the liquid phase was dried and then boiled for 1 h with concentrated nitric acid. The solution was separated by centrifugation and diluted with water to a volume of 5 ml, and the copper in this solution was determined by atomic-absorption spectrophotometry. In order to carry out blank experiments, a bed from a column that had only been purged with helium was used.

RESULTS AND DISCUSSION

The sorption of copper trifluoroacetylacetonate in the column during conditioning was first studied. Fig. 1 shows the curves of the distribution of copper-64 over the length of the column for various amounts of CuL_2 injected into the column, using Chromaton NAW and Polychrome-1 containing 5% of SE-54 as beds. The temperatures of the columns were 120° and 140°, respectively. The conditions selected for the chromatographic analysis were the optimum for observing most clearly the process of column conditioning. When the first portion of copper chelate (25 μg of CuL_2 or 4.3 μg of Cu) was injected the compound was completely sorbed in the column, mainly in the top part. During subsequent injections a gradual equilibration of the concentration of sorbed compounds along the length of the column occurred. On injection into the column packed with 5% SE-54 on Chromaton NAW (and also on Chromaton NAW DMCS) of more than 400 μg of CuL_2 at 140°, stabilization of the form and area the peaks of copper trifluoroacetylacetonate was observed, although chelate sorption in the column was not completed.

In the study of the distribution of the copper compound in the column, significant sorption of the compound by glass-wool plugs was observed, reaching 25–30% of the total amount of the compound sorbed. Replacement of glass-wool with PTFE-wool resulted in less sorption of the chelate (10–12%) on the plugs. The maxima on the curves of the distribution of copper-64 (Fig. 1a and 1b) correspond to

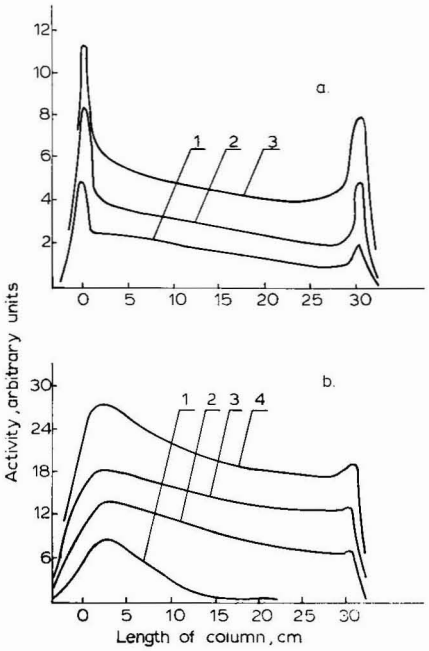


Fig. 1. Distribution of copper-64 in a chromatographic column containing 5% of SE-54 on (a) Chromaton NAW and (b) Polychrome-1 during conditioning. Amount of copper trifluoroacetylacetonate injected into the column: (a) 1 = 25 μg; 2 = 50 μg; 3 = 250 μg; (b) 1 = 25 μg; 2 = 125 μg; 3 = 250 μg; 4 = 1000 μg. Temperature of column: (a) 120°; (b) 140°. Amount of the bed in the column: (a) 1 g; (b) 2 g.

sorption of the chelate by PTFE-wool plugs at each end of the column. This substantial sorption of copper trifluoroacetylacetonate by glass-wool and even of PTFE-wool plugs is not only of interest from the methodological point of view, but may also explain some of the anomalous effects that have been observed during the gas chromatography of chelates of metals. Thus, Uden and Jenkins[1] noticed that during displacement of aluminium trifluoroacetylacetonate, sorbed in a column, by a corresponding iron chelate, the retention time of the complex being displaced during a series of successive injections of iron chelate did not change. The same effect was observed on replacement of the bed of the column, silanization of the solid carrier and use of PTFE instead of a diatomite carrier. This effect may be explained by the sorption of the aluminium chelate by glass-wool in the top part of the column.

Figs. 2 and 3 give data that illustrate the influence of various factors upon the sorption of copper trifluoroacetylacetonate in the column, namely the amount of chelate introduced into the column, the content of the liquid phase on the carrier, the nature of the carrier and the temperature. With an increase in the amount of chelate introduced into the column, gradual saturation of the bed occurs, a gradual decrease in the amount of compound sorbed with respect to the amount of chelate introduced being observed. Table I gives data that characterize the sorption in a column packed with 5% of SE-54 on Chromaton NAW (140°) on the successive introduction of various amounts of chelate. The values given were obtained by taking into account sorption of the chelate by PTFE-wool plugs.

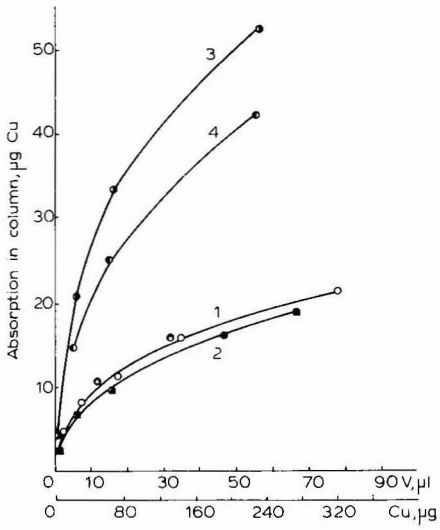


Fig. 2. Sorption of copper trifluoroacetylacetonate in columns containing SE-54 on (1) and (4) Chromaton NAW, (2) Chromaton NAW DMCS and (3) Polychrome-1 (2 g) supports at 140°. SE-54 content: (1) and (3) 5%; (4) 20%. Curve 1 was constructed according to data obtained on two columns. Abscissa: total volume of solution under investigation and total amount of copper injected into the column.

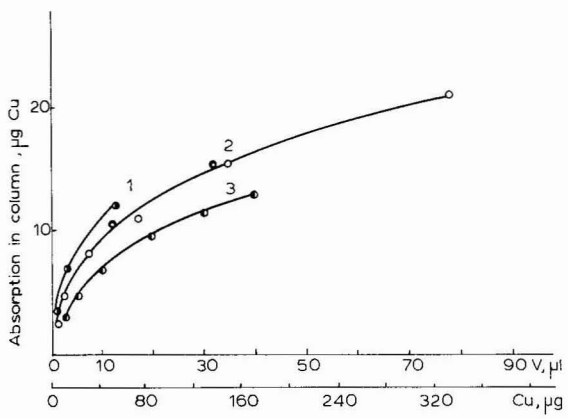


Fig. 3. Influence of temperature on sorption of copper trifluoroacetylacetonate. Temperature: (1) 120°; (2) 140°; (3) 160°. Abscissa: as in Fig. 2.

The adsorption of copper trifluoroacetylacetonate in the column occurs as a result of its interaction with the bed. This was also confirmed by radiometric measurements on the portion of the tube in the evaporator: the amount of copper found in this portion was less than 1% of the amount injected.

As can be seen from a comparison of curves 1 and 4 in Fig. 2, the sorption of copper trifluoroacetylacetonate depends on the amount of liquid phase in the column. With an increase in the content of SE-54 from 5 to 20% (on Chromaton NAW), the sorption of the chelate increased almost 2.5-fold.

TABLE I

SORPTION IN COLUMN PACKED WITH 5% OF SE-54 ON CHROMATON NAW

Volume of solvent injected (μl)	*Amount of* CuL_2 *injected into the column* (μg)	*Sorption of* CuL_2 (% *of amount injected*)
3	15	100
2	50	34
5	125	20
10	250	7.8
2 × 10	500	6.1
4 × 10	1000	3.5

The nature of the solid carrier also influences the sorption of the copper chelate in the column. Although the difference in extent of sorption of the chelate when non-silanized and silanized Chromaton NAW is used as the carrier was not great, when polychrome-1 was used a significant increase in sorption was observed. This effect was unexpected because Polychrome-1 is usually considered as the most inert solid support.

The determination of copper in the bed by atomic-absorption spectrophotometry showed that after removal of the liquid phase by treating the bed with chloroform a noticeable amount of copper remained in some form on the solid support, and could be removed from the support only by treating it with concentrated nitric acid. This appears to indicate partial decomposition of the copper chelate in the column. Using 5% of SE-54 on Chromaton NAW DMCS, the weight of the bed being 1 g, 770 μg of CuL_2 being injected into the column and the temperature of the column being 140°, the amount of copper determined in the liquid phase was 12.1 μg by atomic-absorption spectrophotometry and 13.4 μg by the radiometric method.

Experiments on the influence of temperature on the sorption of copper trifluoroacetylacetonate (Fig. 3) showed that as the temperature was increased the extent of sorption decreased. On purging the column, conditioned with a fixed amount of chelate at 140°, with a carrier gas for 1–2 h at the same temperature, no decrease in the amount of copper in the column was observed. However, on purging at 160° the amount of copper in the column rapidly decreased to a level corresponding to the extent of sorption during conditioning of the column at 160°. From this result it can be concluded that the sorption of the chelate in the column is not completely irreversible. Further experiments were carried out in order to elucidate the question of whether purging from the column of a reversibily sorbed chelate at a constant temperature of the column occurs. For this purpose, columns with removable glass tubes at the ends were used. After injection into the column of a series of samples of copper chelate labelled with copper-64 (ten injections, each of 5 μl or 1250 μg of CuL_2), the removable tube at the end of the column was replaced with an exit tube to the cold zone and provided with a plug of glass-wool. The column was purged with carrier gas at a flow-rate of 30 ml/min for 5 h and then radiometric measurements on the removable tube with the plug were made.

The results showed that during this period 5–10% of the chelate sorbed in the column was purged from the column. Thus sorbed chelate is continuously purged from the column, liberation of active centres of the bed occurring which may sorb

the chelate again on subsequent injection of a sample. This effect probably explains the "disappearance" from columns of small amounts of chelates of metals such as copper, iron and rare earths making their determination in microamounts impossible.

The question of the role of the liquid phase in the gas chromatography of chelates of metals has previously been poorly studied. In most instances silicone phases of the type SE-30, XE-60, OV-17 or OV-1 were used. In this work, the silicone SE-54 was studied, and also for comparison, the sorption of copper chelate on 5PUE polyphenyl ether, which contains hydrogen bridges in the chain. Such phases are considered to be unsuitable for the gas chromatography of chelates of metals. Experiments have shown that, when 1500 μg of copper chelate are injected into a column packed with 5PUE on Chromaton NAW (Fig. 4), the compound is completely absorbed in the column and a strongly pronounced dependence of the amount of chelate sorbed on the amount of liquid phase in the column is observed.

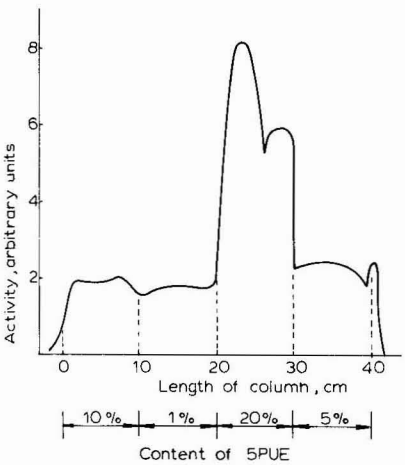


Fig. 4. Distribution of copper trifluoroacetylacetonate during sorption in a column containing 5PUE on Chromaton NAW. Temperature of column, 140°; amount of copper chelate injected into the column, 1500 μg.

REFERENCES

1 P. C. Uden and C. Jenkins, *Talanta*, 16 (1969) 893.
2 D. N. Sokolov and G. N. Nesterenko, *Zavod. Lab.*, 39 (1973) 939.
3 C. A. Burgett and J. S. Fritz, *J. Chromatogr.*, 77 (1973) 265.
4 K. Utsunomiya, *Anal. Chim. Acta*, 59 (1972) 147.
5 R. Belcher, W. I. Stephen, I. J. Thomson and P. C. Uden, *J. Inorg. Nucl. Chem.*, 33 (1971) 1851.
6 C. A. Burgett and J. S. Fritz, *Anal. Chem.*, 44 (1972) 1738.
7 C. A. Burgett and J. S. Fritz, *Talanta*, 20 (1973) 363.
8 R. Belcher, R. J. Martin, W. I. Stephen, D. E. Henderson, A. Kamalizad and P. C. Uden, *Anal. Chem.*, 45 (1973) 1197.
9 H. Kaweguchi, T. Sakamoto, Y. Tuji and A. Mizaike, *Bunseki Kagaku (Jap. Anal.)*, 22 (1973) 1434.
10 V. G. Berezkin, V. P. Pakhomov and K. I. Sakodynsky, *Solid Carriers in Gas Chromatography*, Khimia Moscow, 1975, p. 128.

Journal of Chromatography, 155 (1978) 249–259

CHROM. 10,857

COMPUTER PROGRAM FOR PROCESSING DATA ACQUIRED FROM GAS–LIQUID CHROMATOGRAPHIC ANALYSIS OF AMINO ACIDS

BABOO M. NAIR

Department of Applied Nutrition, University of Lund, Chemical Centre, Box 740, S-220 07-Lund (Sweden)

(First received October 21st, 1977; revised manuscript received December 28th, 1977)

SUMMARY

In order to automate the gas–liquid chromatographic analysis of amino acids and reduce the cost, which is essential in large screening programms, a data processing program was formulated. The crude integrator data containing retention times and peak area values were collected on punched paper tape. The data from the punched tape were transferred to a magnetic tape and processed further in a Univac 1108 computer with the help of a Fortran program. The structure of the data obtained and the procedure for processing and presentation of results are described.

INTRODUCTION

In an earlier paper, a modification of the technique for the gas–liquid chromatographic (GLC) analysis of N-trifluoroacetyl-*n*-butyl (TAB) derivatives of amino acids was presented[1]. Instead of utilizing the signals from the two detectors and electrometers for complete and simultaneous quantitation in two independent digital integrators of all relevant peaks or in one set of equipment recording the results from the two columns successively, we utilized first the signal from the detector of the EGA column until the elution of TAB aspartic acid was completed and then that from the OV-17 column on a single integrating and recording unit. In this way, a saving of analysis time or investment cost has been accomplished. As every amino acid has to be calculated with separate calibration factors, the calculation, tabulation and presentation of data acquired during a large project would obviously be very tedious.

In order to automate the analysis further and reduce the costs, which is essential in large screening programme such as in plant breeding or nutritional surveys, an automatic data-processing system to replace the time-consuming calculations of the "crude" integrator data was sought. One solution to this problem is presented in this paper.

DATA COLLECTION

The column system and the flow of signals are illustrated in Fig. 1. The EGA column was connected to the detector A and the OV-17 column to the detector B. Both detectors were then connected to the teleprinter through a dual differential electrometer–digital integrator system and to the potentiometric recorder. With this arrangement, signals from each of the two detectors could be carried to the integrator and the recorder as and when desired by changing the mode-selector position between A and B.

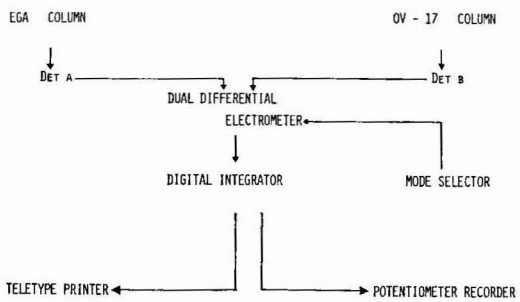


Fig. 1. Diagram showing the flow of detector signals in a dual-column system for amino acid analysis by GLC.

First, a standard sample was injected into the EGA column and then, after a complete run, into the OV-17 column. By comparing the chromatographic charts from both we could select an optimal time for a switchover of the mode selector from A (EGA) to B (OV-17). We chose to change the mode selector from A to B after the elution of TAB aspartic acid from the EGA column, which was probably the best place because we could exploit the EGA column more satisfactorily. The results from our experiment in which first a sub-sample was injected into the EGA column and then another sub-sample into the OV-17 column, as presented in Fig. 2, ensured that a chromatogram containing of all the amino acid peaks was obtained.

We used norleucine as the internal standard in the EGA column and butyl stearate in the OV-17 column for more accurate calibration and further calculation of the data. On certain occasions some difficulty occurred in obtaining satisfactory separation of norleucine from neighbouring peaks. Our experiment in search of a new internal standard resulted in the use of α-aminocaprylic acid. As can be seen from Fig. 2, a simultaneous separation of all of the amino acids could be carried out within about 35 min using the improved method described above. Nevertheless, if only the amino acids separated on the EGA column are of interest, it is possible to run it alone. Thus, a choice between the EGA column alone and the EGA + OV-17 dual-column system was available.

There are principally five types of systems that could be used for the automatic data processing[2–8]: (1) off-line systems; (2) hybrid systems; (3) time-shared computer system; (4) dedicated computer system; and (5) multichannel dedicated computer system.

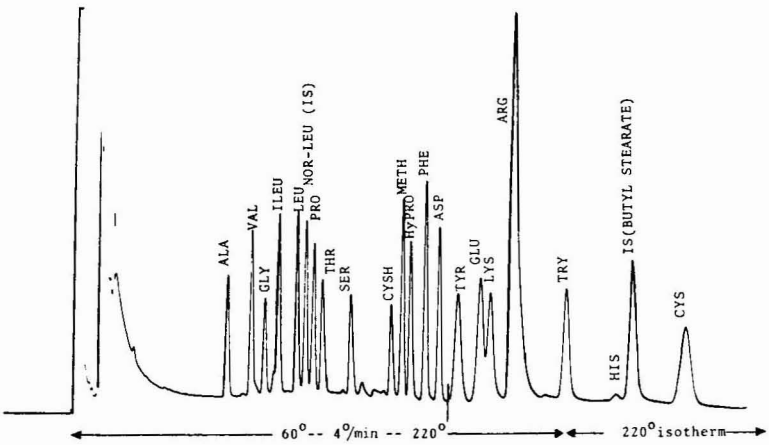


Fig. 2. Separation of all the protein amino acids from a dual-column system. A 0.5-μg sample of each amino acid, norleucine and butyl stearate as the internal standards was injected into the EGA column first and then into the OV-17 column simultaneously within a period 2 min before the beginning of the temperature program (60 to 220° at 4°/min). Argon flow-rate, 50 ml/min. Attenuation, $10^{-10} \times 2$.

(1) An off-line system basically consists of, in addition to a gas–liquid chromatograph, an integrator, which converts the analogue data into digital data, attached to a printer, and with cards, paper-tape or magnetic-tape facilities for data storage. These stored data can then be processed in the computer when it is available and the amount of "crude" data accumulated is adequate for an economically sound used of the computer. This system would be the least expensive and in addition offers a smooth means of adapting to fully computerized GLC systems.

(2) In the hybrid system, many GLC integrator units could be connected to and controlled by one computer-printing device. A major disadvantage is the risk of a serious delay in the analytical work if the computer stops funtioning, as a consequence of which all the GLC integrator units that depend on it would also be out of use.

(3) With the time-shared computer system, one computer with a larger memory is directly connected to many analytical instruments. The large memory would be used for storage of extensive data, tables, statistical data, routines for data interpretation and other programs.

(4) and (5) Both dedicated systems require very large investments and are practicable only for laboratories that have many analytical instruments functioning simultaneously.

The alternative for data processing assessed as optimal under our working conditions was an off-line system (Fig. 3). The analogue signals were carried to an electronic digital integrator when supplying the teletype printer with data on retention times and peak areas. The teletype, in addition to its own printout, was also equipped with a paper-tape punching mechanism. This may not be generally acceptable to the analyst. However, where long calculations are involved, especially when debugging and internal standard identification instructions are to be supplied, there is often much convenience in using this approach.

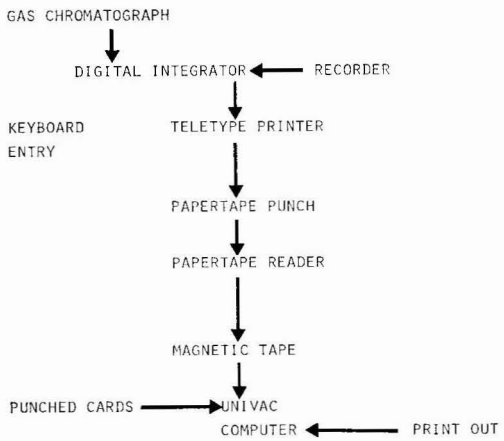


Fig. 3. Schematic presentation of a data-handling system for amino acid analysis by GLC.

Before the sample was injected, information about the sample (weight, nitrogen content, etc.) was introduced to the tape through the keyboard as presented in Table I (points 1–5). The teletype was then switched on to the "on-line" position and a chromatographic analysis was run. Data on retention times and peak areas were obtained as listed under 6 in Table I. At the end of the run, debugging instructions were entered through the keyboard (points 7 and 8). Further, the retention data for the internal standards were introduced through the keyboard as observed on the printout and chromatogram. The actual record of a complete run is shown in Fig. 4. The accumulated data on the paper tape were stored there and transferred before processing to magnetic tape using a tape reader (RC-2000).

DATA PROCESSING

The program for the data processing was developed for the Univac 1108 computer in Fortran V (see Appendix). The program for processing of punched tape contains eight operational subroutines and a main program:

1. BINARS: Binary search
2. MSORT: Sorting
3. KROM: Punched tape read
4. RINT: Read whole numbers
5. RREAL: Read real numbers
6. RSTR: Read strings
7. INTAB: Read conversion tables
8. INFILE: File definition

PARAM: Main program

In addition to the data contained in the magnetic tape, which are transferred from paper tape, three sets of parameter punched cards are used for processing and presentation (see Appendix). The first set carries the number of the sample to be

```
(1) Amino Acid analysis by GLC. Data collection and processing. Dual column
    data for programme test. Norleucine and butyl stearate as internal
    standards.
    -72 06 07- -001-;
(2) 0.094 0.117 0.127 0.115 0.115 0.000 0.185 0.255 0.161 0.000 0.000 0.000
    0.120 0.128 0.000 0.351 0.111 0.129 0.120 0.150 0.135 0.130 -1
(3) (4) (5)
    9.45 0.165 0.187
(6) 097:0039834
    113:0059108
    127:0043540
    135:0041325
    146:0058973
    152:0037225
    156:0085748
    165:0050852
    182:0055043
    218:0015345
    225:0029789
    234:0048466
    242:0082941
    274:0028600
    290:0131637
    302:0056092
    319:0025318
    351:0023300
    392:0027403
    408:0036224
    445:0025560
    -1
(7) 000 000 000 000 -1
(8) 000 000 000 -1
(9) 000 152 408 260
```

Fig. 4. Printout from the teletype after a single GLC run. The circled figures refer to the text in Table I

TABLE I

STRUCTURE OF THE DATA OBTAINED AS IN FIG. 4

Data *Through keyboard*	*From GC integrator*
(1) Text, information introduced into the punched tape through the keyboard of the teletype printer. This could include a description of the sample, internal standards used, date and sample number. Entered as below: – 72 06 02 – – 001 –	(6) Values for retention time and peak area 0.97:003984 113:005988 m : : : 445:0025560
(2) Relative molar ratio of all the amino acids entered in the order they are eluted, starting with value for alanine, etc.: 0.094 0.117 0.127 0.130 – 1	
(3) Weight of the sample (mg)	
(4) Weight of the internal standard 1 (mg) 9.45 0.165	–1
(5) Weight of the internal standard 2 (mg) 0.187	
(7) 000 000 000 –1 (retention time of peak to be deleted)	
(8) 000 002 001 –1 (000, retention time of the first half of the split peak; 002, retention time of the second half; 001, retention time of the added peak)	
(9) 001 002 008 005: 001, retention time of the internal standard in the single column 002, retention time of the internal standard in the EGA column 008, retention time of the internal standard in the OV-17 column 005, time at which the switch was turned over from EGA to OV-17	

AMINO ACID ANALYSIS BY GLC.DATA COLLECTION AND PROCESSING.DUEL COLUMN DATA FOR PROGRAMME TEST.NORLEUCINE AND BUTYL STEARATE AS INTERNAL STANDARDS. -77 06 07- -001-

DATE: 770607 SAMPLE WEIGHT 9.45 MGS PROTEIN CONTENT 32.19 %

SAMPLE: 1 MOISTURE CONTENT 7.39% NITROGEN CONTENT 5.15 %

S.NO	AM.ACID	REL.RETENT.	AAA/AIS	RMRFMGAA IN SAMP.	MGAA/GR SAMP.	GAA/16GR NITGRAA	/100GR PRMMOL AA/GR SAMPLE		
1	ALA	.638	1.070	.094	.101	10.644	3.307	3.307	.119
2	VAL	.743	1.586	.117	.186	19.659	6.108	6.108	.168
3	GLY	.836	1.170	.127	.149	15.719	4.884	4.884	.210
4	ILEU	.888	1.110	.115	.128	13.510	4.197	4.197	.103
5	LEU	.961	1.584	.115	.182	19.279	5.990	5.990	.147
6	PRO	1.026	2.304	.185	.426	45.095	14.010	14.010	.392
7	THR	1.086	1.366	.255	.348	36.662	11.452	11.452	.310
8	SER	1.197	1.479	.161	.238	25.192	7.827	7.827	.240
9	METH	1.434	.412	.000	.000	.000	.000	.000	.000
10	HYPRO	1.480	.558	.000	.000	.000	.000	.000	.000
11	PHE	1.539	1.302	.120	.156	16.533	5.136	5.136	.100
12	ASP	1.592	2.228	.128	.285	30.180	9.376	9.376	.227
13	TYR	.672	.790	.351	.277	29.325	9.111	9.111	.162
14	GLU	.711	3.634	.111	.403	42.665	13.261	13.261	.290
15	LYS	.740	1.548	.129	.200	21.136	6.567	6.567	.145
16	ARG	.782	.699	.120	.084	8.875	2.757	2.757	.051
17	TRY	.860	.643	.130	.084	8.849	2.749	2.749	.043
18	HIS	.961	.756	.150	.113	12.008	3.731	3.731	.077
19	CYS	1.091	.706	.135	.095	10.080	3.132	3.132	.042

Fig. 5. Printout of the processed data for one sample by the Univac 1108.

processed and the index to the corresponding card in set II carrying the data on moisture content, nitrogen content and the conversion factor for nitrogen to protein, and a second index to a card in set III to identify the relative retention-time window limits with two alternatives, single column (EGA) or dual-column (EGA + OV-17). The cards in set II have the information on moisture content, nitrogen content, etc., for each sample. This information can also be introduced through the keyboard (see point 1 in Table I) if available at the time of the GLC run. For the purpose of calculation, however, the computer cannot use this information under text 1, Table I.

Fig. 5 presents the printout of the final results of processed data. Because there have been many different ways of expressing amino acid analysis data, we have employed most of the common ways that we observed in the literature.

PEAK IDENTIFICATION

The identification of the GLC peaks by the computer is based on the retention times of individual amino acids relative to those of the internal standards. The memory data are from analyses of the TAB derivatives of a standard amino acid mixture.

A very simple logic is used in our program for the peak identifications, namely the use of two limiting values of relative retention time within which any peak is considered as being identified with the derivative that in the memory has a certain value for relative retention time, generally the mean of the two limit values.

As is well known, several of the TAB derivatives of amino acids separate near one another. As the stationary phase of a GLC column could bleed out, the relative retention times of certain amino acid derivatives could change. Also other analytical parameters could be a source of minor variation in relative retention times in a temperature-programmed GLC analysis. Therefore, a more complex logic such as that suggested by the Vidar Corporation[9] may have to be applied.

CALCULATION

For routine analysis, weight response calculation was convenient. Data available from the analysis of each sample and the method of calculation are as follows.

Peak area of amino acid = Aaaa.

Peak area of internal standard = Ais.

Weight (mg) of amino acid = Mg aa.

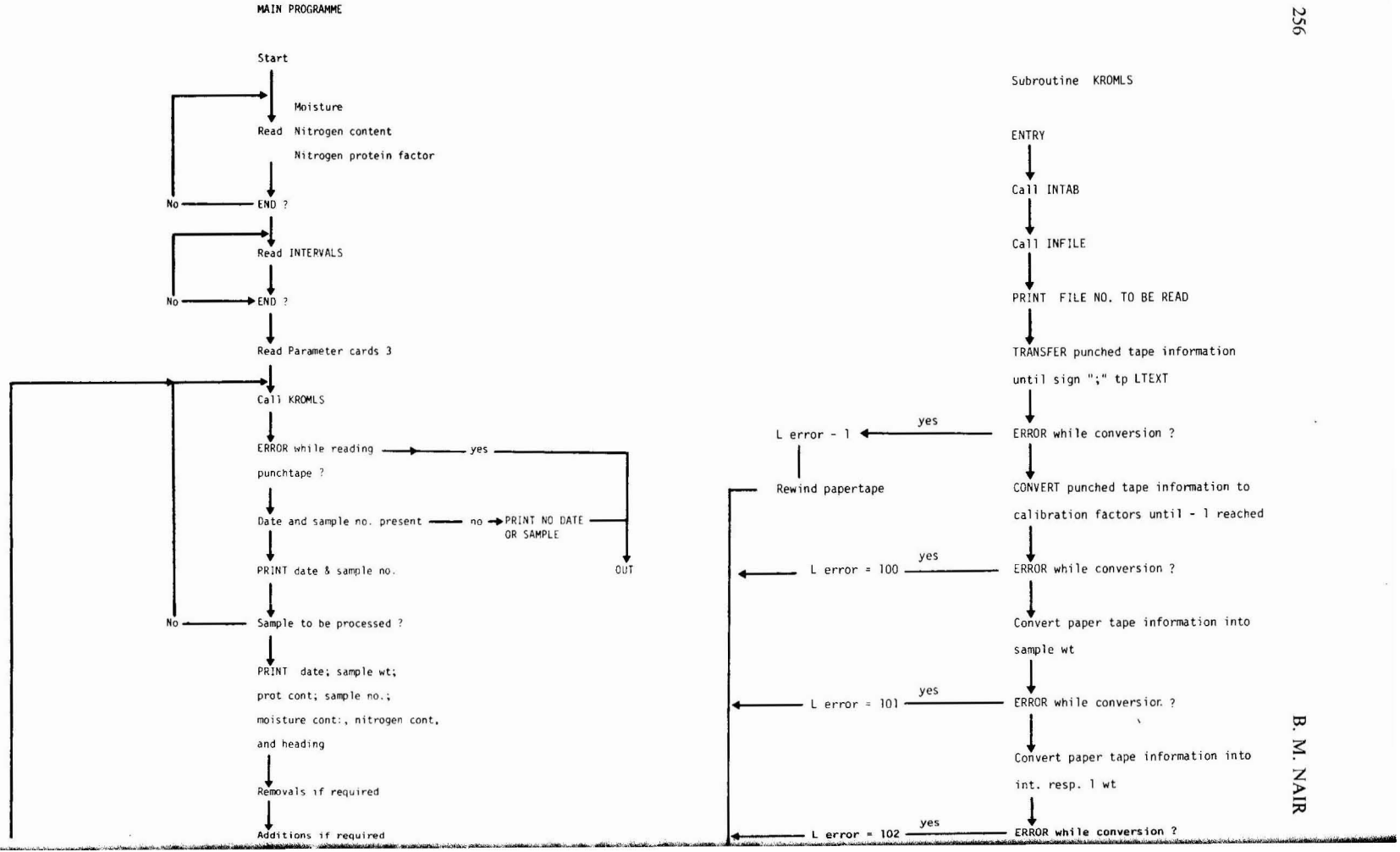
Weight (mg) of internal standard = Mg is.

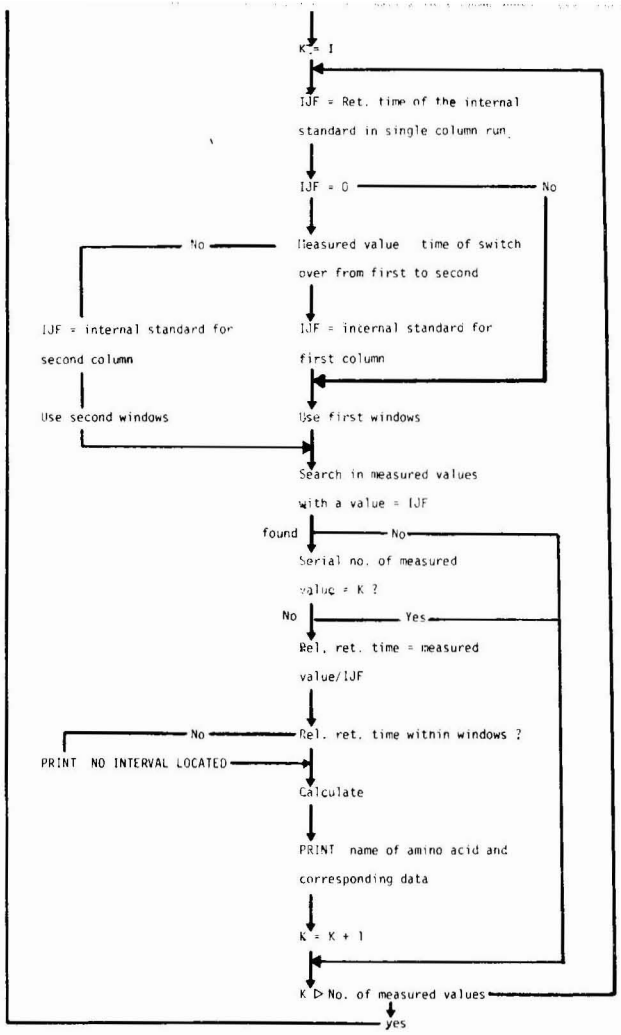
Relative retention time = time aa/time is.

$$\text{Relative molar response factor (RMRF)} = \frac{\text{Ais} \times \text{Mg aa}}{\text{Aaa}}.$$

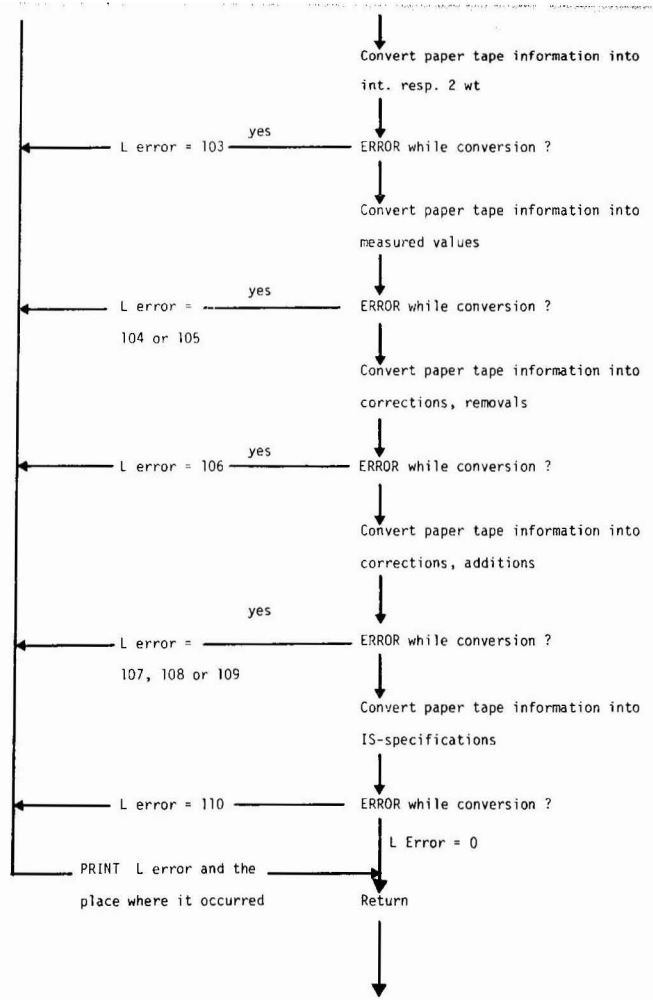
RMRFH = RMRF × correction factor for hydrolysis losses.

Amounts of internal standard in the calibration mixture and in the sample are the same.


MAIN PROGRAMME
Start
Moisture
Read Nitrogen content
Nitrogen protein factor
No
END ?
Read INTERVALS
No
END ?
Read Parameter cards 3
Call KROMLS
ERROR while reading punchtape ?
yes
Date and sample no. present
no
PRINT NO DATE OR SAMPLE
OUT
PRINT date & sample no.
No
Sample to be processed ?
PRINT date; sample wt; prot cont; sample no.; moisture cont:, nitrogen cont, and heading
Removals if required
Additions if required
Subroutine KROMLS
ENTRY
Call INTAB
Call INFILE
PRINT FILE NO. TO BE READ
TRANSFER punched tape information until sign ";" tp LTEXT
ERROR while conversion ?
yes
L error - 1
Rewind papertape
CONVERT punched tape information to calibration factors until - 1 reached
ERROR while conversion ?
yes
L error = 100
Convert paper tape information into sample wt
ERROR while conversion ?
yes
L error = 101
Convert paper tape information into int. resp. 1 wt
ERROR while conversion ?
yes
L error = 102



Scheme A.



Scheme B.

Sample number.

Date of run.

Sample weight: SW mg.

Moisture: M%.

Protein: P%.

Nitrogen: N%.

NPF = nitrogen to protein conversion factor.

$$\text{Amount of amino acid (mg) in the sample (Mg aa)} = \frac{\text{Aaa} \times \text{RMRFH}}{\text{Ais}}.$$

$$\text{Amount of amino acid (mg) in 1 gram of sample (W)} = \frac{\text{Mg aa} \times 1000}{\text{SG}}.$$

$$\text{Protein (P)\%} = \%\,\text{N} \times \text{NPF}.$$

$$\text{Grams amino acid/100 g protein} = \frac{\text{W} \times 10}{\text{P}}.$$

$$\text{Milligrams amino acid/16 g nitrogen} = \frac{\text{W} \times 16 \times 100}{\text{N}}.$$

$$\text{Grams amino acid/16 g nitrogen} = \frac{\text{W} \times 16}{\text{N} \times 10}.$$

FURTHER AUTOMATION OF THE GLC ANALYSIS OF TAB AMINO ACIDS

The introduction of some information about the sample and additional information for debugging through the keyboard of the teletype for each sample before and after the run could be a handicap when using automation for sample introduction and data collection. There would not be space in the punched tape for introducing this information after the run of a series of samples. This problem could be resolved either (1) by leaving a gap on the tape between the runs that is large enough to take the necessary information, which could be introduced later on when the series are ready, or (2) by providing a reference sample number and making provision in the program for a set of cards containing this information.

APPENDIX

See Schemes A and B.

Subroutine KROMLS

The subroutine KROMLS uses standard library programs INTAB and INFILE for conversion of information from punched tape to the computer's internal code. If something goes wrong during conversion, it prints the place where it occurred. Subroutine INTAB gives the conversion table. After INFILE has been called, conversion of text string, real numbers and integral numbers is achieved by calling RSTR, RREAL and RINT, respectively.

Subroutine BINARS

Subroutine BINARS does binary searching. If the given value is located in the array, the variable IX gets the value corresponding to its location and the variable IND gets value 2. IND gets other values if the given value is not located.

Subroutine MSORT

Subroutine MSORT sorts out a two dimensional array in ascending order according to the second index.

Punched cards

Set I. Variables.
Sample number to be processed.
Index to variables in set II.
Index to variables in set III.
Last card −1 in column 15–16.
Set II. Variables:
M: moisture (%).
N: nitrogen (%).
NPF: nitrogen to protein factor.
One card for each sample with sample number as reference.
Format 3 F 7.2. Last card in the set $\frac{7}{8}$ b EØF.
Set III. Variables:
Names of the amino acid.
Relative retention time window limits.
Molecular weights.

Each card contains two sets of values for retention time window limit, one for single-column run and the other for dual-column run. Last card $\frac{7}{8}$ b EØF.

Format (2 (16 218, 15)).

ACKNOWLEDGEMENTS

Generous grants from the Swedish Board for Technical Development and the assistance of H. Kvist and A. Roy are gratefully acknowledged.

REFERENCES

1 L.-A. Appelqvist and B. M. Nair, *J. Chromatogr.*, 124 (1976) 239.
2 J. M. Gill and H. W. Habgood, *J. Chromatogr. Sci.*, 5 (1967) 595.
3 J. M. Gill and S. P. Perone, *J. Chromatogr. Sci.*, 7 (1969) 709.
4 L. L. Hegedus and E. E. Petersen, *J. Chromatogr. Sci.*, 9 (1971) 551.
5 J. M. Gill, *J. Chromatogr. Sci.*, 7 (1969) 731.
6 D. R. Deans, *J. Chromatogr. Sci.*, 9 (1971) 729.
7 C. E. West and T. R. Rowbotham, *J. Chromatogr. Sci.*, 8 (1970) 688.
8 H. E. Pattee, E. H. Wiser and J.-A. Singhton, *J. Chromatogr. Sci.*, 8 (1970) 668.
9 Autolab-Spectraphysics, Technical Bulletin No. 107-72, 1972.

Journal of Chromatography, 155 (1978) 261–271

CHROM. 10,876

LIQUID CHROMATOGRAPHY OF POLAR AROMATIC COMPOUNDS ON CATION-EXCHANGE RESINS AND POROUS POLYMER GELS

T. HANAI, H. F. WALTON, J. D. NAVRATIL and DORIS WARREN
University of Colorado, Boulder, Colo. 80309 (U.S.A.)
(Received January 19th, 1978)

SUMMARY

The retention and separation of some 12 polar aromatic compounds, some of them weak acids and ampholytes, have been studied on cation-exchange resins and a non-ionic macroporous styrene–divinylbenzene polymer. The effects of pH, counter-ions and ion-pairing reagents have been measured. Ammonium ions extend the absorption of weak acids (pK_a above 8) to higher pH values on an ion-exchange resin. The porous polymer has more selectivity than the ion-exchange resins, but plate numbers are less. Gradient elution may be used with the porous polymer, but radical solvent changes affect the quality of the packing.

INTRODUCTION

Cation-exchange resins serve as stationary phases for the chromatography of non-ionic organic compounds[1–3] and of weak acids, both aromatic and aliphatic[4–6]. The acids are absorbed by the resin polymer matrix in their non-ionized forms and excluded in their ionized or ionic forms. Retention depends on the ionization constants and the pH of the solution, and in mixtures of acids of differing ionization constants the elution sequence can be changed by changing the pH. "Ion-exclusion chromatography", the separation of acids on a cation-exchange resin at selected pH values, has been used to advantage in the analysis of nucleic acid derivatives[7,8]. Because many of the compounds to be separated have a limited solubility in water, it is customary to use mixtures of water with another solvent, such as alcohol or methyl cellosolve[5]. The composition of the solvent mixture strongly affects the retention and may affect the elution sequence.

Fast mass transfer requires resins of low cross-linking. Polystyrene-based resins with 2%[4] and 4%[2,3,6] divinylbenzene have been used for this type of chromatography. Resins with small particle sizes and low cross-linking give excellent theoretical plate heights, routinely 0.1 mm and less, with symmetrical elution peaks even at high loadings. Their great drawback is their softness, which limits the solvent flow-rates to about 1 cm/min and limits the length of the column to some 30 cm.

The solutes are bound to the resin by non-polar, hydrophobic interaction with the resin polymer matrix and the primary, perhaps the only function of the ionic

groups is to be hydrated and to cause the resin to be swollen and permeable. The hydrophobic interactions can be exploited in styrene–divinylbenzene polymers with no ionic groups, provided they have a suitably porous structure. Macroporous polymers like the Amberlite XAD resins (Rohm & Haas, Philadelphia, Pa., U.S.A.) have been used for liquid chromatography of polar organic compounds, including weak acids[9,10]. They absorb the non-ionic, protonated acids, and to a slight, but measurable extent they also absorb sodium salts of these acids. For chromatographic use, commercial resin beads are ground and screened, but the large and irregular particles of the ground resins cause the theoretical plate heights to be unduly large. They do, however, have the advantage of rigidity.

Macroporous polymers specially prepared for liquid chromatography, with particle diameters of 10 μm and less, are now produced commercially in Japan[11,12]. In this report we compare their performance with that of cation-exchange resins having comparable particle diameters.

EXPERIMENTAL

Apparatus

Liquid chromatography pumps of various kinds were used, principally the Model 6000 pumps from Waters Assoc. (Milford, Mass., U.S.A.), with ultraviolet absorbance detectors from Spectra-Physics (Santa Clara, Calif., U.S.A.). Columns were either of glass (Glenco, Houston, Texas, U.S.A.; high-pressure model), 6.3 mm I.D., or of stainless steel, 4 mm I.D. All columns were water-jacketed and maintained at a constant temperature of 55°.

Materials

Ion-exchange resins. Aminex 50W-X4, 20–30 μm, was obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.). Other resins of 10–15 μm diameter and 4% or 7% cross-linking were supplied by Hamilton (Reno, Nev., U.S.A.). All were cation exchangers with sulfonic acid groups on a styrene–divinylbenzene matrix.

Porous polymer gel. This was a styrene–divinylbenzene copolymer of macroporous structure without ionic groups, particle diameters 5 and 10 μm made by Toyo Soda (Tokyo, Japan).

Chemicals. High-quality products from various suppliers were used, and were recrystallized when necessary. Salicylamide, whose behaviour was studied in some detail, was recrystallized from water.

Solvent. Mixtures of ethyl alcohol with water were used. Commercial 95% alcohol was used, as it had excellent spectral purity. By "25% alcohol" we mean a mixture of 25 parts of 95% alcohol with 75 parts of water by volume. The volume ratio was controlled as carefully as possible; however, the composition of the (nominally) 95% alcohol stock may have varied from one bottle to another. Tests described below showed that the net retention volume of caffeine fell by 5% if the alcohol concentration rose by 1% (that is, from 25% to 26% by volume).

Column packing

Ion-exchange resins were packed as slurries in 25% alcohol, allowing them to settle by gravity and then applying moderate pressure. The porous polymer gel

was packed as a slurry in the same solvent that would be used in the chromatographic runs, namely 25% alcohol. It was packed down-flow in a pulsing mode.

RESULTS AND DISCUSSION

pH measurements and ionization constants

Though the columns were run at 55°, pH measurements were made at room temperature. Portions of effluents were collected and compared with standard aqueous buffers, generally 0.05 *M* phthalate or phosphate. The pH values of these buffers change little with temperature[13]. A greater uncertainty comes from comparing pH measurements in aqueous buffers with those in effluents containing 25% ethanol; however, the studies in which an accurate knowledge of pH was most important were made with salicylamide and we determined the ionization constant of this acid by titrating it in 25% alcohol containing 0.10 *M* potassium chloride at 25° and 55°, again calibrating the electrodes with aqueous buffers. We measured the ionization constant of acetaminophen (*p*-hydroxyacetanilide) in the same way. The ionization constants of the acids used in our study are shown in Table I.

TABLE I

IONIZATION CONSTANTS OF ACIDS USED IN THIS STUDY (pK_a UNITS AT 25°, REF. 14)

Acid	pK_a
Salicylamide*	8.45 (25°), 8.46 (55°)
p-Hydroxyacetanilide*	9.9
Benzoic acid	4.2
Caffeic acid	4.7
Cinnamic acid	4.45
p-Aminobenzoic acid	2.4 (pK_1), 4.9 (pK_2)
Nicotinic acid	4.8 (pK_1), 12.0 (pK_2)
Salicylic acid	2.9 (pK_1), 12.4 (pK_2)
Xanthine	7.7

* Our values.

Analgesic drugs on 4% cross-linked resin

These data were obtained on a column of Aminex 50W-X4 cation-exchange resin with eluents containing 25% ethyl alcohol. All were 0.10 *M* in Na^+ or NH_4^+ and were buffered with formic, acetic, phosphoric, citric or boric acid. The nature of the anion did not affect the retention, and the ionic strength had little effect.

Fig. 1 shows elution curves at two pH values, and Fig. 2 shows the effect of pH on retention volume for sodium and ammonium buffers. The pH has no effect on the elution of non-ionized compounds like caffeine and phenacetin, but with weak acids, the elution volume falls as the pH rises and becomes equal to the void volume at high pH. The uncharged acid molecules are retained, but the anions are not. In such cases the pH of half retention, where the capacity factor is half of its maximum, should equal pK_a[9,10,15]. Salicylic acid is stronger than acetylsalicylic acid by about 0.5 pK_a, and this fact makes it possible to separate these two acids below pH 5.

The case of salicylamide is interesting. From Fig. 2, its pH of half retention

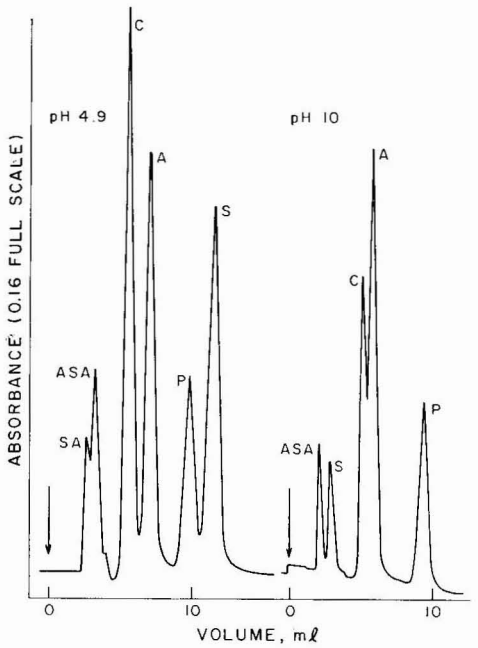


Fig. 1. Elution of analgesic drugs from the cation-exchange resin, Aminex 50W-X4-NH_4^+. Column, 20 × 0.63 cm; eluent, 25% alcohol, 0.1 *M* buffers (see text); temperature, 55°; flow-rate, 24 ml/h. SA = salicylic acid; ASA = acetylsalicylic acid; C = caffeine; A = *p*-hydroxyacetanilide; P = phenacetin (*p*-ethoxyacetanilide); S = salicylamide.

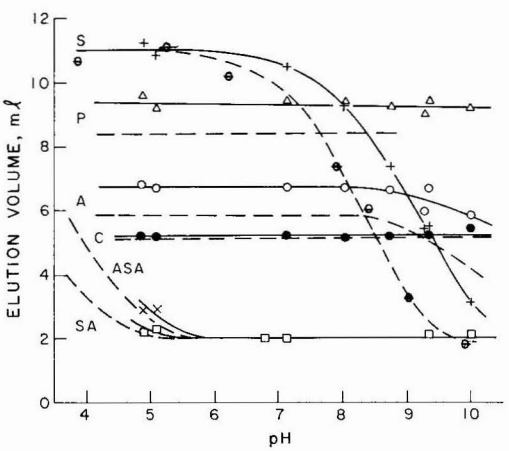


Fig. 2. Elution of analgesic drugs from Aminex 50W-X4; pH and counter-ion effects. Same column and conditions as Fig. 1. Dashed lines, Na^+ resin and buffers; solid lines, NH_4^+ resin and buffers. Abbreviations as in Fig. 1.

on the sodium-form resin was 8.2, which is somewhat less than the measured pK_a value. With ammonium counter-ions, however, the half-retention pH was one unit higher. The same conclusion was reached from a separate set of experiments (Fig. 3) with a different column of the same resin, using ammonia–ammonium nitrate

buffers. Potassium-loaded resin gave the same elution volumes as sodium-loaded, but the half-retention pH values were 8.1 for Na^+ and K^+, 9.05 for NH_4^+. The maximum retention (below pH 6) was the same for all three counter-ions, and the minimum in each case was zero, that is, the compound eluted at the void volume. In the intermediate pH range (8–9) the retention was very slightly greater in 0.1 *M* ammonium salt than in 0.02 *M*, namely, 0.1 ml out of 4–5 ml.

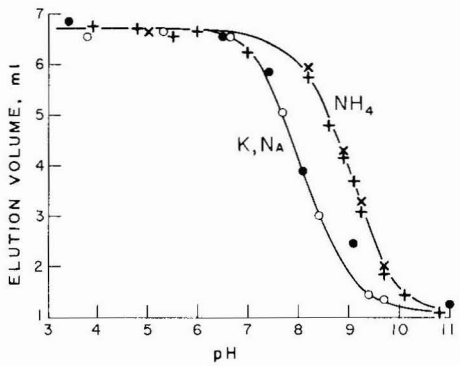


Fig. 3. Elution of salicylamide from Aminex 50W-X4; pH and counter-ion effects. Column, 12.5 × 0.63 cm; temperature, 55°; flow-rate, 24 ml/h; solvent, 25% alcohol. Counter-ions: ○, Na^+, 0.10 *M*; ●, K^+, 0.10 *M*; +, NH_4^+, 0.02 *M*; ×, NH_4^+, 0.10 *M*.

The reason for this behavior undoubtedly involves the acid nature of the ammonium ion. If stable $NH_4^+X^-$ ion-pairs were formed, one would expect significant retention at high pH, and this is not observed. The micellar fluid, that is, the solution within the swollen resin beads, has a high NH_4^+ concentration and a very low OH^- concentration because of Donnan exclusion. It must therefore have a relatively low pH, compared with the external solution, and the proportion of uncharged salicylamide, HX, to anions X^- must be relatively high.

There are signs of a similar behavior with the drug acetaminophen (*p*-hydroxyacetanilide), which we found to be a weak acid ($pK_a = 9.9$). The data of Fig. 2 do not extend to a high enough pH to determine its pH of half-retention in ammonium-form resin, but obviously it is higher than that in sodium-form resin.

In the low pH range, ammonium ions do not seem to have this effect. Tests performed with benzoic and cinnamic acids indicated the pH of half retention was about the same with sodium, potassium and ammonium counter-ions. For cinnamic acid this pH was 4.6.

The counter-ion effect was studied in detail with all the solutes shown in Fig. 2, as well as with chlorinated biphenyls[3]. Counter-ions used were Na^+, K^+, NH_4^+, and Ca^{2+}. The effects on retention were not great, and for some solutes, notably caffeine, they were almost nil. Retention increased in the sequence Na^+, NH_4^+, K^+, Ca^{2+} (except for the special effect of NH_4^+ at high pH, mentioned above). This was the inverse order of the bed volumes. Calcium-loaded resins showed the least swelling and the retention effect may simply be correlated with the lower water content and greater hydrophobic character of the calcium resins. For salicylamide and phenacetin the theoretical plate heights increased considerably when Ca^{2+} was substituted for

NH_4^+ or Na^+, a natural consequence of decreased swelling and retarded diffusion.

Fig. 4 shows the effect of pH on the retention of several compounds on Aminex 50W-X4-Na and Fig. 5 shows a chromatogram obtained with the Hamilton 4% cross-linked resin. The plate number for phenacetin in Fig. 5 is 3500 for a 24-cm column. The Hamilton 7% cross-linked resin gave a similar chromatogram with somewhat broader bands, showing better resolution of caffeic acid and xanthine. Salicylamide eluted before *p*-aminobenzoic acid.

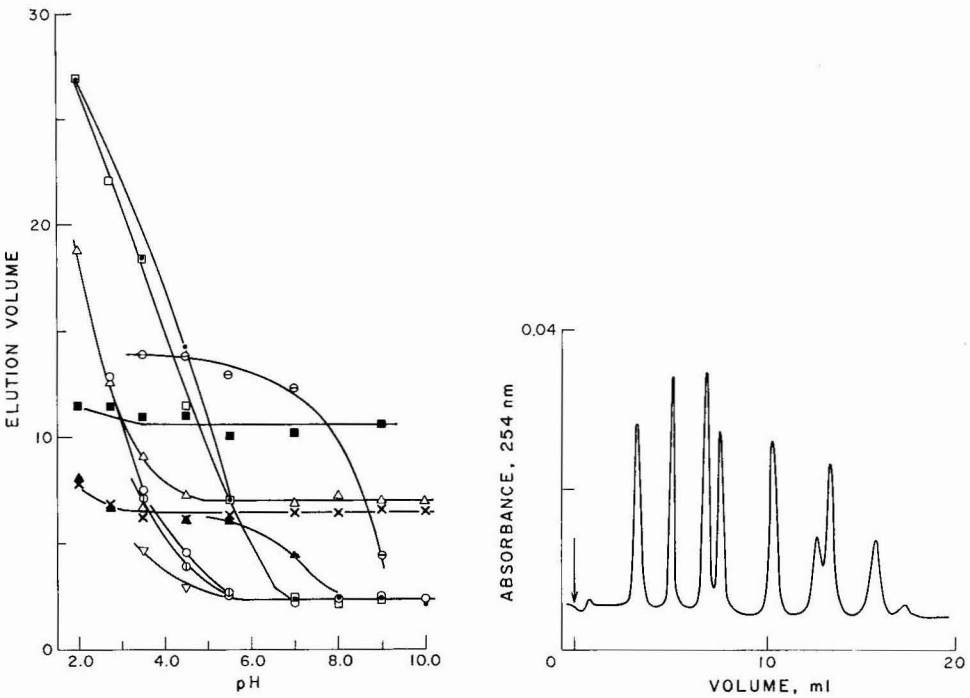


Fig. 4. Elution from Aminex 50W-X4-Na; pH effect. Column, 22 × 0.63 cm; temperature, 55°; solvent, 25% alcohol with 0.1 *M* Na^+, variable phosphate. Solutes: ●, caffeic acid; ○, nicotinic acid; □, *p*-aminobenzoic acid; ⊖, salicylamide; ■, phenacetin; △, trigonellin, ×, caffeine; ▲, xanthine; ⊕, acetylsalicylic acid; ▽, salicylic acid.

Fig. 5. Elution from Hamilton 4% cross-linked resin, Na^+, 10–15 μm. 0.1 *M* Na^+, phosphate at pH 4.5 in 25% alcohol at 55°; flow-rate, 12 ml/h. Column, 24 × 0.63 cm. Peaks, in order of appearance, are: salicylic acid, nicotinic acid, xanthine plus caffeine, trigonellin, phenacetin, *p*-aminobenzoic acid, salicylamide, cinnamic acid, small unknown peak. Quantities injected were between 0.1 and 2.0 μg. Plate number was 3500 for phenacetin, 5000 for salicylamide.

Fig. 4 shows the effect of protonation on the retention of trigonelline (N-methylpyridinium-3-carboxylate), which above pH 5 is a dipolar ion. At lower pH values the carboxylate ion is protonated, and the molecule has a net positive charge, which makes it absorb as a cation through ion exchange. The dipolar ion is absorbed also, but more weakly. The cases of *p*-aminobenzoic acid ($pK_1 = 2.4$, $pK_2 = 4.9$)[14] and nicotinic acid ($pK_1 = 4.9$, $pK_2 = 12$) are similar. It appears that the uncharged form of *p*-aminobenzoic acid is retained with k' of about 8, while the uncharged form of nicotinic acid is hardly retained at all.

Figs. 6 and 7 show the effect of pH on retention by the 7% cross-linked resin in the presence of sodium sulfate. The buffering ions are phosphate, and the total sodium-ion concentrations range from 0.25 to 0.30 *M*. Extensive experiments showed that the retention was the same in the presence as in the absence of sodium sulfate; 0.005 *M* phosphate gave the same retention as 0.05 *M* phosphate. Retention is, therefore, insensitive to ionic strength. Figs. 6 and 7 cover a wider pH range than Fig. 4, and show that the pH of half-retention for the uncharged, monoprotic acids, benzoic, cinnamic, caffeic, and xanthine are very close to their pK_a values. There is no evidence of ion-pair formation.

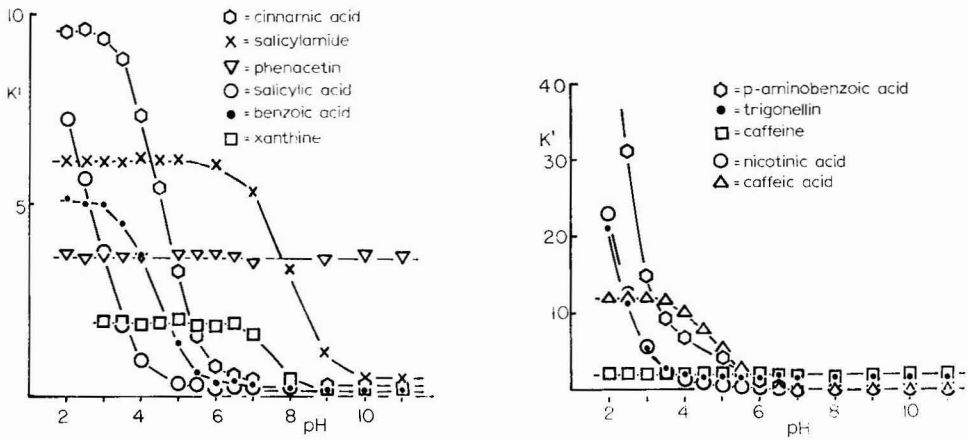


Fig. 6. Elution from Hamilton 7% cross-linked resin, Na^+; effect of pH. 25% alcohol, 0.1 *M* in Na_2SO_4 and 0.05 *M* in Na_2HPO_4; pH adjusted by adding NaOH or H_3PO_4; temperature, 55°, flow-rate 16 ml/h.

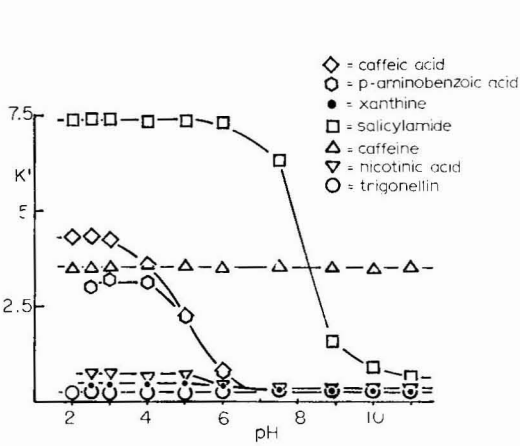
Fig. 7. Elution from Hamilton 7% cross-linked resin, Na^+; effect of pH. Same column, flow conditions, buffers and temperature as in Fig. 6.

Tests were also made in the presence of 0.017 *M* sodium dodecyl sulfate. This compound quadrupled the retention of nicotinic acid and trigonelline at pH 3, and almost doubled the retention of *p*-aminobenzoic acid, while having little or no effect on the other compounds. Evidently it forms ion pairs with the cationic forms of the three compounds that it affects.

Studies with porous polymer gel

Figs. 8 and 9 show the retention of two groups of compounds on the macroporous non-ionic polymer, TSK-LS110. The same pH dependence is found as was found with the cation-exchange resin, but there is a striking difference between the strengths of retention of the compounds shown in Figs. 8 and 9. Cinnamic and salicylic acids are held much more strongly on the porous polymer than on the ion-exchange resins. Phenacetin is held twice as strongly as caffeine on the ion-exchange resins, but 7–8 times as strongly on the porous polymer. On the other hand, trigonelline and nicotinic acid, which exist as dipolar ions over large pH ranges, are hardly absorbed at all by the porous polymer, and *p*-aminobenzoic acid is absorbed only weakly. One would not expect dipolar ions to be strongly absorbed by a non-

polar, non-ionized medium of low dielectric constant such as polystyrene; however, significant sorption of *o*-aminobenzoic acid (anthranilic acid) on octadecyl-coated silica has been observed[15]. The caffeine–phenacetin retention ratios suggest that the porous polymer gel has a preference for benzene-ring aromatic compounds, as opposed to heterocyclic structures like xanthines.



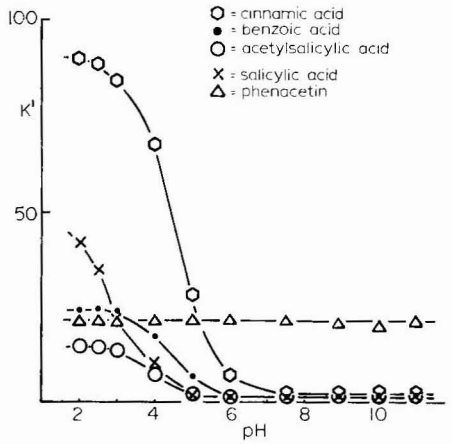


Fig. 8. Elution from porous polystyrene gel. Column, 25 × 0.4 cm; same buffers, flow-rate and temperature as in Fig. 6.

Fig. 9. Elution from porous polystyrene gel. Same column and conditions as in Fig. 8.

Retention of acidic compounds drops to zero at high pH. Under the conditions of Figs. 8 and 9 there is no sign of anion absorption or ion-pair formation, in contrast to the observations of Pietrzyk and Chi-Hong Chu[10].

Ion pairing in porous polymer gel

To examine the role of ion-pairs, tests were made with eluents containing sodium dodecyl sulfate (0.017 *M*) and tetrabutylammonium ions (0.020 *M*). Tetrabutylammonium ions (TBA) were added as the hydroxide, and the pH was adjusted to the desired value by adding concentrated phosphoric acid. Sodium dodecyl sulfate increased the retention of cationic species at low pH, as expected. The behaviour of TBA is shown in Fig. 10.

Without added TBA, salicylamide is not retained at high pH, but emerges at the void volume. The retention rises to a maximum as the pH falls and the compound assumes its uncharged form. The pH of half retention is 8.1, the same value found with sodium-form ion-exchange resin (Figs. 2 and 3). Theoretically the pH of half retention should equal pK_a. We found pK_a to be 8.4 (see above). The discrepancy is small and may or may not be significant.

In the presence of TBA, salicylamide is retained at high pH, presumably as an ion-pair, and k' is about half that of uncharged salicylamide. Retention is halfway between the high and low pH values at pH 8.3. Theoretically this should occur at pH = pK_a (eqn. 4 in ref. 10).

Nicotinic acid is affected by TBA in an unexpected way. In the absence of

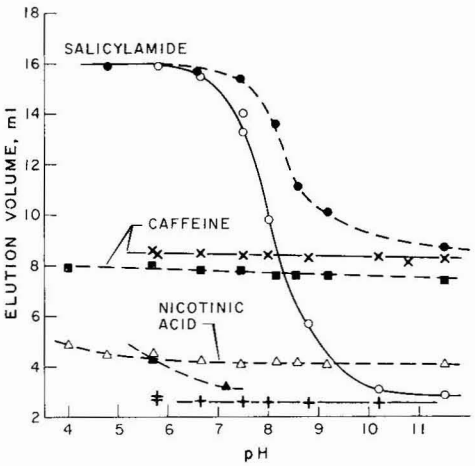


Fig. 10. Elution from porous polystyrene gel; effect of ion pairing. Same column as in Fig. 8; solvent, 25% alcohol, 0.10 *M* in Na_2HPO_4; flow-rate, 30 ml/h; temperature, 55°. ———, without ion-pairing reagent; – – –, eluent 0.020 *M* in $N(C_4H_9)^+$ (TBA). Phenacetin (not shown) eluted at 38.0 ml, independent of pH or TBA; trigonellin (not shown) always eluted at the void volume, 2.4–2.5 ml. ○ and ●, salicylamide; × and ■, caffeine; + and △, nicotinic acid; ▲, *p*-aminobenzoic acid.

TBA it is not retained, but emerges at the void volume. It exists as a neutral species, probably the dipolar ion $C_5H_4NH^+COO^-$, over the whole pH range shown in Fig. 10. With TBA present it is retained with $k' = 0.6$. Similar behaviour is shown by *p*-aminobenzoic acid, in which the dipolar ion predominates between pH 2.4 and 4.9. It is not clear how TBA can promote the sorption of a dipolar ion. Trigonelline, which exists as a dipolar ion over the pH range of Fig. 10, is unretained whether TBA is present or not.

The retention of caffeine is reduced slightly by TBA, that of phenacetin is unaffected.

Plate heights and resolution

For the conditions of Figs. 8 and 9 with linear velocity 2.4 cm/min, the plate heights for an unretained solute (xanthine) was less than 0.1 mm, whereas the plate height for caffeine was 0.2 mm with some tailing. Plate heights are not as good as those found with the ion-exchange resins; however, the resolution is generally better because of larger differences in retention. To exploit these retention differences one must use a gradient. Fig. 11 shows the separation of 12 compounds on a column of TSK gel, using a convex pH gradient with rapid composition change at first and slower change later. The alcohol concentration was constant, 25% by volume, and the pH of the phosphate buffer changed from 2.5 to 8.5 over 2 h.

With an alcohol concentration gradient from 25% to 95% we found that the column packing was damaged. It seemed that the gel particles expanded and then, when the solvent was changed back to its original composition, the particles did not rearrange themselves to their original distribution; the quality of packing, tested by the width of an unretained peak, was now very poor. The gel itself was not irreversibly spoiled, as the column could be emptied and repacked to give good performance

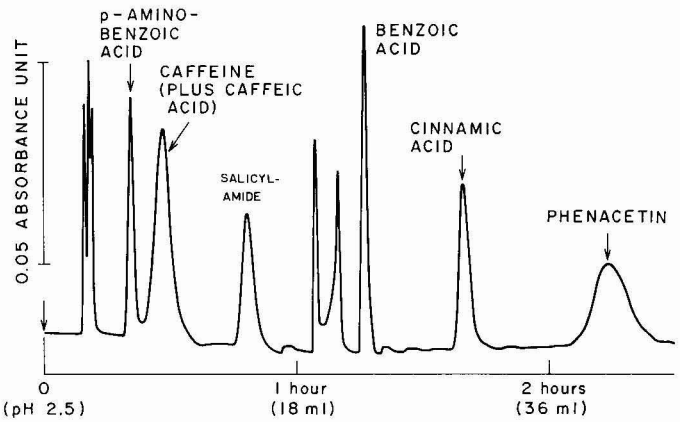


Fig. 11. Separation of polar aromatic compounds on porous polystyrene gel. Same column as in Fig. 8; flow-rate, 18 ml/h; pressure, 70 bar; eluent, phosphoric acid (0.2 *M*) to Na_2HPO_4 (0.2 *M*) over 2 h; gradient No. 3 on Waters Assoc. programmer (see text). Peaks in order of appearance are: trigonellin, xanthine, nicotinic acid (close together); *p*-aminobenzoic acid; caffeic acid and caffeine (together); salicylamide, acetylsalicylic acid, salicylic acid, benzoic acid, cinnamic acid, phenacetin. Quantities injected were 0.5–7.5 μg.

again but it is evident that the use of solvent gradients is limited with this packing. Perhaps one would get better performance in a wider column.

Effect of solvent composition

The effect of alcohol concentration on retention was measured for phenacetin and caffeine on the porous polymer gel and on the Hamilton 7% cross-linked resin, from 20% to 30% of commercial (95%) alcohol by volume, that is, from 19% to 28.5% of ethanol. In all cases, straight-line plots of $\log k'$ against volume fraction of alcohol were obtained. The retention of phenacetin fell by a factor of 2.2 on the polymer, 2.1 on the ion-exchange resin, over the interval named; that of caffeine fell by a factor of 1.65 on both sorbents. The linear relation between the free energy of sorption and the volume fraction of alcohol in the solvent has been noted by several workers, including Karger *et al.*[16], who have made a study of the hydrophobic effect in liquid chromatography.

CONCLUSION

Most liquid chromatography today is "reversed-phase", in which the stationary phase is non-polar and hydrocarbon-like. The most popular material is porous silica coated with octadecyl groups. Horváth and co-workers[15,17] have used solubility theory and the theory of solvophobic interactions to predict distribution ratios of molecules that have a hydrocarbon, or hydrophobic, part and a polar, or hydrophilic, part. Among the solutes they studied experimentally were benzoic and cinnamic acids. The effect of pH on the distribution of weak organic acids, bases and ampholytes was studied experimentally and theoretically[15], using octadecyl–silica as the stationary phase.

The drawback to octadecylsilica is that it can only be used in a limited pH

range, at most 2–8. Porous polymer gels can be used over the entire pH range, as can ion-exchange resins. Non-ionic porous polymer gels are easier to interpret than ion-exchanging polymers; they show greater differences in distribution ratios between different solutes, and they can be used in columns with fairly high pressure gradients. Ion-exchange resins with low cross-linking allow faster mass transfer than porous polymers because of their looser internal structure, and therefore they give smaller theoretical plate heights. Ionic attractions and repulsions are superimposed on solvophobic interactions, and therefore the retention of ionogenic solutes depends on pH in a different way. In certain cases the counter-ion affects the retention. Ion-exchange resins and non-ionic porous polymers can thus supplement each other in solving analytical problems. The disadvantage of ion-exchange resins is their softness.

Styrene–divinylbenzene porous polymer gels have recently been used for chromatography of fatty acids[18], aromatic hydrocarbons and benzoate esters[19].

ACKNOWLEDGEMENTS

This work was supported by the National Science Foundation Grant CHE76-08933. We are grateful to Toyo Soda, Tokyo, Japan for the gift of porous polymer gel.

REFERENCES

1 P. Larson, E. Murgia, Tong-Jung Hsu and H. F. Walton, *Anal. Chem.*, 45 (1973) 2306.
2 E. Murgia, P. Richards and H. F. Walton, *J. Chromatogr.*, 87 (1973) 523.
3 T. Hanai and H. F. Walton, *Anal. Chem.*, 49 (1977) 764.
4 J. Lehotay and M. Traiter, *J. Chromatogr.*, 91 (1974) 261.
5 Y. Takato and Y. Arikawa, *Bunseki Kagaku (Jap. Anal.)*, 23 (1974) 1522.
6 M. Richards, *J. Chromatogr.*, 115 (1975) 259.
7 R. P. Singhal, *Arch. Biochem. Biophys.*, 152 (1972) 800.
8 R. P. Singhal and W. E. Cohn, *Biochemistry*, 12 (1973) 1532.
9 Chi-Hong Chu and D. J. Pietrzyk, *Anal. Chem.*, 46 (1974) 330.
10 D. J. Pietrzyk and Chi-Hong Chu, *Anal. Chem.*, 49 (1977) 757; 49 (1977) 860.
11 K. Fuzita, Y. Arikawa and S. Ganno, *Nippon Kagaku Kaishi*, (1975) 463.
12 N. Takai and T. Yamabe, *Seisankenkyu*, 26 (1974) 278; 26 (1974) 343.
13 R. G. Bates, *Determination of pH: Theory and Practice*, Wiley-Interscience, New York, 2nd ed., 1973.
14 G. Kortum, W. Vogel and K. Andrussov, *Dissociation Constants of Organic Acids in Aqueous Solution*, Butterworths, London, 1961.
15 C. Horváth, W. Melander and I. Molnar, *Anal. Chem.*, 49 (1977) 142.
16 B. L. Karger, J. R. Gant, A. Hartkopf and P. H. Weiner, *J. Chromatogr.*, 128 (1976) 65.
17 C. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129.
18 M. Uchida and T. Tanimura, *J. Chromatogr.*, 138 (1977) 17.
19 A. Nakae and G. Muto, *J. Chromatogr.*, 120 (1976) 47.

Journal of Chromatography, 155 (1978) 273–283

CHROM. 10,905

COMBINED HIGH-PRESSURE LIQUID CHROMATOGRAPHY AND RADIOIMMUNOASSAY METHOD FOR THE QUANTITATION OF Δ^9-TETRAHYDROCANNABINOL AND SOME OF ITS METABOLITES IN HUMAN PLASMA

P. L. WILLIAMS and A. C. MOFFAT*

Home Office Central Research Establishment, Aldermaston, Reading, Berkshire RG7 4PN (Great Britain)

and

L. J. KING

Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH (Great Britain)

(Received January 26th, 1978)

SUMMARY

A high-pressure liquid chromatography–radioimmunoassay (HPLC–RIA) method for the measurement of cannabinoid levels in plasma is described. The method is capable of quantifying 0.1 ng of a cannabinoid in 1 ml of plasma. The experimental procedure consists of an initial separation of cannabinoids in a plasma extract by HPLC followed by collection of the HPLC eluate and RIA. A chromatogram consisting of the cross-reacting cannabinoids in plasma may then be constructed. The plasma concentrations of cannabinoids with retention volumes equivalent to those of Δ^9-tetrahydrocannabinol, cannabinol and mono-hydroxylated metabolites have been measured by this technique.

INTRODUCTION

Over the last decade there has been increasing interest from a research and forensic science viewpoint in qualitative and quantitative analyses of cannabinoids in blood. There is now a wide variety of methods available, most of which are based upon some form of chromatography.

The most popular techniques for the analysis of tetrahydrocannabinol (THC) in plasma have been chromatographic methods. Thin-layer chromatography has been used[1] and gas chromatography (GC) following derivatisation to a species suitable for electron-capture or flame photometric detection[2–4]. Linked GC–mass spectrometric (MS) methods have been described[5–7] as well as HPLC[8]. Methods for quantitation of 11-hydroxy-Δ^9-THC and Δ^9-THC-11-oic acid using GC–MS have been reported[9,10] and applied to the analysis of human plasma[11].

* To whom correspondence should be addressed.

A major problem encountered with these chromatographic methods has been the presence of endogenous interfering substances in plasma which usually required the use of an extensive extraction procedure for their exclusion. Immunoassay methods, because of the specific nature of the immune-reaction involved, avoid many of these difficulties and the simplicity of the immunoassay method results in shorter analyses times. Cannabinoid RIAs have been described[12–15] as well as a free-radical immunoassay[16]. These immunoassay methods, unlike the chromatographic methods, usually give a combined result obtained from several cannabinoids present in a body fluid and this lack of specificity makes interpretation of RIA levels difficult. A more detailed examination of cannabinoid levels could be made either by using a series of RIA procedures specific for individual cannabinoids similar to those specific for Δ^9-THC and Δ^9-THC-11-oic acid[17] or by combining a chromatographic separation stage with the normal cannabinoid RIA. For the latter method a plasma extract may be chromatographed by HPLC and the eluate monitored by RIA to measure the quantities and elution volume of cross-reacting cannabinoids in the sample.

This report describes such an HPLC–RIA method. The analysis time for the method is comparable with other chromatographic techniques but will quantitate THC and some of its metabolites in one analysis at levels down to 0.1 ng/ml.

EXPERIMENTAL

Radioimmunoassay

Materials and equipment. Antiserum (133Y/22/5) for the assay was obtained from Dr. J. D. Teale, Department of Biochemistry, University of Surrey, Great Britain. Δ^9-THC and other cannabinoid compounds were generously provided by the National Institute on Drug Abuse, Rockville, Md., U.S.A. Δ^9-(G-^{3}H)-THC was purchased from the Radiochemical Centre, Amersham, Great Britain, polyvinylpyrrolidone-40 and charcoal (Norit A) from Sigma, St. Louis, Mo., U.S.A., Dextran T70 from Pharmacia, Uppsala, Sweden, and methanol (AnalaR) and all other chemicals and solvents were obtained from BDH, Poole, Great Britain. An Intertechnique SL30 was used for liquid scintillation counting.

Method. Three volunteers smoked tobacco cigarettes impregnated with 10 mg (subject 1 and 2) and 8 mg (subject 3) of Δ^9-THC over a 10-min period. Blood samples (10 ml) were taken at timed intervals after the subject had finished smoking. Anticoagulant (K_2EDTA, 10 mg) was added to the blood which was centrifuged to separate the plasma. This was stored at −20° until analysis.

The RIA method was based upon that described by Teale *et al.*[18] but was modified by the replacement of the THC solubilising agent Triton X-405 with methanol and with the replacement of bovine γ-globulin in the assay buffer with polyvinylpyrrolidone-40. Antiserum (133Y/22/5) was stored as aliquots in buffer (0.1 *M* phosphate buffer pH 7.5, containing 0.2% polyvinylpyrrolidone-40) at −20° and diluted to 1:300 before use. Solutions of Δ^9-THC used to calibrate the assay were made up in aqueous methanol (50%, v/v, pH 7.5) at concentrations ranging from 500 pg/ml to 50 ng/ml and stored at −20°. Δ^9-(^{3}H)-THC was also stored at −20° at a concentration of 0.25 μCi/ml (12 Ci/mmole) in aqueous methanol (50%, v/v, pH 7.5) ready for use.

The plasma sample to be assayed was mixed with three volumes of methanol,

vortexed and allowed to stand for 30 min. It was then centrifuged and the supernatant added directly to the assay tubes. Normal human plasma similarly treated was used in the total, non-specific binding and zero tubes and for assay dilutions. Using the protocol in Table I, reagents were added to a series of assay tubes (duplicated), the antiserum was added last. These were then allowed to stand at room temperature for 1 h. Dextran-coated charcoal (pH 9.5)[18] was added, the tubes were centrifuged and after 2 min contact time, 500 μl of the supernatant from each tube were counted.

TABLE I

RADIOIMMUNOASSAY PROTOCOL

Reagent	*Volume (μl)*				
	Total counts tube	*Non-specific binding tube*	*Zero tube*	*Standard tube*	*Sample tube*
^{3}H-THC (50% MeOH)	50	50	50	50	50
THC standard (50% MeOH)	—	—	—	100	—
50% MeOH	100	100	100	—	100
Plasma sample extract	—	—	—	—	100
Normal plasma extract	100	100	100	100	—
Diluent buffer	250	350	250	250	250
Antiserum	100	—	100	100	100
	Incubated for 1 h at room temperature				
Diluent buffer	200	—	—	—	—
Dextran-coated charcoal (2.5%)	—	200	200	200	200
	Centrifuged and 500 μl of supernatant counted				

High-pressure liquid chromatography–radioimmunoassay

Materials and equipment. A constant-flow pump (M-6000A Waters Assoc., Milford, Mass., U.S.A.) was used to deliver a methanol–water eluent to a stainless-steel HPLC column (10 cm × 4.6 mm I.D.) slurry-packed with Spherisorb-5-ODS (Phase Separations, Flintshire, Great Britain). Samples were introduced onto the HPLC column with a six-port injection valve (Spectroscopy Accessory, Sideup, Great Britain) fitted with a 10-ml injection loop. Column eluate was either monitored with an ultraviolet (UV) detector at 280 nm (Cecil CE212) or collected with a Struers Samplomat fraction collector (Camlab, Cambridge, Great Britain). A freezedrier (Model SB4, Chemlab. Instruments, Ilford, Great Britain) was used to remove solvents.

Method. Plasma (0.2–1 ml) was mixed with three volumes of methanol, vortexed, left to stand for 30 min and centrifuged. The supernatant was removed, the residue mixed with methanol and the sample again centrifuged. Water was added to the combined supernatants to give a methanol concentration of 50% (v/v). The HPLC pump, sample loop and column were flushed with aqueous methanol (50%, v/v) and the sample solution was injected onto the column. A stepped solvent elution programme was used: 10 ml of a mixture of methanol–water (50:50), 10 ml of methanol–water (62.5:37.5), 20 ml of methanol–water (72.5:27.5, 1 ml/min), and eluent fractions were taken every 30 sec. Solutions containing the appropriate THC standards and the HPLC fractions were freezedried. The freezedrier was flushed with argon before and after drying to prevent atmospheric oxidation of sensitive metab-

olites. A solution of Δ^9-(^{3}H)-THC (0.025 μCi in 500 μl of a mixture of methanol–diluent buffer, 30:70) was added to each of the dried tubes with a solution of antiserum in diluent buffer (100 μl of 1:300 antiserum solution). The non-specific binding tubes received diluent buffer in place of antiserum solution. The subsequent RIA procedure was the same as that described above.

Cannabinoid retention volumes were determined by monitoring the eluate with either RIA for cross-reacting compounds, or UV absorption (280 nm) for non-cross-reacting compounds. UV detection required μg quantities compared with the ng quantities used with RIA detection.

High-pressure liquid chromatography–gas chromatography–mass spectrometry

Materials and equipment. HPLC equipment was the same as that described in the previous section. GC–MS was conducted using a Pye 104 GC equipped with an OV-17 column (0.3 m × 2 mm I.D., Gas-Chrom Q, 80–100 mesh) interfaced to a VG Micromass 16F Mass Spectrometer. The internal standard (5′-$^2H_3$)-Δ^9-THC was kindly supplied by Dr. S. Agurell, Stockholm.

Method. The GC–MS method used was similar to that described by Agurell *et al.*[5] (5′-$^2H_3$)-Δ^9-THC (10 ng) in methanol was added to the plasma sample (1 ml) and the mixture extracted with methanol as described previously. The plasma extract was chromatographed using a 10-cm ODS column (4.6 mm I.D.) and methanol–water (67.5:32.5) as the eluent. The THC fraction (at a retention volume of approximately 20 ml) was collected, freezedried and the residue dissolved in hexane. This was gas chromatographed with a mass spectrometer in the multiple ion-detection mode tuned to ions of *m/e* 299 and 314 for THC and 302 and 317 for tri-deuterated THC. The THC retention time was 4 min with a helium flow-rate of 20 ml/min at 190°. The THC plasma concentration was calculated from the ratio of ion intensities for THC and deuterated THC using a previously prepared calibration graph.

RESULTS AND DISCUSSION

The assay method was similar to that described by Teale *et al.*[18] except that methanol replaced Triton X-405 as the THC solubilising agent. This modification increased the solubility of THC and reduced non-specific binding in the assay. A reduction of blank levels was obtained with normal plasma samples together with improved assay sensitivity.

Teale *et al.*[18] investigated antiserum specificity and found no cross-reaction with 24 non-cannabinoid drugs. In our study 44 non-cannabinoid drugs were examined (Table II) at a concentration equivalent to 400 μg/ml plasma and again no cross-reaction was observed with any of the compounds. Antiserum specificity to cannabinoid compounds using the modified RIA procedure was examined (Table III). Results were similar to those obtained by Teale *et al.*[18] in that the antiserum cross-reacted with the same avidity to THC as it did to some of the closely related THC metabolites.

The levels of cross-reaction in plasma samples obtained from three subjects who smoked THC impregnated cigarettes are given in Table IV. Levels of cross-reaction were highest for those samples obtained immediately after smoking while values obtained for subsequent samples fell to a constant level for the remainder of

TABLE II

MISCELLANEOUS DRUGS TESTED FOR CROSS-REACTIVITY IN THE RADIOIMMUNOASSAY

Adrenaline	Methaqualone hydrochloride
Amitriptyline hydrochloride	Morphine sulphate
Amphetamine sulphate	Nicotine hydrogen tartrate
Ascorbic acid	Nitrazepam
Aspirin	Oestradiol
Barbitone	Papaverine hydrochloride
Bromodiphenhydramine hydrochloride	Paracetamol
	Pentobarbitone
Caffeine	Phenobarbitone
Codeine phosphate	Phenmetrazine theoclate
Cocaine hydrochloride	Phenylbutazone
Chlorodiazepoxide	Progesterone
Chloropromazine hydrochloride	Promazine hydrochloride
N,N-Dimethyltryptamine	Spironolactone
Diphenhydramine hydrochloride	Stilboestrol
Doxepin	Sulphadimidine
Ephedrine hydrochloride	Sulphamethoxypyridazine
Ergometrine maleate	Tetracycline hydrochloride
Ethylenediamine tetraacetic acid	Thiopentone sodium
Imipramine	Trifluoperazine
Lignocaine	Tryptamine hydrochloride
Mescaline sulphate	Tyramine hydrochloride
Methadone hydrochloride	

TABLE III

CROSS-REACTIVITY OF Δ^9-THC METABOLITES AND RELATED COMPOUNDS IN THE RADIOIMMUNOASSAY

THC = Δ^9-tetrahydrocannabinol, CBN = cannabinol, CBD = cannabidiol.

Compounds	*Amount required for 50% depression of binding (ng)*
Δ^9-THC	0.6
Δ^8-THC	0.6
CBN	0.6
11-Hydroxy-Δ^9-THC	0.6
Δ^9-THC-11-oic acid	0.6
11-Hydroxy-CBN	0.6
CBN-11-oic acid	0.6
Hexahydro-CBN	0.6
$\Delta^{9,11}$-THC (Exo-cyclic compound)	0.6
8α-Hydroxy-Δ^9-THC	0.6
8β-Hydroxy-Δ^9-THC	3.0
8α,11-Dihydroxy-Δ^9-THC	2.0
8β,11-Dihydroxy-Δ^9-THC	9.0
1′-Oxo-CBN	3.0
1′-Hydroxy-CBN	10 0
5′-Hydroxy-Δ^9-THC	30.0
CBD	$>$50.0
Cannabicyclol	$>$50.0
Cannabichromene	$>$50.0
Cannabigerol	$>$50.0

TABLE IV

THE PLASMA CONCENTRATION OF Δ^9-THC IN VOLUNTEERS WHO HAD SMOKED Δ^9-THC DETERMINED BY RIA, HPLC AND GC–MS

ND = Not determined.

Subject	*Time after smoking (min)*	*Plasma concentration of Δ^9-THC (ng/ml)*		
		*Direct RIA**	*RIA after separation by HPLC*	*GC–MS*
1	Control	23**	0	0
	2	67	47	55
	12	48	15	18
	24	47	7	9
	34	48	5	8
	64	47	3	5
	126	48	1	2
2	Control***	ND**	ND	ND
	3	63	45	58
	13	44	20	30
	24	53	9	16
	34	47	6	9
	64	58	4	5
	124	50	2	1
3	Control	0	0	0
	2	37	26	26
	22	7	4	3
	31	5	1.1	ND
	60	9	0.9	ND
	120	6	0.8	ND

* These values include a contribution from THC metabolites.

** Subject 1 and 2 were both cannabis users and cannabinoid material may have been present in the plasma before the experiment.

*** A control sample was taken for subject 2 but this was lost during centrifugation.

the 2-h experiment. The absence of a continuing decline of plasma cross-reacting cannabinoids over the course of the experiment could not be fully explained without a greater understanding of the changes of plasma concentrations of the THC metabolites. These data were obtained by using an HPLC separation stage prior to RIA.

Chromatograms representing the elution of cross-reacting compounds from an HPLC column were constructed for each plasma sample by plotting the RIA results obtained for consecutive HPLC eluent fractions against their retention volumes. Two of the chromatograms produced by this method are represented in Figs. 1 and 2. Individual components were quantified by summing the data points for each area of cross-reaction and relating these to the original volume of plasma used. Plasma THC levels for the three subjects were measured by this method and compared with THC levels obtained by the GC–MS method (Table IV). These results showed a biphasic elimination pattern for THC (*e.g.*, Fig. 3), but values obtained by the HPLC–RIA method were consistently lower than those obtained by GC–MS (Table IV). This discrepancy could not be fully explained by losses that occurred during the plasma extraction and chromatographic stages of the HPLC–RIA procedure which were found to be approximately 10%. The HPLC–RIA results for subjects 1 and 2 were obtained approximately six months after the GC–MS analyses were performed. It it possible that decomposition of the THC had occurred during this period.

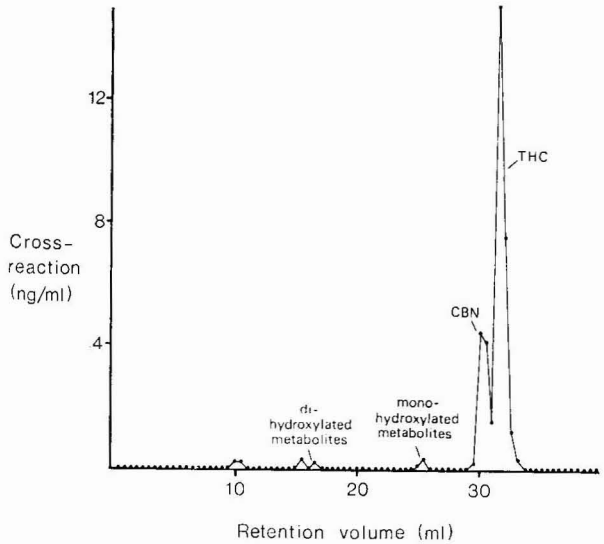


Fig. 1. HPLC–RIA chromatogram of a plasma sample from subject 3 taken 2 min after smoking Δ^9-THC (8 mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.

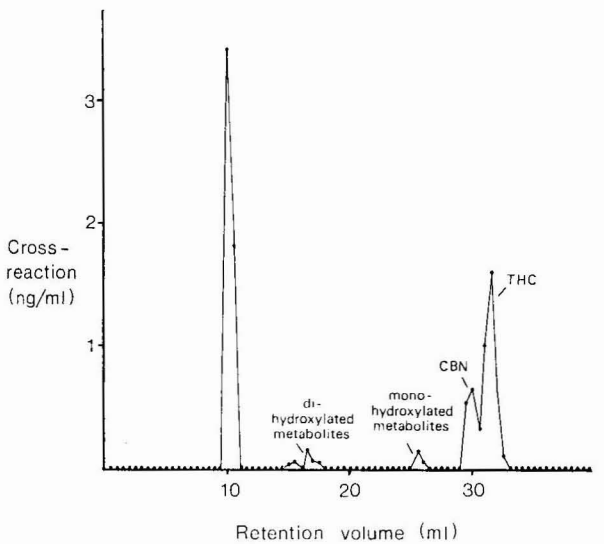


Fig. 2. HPLC–RIA chromatogram of a plasma sample from subject 3 taken 22 min after smoking Δ^9-THC (8 mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.

The presence of cross-reacting compounds in the plasma samples other than THC was also indicated by the HPLC–RIA data. A comparison of cannabinoid retention volumes (Table V) with the HPLC–RIA data, showed the presence of cross-reacting compounds in the plasma samples with retention volumes corresponding to

those of cannabinol (CBN), mono-hydroxylated metabolites and di-hydroxylated metabolites (Figs. 1 and 2). Control plasma samples contained no indication of the presence of these cannabinoids. Subject 1 was a regular user of cannabis and the control sample contained cross-reacting material which had a retention volume of 10 ml. This was probably due to the consumption of cannabinoid material at some time before the experiment. Subject 3 was not a cannabis-user and his control sample contained no indication of cross-reacting material.

TABLE V

CANNABINOID HPLC RETENTION VOLUMES

Compounds	*Retention volume (ml)* *
Δ^9-THC	31.5
Δ^8-THC	31.0
CBN	30.0
11-Hydroxy-Δ^9-THC	25.5
Δ^9-THC-11-oic acid	10.0
11-Hydroxy-CBN	25.5
CBN-11-oic acid	10.0
8α-Hydroxy-Δ^9-THC	25.5
8β-Hydroxy-Δ^9-THC	20.5
8α,11-Dihydroxy-Δ^9-THC	17.0
8β,11-Dihydroxy-Δ^9-THC	16.5
1′-Oxo-CBN	29.0
1′-Hydroxy-CBN	22.5

* For the system described in the text.

CBN has been reported as present in plasma samples after THC administration[19] which suggested that CBN was a metabolic product of THC. However, Wall *et al.*[11] found almost no CBN in plasma after the intravenous administration of THC to human subjects and concluded that CBN was not a THC metabolite. Plasma concentrations of the compound eluting with the retention volume of CBN found in this study (Table VI) appeared to follow a biphasic elimination pattern similar to that of THC (Fig. 3). This suggests that a metabolic explanation for the presence of CBN in these samples is unlikely.

One of the other areas of cross-reaction observed with the HPLC–RIA data had a retention volume of 25.5 ml. The metabolites 11-hydroxy-Δ^9-THC, 8α-hydroxy-Δ^9-THC and 11-hydroxy-CBN all have this retention volume with the HPLC system used and it is possible that the observed level of cross-reaction may represent a contribution from each of these metabolites. Levels of cross-reaction at this retention volume reached a maximum shortly after Δ^9-THC smoking for the three subjects, falling slowly over the remainder of the experiment (Fig. 3). The concentrations and elimination curves for this area of cross-reaction are similar to those of 11-hydroxy-Δ^9-THC reported in plasma samples obtained from subjects receiving intravenously administered Δ^9-THC[11].

An area of cross-reaction at the retention volumes corresponding to the dihydroxylated metabolites 8α,11-dihydroxy-Δ^9-THC and 8β,11-dihydroxy-Δ^9-THC was also observed with the HPLC–RIA data (Figs. 1 and 2). These metabolites were

TABLE VI

THE PLASMA CONCENTRATIONS OF CROSS-REACTING CANNABINOIDS OTHER THAN Δ^9-THC IN VOLUNTEERS WHO HAD SMOKED Δ^9-THC DETERMINED BY HPLC–RIA

Subject	*Time after smoking (min)*	*Plasma concentration as determined by RIA and expressed as ng/ml of cross-reacting cannabinoid*	
		Peak elution at the position of mono-hydroxylated metabolites	*Peak elution at the position of CBN*
1	Control	0	0
	2	0.6	9.0
	12	1.2	2.1
	24	0.6	1.4
	34	0.5	0.5
	64	0.2	0.2
	126	0.1	0.1
2	3	1.0	5.0
	13	1.0	2.5
	24	1.4	1.7
	34	0.5	0.7
	64	0.3	0.2
	124	0.4	0.1
3	Control	0	0
	2	0.3	9.0
	22	0.3	1.2
	31	0.1	0.4
	60	0.1	0.3
	120	0.1	0.2

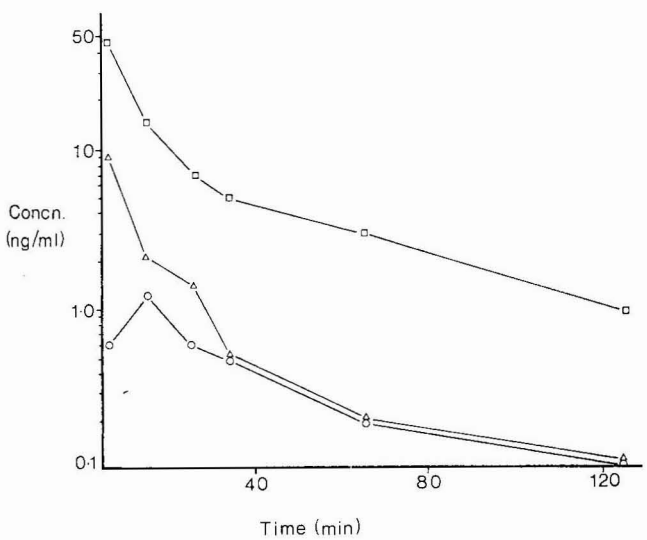


Fig. 3. Elimination from plasma of cannabinoids with retention volumes equivalent to Δ^9-THC (□), CBN (△) mono-hydroxylated metabolites (○) for subject 1.

not well resolved with the chromatographic system used (Table V) and do not cross-react in the RIA to the same extent as each other or to Δ^9-THC (Table III). Quantitative results for these compounds were not therefore, obtained from the HPLC–RIA data.

A further feature of the HPLC–RIA data was the presence of an area of cross-reaction eluting from the HPLC column at a retention volume of 10 ml (Figs. 1 and 2). This corresponds to the retention volume of metabolite conjugates observed in human urine samples obtained after Δ^9-THC administration as well as to the mono-carboxylated metabolites Δ^9-THC-11-oic acid and CBN-11-oic acid (Table V). This area of cross reaction may therefore represent a mixture of cross-reacting metabolite conjugates and the mono-carboxylated metabolites. These cross-reacting components may be separated by acidifying the HPLC eluate. The contribution of this area of cross-reaction to the total RIA result increased as the THC concentration decreased so that the total RIA result remained approximately constant for the 2-h period after THC administration (Table IV). The absolute identification of the structure of cross-reacting compounds will require the isolation of sufficient quantities of material to obtain MS data.

HPLC–RIA provides a convenient method for separating, presumptively identifying and quantifying THC and some of its metabolites in plasma. It has the advantage over previously reported methods of using a small volume of sample to quantify simultaneously THC and some of its metabolites. The application of this method to samples submitted for forensic examination has been reported[20].

ACKNOWLEDGEMENTS

We thank Professor J. W. Thompson (University of Newcastle Upon Tyne) for supplying all the plasma samples, Dr. S. Agurell and Miss A. Ohlsson (Astra Lakemedel, Sodertalje, Sweden) for the samples of deuterated THC, Dr. R. Ardrey (HOCRE) for help with the mass spectral studies, Dr. J. D. Teale (University of Surrey) for the antisera and Miss J. Bryant (National Institute on Drug Abuse) for samples of cannabinoids and their metabolites.

REFERENCES

1 J. A. Vinson, D. D. Patel and A. H. Patel, *Anal. Chem.*, 49 (1977) 163.
2 D. C. Fenimore, R. R. Freeman and P. R. Loy, *Anal. Chem.*, 45 (1973) 2331.
3 E. R. Garrett and C. A. Hunt, *J. Pharm. Sci.*, 62 (1973) 1211.
4 N. K. McCallum, *J. Chromatogr. Sci.*, 11 (1973) 509.
5 S. Agurell, B. Gustafsson, B. Holmstedt, K. Leander, J. E. Lindgren, I. Nilsson, F. Sandberg and M. Asberg, *J. Pharm. Pharmacol.*, 25 (1973) 554.
6 J. Rosenfeld, B. Bowins, J. Roberts, J. Perkins and A. S. Macpherson, *Anal. Chem.*, 46 (1974) 2232.
7 R. Detrich and R. L. Foltz, *Res. Monogr. Ser., Nat. Inst. Drug Abuse (U.S.A.)*, 7 (1976) 88.
8 J. L. Valentine, P. J. Bryant, P. L. Gutshall, O. H. M. Gan, E. D. Thompson and H. C. Niu, *Res. Monogr. Ser., Nat. Inst. Drug Abuse. (U.S.A.)*, 7 (1976) 96.
9 J. Rosenfeld and V. Y. Taguchi, *Anal. Chem.*, 48 (1976) 726.
10 M. Nordqvist, J. E. Lindgren and S. Agurell, *Res. Monogr. Ser., Nat. Inst. Drug Abuse (U.S.A.)*, 7 (1976) 64.
11 M. E. Wall, T. M. Harvey, J. T. Bursey, D. R. Brine and D. Rosenthal, *Res. Monogr. Ser., Nat. Inst. Drug Abuse (U.S.A.)*, 7 (1976) 107.

12 J. D. Teale, E. J. Forman, L. J. King and V. Marks, *Nature (London)*, 249 (1974) 154.
13 J. D. Teale, E. J. Forman, L. J. King and V. Marks, *Lancet*, ii (1974) 553.
14 S. J. Gross, J. R. Soares, J.-R. R. Wong and R. E. Schuster, *Nature (London)*, 252 (1974) 581.
15 C. E. Cook, M. L. Howes, E. W. Amerson, C. G. Pitt and D. Williams, *Res. Monogr. Ser., Nat. Inst. Drug Abuse (U.S.A.)*, 7 (1976) 15.
16 M. Cais, S. Dani, Y. Josephy, A. Modiano, H. Gershon and R. Mechoulam, *Febs. Lett.*, 55 (1975) 257.
17 S. J. Gross and J. R. Soares, *Res. Monogr. Ser., Nat. Inst. Drug Abuse (U.S.A.)*, 7 (1976) 10.
18 J. D. Teale, E. J. Forman, L. J. King, E. M. Piall and V. Marks, *J. Pharm. Pharmacol.*, 27 (1975) 465.
19 N. K. McCallum, B. Yagen, S. Levy and R. Mechoulam, *Experientia*, 31 (1975) 520.
20 J. D. Teale, J. M. Clough, L. J. King, V. Marks, P. L. Williams and A. C. Moffat, *J. Forensic Sci. Soc.*, 17 (1977 177).

Journal of Chromatography, 155 (1978) 285–293

CHROM. 10,968

LIGAND-EXCHANGE CHROMATOGRAPHY OF RACEMATES

V. SEPARATION OF OPTICAL ISOMERS OF AMINO ACIDS ON A POLYSTYRENE RESIN CONTAINING L-HYDROXYPROLINE

V. A. DAVANKOV and Yu. A. ZOLOTAREV
Institute of Organo-Element Compounds, Academy of Sciences, Moscow (U.S.S.R.)
(Received November 17th, 1977)

SUMMARY

An asymmetric resin containing copper(II) ions with L-hydroxyproline ligands fixed in a macronet isoporous polystyrene matrix has been used for the separation of optical isomers of amino acids. The resin selectivity and the column efficiency were determined and their dependence on the chromatographic conditions (the degree of saturation of the resin with copper(II) ions, ammonia concentration, column parameters) discussed.

INTRODUCTION

Ligand-exchange chromatography (LEC), which has recently been reviewed[1], has proved to be a powerful method for separating compounds with very similar molecular structures, including geometrical and optical isomers. For example, asymmetric resins with residues of optically active α-amino acids fixed on a polystyrene matrix can be used to separate the enantiomers of amino acids, provided the resin contains metal ions capable of forming complexes with both the fixed and the mobile amino acid ligands[2]. Earlier we have studied some general features characteristic of LEC of enantiomers, including its dependence on the amount of the complex-forming metal ion[3], on the temperature and concentration of the eluent[4] and on the structure of the fixed ligands[5].

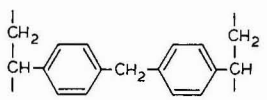
However, in order to evaluate the possibilities of practical application of ligand-exchange separation of optical isomers, a more detailed and quantitative study is needed of the interaction of enantiomers with the resin phase. In the previous papers[6,7] we discussed the method and results of calculating the stability constants of the fixed (stationary) complexes formed by the fixed ligands with the metal ions and of mixed-ligand sorption complexes containing both the fixed and the mobile ligands co-ordinated to the metal ion.

The present report deals with results of a systematic chromatographic study of the interaction of the enantiomers of amino acids with a resin loaded with copper(II) ions and containing L-hydroxyproline as the fixed ligand. The chromatographic

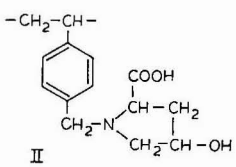
experiments were performed on a scale which would permit evaluation of the technique for use in both analysis and preparation.

EXPERIMENTAL

The asymmetric resin was prepared by aminating a chloromethylated polystyrene containing 11 mol% of cross-links of structure I with methyl L-hydroxyprolinate hydrochloride[6]. According to the nitrogen content and potentiometric titration of the resin obtained, the content of fixed ligand of structure II amounted to 3.44 mmol per gram of dry resin, taken in the zwitterionic form. The resin particles were of irregular shape and had an average size in the swollen state of 100 μm.



I



II

On treatment with excess copper–ammonia solution, the resin was saturated with copper(II) ions to an extent of 92% of the theoretical capacity calculated for the fixed complexes containing two fixed ligands per copper(II) ion. The equilibrium water content of the copper-containing resin in neutral media amounted to 200%. To maintain the copper saturation of the resin at 92%, the eluents used contained copper(II) ions in the following concentrations: $1.2 \cdot 10^{-5}$ M in 0.1 M NH_4OH for the chromatography of neutral amino acids; $2.5 \cdot 10^{-5}$ M in 0.025 M $Na(NH_4)_2PO_4$ solution of pH 8.3 for acidic amino acids and $2.0 \cdot 10^{-4}$ M in 1.5 M NH_4OH for basic amino acids.

The resin (6.3 ml) was packed into a column of 140×7.8 mm I.D. Portions of 1.0–1.5 mg of optically active or 2–3 mg of racemic amino acids were introduced into the column and eluted at a rate of 10 ml/h. Elution curves were recorded with a Uvicord III (LKB) detector at 206 nm. The void column volume of 4.7 ml was determined using acetone, which was assumed to be able to enter the resin phase without being strongly retained.

The retention volumes, V_L and V_D, of amino acid enantiomers L and D were determined from the distance between their peak maxima and that of acetone and expressed in void column volumes. The ratio of V_D to V_L is the enantioselectivity, α, of the sorption process and was used for calculating the difference, $\delta\Delta G^\circ$, between the free energies of the two diastereomeric sorption complexes formed in the resin phase:

$$\delta\Delta G^\circ = \Delta G^\circ_{R-Cu-D} - \Delta G^\circ_{R-Cu-L} = -RT \ln \alpha = -RT \ln \frac{V_D}{V_L}$$

If insufficient resolution of the racemate rendered a precise determination of the maxima positions or calculation of HETP values impossible, a separate chromatography of D- and L-enantiomers was performed.

RESULTS AND DISCUSSION

Modern chromatographic methods can separate components that differ in sorption energy, $\delta\Delta G^\circ$, by as little as 10 cal/mol (sorption selectivity $\alpha = 1.01$). For separation on a preparative scale, it is desirable that α should be not less than 1.5 (or $\delta\Delta G^\circ \geqslant 250$ cal/mol). It is helpful to bear these figures in mind when considering the results (Table I) of the LEC of amino acid enantiomers on the resin containing L-hydroxyproline.

Aliphatic amino acids

As illustrated by Table I and Fig. 1, the retention volumes of the L-enantiomers increase steadily as the size of the α-C-atom substituent increases from methyl in alanine to butyl in norleucine. The retention times of the D-isomers increase faster still. This leads to a rise in the selectivity factor from 1.04 to 2.20, corresponding to $\delta\Delta G^\circ$ values of 24 and 460 cal/mol. Among amino acids with the same number of carbon atoms, racemic compounds with a linear side-chain (norvaline; norleucine) are resolved better than their isomers with a branched one (valine; leucine, isoleucine). In the latter instance branching at the β-carbon atom (isoleucine) is more favourable than that in the γ-position (leucine). However, the presence of two substituents on the same α-C-atom has an adverse effect on the separation of enantiomers (compare isovaline with valine and norvaline).

The retention volumes of glycine (6.44) and β-alanine (0.28) clearly show the large difference in stability between five-membered and six-membered chelate rings.

Hydroxyamino acids

Substitution of a β-H-atom in alanine and aminobutyric acid by a hydroxy group (to give serine and threonine) results in a significant decrease in the retention volume (Fig. 2), probably reflecting the lower stability of copper(II) complexes of serine and threonine as compared with those of alanine and aminobutyric acid. However, the presence of a hydroxy group in the β-position enhances the enantioselectivity of the sorption process so that the $\delta\Delta G^\circ$ values rise to 150 and 245 cal/mol for serine and threonine from 24 and 120 cal/mol for alanine and aminobutyric acid, respectively. Contrary to this, a hydroxy group in the γ-position has almost no influence on either the retention volumes of the enantiomers or their separation (compare homoserine with aminobutyric acid). Other functional groups in the γ-position (as in methionine and asparagine), just like the hydroxy group, do not increase the enantioselectivity above the value for aminobutyric acid. It is likely that β-hydroxy groups alone have the ability to participate in the coordination process. This suggestion explains the noticeable role of the β-C-atom configuration (compare threonine with allo-threonine) and the unusually high values of HETP for serine and threonine.

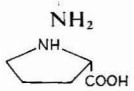
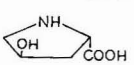
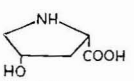
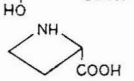
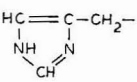
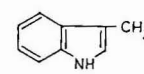
Aromatic amino acids

Amino acids containing a phenyl ring demonstrate comparatively high resolution ability and a high affinity towards the resin (large retention volumes). Some additional interactions with the polymer matrix must be increasing the stability of sorption complexes of these amino acids, especially β-phenyl-α-alanine (Fig. 3). Its

TABLE I

ELUTION PARAMETERS OF AMINO ACIDS ON THE L-HYDROXY-PROLINE RESIN IN THE COPPER(II) FORM

Eluents: 0.1 *M* NH_4OH (N = 1–28); 1.5 *M* NH_4OH (N = 29–32); 0.025 *M* $Na(NH_4)_2PO_4$, pH 8.3 (N = 33–36).

N	*Amino acid*	*α-Radicals or molecular structure*	*V*		*α*	*ΔG°* (*cal/mol*)	*HEEP* (*cm*)	
			L	D			L	D
1	Glycine	H–	6.44				0.31	
2	β-Alanine	$H_2NCH_2CH_2COOH$	0.28				~100	
3	Alanine	CH_3–	5.82	6.04	1.04	24	0.38	0.56
4	Aminobutyric acid	CH_3CH_2–	6.48	7.95	1.22	120	0.29	0.32
5	Norvaline	$CH_3CH_2CH_2$–	11.2	19.9	1.65	290	0.30	0.21
6	Norleucine	$CH_3CH_2CH_2CH_2$–	21.4	47.4	2.20	460	0.26	0.17
7	Valine	$CH_3CH(CH_3)$–	7.27	11.8	1.61	280	0.31	0.43
8	Isovaline	CH_3CH_2–; CH_3–	6.8	8.5	1.25	130		
9	Leucine	$CH_3CH(CH_3)CH_2$–	14.2	24.2	1.70	310	0.44	0.51
10	Isoleucine	$CH_3CH_2CH(CH_3)$–	11.1	20.9	1.89	370	0.53	0.49
11	Serine	$HOCH_2$–	3.47	4.48	1.29	150	0.94	0.90
12	Threonine	$HOCH(CH_3)$–	3.47	5.27	1.52	245	1.03	0.63
13	allo-Threonine	$HOCH(CH_3)$–	2.65	3.85	1.45	220		
14	Homoserine	$HOCH_2CH_2$–	5.32	6.65	1.25	130	0.35	
15	Methionine	$CH_3SCH_2CH_2$–	11.7	14.3	1.22	120	0.53	0.53
16	Asparagine	H_2NCOCH_2–	4.60	5.37	1.17	90	0.39	0.38
17	Glutamine	$H_2NCOCH_2CH_2$–	2.46	3.70	1.50	240	0.52	
18	Phenylglycine	C_6H_5–	6.15	13.6	2.22	465	0.70	0.74
19	Phenylalanine	$C_6H_5CH_2$–	33.8	97.6	2.89	620	0.59	0.62
20	α-Phenyl-α-alanine	C_6H_5–; CH_3–	11.9	12.5	1.07	39	1.25	1.25
21	Tyrosine	$HOC_6H_4CH_2$–	8.95	19.8	2.23	465	1.25	0.83
22	Phenylserine	$C_6H_5CH(OH)$–	22.6	41.1	1.82	350	0.98	0.94
23	β-Phenyl-β-alanine	$C_6H_5CHCH_2COOH$	1.25	2.23	1.79	340		
24	Proline		14.6	57.8	3.95	800	0.60	0.88
25	Hydroxyproline		9.18	29.1	3.17	680	0.67	0.48
26	allo-Hydroxy-proline		29.4	17.7	1.65	290	0.68	0.51
27	Azetidinecarboxylic acid		14.0	31.5	2.25	475	1.02	0.92
28	Ornithine	$H_2NCH_2CH_2CH_2$–	34.4					
29	Ornithine	$H_2NCH_2CH_2CH_2$–	2.0	2.0	1.0	0	1.2	1.2
30	Lysine	$H_2NCH_2CH_2CH_2CH_2$–	2.5	3.04	1.22	120	0.84	0.87
31	Histidine		14.6	5.22	2.80	600	0.49	0.51
32	Tryptophan		20.7	36.5	1.77	330	0.81	0.63
33	Aspartic acid	$HOOCCH_2$–	11.5	11.5	1.0	0	1.34	1.45
34	Glutamic acid	$HOOCCH_2CH_2$–	2.2	1.8	1.22	120	1.63	1.42
35	Iminodiacetic acid	$HN(CH_2COOH)_2$	32.6				1.28	
36	Serine	$HOCH_2$–	42.5					

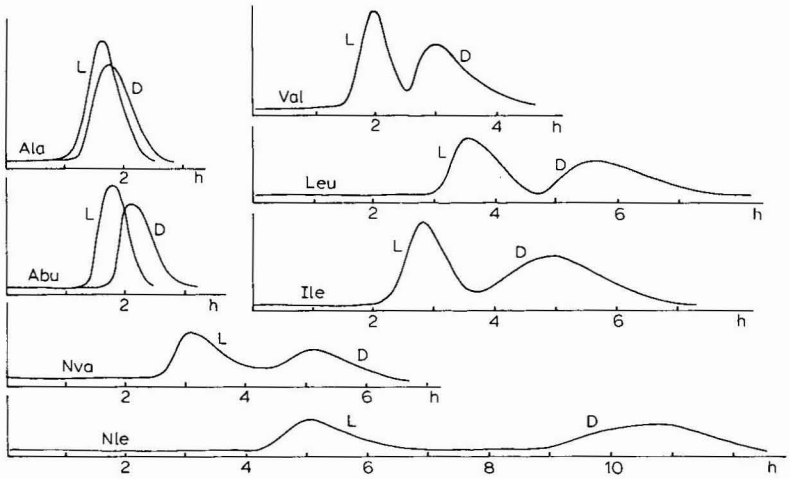


Fig. 1. Chromatography of the enantiomers of alanine (α = 1.04), aminobutyric acid (α = 1.22), norvaline (α = 1.65), norleucine (α = 2.20), valine (α = 1.61), leucine (α = 1.70) and isoleucine (α = 1.89). Column 7.8 × 140 mm; 0.1 *M* NH_4OH; 10 ml/h. The degree of saturation of the L-hydroxyproline resin by copper(II) ions was 92%. Particle size *ca.* 100 μm.

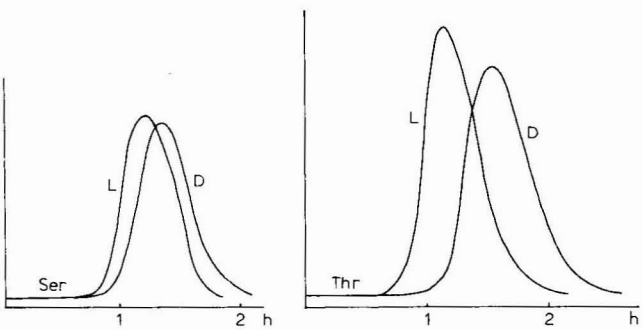


Fig. 2. Chromatography of the enantiomers of serine (α = 1.29) and threonine (α = 1.52). Conditions as given in Fig. 1.

hydroxy analogue, tyrosine, is partially ionized under the chromatographic conditions (0.1 *M* NH_4OH) and, to a certain extent, expelled from the negatively charged resin phase. A very low retention time of the β-phenyl-β-alanine isomers forming a six-membered chelate ring (but nevertheless resolving with a factor of α = 1.79) indicates that there are no additional hydrophobic interactions between the aromatic sorbate and the resin matrix, other than in the coordination sphere of the sorption complex.

Among the aromatic amino acids, α-phenyl-α-alanine alone contains two substituents at the α-carbon atom. As was the case with isovaline, it is poorly resolved.

Cyclic amino acids

Proline enantiomers show the highest sorption selectivity on the resin containing L-hydroxyproline (α = 3.95; $\delta\Delta G^\circ$ = 800 cal/mol). Hydroxyproline (α = 3.17) is also completely resolved into its enantiomers. Its diastereomer, allo-hydroxyproline,

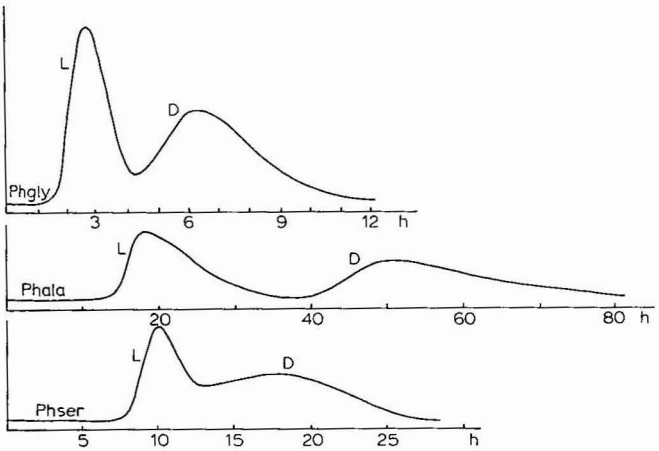


Fig. 3. Chromatography of the enantiomers of phenylglycine (α = 2.22), phenylalanine (α = 2.89) and phenylserine (α = 1.82). Conditions as given in Fig. 1.

displays a lower selectivity (α = 1.65) and surprisingly, a reversed elution order of the components: the D-isomer is eluted before the L-isomer (Fig. 4). The β-hydroxy group of the latter is undoubtly coordinated to the axial position of the copper(II) ion. The D-enantiomer cannot do this because the corresponding axial position is blocked by the *N*-benzyl group of the fixed ligand.

Azetidinecarboxylic acid, a four-membered heterocyclic homologue of proline, shows a sufficiently high selectivity ($\delta\Delta G^\circ$ = 475 cal/mol) but unusually broad elution peaks (HETP *ca.* 1 cm).

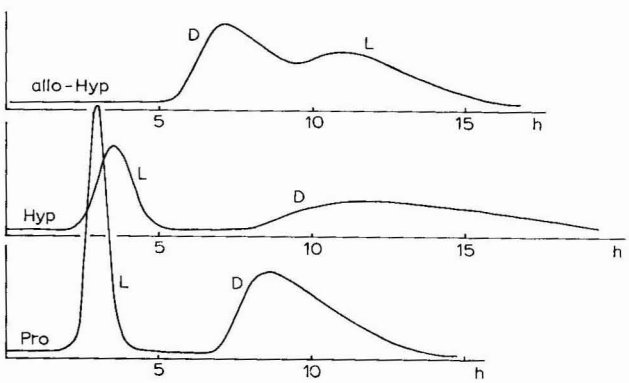


Fig. 4. Chromatography of the enantiomers of allo-hydroxyproline (α = 1.65) and hydroxyproline (α = 3.17) in 0.2 *M* NH_4OH at a flow-rate of 20 ml/h and proline (α = 3.95) in 0.5 *M* NH_4OH at a flow-rate of 8 ml/h. Other conditions as given in Fig. 1.

Basic amino acids

Basic amino acids are strongly retained by the resin, so 1.5 *M* NH_4OH is used for their elution. This is probably due to the partial positive charge of these com-

pounds, which can strongly influence their distribution between the resin and the solution, rather than to the stability of the sorption complexes. Arginine, which contains a positively charged side-chain is not eluated even if 6 *M* NH_4OH is used.

Ornithine isomers are not resolved, and lysine isomers only poorly so. Tryptophan shows a high selectivity factor ($\alpha = 1.77$), but its peaks are too broad to be completely resolved under standard conditions (Fig. 5). The elution order of histidine isomers is reversed, which can be accounted for in terms of axial coordination of the imidazole group of the L-isomer.

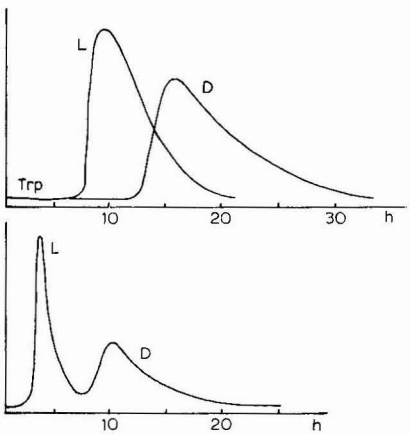


Fig. 5. Chromatography of the enantiomers of tryptophan. *Above:* in 1.5 *M* NH_4OH, other conditions as given in Fig. 1; $\alpha = 1.77$. *Below:* in 0.2 *M* NH_4OH at a flow-rate of 6 ml/h on a column of 4.5 × 280 mm with the L-hydroxyproline resin saturated by copper(II) ions to an extent of 15%, particle size 20–30 μm; $\alpha = 3.09$.

Monoaminodicarbonic acids

Acid compounds are eluted by ammonia within the void column volume in spite of the fact that the stability of their copper complexes does not yield to that of aliphatic amino acids — negatively charged species are expelled from the resin phase. However, the distribution coefficients of amino acids rise significantly at lower pH values[7]. Thus, chromatography of acid compounds can be carried out in an ammonium phosphate buffer solution at a pH of 8.3. The retention volumes increase in the series: glutamic acid, aspartic acid, iminodiacetic acid, in accordance with the increasing stability constants of their copper complexes. (On chromatography in the phosphate buffer, neutral amino acids show very high retention volumes.)

Unfortunately, no resolution was observed in the case of aspartic acid and only a small one ($\delta\Delta G^\circ = 120$ cal/mol) in the case of glutamic acid. From unknown reasons, D-glutamic acid forms more stable sorption complexes than the L-isomer does.

General remarks

Though there is a general correlation between the stability of copper(II) complexes of different amino acids and their retention volumes on the L-hydroxyproline resin, contributions from electrostatic interactions and hydrophobic interactions

undoubtedly play an important role in the resin phase. Besides, only mixed ligand (amino acidato) (N-benzyl-L-hydroxyprolinato) copper(II) complexes can be considered as adequate low molecular weight models for sorption complexes.

Sorption selectivity of many amino acid enantiomers on the L-hydroxyproline resin is sufficiently high to permit their complete separation under the conditions used and to indicate that the process can be successfully carried out on a preparative scale.

The efficiency of the column used was rather poor: the values of HETP amounted to 0.3–0.5 cm for aliphatic amino acids, 0.6–1.2 cm for aromatic, basic and hydroxy amino acids, and greater than 1 cm for acid compounds. However, the efficiency can be substantially improved by using resins with a smaller particle size (Fig. 6), which reduces the values of HETP by a factor of 2 or 3 and results in complete resolution of valine, leucine, tryptophan, phenylglycine and some other amino acids.

The LEC process can be improved in other ways. The very high retention volumes of basic amino acids can be reduced by lowering the degree of saturation

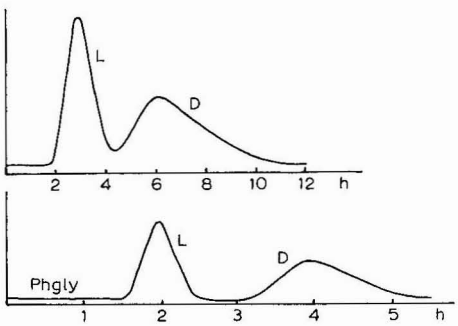


Fig. 6. Chromatography of the enantiomers of phenylglycine. *Above:* under standard conditions (see Fig. 1); HETP = 7 mm, $\alpha = 2.22$, $R_s = 0.75$. *Below:* on a column of 3.5 × 190 mm at a flow-rate of 8.2 ml/h, particle size 20–30 μm; HETP = 2.8 mm, $\alpha = 2.22$, $R_s = 1.22$.

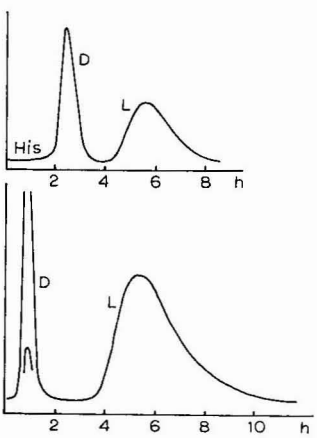


Fig. 7. Chromatography of the enantiomers of histidine. *Above:* in 1.5 *M* NH_4OH, other conditions as given in Fig. 1; $\alpha = 2.80$. *Below:* in 0.5 *M* NH_4OH at a flow-rate of 20 ml/h; the degree of resin saturation by copper(II) ions was 70%; $\alpha = 8.0$.

of the resin by copper(II) ions[3,7]. More dilute ammonia solutions can then be used for chromatography. Unexpectedly, a significant increase in sorption selectivity was observed in dilute ammonia for histidine (Fig. 7), tryptophan (Fig. 5) and proline. Obviously, this phenomenon indicates that ammonia molecules can take part in the formation of mixed-ligand sorption complexes, sometimes lowering the enantioselectivity.

Fig. 8 shows an example of how elution profiles can be improved by the use of ammonia gradients.

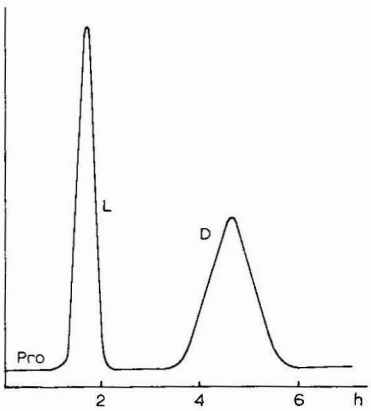


Fig. 8. Chromatography of the enantiomers of proline in a 0.5–1.5 *M* gradient of ammonia. Other conditions as given in Fig. 1.

Following further improvement of the LEC process, we hope to develop a simple and rapid method for the analysis of the enantiomeric composition of many amino acids, which would differ from the GLC method by making superfluous the preparation of volatile derivatives of amino acids.

REFERENCES

1 V. A. Davankov and A. V. Semechkin, *J. Chromatogr.*, 141 (1977) 313.
2 S. V. Rogozhin and V. A. Davankov, *U.S. Pat.*, 3, 853, 906; *U.S.S.R. Pat. appl.*, 01.07.1968; *C.A.*, 72 (1970) 90,875c.
3 V. A. Davankov, S. V. Rogozhin, A. V. Semechkin and T. P. Sachkova, *J. Chromatogr.*, 82 (1973) 359.
4 V. A. Davankov, S. V. Rogozhin, A. V. Semechkin, V. A. Baranov and G. S. Sannikova, *J. Chromatogr.*, 93 (1974) 363.
5 A. V. Semechkin, S. V. Rogozhin and V. A. Davankov, *J. Chromatogr.*, 131 (1977) 65.
6 Yu. A. Zolotarev, A. A. Kurganov and V. A. Davankov, *Talanta*, (1978) in press.
7 Yu. A. Zolotarev, A. A. Kurganov, A. V. Semechkin and V. A. Davankov, *Talanta*, (1978) in press.

Journal of Chromatography, 155 (1978) 295–302

CHROM. 10,969

LIGAND-EXCHANGE CHROMATOGRAPHY OF RACEMATES

VI. SEPARATION OF OPTICAL ISOMERS OF AMINO ACIDS ON POLYSTYRENE RESINS CONTAINING L-PROLINE OR L-AZETIDINE CARBOXYLIC ACID

V. A. DAVANKOV and Yu. A. ZOLOTAREV
Institute of Organo-Element Compounds, Academy of Sciences, Moscow (U.S.S.R.)
(Received February 14th, 1978)

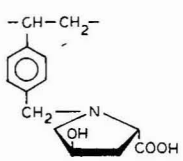
SUMMARY

Optical isomers of amino acids have been separated on the copper form of asymmetric resins containing residues of heterocyclic L-α-amino acids (proline and azetidine carboxylic acid) on a cross-linked polystyrene matrix. The enantioselectivity of the sorbents with respect to the optical isomers of many amino acids is high enough (200–800 cal/mole) to allow their quantitative separation. The separating capacity of the sorbent containing L-proline residues is generally higher than that of the sorbent based on L-azetidine carboxylic acid.

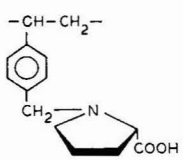
INTRODUCTION

Ligand-exchange chromatographic resolution of racemates of amino acids on a polystyrene resin containing L-hydroxyproline residues (I) as fixed ligands and charged with copper ions has been described in our previous paper[1]. The results are provided evidence that ligand-exchange chromatography (LEC) of enantiomers is a very promising technique for both analysis and preparation.

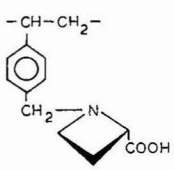
The present paper is the next step in determining the influence of the fixed ligand structure on LEC of racemic amino acids. It describes the resolution of racemates on sorbents containing fixed five- and four-membered heterocyclic ligands that are residues of L-proline (II) and L-azetidine carboxylic acid (III).



I



II



III

EXPERIMENTAL

The asymmetric resins were prepared by aminating the chloromethylated macronet polystyrene matrix containing 11 mol% of cross-links of diphenylmethane structure by methyl esters of proline or azetidine carboxylic acid, as described in ref. 2. The sorbent capacity was 2.78 (for II) and 2.40 (for III) mmol fixed ligands per gram of dry resin in its zwitterionic form. The resin particles were of irregular shape, and their size in the swollen form was *ca.* 100 μm. The resins were charged with copper(II) ions from a copper–ammonia solution, until they contained 80% of the theoretical amount of copper corresponding to the formation of fixed complexes containing two fixed ligands per copper ion. The equilibrium water content in the copper form of the resin was 170% for sorbent II and 140% for sorbent III.

As in the previous study[1], the chromatographic column (14 cm × 7.8 mm I.D.) contained 6.3 ml resin. The detector used was Uvicord III (LKB) with the 206 nm light filter. Elution at the rate 10 ml/h was carried out at room temperature by ammonia solutions of concentration 0.1, 0.3 and 1.5 *M* containing $1.2 \cdot 10^{-5}$, $3.8 \cdot 10^{-5}$ and $2.0 \cdot 10^{-4}$ *M* $CuSO_4$ respectively, and by an 0.017 *M* ammonium phosphate solution (pH 8.8) containing $2.5 \cdot 10^{-5}$ *M* $CuSO_4$. In each experimental run either 2–3 mg of racemic amino acid or 1.0–1.5 mg of each enantiomer were chromatographed.

RESULTS AND DISCUSSION

Tables I and II give retention parameters for D- and L-amino acid enantiomers in LEC on columns filled with copper complexes of sorbents II and III, respectively. The retention volumes, V_L and V_D, which are expressed in multiples of the void volume of the column (4.7 ml), were used to calculate the column selectivity, α, and the difference in standard free energies, $\delta\Delta G^\circ$, of two diastereomeric sorption complexes formed by coordination of amino acid enantiomers to the chiral resin chelate according to the relation:

$$\delta\Delta G^\circ = -RT \ln \alpha = -RT \ln \frac{V_D}{V_L}$$

Aliphatic amino acids

As indicated by Tables I and II, the larger substituent on the α-carbon atom in the mobile amino acid ligand, the longer the retention time of enantiomers and the better their separation.

The steric structure of the chiral sorption centres of the resin is also very important. Even minor changes in the fixed ligand structure markedly affect the sorption enantioselectivity. For instance, chromatography of D,L-Val on resin I with hydroxyproline residues results in a value of $\delta\Delta G^\circ$ almost twice as high as for sorbent II with proline residues, in spite of the fact that for spatial reasons the hydroxy group of the fixed hydroxyproline ligand cannot directly interact with the chelated copper ion and the mobile ligand attached to it. A decrease in the size of the fixed ligand ring by one methylene group on going from sorbent II to sorbent III leads to a considerable increase in valine sorption enantioselectivity (from 150 to 320 cal/mol).

TABLE I

ELUTION PARAMETERS OF AMINO ACIDS ON THE L-PROLINE RESIN IN THE COPPER(II) FORM

Eluents: 0.1 *M* NH_4OH (N = 1–15); 0.3 *M* NH_4OH (N = 16–24); 1.5 *M* NH_4OH (N = 25–29); 0.017 *M* $(NH_4)_3PO_4$, pH 8.8 (N = 30–32).

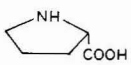
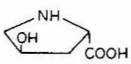
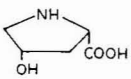
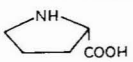
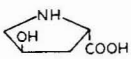
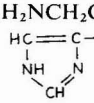
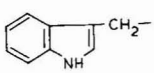
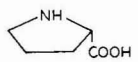
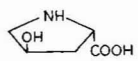
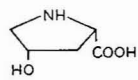
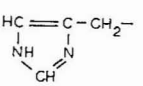
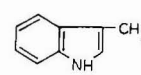
N	*Amino acid*	*α-Radicals or molecular structure*	*V*		*α*	*δΔG°* *(cal/mol)*	*HEEP (cm)*	
			L	D			L	D
1	Glycine	H–	5.0		—	—	0.66	
2	Alanine	CH_3–	6.75	7.25	1.08	46	0.67	0.64
3	Aminobutyric acid	CH_3CH_2–	7.20	8.50	1.17	92	0.70	0.68
4	Norvaline	$CH_3CH_2CH_2$–	14.2	18.2	1.28	145	0.68	0.70
5	Norleucine	$CH_3CH_2CH_2CH_2$–	25.5	39.4	1.54	225	0.65	0.67
6	Valine	$CH_3CH(CH_3)$–	9.0	11.6	1.29	150	1.00	0.95
7	Serine	$HOCH_2$–	4.0	4.35	1.09	52	0.85	0.90
8	Threonine	$HOCH(CH_3)$–	4.0	5.5	1.38	190	1.00	0.95
9	allo-Threonine	$HOCH(CH_3)$–	3.25	5.0	1.55	260	1.05	1.00
10	Asparagine	H_2NCOCH_2–	4.25	5.0	1.18	97	0.70	0.62
11	Glutamine	$H_2NCOCH_2CH_2$–	3.75	4.5	1.20	110	0.90	0.74
12	Proline		17.0	70	4.10	825	0.62	0.60
13	Hydroxyproline		9.9	38.2	3.85	790	0.42	0.46
14	allo-Hydroxyproline		43.5	18.8	2.32	490	0.96	0.92
15	Phenylglycine	C_6H_5–	11.3	18.8	1.67	300	1.40	1.20
16	Norvaline	$CH_3CH_2CH_2$–	4.10	5.75	1.40	200	0.66	0.68
17	Norleucine	$CH_3CH_2CH_2CH_2$–	12.0	18.5	1.54	250	0.65	0.68
18	Leucine	$CH_3CH(CH_3)CH_2$–	13.0	16.5	1.27	140	0.56	0.58
19	Isoleucine	$CH_3CH_2CH(CH_3)$–	7.0	10.5	1.50	240	0.49	0.48
20	Phenylalanine	$C_6H_5CH_2$–	31.5	51.5	1.63	286	1.05	0.98
21	Tyrosine	$HOC_6H_4CH_2$–	2.65	6.5	2.46	530	1.20	1.06
22	Proline		6.25	25.0	4.00	815	0.67	0.62
23	Hydroxyproline		3.50	13.5	3.85	790	0.37	0.43
24	Methionine	$CH_3SCH_2CH_2$–	6.25	6.5	1.04	24	0.46	0.48
25	Lysine	$H_2NCH_2CH_2CH_2CH_2$–	2.5	2.75	1.10	57	1.80	1.64
26	Ornithine	$H_2NCH_2CH_2CH_2$–	2.5	2.5	1.0	—	2.6	2.2
27	Histidine		15.5	5.75	2.70	365	1.00	0.95
28	Tryptophan		5.5	7.8	1.40	200	1.1	1.03
29	Phenylalanine	$C_6H_5CH_2$–	6.0	9.25	1.54	255	0.95	0.96
30	Aspartic acid	$HOOCCH_2$–	4.25	3.75	1.10	57	1.6	1.4
31	Glutamic acid	$HOOCCH_2CH_2$–	2.0	1.25	1.60	275	1.75	1.6
32	Iminodiacetic acid	$HN(CH_2COOH)_2$	7.5		—	—	1.2	

TABLE II

ELUTION PARAMETERS OF AMINO ACIDS ON THE AZETIDINE-CARBOXYLIC ACID RESIN III IN THE COPPER(II) FORM

Eluents: 0.1 *M* NH_4OH (*N* = 1–6); 0.3 *M* NH_4OH (*N* = 7–17); 1.5 *M* NH_4OH (*N* = 18–23); 0.017 *M* $(NH_4)_3PO_4$, pH 8.8 (*N* = 24–31).

N	*Amino acid*	*α-Radicals or molecular structure*	*V*		*α*	*δΔG°* *(cal/mol)*	*HEEP (cm)*	
			L	D			L	D
1	Glycine	H–	7.2		–		1.72	
2	Alanine	CH_3–	11.2	11.9	1.06	35	1.68	1.70
3	Aminobutyric acid	CH_3CH_2–	15.0	19.2	1.28	145	1.91	1.84
4	Valine	$CH_3CH(CH_3)$–	24.0	41.0	1.78	315	1.87	1.93
5	Norvaline	$CH_3CH_2CH_2$–	52	64	1.23	120	1.66	1.74
6	Tyrosine	$HOC_6H_4CH_2$–	9.6	19.0	1.78	335	2.04	2.01
7	Valine	$CH_3CH(CH_3)$–	3.1	5.4	1.74	320	1.92	1.96
8	Aminobutyric acid	CH_3CH_2–	1.85	2.4	1.30	155	1.97	1.93
9	Methionine	$CH_3SCH_2CH_2$–	7.2	9.3	1.29	150	1.59	1.67
10	Proline		7.5	18.6	2.48	530	1.93	1.84
11	Hydroxyproline		3.6	8.1	2.25	475	1.86	1.85
12	allo-Hydroxyproline		8.3	5.7	1.46	220	1.84	1.93
13	Leucine	$CH_3CH(CH_3)CH_2$–	18.2	22.5	1.24	125	1.62	1.69
14	Isoleucine	$CH_3CH_2CH(CH_3)$–	15.1	25.5	1.68	305	1.57	1.63
15	Norvaline	$CH_3CH_2CH_2$–	9.12	11.4	1.25	130	1.69	1.74
16	Norleucine	$CH_3CH_2CH_2CH_2$–	25.2	35.4	1.41	200	1.63	1.60
17	Phenylglycine	C_6H_5–	4.8	6.6	1.38	190	1.94	1.97
18	Ornithine	$H_2NCH_2CH_2CH_2$–	2.1	2.1	1.0		2.4	2.4
19	Lysine	$H_2NCH_2CH_2CH_2CH_2$–	1.8	1.91	1.06	35	2.3	2.25
20	Histidine		27.6	15.3	1.80	340	2.1	2.2
21	Tryptophan		33.2	37.4	1.13	70	2.25	2.16
22	Phenylalanine	$C_6H_5CH_2$–	7.25	13.5	1.86	360	2.24	2.20
23	Norleucine	$CH_3CH_2CH_2CH_2$–	3.10	4.28	1.38	190	1.80	1.73
24	Serine	$HOCH_2$–	6.0	12.9	2.15	445	1.92	1.96
25	Threonine	$HOCH(CH_3)$–	13.7	10.7	1.28	145	1.94	1.92
26	Asparagine	H_2NCOCH_2–	13.8	9.6	1.44	210	1.78	1.83
27	Glutamine	$H_2NCOCH_2CH_2$–	17.4	21.8	1.25	130	1.84	1.96
28	Glycine	H–	36		–	–	1.78	
29	Aspartic acid	$HOOCCH_2$–	3.05	2.7	1.13	70	2.15	2.23
30	Glutamic acid	$HOOCCH_2CH_2$–	9.0	7.2	1.29	150	2.08	2.18
31	Iminodiacetic acid	$HN(CH_2COOH)_2$	2.4		–	–	2.04	

It is also noteworthy that sorbent III is more sensitive to an increase in the size of the hydrocarbon radical of the mobile amino acid ligand. When passing from non-substituted Gly to L-Nva, the retention volume increases on this sorbent by almost 7 times, but by only 3.5 times on sorbent II.

Hydroxyl-containing amino acids

It was found for sorbent I that introduction of a hydroxy group on to the β-C-atom of the mobile amino acid ligands decreases their retention time and increases the sorption enantioselectivity. The same proved true for the sorbents II and III. The most pronounced decrease in retention time is exhibited by the sorbent III with azetidine carboxylic acid residues. In 0.1 *M* NH_4OH, L-Ser very weakly interacts with this sorbent; elution by phosphate buffer solution indicates that retention of this amino acid is about 10 times weaker than that of alanine. Nevertheless, serine sorption enantioselectivity in this system is very high ($\alpha = 2.15$). Retention of threonine on all three sorbents is greater than that of serine. Surprisingly, the order of elution of threonine enantiomers (D, L) on sorbent III is reversed.

Resolution of allo-threonine on sorbent II is better than that of threonine whereas on sorbent I it is somewhat worse.

Aromatic amino acids

The sorbents II and III, with heterocyclic fixed ligands, exhibit high affinity and enantioselectivity towards aromatic amino acids. On these sorbents, the separation factors for Phgl and Phe are lower than on sorbent I. For Tyr, which differs from Phe by a hydroxy group in the *p*-position of the aromatic ring, the separation factor proved to be the highest on sorbent II ($\alpha = 2.46$).

Cyclic amino acids

The highest enantioselectivity of the sorbents studied was observed in resolutions of cyclic amino acids. The retention volumes, the order of enantiomer elution and enantioselectivity values for Pro, Hyp and aHyp on the sorbents I and II (with proline and hydroxyproline residues) proved to be close to each other. The separation factors on the sorbent III (with azetidine carboxylic acid residues) are *ca.* 2 times lower. These results are consistent with the appreciably lower enantioselectivity of sorbent I towards racemic azetidine carboxylic acid than towards racemates of proline or hydroxyproline[1]. The lower stability of mixed-ligand sorption complexes with D-aHyp on all three sorbents, which leads to the inverse order of elution of isomers of aHyp, is due to the fact that contrary L-aHyp D-aHyp cannot act in a tridentate manner in complex formation with fixed ligands of L-configuration. We have already explained the reasons for this behaviour with reference to sorbent I[1].

Basic and acidic amino acids

As basic amino acids display a particularly strong affinity for asymmetric sorbents II and III, their elution was carried out in ammonia solutions of high concentration (1.5 *M*). The sorption enantioselectivity proved somewhat lower than in the case of sorbent I. As typical tridentate ligands, His isomers displayed an inverse order of elution on all three sorbents.

On the contrary, acidic amino acids possess a weak affinity for the sorbents

in alkaline eluents. Thus, their chromatography requires the use of a phosphate buffer (pH 8.8). D-Isomers of glutamic and aspartic acids are eluted first, this being a possible indication that L-enantiomers can participate in the sorption complexes as tridentate ligands. The highest enantioselectivity towards dicarboxylic amino acids is manifested by sorbent II with proline residues.

In the series glutamic, aspartic, iminodiacetic acid the retention time on sorbent III falls, whereas the affinity towards sorbents I and II increases.

General remarks

LEC of racemic amino acids using sorbents II and III, containing L-proline and L-azetidine carboxylic acid residues, is highly enantioselective. Resin II provides better resolution of racemic allo-threonine, proline, hydroxyproline, allo-hydroxyproline, tyrosine and glutamic acid than does sorbent I. The average enantioselectivity of sorbent III is somewhat lower. However, it provides better resolution of aminobutyric acid, valine, methionine, serine, asparagine and aspartic acid. Fig. 1 illustrates the high resolution capacity of asymmetric resins in LEC of racemic amino acids —the capacity that is adequate for preparative separations.

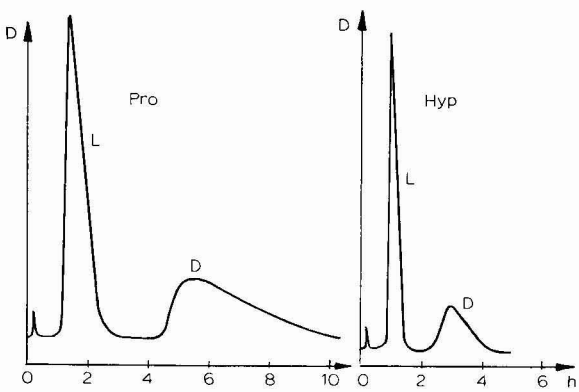


Fig. 1. Chromatography of racemic proline and hydroxyproline. Column 7.8 × 140 mm; 0.3 *M* NH_4OH; 10 ml/h. The degree of saturation of the L-proline resin by copper(II) ions was 80%. Particle size *ca.* 100 μm. Degree of cross-linking 11%.

When copper(II) ions are used as the complex-forming agent, all three resins with fixed ligands of the L-configuration exhibit higher affinity for D-amino acids. However, the tridentate amino acid ligands (histidine, allo-hydroxyproline, aspartic acid and glutamic acid) are an exception. This may be because the D-isomers of these amino acids cannot act as tridentates in the mixed-ligand sorption complexes of the *trans* structure in the coordination square-plane of the copper(II) ion because of steric interactions of their side-groups with the *N*-benzyl radical of the L-fixed ligands[1]. This consideration may also account for the inverse elution order of threonine enantiomers on sorbent III.

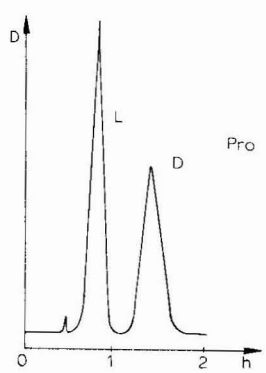
We shall show in a following paper that sorption of amino acids on the copper form of the sorbents obeys gel-phase diffusion kinetics. Therefore the sooner interfacial equilibrium is reached, the greater are the dispersity and swelling ability of

the resin. The latter property sharply increases with the sorbent exchange capacity, thus providing for higher efficiency in LEC.

The swelling ability of sorbent I was 200%, and its capacity was 3.44 mmol L-hydroxyproline residues per gram of dry sorbent (in its zwitterionic form), the copper ion content being 92% of the theoretical value. The heights equivalent to effective plate (HEEP) for aliphatic amino acids separated on this sorbent were 0.3–0.5 cm, so that a number of racemates could be completely resolved on a column of 14 cm[1]. Complete separation of enantiomers at a sorption enantioselectivity of *ca.* 300 cal/mol requires *ca.* 50 theoretical plates.

The capacities of sorbents II and III (2.78 and 2.40 mmol/g), as well as their swelling abilities (170 and 140%), were lower than those of the sorbent I. This apparently was the reason for increased HEEP values (Tables I and II) under identical chromatography conditions. In case of sorbent II, a column 14 cm long corresponded (for aliphatic amino acids) to 25–15 theoretical plates, but in case of sorbent III to just 7–9. Therefore, the ultimate separation of enantiomers on sorbents II and III was poorer than that on similar columns with sorbent I. However, the chromatographic efficiency can be enhanced. We have synthesized a sorbent with L-proline fixed ligands on spherical polystyrene beads containing 1% divinylbenzene and displaying a narrow distribution of particle size (0.64–0.72 μm). The capacity of this sorbent is 3.5 mmol per gram of dry resin. The HEEP values appeared to be 2.5–3 times lower than those given in Table I. Fig. 2 illustrates how this variation in the sorbent performance accelerates and improves the resolution of D,L-proline without decreasing the sorbent enantioselectivity. However, further enhancement of the efficiency is still needed for the complete resolution of aromatic amino acids, which have especially broad elution bands (Fig. 3).

It is of interest that for proline and hydroxyproline resins of equal capacity, swelling ability, and granule size, the efficiency parameters are almost equal.



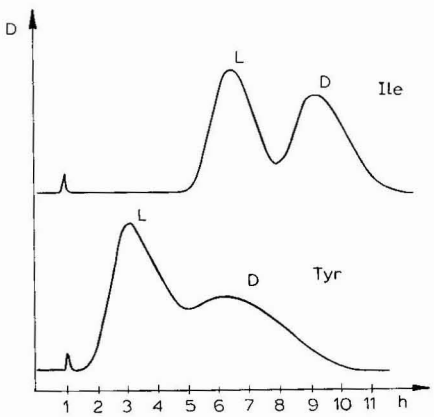


Fig. 2. Chromatography of racemic proline. Column 7.8 × 140 mm; 1.0 *M* NH_4OH; 10 ml/h. The degree of saturation of the L-proline resin by copper(II) ions was 60%. Particle size *ca.* 70 μm. Degree of cross-linking 1%.

Fig. 3. Chromatography of racemic isoleucine and tyrosine. Column 7.8 × 140 mm; 0.1 *M* NH_4OH; 5 ml/h. The degree of saturation of the L-proline resin by copper(II) ions was 70%. Other resin parameters as given in Fig. 2.

ACKNOWLEDGEMENTS

We gratefully acknowledge the gift of a sample of L-azetidine carboxylic acid from Dr H. Knobloch, REXIM, Paris.

REFERENCES

1 V. A. Davankov and Yu. A. Zolotarev, *J. Chromatogr.*, 155 (1978) 285.
2 Yu. A. Zolotarev, A. A. Kurganov, A. V. Semechkin and V. A. Davankov, *Talanta*, (1978) in press.

Journal of Chromatography, 155 (1978) 303–310

CHROM. 10,970

LIGAND-EXCHANGE CHROMATOGRAPHY OF RACEMATES

VII. SEPARATION OF OPTICAL ISOMERS OF AMINO ACIDS ON A POLYSTYRENE RESIN CONTAINING L-ALLO-HYDROXYPROLINE AS THE FIXED LIGAND

V. A. DAVANKOV and Yu. A. ZOLOTAREV
Institute of Organo-Element Compounds, Academy of Sciences, Moscow (U.S.S.R.)
(Received February 14th, 1978)

SUMMARY

An asymmetric sorbent has been synthesized containing L-allo-hydroxyproline groupings in a macronet polystyrene matrix. The γ-hydroxy group in allo-hydroxyproline, like the carboxylic and the amino group, is capable of coordinating copper(II) ions. The sorbent thus forms more stable bis-chelate complexes with copper(II) than the analogous sorbent containing L-hydroxyproline residues.

The sorption selectivity of proline enantiomers on the copper(II) forms of the two resins has been studied as a function of copper ion content. Enantioselectivity and the retention parameters for various amino acids on the sorbent containing allo-hydroxyproline have been evaluated using ligand-exchange chromatography. The resolving power of this sorbent with respect to racemates of acidic and basic amino acids, as well as methionine and phenylalanine, is higher than that of similar resins described earlier.

INTRODUCTION

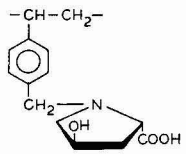
Ligand-exchange chromatography (LEC), which has recently been reviewed[1] makes the efficient separation of amino acid enantiomers possible on both analytical and preparative scales[1–4] without the preliminary modification of their amino and carboxy groups, that is necessary in gas chromatography.

The two previous papers in this series[3,4] were concerned with LEC of amino acid racemates on sorbents containing residues of L-hydroxyproline (I), L-proline (II) and L-azetidine carboxylic acid (III), and loaded with copper(II) ions. We determined the enantioselectivity of the resins and the efficiency of the chromatographic columns and discussed the dependence of these parameters on conditions of the process.

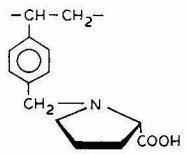
Influence of the fixed-ligand structure on LEC of amino acid racemates is a particularly interesting subject.

It was shown on low-molecular-weight model species[5] that in copper(II) complexes with N-benzyl-L-allo-hydroxyproline the γ-hydroxy group of the amino acid

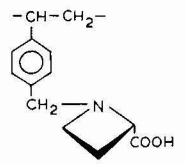
takes part in complex formation. On the contrary, N-benzylhydroxyproline cannot act as a tridentate ligand. In order to reveal the influence of the configuration of the fixed-ligand γ-carbon atom on the sorbent properties we have compared statics and dynamics of the sorption of enantiomeric amino acids on resins with hydroxyproline (I) and allo-hydroxyproline residues (IV).



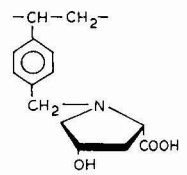
I



II



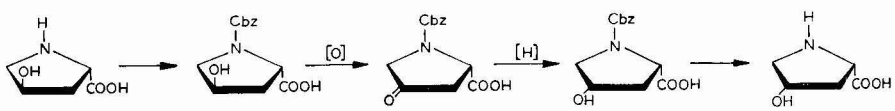
III



IV

EXPERIMENTAL

The asymmetric sorbent IV was prepared by aminating chloromethylated polystyrene containing 11 mol% cross-links of diphenylmethane structure[6] using methyl-L-allo-hydroxyprolinate hydrochloride, according to a procedure described in ref. 6 for methyl-L-prolinate hydrochloride. The initial L-allo-Hyp was synthesized from L-Hyp according to a scheme[7] involving inversion of configuration at the γ-carbon atom:



According to elemental analysis and potentiometric titration, the sorbent IV contains 2.82 mmol of fixed ligands per gram of dry resin in its zwitterionic form. The resin particles have an irregular shape, with an average size of *ca.* 100 μm when swollen. The resin was charged with copper ions from a copper–ammonia solution, resulting in quantitative formation of complexes containing two fixed ligands per copper ion. The equilibrium water content in the copper-saturated resin was 140%.

The chromatographic technique is described in detail in ref. 3. The column was 14 cm $\times$ 7.8 mm I.D. and contained 6.3 ml of resin. The eluents used were ammonia solutions of concentration 0.1, 0.3 and 1.5 *M*, containing $1.2 \cdot 10^{-5}$, $3.8 \cdot 10^{-5}$ and $2.0 \cdot 10^{-4}$ *M* $CuSO_4$, respectively, as well as a 0.017 *M* ammonium phosphate solution (pH 8.8) containing 2.0×10^{-5} *M* $CuSO_4$. Aliquots of 1.5–2.0 mg of the amino acid enantiomers introduced into the column were eluted at a flow-rate of 10 ml/h. The detector used was Uvicord-III with a 206-nm light filter.

The complex-formation properties of the resins were estimated by potentiometric titration[6], by studying the sorption of copper from copper–ammonia solutions[8] and by measuring the pH decomplexation values (DpH) of copper(II) ions, as described by Hering[9]. The sorption constant of L-proline on resins I and IV was studied and calculated according to the procedure given in ref. 8.

The enantioselectivity of the sorption of proline isomers under static conditions was estimated from the equilibrium distribution of the amino acid enantiomers

between the aqueous phase and the asymmetric sorbent in batch experiments. Equilibration was carried out in 15-ml vials equipped with a porous glass filter and capable of being stoppered at both ends[6]. Into the vials were placed 0.300 g of air-dried resin containing 0.540 mmol of functional groups, 1 ml of a 0.5 *M* solution of D,L-Pro, differing amounts of copper nitrate (0.05–0.5 mmol) and 2 ml of 0.520 *M* KOH. After the volume was made up to 10 ml with water, the vials were closed and shaken for 72 h at 25°.

The resin phase was then separated by centrifugation at 1600 *g* for 15 min. To analyse the resin phase copper ions and L-proline were desorbed by washing the sorbent in the same vial with 25 ml of 5 *M* HCl.

Copper in both phases was determined spectrocolorimetrically with sodium N,N-diethyldithiocarbamate using a Specol spectrocolorimeter at $\lambda = 440$ nm.

Proline was determined by the technique of Pope and Stevens[10], modified by Woiwod[11], *i.e.* the pH of the solution was increased to 8.0 and then a four-fold volume of a colloidal suspension of copper hydroxy phosphate in 0.2 *M* Na_2HPO_4 was added. The resulting mixture was kept at 80° for 30 min. The residual copper phosphate was then filtered off and the copper content in the filtrate, which is equivalent to the proline content, was determined by the method cited above. The predominance of one proline enantiomer over the other (in both phases) was determined polarimetrically, the specific rotation of L-Pro in 5.0 *M* HCl being assumed to be $[\alpha]_{436}^{20} = -123°$.

The quantitative analysis of both phases makes it possible to calculate the difference between the standard free energies of the two diastereomeric mixed-ligand sorption complexes, R–Cu–D-Pro and R–Cu–L-Pro, according to the equation

$$\delta\Delta G^0 = -RT \ln \frac{[\text{R–Cu–D-Pro}]}{[\text{R–Cu–L-Pro}]} \cdot \frac{[\text{L-Pro}]}{[\text{D-Pro}]}$$

RESULTS AND DISCUSSION

Thermodynamics of sorption of copper(II) ions and proline molecules

Owing to the additional interaction of the γ-hydroxy group of allo-hydroxyproline with the axial position of the copper(II) chelated ion, the complexes of both the amino acid itself and its N-benzyl derivative have higher stability constants than those of hydroxyproline and its derivative (Table I).

The same is true for polymer complexing agents. The sorption isotherms for copper(II) ions from ammonia solutions by the asymmetric resins I and IV (Fig. 1) indicate that the sorbent containing L-allo-hydroxyproline residues exhibits stronger complexing capacity. The stability constants of R–Cu and R–Cu–R complexes, found from potentiometric titration, are also higher for R = allo-Hyp than for R = Hyp (Table I).

The complexing properties of the resins were estimated, also according to Hering[9]. As we have shown earlier[6], his method for calculating stability constants of complexes of the Dowex A-1 type resins, based on measuring the pH values of metal decomplexation (DpH), is inapplicable to chelating sorbents that form 2:1 complexes. However, for a series of sorbents having similar fixed ligands the experimental DpH values are in a good qualitative agreement with the complex-forming

TABLE I

POTENTIOMETRICALLY DETERMINED STABILITY CONSTANTS OF COPPER(II) COMPLEXES WITH LOW MOLECULAR WEIGHT LIGANDS AND POLYMERIC LIGANDS

Ligand	*lg* β_1	*lg* β_2	*DpH*	*Capacity (mmol/g)*
L-Hyp	8.22	15.40		
L-allo-Hyp	8.72	16.81	—	—
N-Bzl-L-Hyp	6.53	11.54	—	—
N-Bzl-L-allo-Hyp	7.97	14.92	—	—
L-Hyp resin I	7.5	12.3	2.46	3.44
	—	—	2.80	2.46
	6.2	10.5	3.41	1.48
L-allo-Hyp Resin IV	7.8	13.3	1.98	2.82

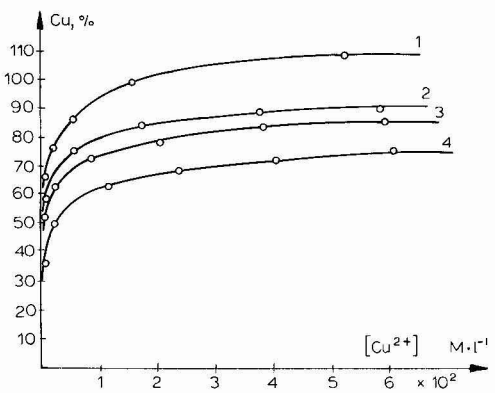


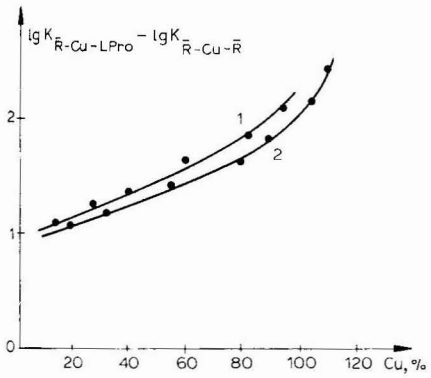
Fig. 1. Sorption isotherms of copper(II) ions from 2 *M* NH_4OH by the asymmetric sorbent IV containing L-allo-hydroxyproline (2.82 mmol/g) groups (1) and the asymmetric sorbent I containing L-hydroxyproline groups in amounts of 3.44 mmol/g (2), 2.46 mmol/g (3) and 1.48 mmol/g (4).

properties of the resins. As seen from the results of DpH measurements (Table I), desorption of copper(II) ions from resin IV requires more acidic eluents than in the case of sorbent I.

Comparison of three samples of hydroxyproline-containing resin shows (Table I and Fig. 1) that the stability constants of 2:1 polymer complexes, estimated by various methods, definitely depend on the exchange capacity of the resins. Therefore, it is correct to compare the complexing properties of different fixed ligands only for resins having an identical matrix structure and an equal content of functional groups. In this case, the exchange capacity of sorbent IV is inside the limits of capacity of the sorbent I samples studied. We can thus unambiguously conclude that hydroxyproline fixed ligands form weaker complexes with copper ions than allo-hydroxyproline ligands, so that the latter appear to be tridentate.

The high affinity of copper(II) ions for allo-hydroxyproline fixed ligands results in a decrease in the affinity of the sorbent chelate for mobile ligands, *e.g.* L-proline. This affinity is characterized by the sorption constant of the mobile ligand,

which can be determined by the difference between the stability constant of the ultimate sorption complex R–Cu–L-Pro and the constant of the initial fixed complex R–Cu–R[8]. Fig. 2 indicates that the affinity of L-proline for sorbent IV is lower than that for the sorbent I, the degree of saturation of both resins with copper ions being taken to be the same.



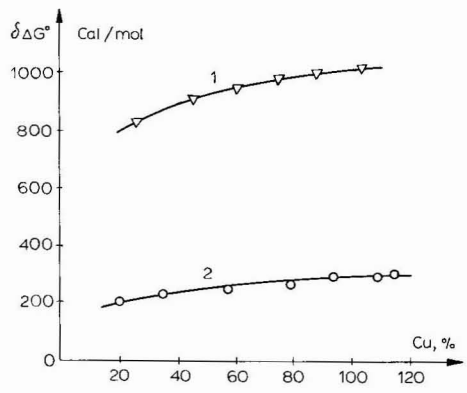


Fig. 2. Sorption constants of L-Pro on the copper forms of sorbent I (1) (capacity 3.44 mmol/g) and sorbent IV (2) as a function of the degree of saturation of the resins by copper(II) ions.

Fig. 3. Enantioselectivity of the sorption of proline enantiomers on sorbent I (1) and sorbent IV (2) as a function of the degree of saturation of the resins by copper(II) ions.

It is interesting to compare the selectivity of sorption of amino acid enantiomers on resins I and IV. It appears from Fig. 3 that coordination of the γ-hydroxy group of allo-hydroxyproline in the axial position of the copper ion results in racemic proline being resolved less well on sorbent IV.

Chromatography of racemates

The results of chromatographic studies of the affinity of sorbent IV for various amino acids and its enantioselectivity are presented in Table II. In general, the properties of sorbent IV have much in common with those of sorbents I, II and III, which may be due to the similar factors underlying the interaction between fixed complexes of these resins with amino acid enantiomers.

In the case of aliphatic amino acids those with larger *n*-alkyl substituents on the α-carbon atom exhibit longer retention times and higher enantioselectivity. However, when passing from Nva to Nle the increase in the value of $\delta\Delta G^0$ is not so great for sorbent IV as for sorbent I.

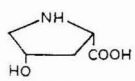
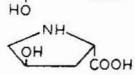
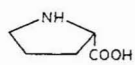
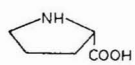
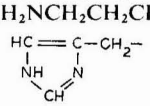
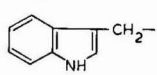
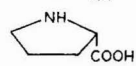
Enantioselectivity and the order of elution of amino acids with branched α-radicals are the same for sorbents I and IV. The resolution factor for Ile which is branched at the β-carbon atom, is higher than that of Leu, which is branched at the γ-carbon atom. The presence of hydroxy groups at the β-carbon atoms of Ser and Thr results in higher enantioselectivity and lower retention times of these amino acids compared with their aliphatic analogues (the same was observed for sorbent I).

Enantioselectivity effects on sorbents I and IV in the case of Asp and Glu were found to be close to each other. However, whereas Asp enantiomers are re-

TABLE II

ELUTION PARAMETERS OF AMINO ACIDS ON THE COPPER(II) FORM OF THE ALLO-HYDROXYPROLINE RESIN

Eluents: 0.1 *M* NH_4OH (N = 1–16); 0.3 *M* NH_4OH (N = 17–23); 1.5 *M* NH_4OH (N = 24–29); 0.017 *M* $(NH_4)_3PO_4$, pH 8.8 (N = 30–32).

N	*Amino acid*	*α-Radicals or molecular structure*	*V*		*α*	*ΔG°* *(cal/mol)*	*HEEP (cm)*	
			L	D			L	D
1	Glycine	H–	5.55		–	–	1.23	
2	Alanine	CH_3–	8.9	9.2	1.04	24	1.16	1.10
3	Aminobutyric acid	CH_3CH_2–	9.6	11.0	1.14	77	1.36	1.22
4	Norvaline	$CH_3CH_2CH_2$–	13.4	19.0	1.42	205	1.32	1.3
5	Norleucine	$CH_3CH_2CH_2CH_2$–	22.9	33.4	1.46	220	1.2	1.25
6	Valine	$CH_3CH(CH_3)$-	8.6	13.6	1.58	270	1.3	1.25
7	Leucine	$CH_3CH(CH_3)CH_2$–	21.6	33.7	1.52	245	1.43	1.35
8	Isoleucine	$CH_3CH_2CH(CH_3)$–	16.2	28.2	1.74	325	1.1	1.0
9	Serine	$HOCH_2$–	4.38	5.25	1.24	125	1.43	1.41
10	Threonine	$HOCH(CH_3)$–	4.82	7.15	1.48	230	1.46	1.35
11	Asparagine	H_2NCOCH_2–	4.38	5.25	1.20	110	1.48	1.56
12	Glutamine	$H_2NCOCH_2CH_2$–	3.94	5.52	1.40	200	1.86	1.84
13	Phenylglycine	C_6H_5–	9.35	16.7	1.78	335	1.85	1.75
14	allo-Hydroxyproline		18.7	27.8	1.48	230	1.48	1.40
15	Hydroxyproline		21.3	34.1	1.63	285	1.2	1.3
16	Proline		52.5	96.0	1.83	355	1.1	1.05
17	Phenylalanine	$C_6H_5CH_2$–	15.2	47.2	3.10	660	1.98	1.85
18	Tyrosine	$HOC_6H_4CH_2$–	3.21	7.58	2.36	505	2.6	2.5
19	Methionine	$CH_3SCH_2CH_2$–	8.62	13.1	1.52	245	1.75	1.70
20	Proline		20.5	38.1	1.85	360	1.0	0.95
21	Leucine	$CH_3CH(CH_3)CH_2$–	8.8	13.8	1.56	325	1.40	1.38
22	Isoleucine	$CH_3CH_2CH(CH_3)$–	7.03	12.2	1.74	325	1.05	1.0
23	Aminobutyric acid	CH_3CH_2–	4.1	6.0	1.21	112	0.9	0.85
24	Lysine	$H_2NCH_2CH_2CH_2CH_2$–	2.25	3.0	1.33	165	1.45	1.0
25	Ornithine	$H_2NCH_2CH_2CH_2$–	1.0	1.2	1.2	110	1.75	1.65
26	Histidine		6.8	9.1	1.32	160	1.3	1.1
27	Tryptophan		63.0	68.8	1.1	57	1.88	1.7
28	Proline		3.15	5.75	1.82	350	1.0	1.0
29	Leucine	$CH_3CH(CH_3)CH_2$–	1.3	2.0	1.54	255	1.75	1.75
30	Aspartic acid	$HOOCCH_2$–	9.8	6.8	1.23	120	2.0	1.9
31	Glutamic acid	$HOOCCH_2CH_2$–	16.0	11.0	1.45	215	2.2	2.1
32	Iminodiacetic acid	$HN(CH_2COOH)_2$	8.0		–	–	2.1	

tained by sorbent I about twice as strongly as Glu, the affinity of both amino acids for sorbent IV is approximately the same.

Sorbent IV exhibits a high affinity for Met; the value of $\delta\Delta G^0$ reaches 245 cal/mol, which is much higher than for sorbents I–III.

Sorbent IV is highly selective with respect to aromatic amino acid enantiomers. Resolution of Phgl was somewhat lower than with sorbent I ($\alpha = 1.78$), and separation of Phe and Tyr isomers was better ($\alpha = 3.10$ and 2.36).

As regards cyclic amino acids, sorbent IV manifests essentially poorer enantioselectivity than its analogues I–III. The values of $\delta\Delta G^0 = 350$–360 cal/mol found on chromatography of racemic proline are consistent with the results of investigations of this system under static conditions (Fig. 3).

There is a specific difference in the order of elution of allo-hydroxyproline isomers. Sorbents I–III have a greater affinity for L-allo-Hyp, but the D-isomer is retained for longer on sorbent IV. The γ-hydroxy group of the fixed L-ligand on sorbent IV apparently blocks the upper axial position of the coordination sphere of the copper ion (Fig. 4), so that tridentate mobile ligands like L-allo-Hyp are capable of interacting with the copper ion only by their two donating groups. Therefore, L-allo-Hyp cannot form sorption complexes of abnormally high stability, which accounts for the strong retention of this isomer and the inverse elution order of allo-Hyp isomers on sorbents I–III.

The same reasons are evidently responsible for the absence of anomalies in the elution order of the isomers of His, Lys and Orn on sorbent IV. Lys and Orn are resolved into their enantiomers by sorbent IV better than by sorbents I–III; for

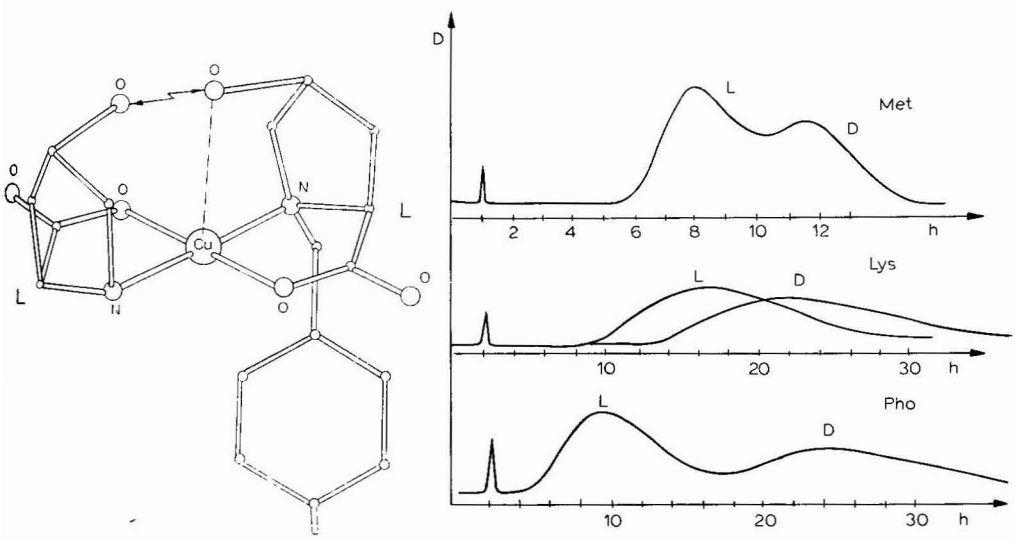


Fig. 4. Repulsion of the two hydroxy groups in the mixed-ligand sorption complex formed by L-allo-hydroxyproline on the asymmetric sorbent IV containing L-allo-hydroxyproline fixed ligands.

Fig. 5. Chromatography of enantiomers of Met, Lys and Phe. The degree of saturation of the L-allo-hydroxyproline resin IV by copper(II) ions was 50%. Column, 5 × 560 mm; 0.1 *M* NH_4OH; 6 ml/h. Particle size, *ca.* 50 μm.

tryptophan the reverse is true. All the basic amino acids are strongly retained by the column and their chromatography requires higher concentration of ammonia in the eluent.

On the contrary, dicarboxylic amino acids display a low affinity for all the sorbents studied. Their chromatography requires a phosphate buffer with a low concentration of the displacing mobile ligand, *i.e.* NH_3. The partially tridentate nature of sorbent IV unexpectedly results in a considerable increase of enantioselectivity for Glu and Asp, the elution order of their enantiomers being reversed, as for sorbents I–III. The L-isomers of these amino acids can presumably form additional hydrogen bonds between the carboxylate groups of their lateral chains and the hydroxy group of the fixed L-ligand coordinated in the axial position.

CONCLUSION

Unlike sorbents I–III, sorbent IV with fixed L-allo-hydroxyproline residues possesses a γ-hydroxy group capable of interacting with the axial position of the chelated copper(II) ion. Consequently, when the L-isomers of allo-Hyp, His, Lys and Orn form sorption complexes, only two of their three donor groups can interact with the metal ion, and the order of elution of their isomers does not differ from that of other bifunctional amino acids.

Sorbent IV exhibits the highest enantioselectivity among all the resins studied (I–IV) with respect to the following amino acid: Phe, Met, Lys, Orn, Asp and Gln. Compared with its diastereomeric analogue, *i.e.* sorbent I (containing hydroxyproline residues), it provides a better separation of Tyr and Glu.

However, the efficiency of chromatographic columns with sorbent IV was somewhat lower, apparently because sorbent IV has a lower swelling ability than sorbent I.

REFERENCES

1 V. A. Davankov and A. V. Semechkin, *J. Chromatogr.*, 141 (1977) 313.
2 E. Tsuchida, H. Nishikawa and E. Terada, *Eur. Polymer J.*, 12 (1976) 611.
3 V. A. Davankov and Yu. A. Zolotarev, *J. Chromatogr.*, 155 (1978) 285.
4 V. A. Davankov and Yu. A. Zolotarev, *J. Chromatogr.*, 195 (1978) 295.
5 A. A. Kurganov, V. A. Davankov, Yu. D. Koreshkov and S. V. Rogozhin, *Zh. Koord. Khim.*, 3 (1977) 667.
6 Yu. A. Zolotarev, A. A. Kurganov and V. A. Davankov, *Talanta*, in press.
7 A. A. Patchett and B. Witkop, *J. Amer. Chem. Soc.*, 79 (1957) 185.
8 Yu. A. Zolotarev, A. A. Kurganov, A. V. Semechkin and V. A. Davankov, *Talanta*, in press.
9 R. Hering, *Chelatbildende Ionenaustauscher*, Akademieverlag, Berlin, 1967, p. 104.
10 C. Pope and M. Stevens, *Biochem. J.*, 33 (1939) 1070.
11 A. Woiwod, *Biochem. J.*, 45 (1949) 412.

Journal of Chromatography, 155 (1978) 311–327

CHROM. 10,956

DETERMINATION OF HYDROCARBONS IN THE PARTS PER 10^9 RANGE USING GLASS CAPILLARY COLUMNS COATED WITH ALUMINIUM OXIDE

WERNER SCHNEIDER, JOHANN C. FROHNE and HARTMUT BRUDERRECK
Zentralbereich Analytik, Veba-Chemie AG, Postfach 45, 4660 Gelsenkirchen-Buer (G.F.R.)
(Received December 23rd, 1977)

SUMMARY

The properties and limitations of a highly efficient gas chromatographic separation column are described. The column is of importance in the analysis of hydrocarbon emissions as it has the following advantages: high separation efficiency; good detection performance owing to a high signal-to-noise ratio; simultaneous determination of aromatics in the presence of other hydrocarbons in the C_1–C_9 range in a short time and without the need for a multi-column technique; simple on-line computer connection even in the parts per 10^9 range.

The preparation of the column and its applications and use with suitable measuring equipment are described in detail.

INTRODUCTION

The measurement of hydrocarbons in air is of importance in environmental monitoring. A measurement and evaluation procedure involving gas sampling in the field and subsequent gas chromatographic analysis in the laboratory was elaborated for measuring the emissions in the neighbourhood of refineries and petrochemical plants[1,2].

Aluminium was used at an early stage in gas chromatography as a separating agent for hydrocarbons[3,4]. Considerable improvements in the separation efficiency, in particular a reduction in the tailing effect, were achieved by modifying the aluminium oxide with water[5–7], organic liquids[8] or inorganic salts[9]. The possibility of coating the inner wall of metal capillaries by applying a thin layer of solid material was described by Halász and Horváth[10].

The application of aluminium oxide in packed capillaries was reported by Halász and Heine[11,12]. Further variations include the generation of oxide layers in aluminium capillaries[13] and coating the inner walls of the tube by using aqueous or organic dispersions[14–17].

The separation column described in this paper combines the favourable separation properties of aluminium oxide with the advantages of the open capillary tube. Further, the use of glass as a tube material offers a number of additional advantages:

(1) The activity of the glass surface towards the sample components to be analysed is very small. The "irreversible" adsorption and tailing that are observed in metal tubes, particularly with unsaturated or polar substances, does not occur at levels down to the detection limit.

(2) Fine-grained aluminium oxide adheres well to glass surfaces, forming a thin layer even without any auxiliaries. The method of preparation described here produces firmly adhering layers that are non-spalling when the glass is deformed within its elasticity limits.

(3) The coating process can be controlled visually. Failures are immediately apparent and can be corrected.

(4) In addition, glass capillaries can be prepared cheaply in the laboratory by the user, so that the column dimensions and packing materials can be varied at will.

The aluminium oxide layer was applied to the inner wall of the glass capillary from an aqueous dispersion in the form of aluminium hydroxide and converted *in situ* into aluminium oxide by heat treatment. By varying the heat treatment on the one hand and by blocking unwanted activities with potassium chloride on the other, adjustment to the desired separation characteristics can be achieved.

The porous separating phase thus created consists only of inorganic material. Even at high temperature it will not release substances into the carrier gas that will be detected by a flame-ionization detector (FID), so that also in temperature-programmed applications maximal sensitivity is reached even without compensation provisions. The favourable signal-to-noise ratio is increased by small HETP values of the separation system. Hence the automatic evaluation of chromatograms by electronic instruments even at measuring sensitivities as low as 1 $\mu g \cdot m^{-3}$ per sample can be effected. This allows trace concentrations, for instance in measurements on gaseous emissions, to be determined either directly or after slight enrichment. Fast sampling enables one to resolve rapid fluctuations of emission concentrations according to time, which is not possible with conventional integrating sampling techniques.

EXPERIMENTAL

Apparatus and materials

Measurements are carried out with a conventional gas chromatograph consisting of the following components: an aluminium oxide coated separation column and pre-column, prepared as described below; a flame-ionization detector[18]; a pneumatic control unit providing control within about $\pm 1\%$ during 24 h; an air thermostat with electronic control and regulation facilities (isothermal temperature stability $\pm 0.1°$, programmable at at least 20°/min); an electrometer amplifier (measuring range up to $1 \cdot 10^{-8}$ A, noise $1 \cdot 10^{-14}$ A, time constant (90% value) for all measuring ranges ≤ 0.5 sec); and an inlet valve (Type 2018 P, Carle Instruments, Fullerton, Calif., U.S.A.).

The amplified detector signal can be fed to a compensation recorder and/or an interface of a data system. Additional equipment required for the analysis includes a micro-stopcock, Type 2 MF 1 (Hamilton, Whittier, Calif., U.S.A.) and a syringe, Type 1010 LL (Hamilton).

Sample input is effected via the pre-column, arranged in the sample loop of the six-way valve (Fig. 1). In this pre-column, the sample is first adsorbed at low temperature and, after heating, passed into the separation column. During the heating phase, a solenoid valve leads the carrier gas through a by-pass, circumventing the pre-column.

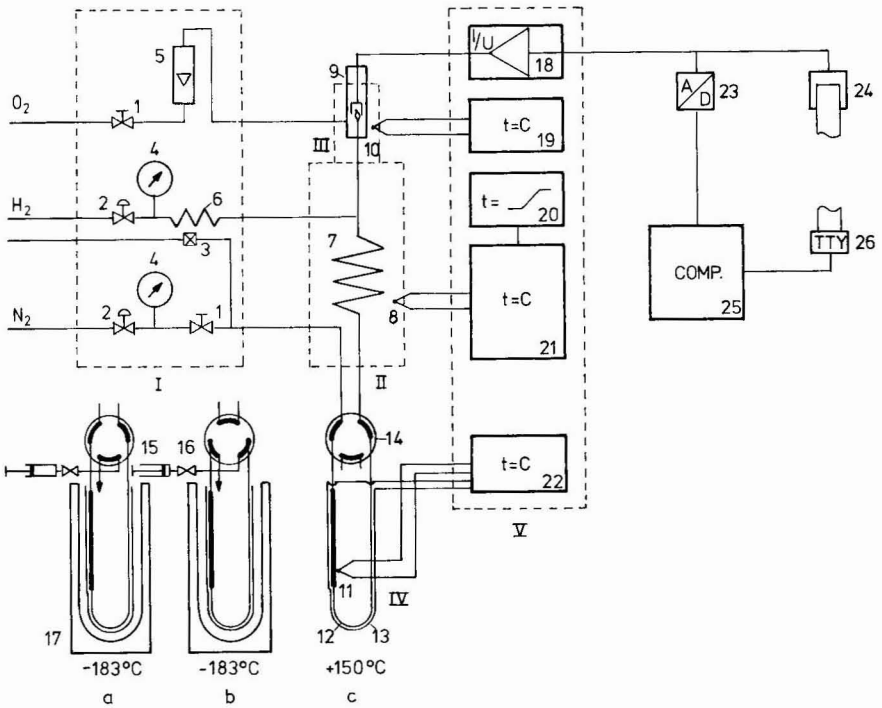


Fig. 1. Schematic layout of the gas chromatographic apparatus. I, Pneumatic supply: 1 = needle valve; 2 = pressure regulator; 3 = solenoid valve; 4 = manometer; 5 = flow meter; 6 = throttle capillary. II, Circulating air thermostat: 7 = separation column; 8 = temperature sensor. III, FID thermostat: 9 = flame-ionization detector; 10 = temperature sensor. IV, Sample inlet and preliminary enrichment: 11 = temperature sensor; 12 = pre-column; 13 = heating conductor; 14 = six-way valve; 15 = gas-tight syringe; 16 = micro-stopcock; 17 = Dewar vessel. V, Power supply: 18 = electrometer amplifier; 19 = power supply and temperature regulator of the FID thermostat; 20 = temperature programmer; 21 = power supply and temperature regulator of the circulating air thermostat; 22 = power supply and temperature regulator of the pre-column; 23 = integrating analogue-digital converter; 24 = recorder; 25 = data evaluation system; 26 = teletype.

To prevent carrier gas and detector supply gases from being contaminated by organic substances, no plastic or rubber material is used in the pneumatic system whenever practicable. Even the diaphragms of the pressure regulators are made of metal.

The connection tubes and constructional units are arranged as to restrict the dead volume to the minimum. The inlet valve is a miniature six-way valve with a Teflon rotating disk of conventional design; the sample loop of this valve is represented by the pre-column.

Pre-column

Aluminium hydroxide (Camag, Muttenz, Switzerland), sieve fraction 0.09–0.10 mm, is used for the production of aluminium oxide (see below).

A 7-cm length of stainless-steel tubing of I.D. 1 mm and 15 cm of stainless-steel tubing of I.D. 0.5 mm are soldered together and bent into an U-shape, as shown in Fig. 2. The tube of I.D. 1 mm is filled to a level of 2 cm with aluminium oxide that has been subjected to a special treatment. The packing is fixed with a wad of quartz-wool.

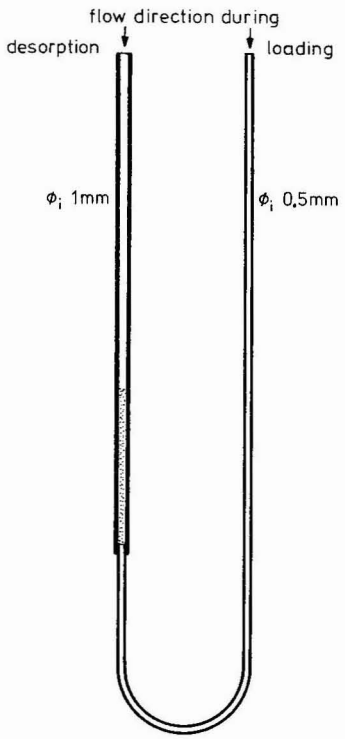


Fig. 2. Pre-column.

Preparing the aluminium oxide for the pre-column. Aluminium hydroxide of grain size 0.09–0.10 mm is heated for 24 h in an open crucible (*e.g.*, nickel) at 400°. Subsequently 3% (w/w) of water is added, followed by heating for 24 h at 300° in a tightly sealed, pressure resistant (100 bar) stainless-steel crucible. The aluminium oxide should fill the stainless-steel container without leaving a dead space.

Separation column

The materials required are as follows: aluminium hydroxide, ≤ 2 μm (Camag); Baymal (colloidal aluminium hydroxide; DuPont, Wilmington, Del., U.S.A.); acetic acid, >96%, reagent grade; acetic acid, 1%; nitrogen; and potassium chloride solution, 2% (w/w) solution. The equipment consists of a 1-ml syringe with a Teflon piston (*e.g.*, Hamilton, Type 1001), a nitrogen source with needle valve (filling >100 bar), a

thermostat (300 ± 1°), a pressure regulator (control range 0–3 bar), a glass-drawing machine as described by Desty *et al.*[19] (*e.g.*, Hewlett-Packard, Avondale, Pa., U.S.A.) and an ultrasonic bath.

By means of the glass-drawing machine, a glass tube (Duran 50; Schott und Genossen, Mainz, G.F.R.) is drawn out to a capillary of O.D. *ca.* 1 mm and I.D. 0.4 mm. This capillary is coiled to give a helix of diameter 12 cm.

Preparation of the coating suspension. Aluminium hydroxide ($\leqslant 2\,\mu m$) is heated in an open metal crucible (*e.g.*, nickel) for 24 h at 300°. A 20-g amount of the aluminium oxide obtained is mixed with 70 ml of 5% (w/w) Baymal solution and 0.3 ml of acetic acid (>96%) and stirred for about 10 min in an ultrasonic bath. Subsequently, the mixture is filtered through a wire sieve of 300 mesh and allowed to stand for 24 h for ageing. The suspension thus prepared shows thixotropic behaviour.

Coating of the capillary. The glass capillary tube to be coated is connected via a polyethylene capillary tube to a length of about 20 m of glass capillary tube of the same diameter to prevent draining disturbances. Through a polyethylene capillary slipped over the other end of the tube, 0.6 ml of the suspension is forced, by means of the syringe, into the capillary tube, which has previously been rinsed and wetted with 1% acetic acid. The polyethylene tube used for filling is connected to the source of nitrogen. The nitrogen flow pushes the suspension plug through the capillary tube, leaving on the inner wall of the tube a layer of uniform thickness which soon solidifies. To achieve complete coverage of a 65-m length of tube, this procedure must be repeated four times. In order to generate a uniform layer, it is essential to apply the same amount of suspension each time. For the coating procedure, the needle valve connected to the source of nitrogen is adjusted to supply gas at a flow-rate of 4 ml·min^{-1}. During this step, the pressure drop at the valve must be high compared with that developing at the capillary while coating is effected. A scheme of the filling equipment is shown in Fig. 3. The 20-m length of glass capillary attached for preventing drainage problems can be cleaned from the suspension by flushing with 1% acetic acid and stored for re-use.

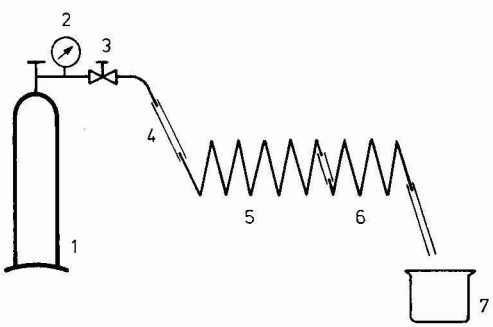


Fig. 3. Coating device. 1 = Storage tank for nitrogen; 2 = manometer; 3 = needle valve; 4 = polyethylene tube; 5 = glass capillary; 6 = auxiliary capillary; 7 = collection vessel.

The coated, moist capillary is stored for at least 10 h and then dried in a flow of nitrogen at ambient temperature. If a nitrogen pressure of 3 bar is applied, drying will take about 1 week. During this time, the whiteness of the coating undergoes changes, allowing one easily to follow the process visually. The nitrogen flowing

through the capillary is water-saturated after having travelled for a short distance, which implies that even small temperature fluctuations along the column will cause condensation, destroying the coating. Storage of the drying columns in well isolated containers, *e.g.*, made of polystyrene, will prevent this effect. After complete drying at ambient temperature the column is heated in a flow of nitrogen for 3 h at 300°. During this treatment, the coating becomes solid, insoluble and activated. The amount of aluminium oxide introduced into the capillary is determined by differential weighing. It can be varied within a wide range by altering the coating parameters, such as modifying the viscosity of the suspension by varying the volume of acetic acid added or by the addition of water, varying the nitrogen flow-rate or varying the amount of suspension introduced in each particular instance. Table I shows the coatings obtained by the above procedure under the same conditions and the reproducibility of the coating strength.

TABLE I

COATINGS ON SEPARATION COLUMNS

Column No.	*Inner diameter (mm)*	*Length (m)*	*Al_2O_3 coating ($mg \cdot m^{-1}$)*
1	0.40	67	5.8
2	0.40	70	5.4
3	0.40	69	5.7
4	0.40	71	5.1
5	0.40	69	5.6
6	0.40	61	5.3
7	0.40	58	5.9
8	0.40	56	5.5
9	0.40	64	5.4
10	0.40	69	5.4
Average			5.5
Standard deviation			±0.24

For activity reduction, the capillary thus prepared is rinsed twice, without intermediate drying, with 2 ml of 2% (w/w) potassium chloride solution. The potassium chloride solution is forced through the column by applying a nitrogen pressure of 2 bar. After drying at ambient temperature with nitrogen (3 bar pressure) and heating for 1 h at 300° under a flow of nitrogen, the column is ready for use.

Sample injection

Sampling for measurements on emissions in air in the field is effected as described earlier[1], using glass gas collection vessels specially developed for this purpose.

Afterwards, in the laboratory, a sample of 10 cm^3 or more is drawn from the gas collection vessel using a gas-tight syringe. About 2 cm^3 of the sample gas are used to scavenge the feed lines to the inlet valve at position (a) in Fig. 1. After switching to position (b), exactly 8 cm^3 are passed into the pre-column. The pre-column is refrigerated by immersion in liquid oxygen. During these steps, the solenoid valve 3 is opened.

Subsequently the coolant is removed, the inlet valve switched to position (c) and the pre-column electrically heated to 150°. The solenoid valve 3 is closed, thus allowing the sample components to be desorbed and passed by the flow of carrier gas

into the separation column. At the moment the solenoid valve is shut, the temperature programme and data evaluation system are started. Fig. 4 shows a time-sequence chart of the analysis. The syringe equipped with injection needle and micro-stopcock, if not in use, is permanently flushed with hydrocarbon-free nitrogen.

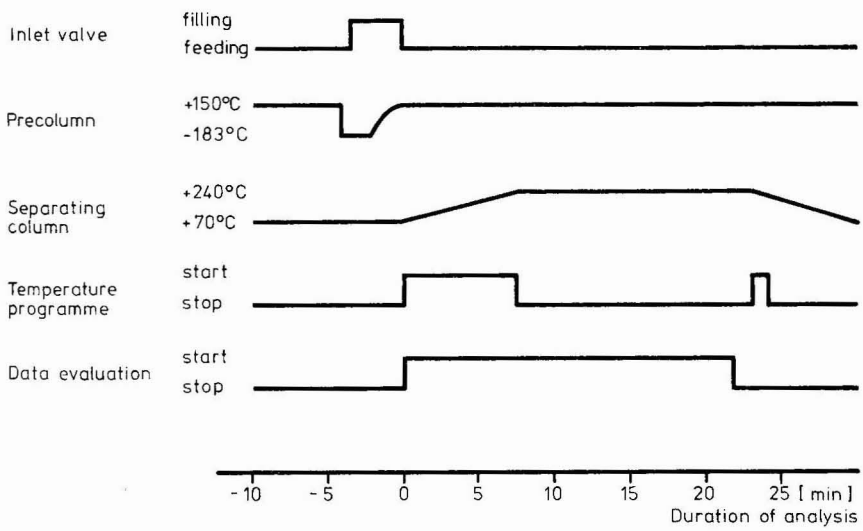


Fig. 4. Temporal sequence of the analysis.

Purity control of the carrier gas is carried out, as mentioned above, under conditions identical with those of the analysis. In position (a) in Fig. 1 carrier gas is passed through the cooled pre-column for 5 min (*ca.* 50 ml), followed by desorption and analysis, as described above. Hydrocarbon peaks must not appear during this procedure.

RESULTS AND DISCUSSION

Example of a measurement

Fig. 5 shows the chromatogram of a mixture of C_1–C_9 hydrocarbons in air, the concentrations of the different components being approximately 0.003–0.5 mg · m^{-3}. As can be seen, 0.014 mg·m^{-3} of benzene, for instance, after a net retention time of about 11 min, yields a readily measurable signal, and 0.008 mg·m^{-3} of *n*-octane gives a signal more than ten times the background noise.

Fig. 6 is the corresponding computer print-out of the results. The values were measured during real-time analysis in an HP 3354 laboratory data system (Hewlett-Packard), which was also used for the subsequent evaluation and documentation. For quantitive evaluation, the analyser system was calibrated with a methane–nitrogen mixture of known methane concentration, applying a previously described procedure[1].

Statistical evaluation

A test mixture similar to that shown in Figs. 5 and 6 was analysed ten times

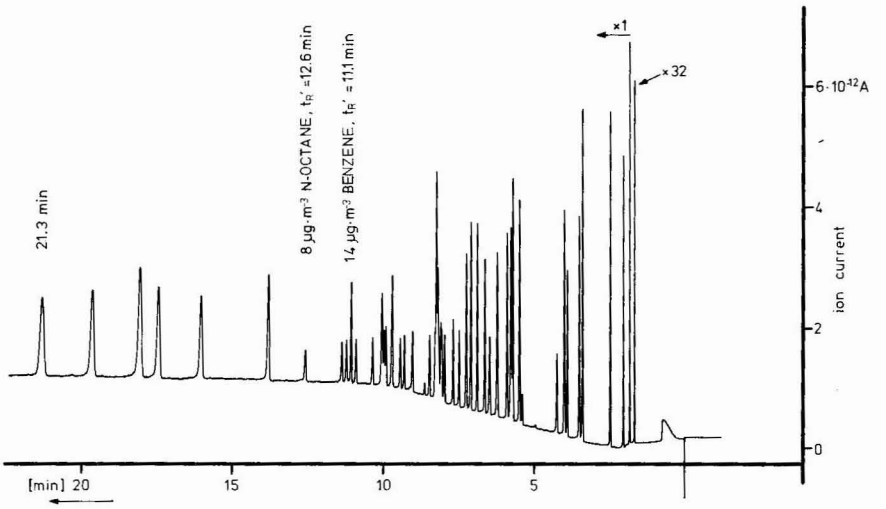


Fig. 5. Analysis of a test mixture using a glass capillary column coated with Al_2O_3. Length, 71 m; I.D., 0.40 mm; coating, 5.1 mg · m^{-1} of Al_2O_3; temperature programme, 70–240° at 20° · min^{-1}. Assignment: see Fig. 6.

consecutively. The mean value, standard deviation and relative standard deviation are given in Table II. Standard deviations for a concentration range of 0.003–0.5 $mg \cdot m^{-3}$ are usually between 2 and 10%. They increase, as is usual with this type of analysis, with decreasing concentration and increasing peak width.

Test of applicability of the apparatus to trace analysis

It is well known that active solids, when considering relatively rapid chromatographic processes, tend to retain "irreversibly" portions of organic substances. The amounts retained are generally small. Consequently, when dealing with high-concentration samples, the resulting error is included within the analytical error and thus is not discovered. However, the amounts involved in the analytical procedure considered here are extremely small. Only $8 \cdot 10^{-11}$ g of a given substance will enter the separation column if its concentration in the injected sample is 0.01 $mg \cdot m^{-3}$. The above-mentioned errors under these circumstances can reach a high percentage of the measured value.

It is therefore imperative to check the method of measurement for its applicability to all classes of substances to be analysed. The test procedure used here has been described earlier[1]. It involves the analysis of known but different amounts of the substance in question and checking for systematic deviations of the measured from the true value. The different test mixtures were prepared by using a Telab pump (Type BF 411/30 + 30 K + H 1/30 + H 2/50; Telab-Labor & Technik, Homburg, G.F.R.) in a dynamic procedure[1]. Table III lists the true values and the analytical results for 10 fillings of a test mixture, their averages, their standard deviations, and their deviations from the true values. When the pre-columns and separation columns were prepared by the method described above, no substantial errors were encountered for concentrations down to a few micrograms per cubic metre. Deviations from the true values are of the same order as the standard deviation.

REPORT NR. 12.31 METHODE LUFT2 A/D-KANAL: 13

PROBE EINGELEITET AM: 13. 4.1977 UM 13:18 UHR

ANALYSENZEIT: 22.4604 MINUTEN

FLAECHENFAKTOR: 3.91223E-05

****

RETENTIONSZEIT MINUTEN	FLAECHE COUNT	KONZENTRATION MG/CBM	KOMPONENTE
1.65	13254	.511	METHAN
1.82	523	.020	AETHAN
2.05	541	.021	AETHEN
2.50	726	.028	PROPAN
3.44	919	.036	CYCLOPROPAN
3.85	614	.024	PROPEN
3.99	424	.017	AETHIN
4.04	769	.033	ISO-BUTAN
4.29	276	.011	N-BUTAN
5.47	83	.003	TR-BUTEN-2
5.56	667	.026	BUTEN-1
5.78	744	.029	ISO-BUTEN
5.83	748	.029	2.2-DIMETHYLPROPAN
5.97	593	.023	CIS-BUTEN-2
6.23	607	.024	ISO-PENTAN
6.52	304	.012	N-PENTAN
6.71	422	.017	PROPIN
6.93	574	.022	BUTADIEN-1.3
7.14	604	.024	3-METHYLBUTEN-1
7.29	520	.020	TR-PENTEN-2
7.53	255	.010	PENTEN-1
7.73	287	.011	CIS-PENTEN-2
8.00	276	.011	2.2-DIMETHYLBUTAN
8.11	299	.012	METHYLCYCLOPENTAN
8.17	425	.017	CYCLOHEXAN
8.24	564	.022	2-METHYLPENTAN
8.32	731	.029	3-METHYLPENTAN
8.43	251	.010	BUTIN-1
8.52	238	.009	N-HEXAN
8.69	80	.003	>C5-VERBINDUNG
9.11	286	.011	TR-HEXEN-2
9.37	203	.008	HEXEN-1
9.51	206	.008	CIS-HEXEN-2
9.77	485	.019	2.2-DIMETHYLPENTAN
9.96	249	.010	3.3-DIMETHYLPENTAN
10.03	233	.009	2.3-DIMETHYLPENTAN
10.11	480	.019	2-+3-METHYLHEXAN
10.41	208	.008	N-HEPTAN
10.95	212	.008	TR-HEPTEN-2
11.13	348	.014	BENZOL
11.26	200	.008	HEPTEN-1
11.42	195	.008	CIS-HEPTEN-2
12.60	217	.008	N-OKTAN
13.82	587	.023	TOLUOL
16.04	821	.032	N-NONAN
17.46	984	.039	AETHYLBENZOL
18.12	1422	.056	M-+P-XYLOL
19.67	1186	.046	O-XYLOL
21.29	1256	.049	CUMOL
	3957	.155	SUMME C4-VERBINDUNGEN
	3325	.130	SUMME C5-VERBINDUNGEN
	6605	.258	SUMME VERBINDUNGEN >C5
	3593	.141	SUMME C6-AROMATEN
	36890	1.443	SUMME KW

Fig. 6. Print-out of the analytical results for a test mixture, produced by the HP 3354 laboratory system. Hydrocarbon concentrations are given in mg · m^{-3}.

TABLE II

REPRODUCIBILITY TEST

10 repeated analyses of the same mixture (test results in mg · m^{-3}, $\bar{x}$ = arithmetic mean; s = standard deviation; v = relative standard deviation or coefficient of variation).

Hydrocarbon	*Number of replicate experiment*										$\bar{x}$	s	v (%)
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*			
Methane	0.515	0.534	0.524	0.538	0.517	0.511	0.503	0.520	0.512	0.516	0.5190	0.0106	2.0
Ethane	0.020	0.021	0.020	0.020	0.021	0.020	0.020	0.020	0.021	0.020	0.0203	0.0005	2.5
Ethene	0.018	0.019	0.019	0.020	0.018	0.021	0.019	0.018	0.018	0.020	0.0190	0.0011	5.8
Propane	0.028	0.028	0.027	0.027	0.029	0.028	0.028	0.029	0.028	0.028	0.0280	0.0007	2.5
Cyclopropane	0.036	0.036	0.034	0.033	0.035	0.036	0.036	0.035	0.036	0.036	0.0353	0.0011	3.1
Propene	0.024	0.024	0.023	0.022	0.024	0.024	0.024	0.024	0.024	0.025	0.0238	0.0008	3.4
Ethyne	0.017	0.017	0.016	0.015	0.017	0.017	0.017	0.017	0.017	0.017	0.0167	0.0007	4.2
Isobutane	0.031	0.030	0.029	0.029	0.030	0.030	0.030	0.030	0.030	0.030	0.0299	0.0006	2.0
n-Butane	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.0110	0.0000	0
trans-2-Butene	0.005	0.004	0.004	0.003	0.004	0.003	0.004	0.005	0.004	0.004	0.0040	0.0007	17.5
1-Butene	0.029	0.028	0.024	0.021	0.029	0.026	0.028	0.027	0.028	0.029	0.0269	0.0026	9.7
Isobutene	0.030	0.029	0.027	0.025	0.030	0.029	0.029	0.030	0.029	0.029	0.0287	0.0016	5.6
2,2-Dimethylpropane	0.029	0.029	0.028	0.027	0.029	0.029	0.029	0.029	0.029	0.028	0.0286	0.0007	2.5
cis-2-Butene	0.023	0.023	0.022	0.020	0.023	0.023	0.023	0.023	0.023	0.023	0.0226	0.0010	4.4
Isopentane	0.024	0.024	0.024	0.023	0.024	0.024	0.024	0.023	0.024	0.024	0.0238	0.0004	1.7
n-Pentane	0.011	0.013	0.012	0.010	0.013	0.012	0.012	0.012	0.011	0.013	0.0119	0.0010	8.4
Propyne	0.017	0.017	0.015	0.013	0.018	0.017	0.017	0.017	0.018	0.017	0.0166	0.0015	9.0
1,3-Butadiene	0.021	0.021	0.018	0.017	0.022	0.022	0.022	0.022	0.022	0.022	0.0209	0.0019	9.1
3-Methyl-1-butene	0.026	0.026	0.022	0.019	0.025	0.024	0.025	0.025	0.026	0.026	0.0244	0.0023	9.4

trans-2-Pentene	0.020	0.021	0.019	0.016	0.021	0.020	0.020	0.021	0.020	0.020	0.0198	0.0015	7.6
1-Pentene	0.010	0.010	0.009	0.008	0.010	0.010	0.010	0.010	0.011	0.011	0.0099	0.0009	9.1
cis-2-Pentene	0.012	0.012	0.010	0.009	0.011	0.011	0.011	0.011	0.012	0.011	0.0110	0.0009	8.2
2,2-Dimethylbutane	0.010	0.012	0.010	0.010	0.011	0.011	0.011	0.010	0.011	0.010	0.0106	0.0007	6.6
Methylcyclopentane	0.011	0.012	0.012	0.012	0.012	0.012	0.011	0.010	0.010	0.010	0.0112	0.0009	8.0
Cyclohexane	0.012	0.015	0.015	0.017	0.014	0.015	0.013	0.014	0.012	0.011	0.0138	0.0018	13.0
2-Methylpentane	0.021	0.022	0.021	0.021	0.022	0.022	0.026	0.026	0.022	0.021	0.0224	0.0020	8.9
3-Methylpentane	0.027	0.027	0.024	0.025	0.027	0.029	0.028	0.025	0.027	0.028	0.0267	0.0016	6.0
1-Butyne	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.007	0.007	0.008	0.0064	0.0007	10.9
n-Hexane	0.009	0.010	0.008	0.010	0.010	0.009	0.009	0.009	0.009	0.009	0.0092	0.0006	6.5
trans-2-Hexene	0.010	0.008	0.008	0.009	0.009	0.011	0.009	0.010	0.009	0.010	0.0093	0.0009	9.7
1-Hexene	0.008	0.008	0.008	0.007	0.008	0.008	0 008	0.009	0.009	0.008	0.0081	0.0006	7.4
cis-2-Hexene	0.008	0.010	0.010	0.007	0.008	0.008	0.009	0.008	0.009	0.008	0.0085	0.0010	11.8
2,2-Dimethylpentane	0.019	0.019	0.019	0.017	0.019	0.019	0.019	0.019	0.019	0.019	0.0188	0.0006	3.2
3,3-Dimethylpentane	0.010	0.010	0.010	0.009	0.010	0.010	0.010	0.010	0.010	0.010	0.0099	0.0003	3.0
2,3-Dimethylpentane	0.009	0.009	0.009	0.008	0.009	0.009	0.009	0.009	0.009	0.009	0.0089	0.0003	3.4
2- + 3-Methylhexane	0.019	0.019	0.018	0.018	0.019	0.019	0.019	0.019	0.019	0.019	0.0188	0.0004	2.1
n-Heptane	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.0080	0.0000	0
trans-2-Heptane	0.009	0.009	0.008	0.008	0.008	0.008	0.009	0.009	0.009	0.008	0.0085	0.0005	5.9
Benzene	0.013	0.014	0.015	0.015	0.014	0.014	0.013	0.014	0.014	0.014	0.0140	0.0007	5.0
1-Heptene	0.008	0.008	0.007	0.006	0.008	0.008	0.007	0.008	0.008	0.008	0.0076	0.0007	9.2
cis-2-Heptene	0.007	0.007	0.007	0.006	0.007	0.008	0.007	0.008	0.007	0.008	0.0072	0.0006	8.3
n-Octane	0.007	0.008	0.008	0.007	0.008	0.008	0.007	0.008	0.008	0.007	0.0076	0.0005	6.6
Toluene	0.025	0.024	0.024	0.023	0.023	0.023	0.023	0.024	0.023	0.028	0.0240	0.0016	6.7
n-Nonane	0.033	0.032	0.032	0.030	0.032	0.032	0.033	0.035	0.032	0.031	0.0322	0.0013	4.0
Ethylbenzene	0.037	0.033	0.036	0.036	0.036	0.039	0.036	0.036	0.039	0.036	0.0364	0.0017	4.7
p- + *m*-Xylene	0.052	0.052	0.052	0.052	0.052	0.056	0.053	0.052	0.053	0.053	0.0527	0.0013	2.5
o-Xylene	0.047	0.046	0.048	0.043	0.045	0.046	0.045	0.046	0.046	0.044	0.0456	0.0014	3.1
Isopropylbenzene	0.045	0.047	0.049	0.047	0.050	0.049	0.049	0.049	0.049	0.047	0.0481	0.0015	3.1

TABLE III

COMPARISON OF TRUE CONCENTRATIONS WITH VALUES MEASURED ON TEN DIFFERENT ANALYTICAL RUNS

Test substance	*True value* ($\mu g \cdot m^{-3}$)	*Measured value* ($\mu g \cdot m^{-3}$)										*Average measured* ($\mu g \cdot m^{-3}$)	*Standard deviation* ($\mu g \cdot m^{-3}$)	*Difference between true and average measured values* ($\mu g \cdot m^{-3}$)
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*			
Ethyne	8	9	8	8	8	9	8	8	9	9	8	8.4	±0.5	−0.4
	33	33	33	32	31	34	32	33	31	31	32	32.2	±1.0	+0.8
	163	161	161	160	159	156	158	159	160	164	165	160.3	±2.7	+2.7
2-Butene	19	20	18	19	19	19	18	18	20	20	18	18.9	±0.9	+0.1
	78	77	77	77	79	77	80	76	79	77	76	77.5	±1.4	+0.5
	399	409	407	412	401	407	409	401	404	412	407	406.9	±3.9	−7.9
Cyclohexane	34	33	32	35	36	30	35	32	30	31	31	32.5	±2.2	+1.5
	143	138	141	143	140	141	140	142	138	142	143	140.8	±1.8	+2.2
	354	354	359	371	358	366	365	360	358	369	357	361.7	±5.7	−7.7
Benzene	27	28	28	29	28	30	28	28	28	30	27	28.4	±1.0	−1.4
	120	120	118	115	121	118	117	116	117	118	115	117.5	±2.0	+2.5
	294	300	302	301	293	300	297	299	293	295	302	298.2	±3.5	−4.2
Octane	5	6	6	5	5	5	6	5	4	5	5	5.2	±0.6	−0.2
	49	46	48	47	47	47	47	47	49	46	51	47.5	±1.5	+1.5
	145	147	143	149	149	143	143	145	150	150	146	146.5	±2.9	+1.5

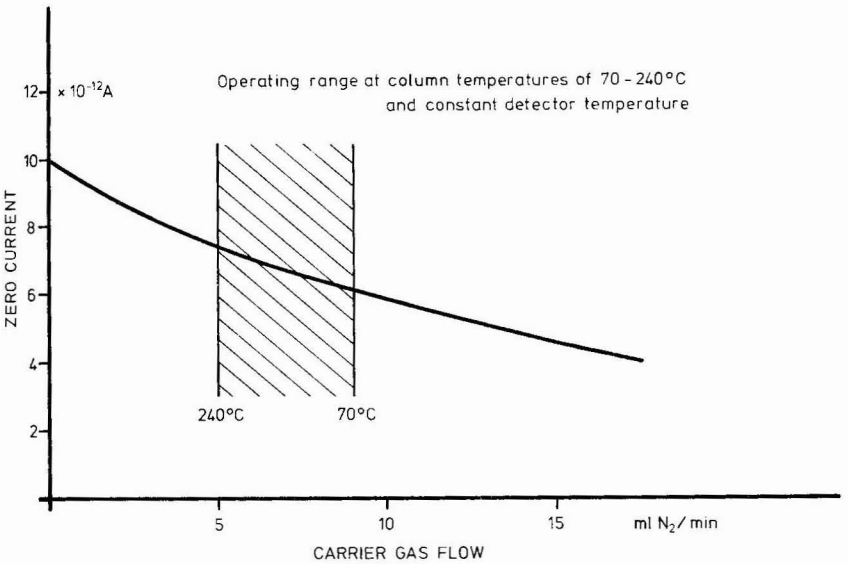


Fig. 7. Zero current of the FID at different carrier gas flow-rates. Combustion gas: H_2, 20 ml · min^{-1}. Scavenging gas: O_2, 500 ml · min^{-1}. Detector temperature: 250°.

Introduction of the sample

The high separation capability of the capillary column used, of course, becomes apparent only if the hydrocarbons enriched from the sample are introduced into the separation system as highly concentrated as possible. *i.e.*, diluted with as small volumes of carrier gas as possible. For this reason, the flow of carrier gas is switched off before heating the loaded pre-column, as described above. If not, double peaks,

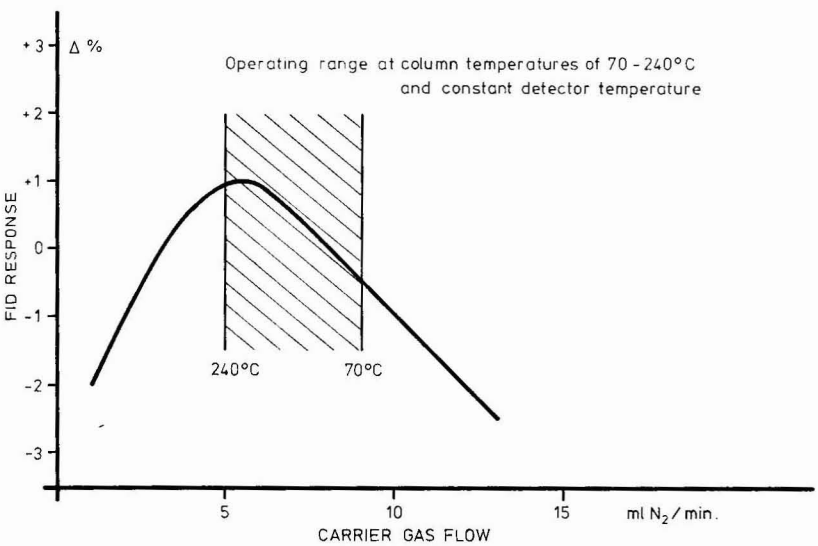


Fig. 8. Change of FID response at different carrier gas flow-rates. Combustion gas: H_2, 20 ml · min^{-1}. Scavenging gas: O_2, 500 ml · min^{-1}. Detector temperature: 250°.

TABLE IV

KOVÁTS RETENTION INDICES MEASURED ON TEN DIFFERENT SEPARATION COLUMNS

Inlet pressure: 2 bar. Column temperature: 50–210°.

Test substance	*Column No.*									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Acetylene	375	376	372	376	375	376	373	374	375	376
Isobutene	454	453	453	458	457	457	455	457	454	458
Pentyne	547	543	545	549	546	550	548	548	544	548
2-Hexene	625	624	623	626	625	626	625	624	626	626
2,2-Dimethylpentane	668	667	667	668	668	668	667	668	667	668
Benzene	734	735	737	737	736	737	736	736	737	736
Toluene	834	835	833	834	834	834	835	834	834	835

peak broadening and tailing will occur. These difficulties are avoided, as can be seen in Fig. 5, by the method of sample introduction described here, *viz.*, with stopped gas flow during heating.

Detector

As illustrated in Fig. 5, the zero current of the detector increases with increasing temperature (70–240°) by about $1.1 \cdot 10^{-12}$ A. This phenomenon is not caused by "bleeding", *i.e.*, an increased release of organic matter from the separation column

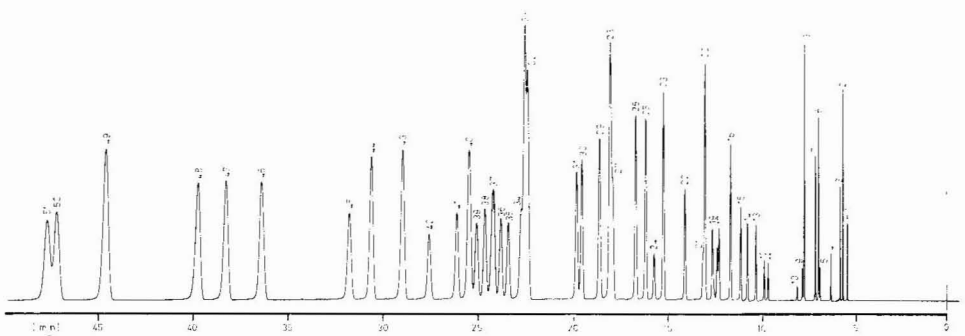


Fig. 9. Analysis of test mixture using a glass capillary coated with Al_2O_3. Length, 71 m; I.D., 0.40 mm; Al_2O_3 coating, 5.1 mg · m^{-1}; column temperature, 130°; inlet pressure, 2 bar. Peaks: 1 = methane; 2 = ethane; 3 = ethene; 4 = propane; 5 = cyclopropane; 6 = propene; 7 = acetylene; 8 = propadiene; 9 = isobutane; 10 = *n*-butane; 11 = *trans*-2-butene; 12 = 1-butene; 13 = isobutene; 14 = *cis*-2-butene; 15 = 2,2-dimethylpropane; 16 = methylcyclobutane; 17 = cyclopentane; 18 = isopentane; 19 = 1,2-butadiene + propyne + *trans*-1,2-dimethylcyclopropane; 20 = 1,1-dimethylcyclopropane; 21 = *n*-pentane; 22 = *cis*-1,2-dimethylcyclopropane + 1,3-butadiene; 23 = ethylcyclopropane; 24 = 3-methyl-1-butene; 25 = cyclopentene; 26 = *trans*-2-pentene; 27 = 2-methyl-2-butene; 28 = 1-pentene + methylenecyclobutane; 29 = 2-methyl-1-butene; 30 = *cis*-2-pentene; 31 = 3-methyl-1,2-butadiene; 32 = 2-butyne; 33 = 2,2-dimethylbutane + 1,1,2-trimethylcyclopropane; 34 = methylcyclopentane + 3,3-dimethyl-1-butene; 35 = ethylcyclobutane; 36 = cyclohexane; 37 = 1-butyne + 2,3-dimethylbutane; 38 = 2-methylpentane; 39 = 3-methylpentane; 40 = 1,2-pentadiene + 2,3-pentadiene; 41 = vinylcyclopropane; 42 = *n*-hexane; 43 = *trans*-4-methyl-2-pentene + isopropylcyclopropane; 44 = 2-methyl-1,3-butadiene; 45 = 1-methylcyclopentene; 46 = 4-methyl-1-pentene; 47 = *cis*-1,3-pentadiene; 48 = *trans*-1,3-pentadiene; 49 = 3-methyl-1-butyne; 50 = isopropylacetylene; 51 = 2-pentyne.

at higher temperature levels, as is usually the case, but by the reduction in the flow of nitrogen (viscosity increase) to the detector as a consequence of the increasing column temperature. This relationship is a well known and more or less pronounced phenomenon, depending on the detector geometry. The general lay-out of the detector used was described elsewhere[18]. Fig. 7 shows the zero current of the detector as a function of the carrier gas flow-rate.

It would require complicated equipment to keep the carrier gas flow-rate constant when capillary columns and rapid temperature changes are involved. On the other hand, the small zero drift does not interfere with the evaluation of chromatograms by manual or modern electronic auxiliaries. Therefore, the carrier gas flow-rate need not be kept constant.

Not only the baseline current, but also the response of a flame-ionization detector, depends on the flow-rate of nitrogen and the ratio of hydrogen to nitrogen in the flame[20]. Depending on the geometry of the detector, this may lead to extensive deviations in response and should be checked carefully. The influence on response

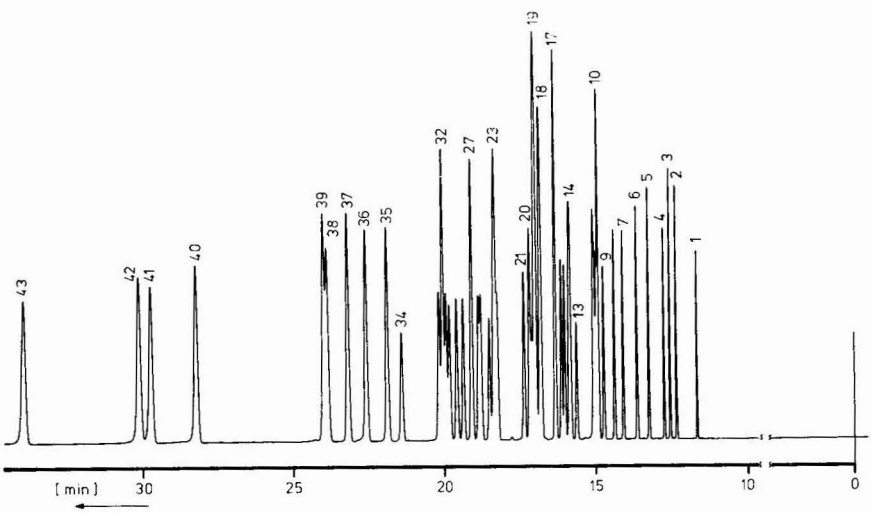


Fig. 10. Analysis of test mixture using a glass capillary coated with Al_2O_3 and squalane. Length, 129 m; I.D., 0.25 mm; coating, Al_2O_3 0.6 mg · m^{-1}; squalane 0.3 mg · m^{-1}; column temperature, 100°; inlet pressure, 5 bar. Peaks: 1 = *n*-pentane; 2 = 2-methylpentane; 3 = 3-methylpentane; 4 = *n*-hexane; 5 = 2,2-dimethylpentane; 6 = 2,2,3-trimethylbutane; 7 = 2-methylhexane; 8 = 3-methylhexane; 9 = *cis*-2,5-dimethyl-3-hexene; 10 = 2,2,4-trimethylpentane + *trans*-2,2-dimethyl-3-hexene; 11 = *trans*-2,5-dimethyl-3-hexene; 12 = benzene + *n*-heptane; 13 = 2,4,4-trimethyl-1-pentene; 14 = 2,4,4-trimethyl-2-pentene + 2,2-dimethylhexane; 15 = *cis*-2,2-dimethyl-3-hexene; 16 = 2,5-dimethylhexane; 17 = 2,4-dimethylhexane + 1,1,3-trimethylcyclopentane; 18 = *trans*-2-methyl-3-heptene + 2,2,3-trimethylpentane; 19 = *trans*-4-ethyl-2-hexene + 3,3-dimethylhexane + 2,5-dimethyl-1-hexene; 20 = 3,4-dimethyl-1-hexene + *trans*-6-methyl-3-heptene; 21 = *trans*-3,4,4-trimethyl-2-pentene; 22 = 3-methylheptane; 23 = *cis*-4-methyl-3-ethyl-2-pentene + 3,4-dimethylhexane; 24 = 3-ethyl-3-hexene; 25 = 2-methyl-1-heptene; 26 = 3-methyl-3-ethylpentane; 27 = 2-methyl-3-ethyl-2-pentene + 1-octene; 28 = *trans*-3-octene; 29 = 2-methyl-2-heptene; 30 = *n*-octane; 31 = toluene; 32 = *trans*-2-octene + *trans*-1-methyl-2-ethylcyclopentane; 33 = 1,1-dimethylcyclohexane; 34 = *trans*-1,3-dimethylcyclohexane; 35 = isopropylcyclopentane; 36 = *cis*-1-methyl-2-ethylcyclopentane; 37 = *n*-propylcyclopentane; 38 = 4-vinylcyclohexene; 39 = ethylcyclohexane; 40 = ethylbenzene; 41 = *p*-xylene; 42 = *m*-xylene; 43 = *o*-xylene.

in the range 5–10 ml·min^{-1} of nitrogen, shown in Fig. 8, was less than $\pm 1\%$ with the detector used. For this and the above reasons, stabilization of the supply of carrier gas to the detector was omitted.

Column

The lifetime of a properly treated column can be several years. A column in use in our laboratories for 18 months has shown no signs of deterioration.

The column described is a powerful tool for measurements relating to emission problems owing to its ability to analyse in one run hydrocarbons from methane to cumene at very low concentrations, with a high separation efficiency for the low-boiling components such as methane, ethane and ethylene.

The Kováts retention indices of several polar substances from ten different separation columns prepared by the same procedure, as listed in Table IV, show that the separation behaviour of the columns is satisfactorily reproducible.

Other applications

The separation column with an aluminium oxide coating described here is applicable not only in trace analyses but, of course, also in the analysis of sample components with concentrations in the per cent range. Fig. 9 shows an example of such an application. In this instance, a test mixture containing C_1–C_6 hydrocarbons in higher concentrations in the percent range was fed into the column. A good separation is obtained, regardless of the concentration.

Figs. 10 and 11 show chromatograms of test mixtures obtained with aluminium

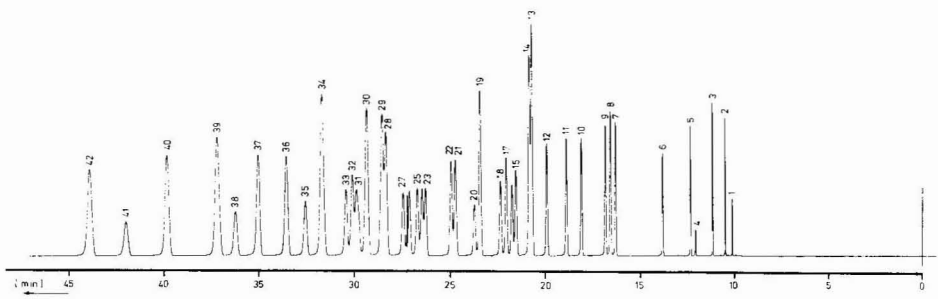


Fig. 11. Analysis of test mixture using a glass capillary coated with Al_2O_3 and Carbowax. Length, 125 m; I.D., 0.25 mm; coating, Al_2O_3 0.5 mg · m^{-1}, Carbowax 0.3 mg · m^{-1}; column temperature, 100°; inlet pressure, 5 bar. Peaks: 1 = *n*-heptane; 2 = *n*-octane; 3 = *n*-nonane; 4 = benzene; 5 = *n*-decane; 6 = toluene; 7 = ethylbenzene; 8 = *p*-xylene; 9 = *m*-xylene; 10 = isopropylbenzene; 11 = *o*-xylene; 12 = *n*-propylbenzene; 13 = 4-ethyltoluene; 14 = 3-ethyltoluene; 15 = *tert.*-butylbenzene; 16 = isobutylbenzene; 17 = 1,3,5-trimethylbenzene; 18 = *sec.*-butylbenzene; 19 = styrene + 2-ethyltoluene + 1-methyl-3-isopropylbenzene; 20 = 1-methyl-4-isopropylbenzene; 21 = neopentylbenzene; 22 = 1,2,4-trimethylbenzene; 23 = 1,3-diethylbenzene; 24 = 1-methyl-2-isopropylbenzene + 1-methyl-3-*n*-propylbenzene; 25 = 1-methyl-4-*n*-propylbenzene; 26 = 1,4-diethylbenzene; 27 = *n*-butylbenzene; 28 = 1-methyl-3-*tert.*-butylbenzene; 29 = 1,3-dimethyl-5-ethylbenzene; 30 = 1-methyl-4-*tert.*-butylbenzene + 1,3-diethylbenzene; 31 = α-methylstyrene; 32 = 1-methyl-2-*n*-propylbenzene + *tert.*-pentylbenzene; 33 = 1,2,3-trimethylbenzene; 34 = 1,3-diisopropylbenzene + 1,4-dimethyl-2-ethylbenzene; 35 = 1,3-dimethyl-5-isopropylbenzene; 36 = 1,2-dimethyl-4-ethylbenzene; 37 = 1,2-diisopropylbenzene + indane; 38 = 1,3-dimethyl-2-ethylbenzene; 39 = 1-methyl-3,5-diethylbenzene + 1,4-diisopropylbenzene; 40 = *n*-pentylbenzene + 1,2-dimethyl-3-ethylbenzene; 41 = 1,2,4,5-tetramethylbenzene; 42 = 1,2,3,5-tetramethylbenzene.

oxide capillaries coated additionally with squalane or polyethylene glycol. It is evident that aluminium oxide is suitable as a base for these two organic separation phases. It is well known that these substances do not adhere well to untreated glass.

These few examples are intended to show that with the separation system described, *viz.*, glass capillaries coated with aluminium oxide, a large number of variations is possible. However, also the operator is not relieved from his responsibility of adapting the separation system to the particular sample under investigation.

REFERENCES

1 W. Schneider and J. C. Frohne, *Staub-Reinhalt. Luft*, 35 (1975) 275.
2 J. C. Frohne and P. Kappe, *Staub-Reinhalt. Luft*, 35 (1975) 307.
3 S. A. Greene and H. Pust, *Anal. Chem.*, 29 (1957) 1055.
4 L. H. Klemm and S. K. Airee, *J. Chromatogr.*, 13 (1964) 40.
5 I. R. Snyder and E. R. Fett, *J. Chromatogr.*, 18 (1965) 461.
6 H. Bruderreck, *Erdöl Kohle*, 16 (1963) 847.
7 R. J. Philippe and R. G. Honeycutt, *Anal. Chem.*, 37 (1965) 928.
8 I. Halász and E. E. Wegner, *Brennst.-Chem.*, 42 (1961) 261.
9 C. G. Scott and C. S. G. Phillips, in A. Goldup (Editor), *Gas Chromatography 1964*, Institute of Petroleum, London, 1965, p. 266.
10 I. Halász and C. Horvath, *Anal. Chem.*, 35 (1963) 499.
11 I. Halász and E. Heine, *Anal. Chem.*, 37 (1965) 495.
12 I. Halász and E. Heine, *Nature (London)*, 194 (1962) 971.
13 D. L. Petitgean and C. J. Leftault, *J. Gas Chromatogr.*, 1 (1963) 18.
14 J. J. Kirkland, in A. Goldup (Editor), *Gas Chromatography 1964*, Institute of Petroleum, London, 1965, p. 285.
15 R. D. Schwarz, D. J. Brasseaux and G. R. Shoemake, *Anal. Chem.*, 35 (1963) 496.
16 J. G. Nikelly, *Anal. Chem.*, 44 (1972) 623.
17 J. G. Nikelly, *Anal. Chem.*, 46 (1974) 290.
18 H. Bruderreck, W. Schneider and I. Halász, *Anal. Chem.*, 36 (1964) 461.
19 D. H. Desty, J. N. Haresnape and B. H. F. Whyman, *Anal. Chem.*, 32 (1960) 302.
20 J. C. Sternberg, W. S. Gallaway and D. T. L. Jones, in N. Brenner (Editor), *Gas Chromatography 1961*, Academic Press, New York, 1962, p. 231.

Journal of Chromatography, 155 (1978) 329–336

CHROM. 10,850

PHENYLBORONIC ACID AS A LIGAND FOR BIOSPECIFIC CHROMATOGRAPHY OF SERINE PROTEINASES

VALERII KH. AKPAROV and VALENTIN M. STEPANOV*
Institute of Genetics and Selection of Industrial Microorganisms, Moscow, M-545 (U.S.S.R.)
(First received August 22nd, 1977; revised manuscript received December 27th, 1977)

SUMMARY

Via attachment of *p*-(ω-aminoethyl)phenylboronic acid to CH-Sepharose in the presence of water-soluble carbodiimide, a new sorbent for the biospecific chromatography of serine proteinases was obtained. The sorbent was shown to be suitable for the purification of subtilisin, α-chymotrypsin and trypsin. It is assumed that the serine hydroxyl group at the active site of the enzyme forms, with the boronic acid moiety of the ligand, a structure that imitates transition enzyme–substrate complex. The presence of glycerol selectively improves the binding of serine proteinases, presumably because of stabilization of the tetrahedral state of the boron atom. Direct isolation of subtilisin from a *Bacillus subtilis* cultural filtrate on phenylboronic acid-containing sorbent gives a virtually homogeneous enzyme (42-fold purification) in a nearly-quantitative yield.

INTRODUCTION

Boronic acid derivatives as inhibitors of serine proteinases were described for the first time by Antonov *et al.*[1], who postulated that the boronic acid residue formed a labile bond at the active site whereas the hydrocarbon moiety interacted with the hydrophobic binding site of the enzyme. Later, Koehler and Lienhard[2] suggested that the complexes with substituted boronic acids reproduced the structure of the transition state at the active site of serine proteinases. This assumption was confirmed by X-ray analysis[3]. It was found that the boronic acid residue formed a covalent bond with the serine residue at the active site. The high specificity of this interaction implied the possibility of its use for the biospecific chromatography of serine enzymes.

MATERIALS AND METHODS

CH-Sepharose was purchased from Pharmacia (Uppsala, Sweden) and α-chymotrypsin, bovine trypsin and subtilopeptidase A (subtilisin BPN') from Serva (Heidelberg, G.F.R.). Subtilisin inhibition with phenylmethylsulphonyl fluoride

* To whom correspondence should be addressed.

(PMSF) was performed as described earlier[4]. The following substrates were used for proteinase assay: N-benzoyl-D,L-arginine *p*-nitroanilide for trypsin (method 2 of Haverback *et al.*[5]), N-3-carboxypropionyl-L-phenylalanine *p*-nitroanilide for α-chymotrypsin[6] and benzyloxycarbonyl-L-alanyl-L-alanyl-L-leucine *p*-nitroanilide for subtilisin[7]. The unit of activity was taken as the amount of the enzyme that splits, under specified conditions, 1 μmole of the substrate per minute.

Thin-layer chromatography (TLC)

TLC was performed on silica gel-coated plates in the following solvent mixtures: A = *n*-butanol–water (86:14); B = toluene–acetone (1:1); C = ethyl acetate; D = propanol-2–ammonia solution–water (7:1:2); and E = propanol-2–hydrochloric acid–water (17:4:5). The spots were rendered visible with iodine vapour[8] or by spraying with a 0.2% solution of diphenylcarbazone in methanol[9]. After spraying with the latter reagent, arylboronic acids appeared as red or violet spots on a pink background.

CHPB-Sepharose synthesis

p-Toluneboronic acid (I) was synthesized by the reaction of *p*-tolylmagnesium bromide with trimethyl borate[10]. After recrystallization from water the yield was 41% (m.p. 243–245°; literature[11] m.p. 245°). The substance gave one spot on TLC with $R_F = 0.89$ in solvent A, 0.57 in solvent B and 0.31 in solvent C.

p-(ω-Bromomethyl)phenylboronic acid (II) was obtained by N-bromosuccinimide bromination of the side-chain in I[12]. The yield was 80% (m.p. 146°; literature[13] m.p. 165–168°) TLC gave $R_F = 0.91$ in solvent A, 0.57 in solvent B and 0.31 in solvent C.

The urotropin complex of II (III) was obtained in quantitative yield from freshly prepared II as described by Pichuzhkina *et al.*[14] and could be used for the following stage without purification. After treatment with hot water-saturated *n*-butanol, on TLC the substance gave one spot with $R_F = 0.0$ in solvent A, 0.10 in solvent B and 0.34 in solvent D.

p-(ω-Aminomethyl)phenylboronic acid (IV) was obtained by decomposition of III with hydrochloric acid. A 1.7-g amount of III was refluxed with 54 ml of ethanol and 20 ml of concentrated hydrochloric acid for 1 h, then the mixture was repeatedly evaporated to dryness *in vacuo* from ethanol. The residue was treated with hot, dry ethanol, ammonium chloride filtered off and the filtrate evaporated to dryness, giving 0.75 g of IV (yield 90%). The substance could be coupled with CH-Sepharose without further purification. For purification, 100 mg of IV were dissolved in sodium hydroxide solution at pH 11 and the solution was poured into a Dowex 1-X1 column (10 × 1 cm) that had been previously washed with 1 m*M* sodium hydroxide solution. The column was washed with 1 m*M* sodium hydroxide solution and water, then IV was eluted with 0.1 *M* hydrochloric acid. Pure IV showed one spot on TLC with $R_F = 0.87$ in solvent A, 0.17 in solvent D and 0.56 in solvent E.

CHPB-Sepharose, the product of the attachment of *p*-(ω-aminomethyl)phenylboronic acid to CH-Sepharose, was obtained as follows. To 2.5 ml of CH-Sepharose (containing about 35 mmole of COOH groups, as certified by the manufacturer) were added 25 mg of IV followed by 41.2 mg of N-cyclohexyl-N′-[2-(4-morpholinyl)ethyl]-carbodiimide *p*-toluenesulphonate. After dissolution of the reagents, the mixture

(pH 4.8) was kept overnight at 20°, then CHPB-Sepharose was collected on a glass filter and washed thoroughly with water. To determine the content of IV in CHPB-Sepharose, 0.1 ml of settled sorbent was treated with 5 ml of 6 *M* hydrochloric acid at 20° until the gel had dissolved, then the concentration of the ligand was calculated from the UV spectrum of the solution using the molar extinction coefficient $\varepsilon^{M}_{225} = 8390$ for IV in 6 *M* hydrochloric acid.

Chromatography on CHPB-Sepharose

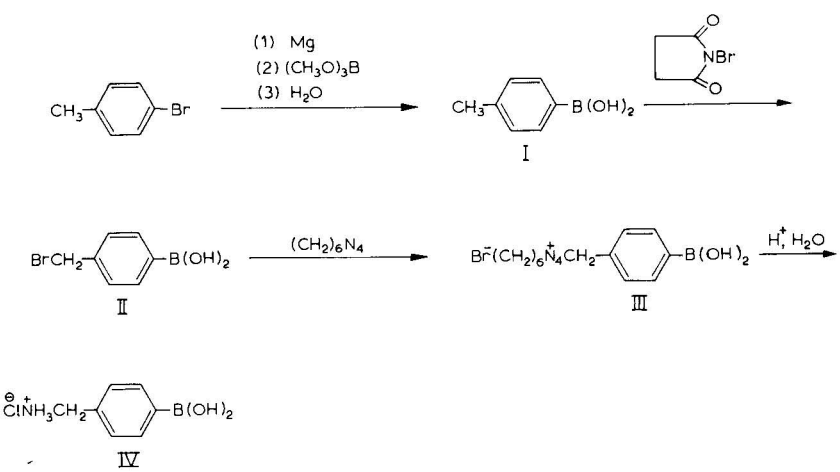
A CHPB-Sepharose column (25 × 1 cm) was equilibrated with 0.05 *M* phosphate buffer of appropriate pH or with 0.5 *M* glycerol solution in the same buffer. Sample volumes did not exceed 2 ml. The column was eluted with equilibrating buffer at 36 ml/h. The protein content was determined on the basis of the absorbance at 280 nm.

Isolation of subtilisin from Bacillus subtilis cultural filtrate

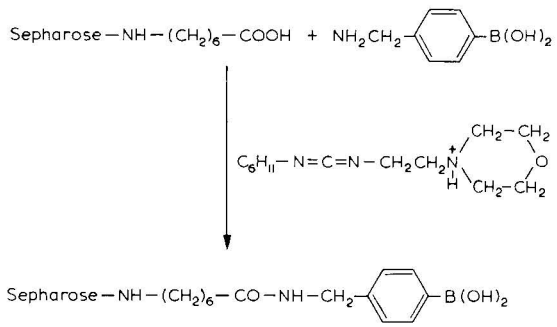
The cultural filtrate of *Bacillus subtilis* strain A-50 was concentrated to 5% of its volume by dialysis against Carbowax 6000, then dialysed against 0.5 *M* glycerol in 0.05 *M* phosphate buffer (pH 7.5). A 2-ml volume of this solution was applied on a 25-ml CHPB-Sepharose column equilibrated with the same buffer. Further operations were carried out as described above.

RESULTS AND DISCUSSION

p-(ω-Aminomethyl)phenylboronic acid was synthesized by the following reaction sequence:



p-(ω-Aminomethyl)phenylboronic acid was attached to CH-Sepharose (Sepharose derivative, containing ε-aminocaproic acid residues with free carboxyl groups) activated by water-soluble carbodiimide:



The sorbent obtained contained 5–10 μmole of the ligand per millilitre of swollen Sepharose, whereas 10–14 μmole of free carboxyl groups per millilitre were claimed by the manufacturer for CH-Sepharose. CHPB-Sepharose was tested as a sorbent for the covalent chromatography of serine proteinases.

Subtilisin BPN', an extracellular serine proteinase of *Bacillus subtilis*, was not firmly bound but only retarded by the sorbent (Fig. 1a). The maximal extent of the retardation was attained at pH 7.5 (Fig. 2), which agreed fairly well with the data on the pH-dependence of the inhibition of serine proteinases by boronic acids[15,16]. Chromatography on CHPB-Sepharose at this pH permits the separation of the active subtilisin from inert proteins and also from PMSF-inhibited subtilisin not retarded by the column (Fig. 1c and e). This clearly shows the importance of the interaction of serine hydroxyl groups with the boronic acid moiety of the ligand for the sorption of subtilisin. The chromatography of a commercial subtilisin preparation on CHPB-Sepharose gave a 1.9-fold purification of the enzyme (Table I). Its specific activity was 1.82 units/mg when tested against the chromogenic substrate, benzyloxycarbonyl-L-alanyl-L-alanyl-L-leucine *p*-nitroanilide, which corresponds to essentially pure enzyme. A high recovery of the enzyme (81 %) was achieved.

TABLE I

CHROMATOGRAPHY OF PROTEINASES ON CHPB-SEPHAROSE

The sorbent was equilibrated and washed with 0.05 *M* phosphate buffer, pH 7.5.

Enzyme preparation	*Glycerol added (M)*	*Column volume (ml)*	*Protein applied (mg)*	*Specific activity (units/mg × 10²)*		*Purification factor*	*Yield (%)*
				Initial	*Final*		
Subtilisin BPN'	0	25	4.25	94	182	1.9	81
Subtilisin BPN'	0.5	25	4.1	94	204	2.2	82
Subtilisin A-50 (cultural filtrate)	0.5	25	50	4.3	180	42	100
Trypsin	0	4	1	22	33	1.5	100
Trypsin	0.5	4	1	22	30	1.4	100
α-Chymotrypsin	0	4	1	2.5	4	1.6	80
α-Chymotrypsin	0.5	4	1	2.5	4	1.6	91

Pentaerythritol, which forms with phenylboronic acid at pH 7.5 a bicyclic complex[17] in which all three hydroxyl groups of the tetradentate boron atom are in covalent bonds with the alcohol, completely suppressed the interaction of subtilisin

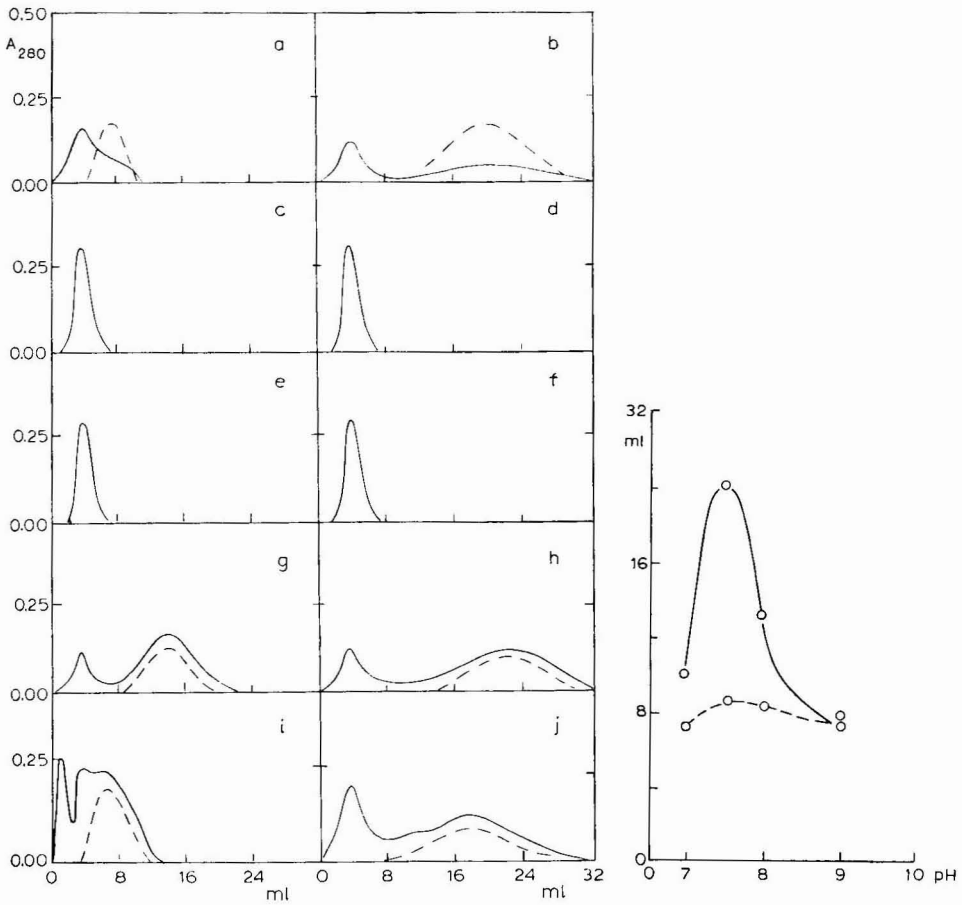


Fig. 1. Chromatography of proteins on CHPB-Sepharose. To a column containing 4 ml of CHPB-Sepharose, 0.1 ml of 1% protein solution was applied. The column was equilibrated and eluted with 0.05 *M* phosphate buffer (pH 7.5) or with 0.5 *M* glycerol in the same buffer. Solid line, absorbance at 280 nm (protein content); broken line, proteolytic activity in arbitrary units. (a) Subtilisin BPN′; (b) subtilisin BPN′ in 0.5 *M* glycerol; (c) bovine serum albumin; (d) bovine serum albumin in 0.5 *M* glycerol; (e) PMSF-inhibited subtilisin BPN′; (f) PMSF-inhibited subtilisin in 0.5 *M* glycerol; (g) α-chymotrypsin; (h) α-chymotrypsin in 0.5 *M* glycerol; (i) trypsin; (j) trypsin in 0.5 *M* glycerol.

Fig. 2. pH dependence of subtilisin BPN′ retention volume on CHPB-Sepharose. To a column containing 4 ml of CHPB-Sepharose, 0.05 ml of 1% subtilisin BPN′ solution was applied. The points correspond to the maxima on the elution curves of the active enzyme. Broken line, column equilibrated and washed with 0.05 *M* phosphate buffer (pH 7.5); solid line, column equilibrated and washed with 0.5 *M* glycerol in the same buffer.

with CHPB-Sepharose. This result confirms that it is the hydroxyl groups of substituted phenylboronic acid that take part in the binding of the enzyme.

The chromatography of other serine proteinases (α-chymotrypsin and trypsin on CHPB-Sepharose gave similar results (Fig. 1g and i; Table I).

Whereas pentaerythritol blocked the binding of serine proteinases by CHPB-Sepharose, glycerol, on the other hand, enhanced the interaction of these enzymes

with the sorbent. Glycerol is known to form at pH 7.5 a complex with phenylboronic acid in which only two hydroxyl groups of the tetradentate boron atom participate in covalent bonds with the alcohol[17], leaving the third hydroxyl group free to interact with the hydroxyl group at the active site of serine proteinases. The complexes of these enzymes with CHPB-Sepharose are even more stable in 0.5 *M* glycerol, which results in increased retardation by the sorbent (Fig. 1b, h and j). Thus, in the presence of glycerol the retention volume of subtilisin BPN′ is 2.5 times, of trypsin 2.2 times and of α-chymotrypsin 1.5 times as large as in its absence.

It has been shown that substituted boronic acid derivatives, when bound to serine proteinase, imitate the tetrahedral transition state of an enzyme–substrate complex[2,3]. One of its hydroxyl groups establishes a covalent bond with the serine residue of the active site, whereas the other two serve as acceptors of the hydrogen bonds with the donor groups belonging to so-called "oxyanion hole" of the enzyme (Fig. 3).

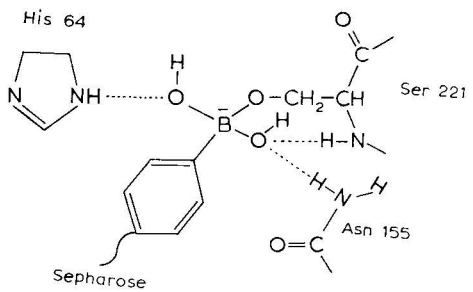


Fig. 3. Hypothetical structure of CHPB-Sepharose complex with the active site of a serine proteinase (amino acid residue numbering is given for subtilisin)[3]. Glycerol presumably binds with both free hydroxyl groups.

It appears that the formation of a phenylboronic acid–glycerol complex does not prevent the building of these hydrogen bonds. On the other hand, the stabilization of the tetrahedral state of the boron atom due to the interaction with glycerol even improves the stability of the enzyme–ligand complex. This hypothesis might explain the positive effect of glycerol on the binding of serine proteinases by CHPB-Sepharose (Fig. 1b, h and j). The presence of glycerol does not lower the selectivity of serine proteinase chromatography. Thus, PMSF-inhibited subtilisin BPN′ was eluted in 0.5 *M* glycerol with the free volume of the column, together with the inert proteins (Fig. 1d and f). No shift in the pH optimum for retardation of serine proteinases was observed in glycerol.

The enhancement of the binding properties of CHPB-Sepharose in glycerol-containing solutions has been used for the direct isolation of subtilisin from *Bacillus subtilis* A-50 cultural filtrate. In a single step by filtration through a CHPB-Sepharose column, equilibrated with 0.5 *M* glycerol in 0.05 *M* phosphate buffer of pH 7.5 (Fig. 4), a subtilisin preparation was obtained with a specific activity of 1.8 units/mg, which can be compared with 0.94 units/mg for a commercial preparation of subtilisin BPN′ and 1.82–2.04 units/mg for subtilisin BPN′ purified on CHPB-Sepharose as described above (Table I). No interference from the components of the cultural filtrate was observed although the interaction of the boronic acid moiety with sugars present in the mixture could adversely affect the procedure. It appears that the content

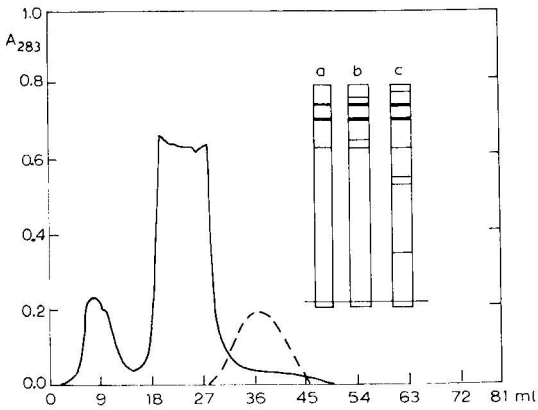


Fig. 4. Isolation of subtilisin from the cultural filtrate of *Bacillus subtilis* A-50. To a CHPB-Sepharose column (volume 25 ml), 2 ml of cultural filtrate were applied. The column was equilibrated and washed with 0.5 *M* glycerol in 0.05 *M* phosphate buffer (pH 7.5). Solid line, absorbance at 280 nm; broken line, proteolytic activity in arbitrary units. The inset shows the disc-electrophoresis pattern in 7.5% polyacrylamide gels (pH 8.9)[18]: (a) subtilisin BPN′ purified on CHPB-Sepharose (see Fig. 1b); (b) subtilisin isolated from *Bacillus subtilis* A-50 cultural filtrate; (c) commercial subtilisin BPN′. Direction of movement, from the top to the bottom; the gels were stained with Coomassie Brilliant Blue R 250. The three most intense bands (R_F = 0.08, 0.16 and 0.30) correspond to the multiple forms of subtilisin BPN′[4].

of sugars, which is usually low at the end of logarithmic growth, was drastically reduced in the course of liquid culture analysis.

Hence, CHPB-Sepharose might be used as a specific sorbent for the chromatography of serine proteinases. It should be mentioned that for some enzymes of this type no satisfactory conditions were found that would permit their purification on CHPB-Sepharose (serine carboxypeptidase from *Aspergillus oryzae* and intracellular serine proteinase from *Bacillus subtilis*). Both of these enzymes possess isoelectric points near pH 4 and it would therefore be reasonable to assume that electrostatic interactions might be operative in the course of the chromatography of enzymes on CHPB-Sepharose, in addition to the specific binding discussed above. Such non-specific factors have to be taken into account and controlled. It appears that CHPB-Sepharose might be of value as a specific sorbent for various serine hydrolases.

REFERENCES

1 V. K. Antonov, T. V. Ivanina, I. V. Berezin and K. Martinek, *FEBS Lett.*, 7 (1970) 23.
2 K. K. Koehler and G. E. Lienhard, *Biochemistry*, 10 (1972) 2477.
3 D. A. Mattews, R. A. Alden, J. J. Birktoft, S. T. Freer and J. Kraut, *J. Biol. Chem.*, 250 (1975) 7120.
4 A. Ya. Strongin, Z. T. Abramov, E. D. Levin and V. M. Stepanov, *Bioorg. Khim.*, 1 (1975) 1441.
5 B. J. Haverback, B. Dyce, H. F. Bundy and H. A. Edmundson, *Amer. J. Med.*, 29 (1960) 424.
6 B. F. Erlanger, F. Edel and A. G. Cooper, *Arch. Biochem. Biophys.*, 115 (1966) 206.
7 L. A. Lyublinskaya, S. V. Belyaev, A. Ya. Strongin, L. F. Matyash, E. D. Levin and V. M. Stepanov, *Anal. Biochem.*, 62 (1974) 371.
8 E. Stahl (Editor), *Dünnschicht-Chromatographie*, Springer, Berlin, 1962, p. 483.
9 R. Neu, *Z. Anal. Chem.*, 176 (1960) 343.
10 W. Seamen and J. R. Johnson, *J. Amer. Chem. Soc.*, 53 (1931) 711.

11 W. König and W. Scharranbeck, *J. Pract. Chem.*, 128 (1930) 152.
12 H. R. Snyder, M. S. Koneeky and W. J. Lennary, *J. Amer. Chem. Soc.*, 80 (1958) 3611.
13 K. Torsell, *Ark. Kemi*, 10 (1957) 507.
14 E. I. Pichuzhkina, I. I. Kolodkina and A. M. Jurkevich, *Zh. Obshch. Khim.*, 43 (1973) 2275.
15 R. N. Lindgust and C. Terry, *Arch. Biochem. Biophys.*, 160 (1974) 135.
16 M. Philipp and M. L. Bender, *Proc. Nat. Acad. Sci. U.S.*, 68 (1971) 478.
17 E. M. Shwarz, *Kompleksy Soedinenii Bora s Polyoksisoedineniyami*, Riga, Zinatne, 1968, p. 40.
18 B. J. Davis, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.

Journal of Chromatography, 155 (1978) 337–348

CHROM. 10,889

THIN-LAYER CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF TERTIARY AROMATIC AMINES AND THEIR N-OXIDES

L. A. DAMANI*, L. H. PATTERSON** and J. W. GORROD***

Department of Pharmacy, Chelsea College, University of London, Manresa Road, London, SW3 6LX (Great Britain)

(Received December 23rd, 1977)

SUMMARY

Thin-layer chromatographic (TLC) systems for the separation of 4-substituted N-ethyl-N-methylanilines and their N-dealkylation and N-oxidation products are described. Various TLC systems for the separation of 2-, 3- and 4-substituted pyridines and their N-oxides are also described. Various detection systems were utilized for revealing the spots of the compounds on the chromatograms. None of the reagents used was specific for the detection of pyridine-N-oxides, but tetracyanoethylene was found to be a very sensitive and specific spray reagent for the detection of N,N-dialkylaniline-N-oxides.

INTRODUCTION

Several workers have described the paper chromatography (PC) and thin-layer chromatography (TLC) of nitrogen oxidation products (hydroxylamines, hydroxamic acids, N-oxides)[1–3] and the possible use of chromogenic reagents for their identification[1,3,4]. These methods have helped to elucidate the widespread natural occurrence of alkaloid-N-oxides in several plant species[5,6]. The realization that the metabolic N-oxidation of certain nitrogenous drugs and foreign compounds leads to the formation of carcinogenic or toxic metabolites has resulted in extensive work on various aspects of nitrogen oxidation[7,8]. Even though chromatographic methods have been used extensively in these studies, hitherto the data were available only from diverse sources. However, a recent review has collated these data on the isolation, identification and quantitation of various types of N-oxidation products using chromatographic techniques[9].

We have studied the metabolism of a range of structural types of nitrogenous compounds with simple structures in order to help to elucidate the occurrence and

* Present address: Laboratory of Toxicology and Pharmacokinetics, U.C.H. Medical School, London WC1E 6JJ, Great Britain.

** Present address: Drug Metabolism Laboratory, Fisons Pharmaceutical Division, Loughborough, Leics, Great Britain.

*** To whom correspondence should be addressed.

enzymology of metabolic N-oxidation[10–12]. These studies invariably required the use of sensitive chromatographic methods for the identification and quantitation of various metabolites. In this paper, the chromatographic systems used for the separation and detection of tertiary amines, their N-oxides and N-dealkylated products are described.

MATERIALS AND METHODS

TLC separations were performed on 20 × 20 cm aluminium sheets pre-coated with a 0.2-mm layer of silica gel 60 F_{254} (E. Merck, Darmstadt, G.F.R.). Stock solutions of compounds to be chromatographed (20 mg in 10 ml of ethanol) were prepared and 10-μl volumes of these solutions were spotted on to the plates using disposable micropipettes (Camlab, Cambridge, Great Britain). Ascending TLC was performed in all instances, in a glass chamber (TLC Chromotank; Shandon, London, Great Britain) saturated with the respective solvent system. The solvent front was run to a height of 15 cm, allowing a 5-min equilibration time before removal of the plate from the tank. The compositions of the solvent systems used are given in Table I. Chloroform, acetone, ethanol and methanol (BDH, Poole, Great Britain) were dried and redistilled prior to use. Cyclohexane, toluene, diethylamine and ammonia solution (specific gravity 0.880) were used as purchased (BDH). All of the spray reagents, except tetracyanoethylene (TCNE) and 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH), were purchased from BDH. TCNE and MBTH were purchased from Aldrich (Gillingham, Great Britain).

TABLE I

SOLVENT SYSTEMS USED IN THE SEPARATION OF PYRIDINES, AROMATIC AMINES AND THEIR N-OXIDES

Compound	*Solvent system*	*Components*
Pyridines and their N-oxides	S1	Chloroform
	S2	Ethyl acetate
	S3	Acetone
	S4	Ethanol
	S5	Chloroform–ethanol–ammonia (sp.gr. 0.88) (95:4:1)
	S6	Ethyl acetate–ethanol–ammonia (sp.gr. 0.88) (90:5:1)
	S7	Acetone–ethanol–ammonia (sp.gr. 0.88) (95:4:1)
	S8	Chloroform–ethanol–diethylamine (80:30:1)
	S9	Chloroform–ethanol–ammonia (sp.gr. 0.88) (100:8:0.5)
	S10	Acetone–ethanol–diethylamine (95:5:1)
Aromatic amines and their N-oxides	S11	Cyclohexane
	S12	Toluene
	S13	Chloroform
	S14	Methanol
	S15	Cyclohexane–toluene (75:15)
	S16	Cyclohexane–toluene–diethylamine (75:15:10)
	S17	Chloroform–methanol (80:20)
	S18	Chloroform–methanol (90:10)
	S19	Chloroform–methanol (60:40)
	S20	Chloroform–methanol–ammonia (sp.gr. 0.88) (80:20:0.5)

The substituted-pyridine series and the 4-substituted-anilines were in part commercial products from Aldrich and Koch-Light (Colnbrook, Great Britain). The pyridine-N-oxide series was synthesized according to methods already described[13]. The N-ethyl- and N-methyl-4-substituted-anilines and the N-ethyl-N-methyl-4-substituted-anilines were prepared by methods developed in this laboratory[11]. The N-ethyl-N-methyl-4-substituted-aniline-N-oxides were synthesized by methods described in the literature[14].

The compounds listed in Table IV were obtained as follows: chlorpromazine and its N-oxide from Mr. G. Navas, Department of Pharmacy, Chelsea College, London, Great Britain; quinoxaline and its N-oxide from Dr. D. Case, ICI, Macclesfield, Great Britain; 2,2′-bipyridyl and its two N-oxides from Dr. J. Haginiwa, Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Japan; and codeine, tropine, atropine and hyoscine and their N-oxides from Dr. J. D. Phillipson, School of Pharmacy, London, Great Britain. The N-oxides of dimethylamphetamine, nicotine, phendimetrazine, N,N-dimethylaniline and N,N-diethylaniline were synthesized in this laboratory.

RESULTS

The results obtained from this investigation on the pyridine and aniline series are summarized in Tables II and III. Whilst, results for some other tertiary amine-N-oxides are given in Table IV. The initial TLC data were obtained using pure solvents (S_1–S_4, S_{11}–S_{14}) arranged in the tables according to their polarity. Solvent systems of intermediate polarity were obtained by mixing different amounts of these pure solvents (S_5–S_{10}, S_{15}–S_{20}). By careful manipulation of the solvents described, the development of solvent systems specific for the separation of the compounds of interest was possible. The following solvent systems were the most useful for TLC: (a) S_9 and S_{10} for the substituted pyridines and their N-oxides, (b) S_{16} for primary, secondary and tertiary anilines and (c) S_{20} for the separation of the tertiary aniline N-oxides from the other anilines. The R_F values reported for the 100 compounds and 20 solvent systems listed in Tables II and III are averages of at least two determinations in each instance. The R_F values obtained clearly reflect the effects that the substituted functional groups have on the adsorption affinity and partitioning behaviour of the series of compounds tested.

The Royal Horticultural Society (RHS) colour chart was used to identify and record the colour reactions for the 15 different reagents used (see Table V). An abbreviated form of the RHS colour chart is shown in Table VI. A − sign in Tables II–IV indicates a negative reaction whereas a −(+) sign is ascribed to a colour too weak to be positively identified. Unless otherwise stated, the colours described were observed 10 min after spraying with the detection reagent.

DISCUSSION

Most of the detection reagents chosen were standard colour reagents that are used mainly for detecting aromatic amines and phenols. The use of Koenig reagent for the detection of pyridines is well documented and was not therefore adopted in this investigation. Although this reagent is very sensitive for pyridines, it does not

TABLE II

TLC SEPARATION AND DETECTION OF A SERIES OF PYRIDINES AND THEIR N-OXIDES

For solvent systems, see Table I. For detection systems, see Tables V and VI.

$R_F \times 100$*										*Compound*
S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	
18	32	70	90	82	74	89	100	85	88	Pyridine
0	0	3	20_T	21	3	16	48	28	14	Pyridine-N-oxide
18	39	73	77	81	78	91	100	86	90	3-Methylpyridine
1	0	3	24_T	27	3	17	65	36	16	3-Methylpyridine-N-oxide
19	46	78	81	82	83	93	100	89	93	3-Ethylpyridine
2	0	4	36_T	35	8	22	80	42	19	3-Ethylpyridine-N-oxide
ND	ND	ND	ND	ND	ND	ND	ND	91	98	3-Fluoropyridine
ND	ND	ND	ND	ND	ND	ND	ND	50	41	3-Fluoropyridine-N-oxide
36	80	100	100	91	100	100	100	94	100	3-Chloropyridine
5	3	20	43_T	47	23	48	83	53	46	3-Chloropyridine-N-oxide
38	80	100	100	92	100	100	100	95	100	3-Bromopyridine
5	3	20	46_T	49	27	50	84	58	48	3-Bromopyridine-N-oxide
16	35	80	65	80	78	91	97	87	90	3-Acetylpyridine
2	1	9	22	27	9	28	57	35	24	3-Acetylpyridine-N-oxide
3	10	45	67	31	50	75	63	36	73	3-Aminopyridine
0	0	1	37_T	3	4	10	19	5	8	3-Aminopyridine-N-oxide
2	6	56	73	20	34	76	70	29	74	3-Acetamidopyridine
0	0	2	30_T	4	3	12	29	8	10	3-Acetamidopyridine-N-oxide
40	67	97	82	90	84	98	100	94	95	3-Cyanopyridine
5	3	36	37_T	42	19	63	68	49	61	3-Cyanopyridine-N-oxide
0	4	50	60	11	22	71	50	18	68	Nicotinamide
0	0	2	17_T	2	2	15_T	19_T	4	13_T	Nicotinamide-N-oxide
0	0	2	8_T	0	0	0	2	0	0	Nicotinic acid
0	0	0	3_T	0	0	0	0	0	0	Nicotinic acid-N-oxide
0	24	78	84	9	31	56	58	16	53	3-Hydroxypyridine
0	0	2	30_T	0	0	0	3	0	0	3-Hydroxypyridine-N-oxide
2	5	56	74	15	23	76	68	26	71	3-Pyridylcarbinol
0	0	3	25_T	2	5	11	24	5	8	3-Pyridylcarbinol-N-oxide
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1-(3-Pyridyl)ethanol
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1-(3-Pyridyl)ethanol-N-oxide
0	25	78	100	44	47	86	100	82	83	3-Dimethylaminopyridine
0	8	10	60	10	3	15	67	32	12	3-Dimethylaminopyridine-N-oxide
5	12	60	62	75	31	82	95	85	81	Niketharnide***
0	0	5	19	31	3	30	70	44	26	Niketharnide-N-oxide
2	4	62	63	35	25	81	78	89	79	N-Monoethylnicotinamide
0	0	5	27	10	2	25	47	46	21	N-Monoethylnicotinamide-N-oxide
3	3	23	34	51	10	54	83	67	51	Cotinine
0	0	2	6	12	1	8	34	17	7	Cotinine-N-oxide
2	3	20_T	23_T	68	18	65	70	64	66	Nicotine
0	0	1	6_T	36	1	16	47_T	30	15	Nicotine-1-N-oxide
15	33	78	83	84	57	91	100	88	87	2-Methylpyridine
3	3	5	37_T	43	5	31	67	44	23	2-Methylpyridine-N-oxide
15	36	65	73	90	55	85	98	89	83	4-Methylpyridine
1	2	3	21	31	2	14	56	38	11	4-Methylpyridine-N-oxide
15	45	71	76	89	60	87	98	89	85	4-Ethylpyridine
1	2	3	30	35	4	19	69	45	15	4-Ethylpyridine-N-oxide
17	64	85	81	91	72	89	100	92	88	Quinoline
3	4	21	48	55	15	54	80	67	49	Quinoline-N-oxide
15	56	79	79	89	67	87	100	89	86	Isoquinoline
2	2	8	46	48	10	37	76	59	32	Isoquinoline-N-oxide

* T = Tailing; ND = not determined.

** Chromatogram heated at 100° for 5 min after spraying.

*** N,N′-Diethylnicotinamide.

Detection system

D_1	D_2	D_3	D_4	D_5**	D_6	D_7	D_8	D_9	D_{10}	D_{11}	D_{12}	D_{13}	D_{14}	D_{15}
102B	86A	–	1D	–				–	29C	4B	–	–(+)	–	112A
95A	86A	–	1D	–	–	–	–	–	–	–	–	–(+)	–	–
102B	86A	–	1D	–	–	–	–	–	29C	4B	–	–(+)	–	112A
95A	86A	–	1D	–	–	–	–	–	–	–	–	–(+)	–	–
102B	86A	–	1C	–	–	–	–	–	29C	4B	–	–(+)	–	112A
95A	86A	–	1C	–	–	–	–	–	–	–	–	–(+)	–	112A
102B	86A	–	1C	–	–	–	–	–	–	4B	–	–	–	–
95A	86A	–	–	–	–	–	–	–	–	155C	–(+)	–	–	–(+)
102B	86A	–	1D	–	–	–	–	–	–	4B	–	–(+)	–	–
95A	86A	–	1D	–	–	–	–	–	–	–	–	–(+)	–	–
102B	86A	–	1D	–	–	–	–	–	–	4B	–	–(+)	–	–
95A	86A	–	1D	–	–	–	–	–	–	–	–	–(+)	–	–
102B	86A	–	1D	173D	–	–	–	–	30D	4B	–	–(+)	–	–
95A	86A	–	1D	177D	199D	–	–	–	30C	–	–	–(+)	–	–
96B	86A	–	1D	173D	37D	5C	50C	–	26B	4B	–(+)	121C	–	112A
102B	86A	166D	1C	202D	–	5B	50B	–	–	–	–(+)	82D	–(+)	–(+)
95A	96B	–	1D	202C	202D	5C	50D	–	165D	4B	–	–(+)	–	–(+)
102B	86A	–	1D	–	–	5C	50C	–	–	–	–	–(+)	–	–(+)
95A	86A	–	–	–	198D	–	–	–	164D	4B	–	–(+)	–(+)	112A
102B	86A	–	1D	–	–	–(+)	–	–	–	–	–	–(+)	–	–
95A	86A	–	1D	202C	–	–(+)	–	–	187C	4B	–	–(+)	–	112A
102B	86A	–	–	–	–	–	–	–	–	–	–	–(+)	–	–
102B	86A	–	–	–	–	–	–	–	164D	4B	–	121D	–	–(+)
95A	86A	–	–	–	–	–	–	–	–	–	–	–(+)	–	–(+)
102B	86A	11C	1D	98B	93B	–	–	–	–	4B	–	121D	–	112A
95A	86A	166D	1D	148C	202D	–	–	–	–	–	–	–(+)	–	–
102B	86A	–	–	–	–	–	–	–	165D	4B	–	–(+)	–	112A
95A	86A	–	–	–	–	–(+)	–(+)	–	–	–	–	124C	–	–
102B	86A	11C	1D	202C	202D	–	–	–	–(+)	4B	–	158C	–	100C
95A	86A	11D	1D	202D	101D	–(+)	50D	–	–	–	–	124D	–	–
102B	86A	202D	1C	202C	–	–	–	–	–	4B	–(+)	82D	137D	–(+)
95A	86A	166D	1D	–	–	–	–	–	–	–	–(+)	–	–	–
102B	86A	–	1D	–	–	–	–	–	187C	4B	–	121D	–	112A
95A	86A	–	1D	–	–	–	–	–	–	–	–(+)	–(+)	–	–(+)
102B	86A	–	1D	202C	–	–	–	–	–(+)	4B	–	–	–(+)	112A
95A	86A	–	1D	–	–	–	–	–	–	–	–	–	–	–
102B	86A	–	–	202C	–	–	–	–	187C	4B	–(+)	–(+)	–(+)	112A
95A	86A	–	–	–	–	–	–	–	–	–	–	124C	–	112A
102B	86A	–	1D	202C	–	–	–	–	187C	4B	–	121C	–	92C
95A	115D	11B	1C	186B	48D	–	–	–	–	155C	–	48D	–(+)	–(+)
102B	86A	–	–	–	–	–	–	–	165D	4B	–	121C	–	92C
95A	86A	–	1D	–	202D	–(+)	–(+)	–	–	–	–	124C	–	112A
102B	86A	–	–	–	–	–	–	–	164D	4B	–	121C	–	92C
95A	86A	–	–	–	–	–(+)	–(+)	–	21C	–	–	–(+)	–	–
102B	86A	–	1D	–	–	–	–	–	–(+)	4B	–	121C	–	112A
95A	86A		–	–		–(+)	–	–	–	–	–	124C	–	–
102B	86A	–	1D	–	–	–	–	–	–(+)	4B	–(+)	121C	–	109D
95A	86A	11B	1C	–	–	–(+)	–(+)	–	–(+)	–	–(+)	162B	173D	112A
102B	86A	–	1D	–	–	–	–	–	168C	4B	–	121C	–	109D
95A	86A	11B	1C	–	–	–(+)	–(+)	–	–(+)	–	–	82D	–	–(+)

TABLE III

TLC SEPARATION AND DETECTION OF A SERIES OF 4-SUBSTITUTED ANILINES AND THEI N-OXIDES

For solvent systems, see Table I. For detection systems, see Tables V and VI.

$R_F \times 100$										*Compound*
S_{11}	S_{12}	S_{13}	S_{14}	S_{15}	S_{16}	S_{17}	S_{18}	S_{19}	S_{20}	
0	13	60	78	3	29	87	89	90	90	Aniline
2	34	76	82	7	63	90	90	90	90	N-Methylaniline
2	37	84	82	8	77	90	90	90	90	N-Ethylaniline
3	43	91	82	13	92	90	90	90	90	N-Ethyl-N-methylaniline
0	0	0	36	0	0	9	24	24	42	N-Ethyl-N-methylaniline-N-oxide
0	9	51	77	3	32	87	88	88	88	4-Toluidine
0	24	69	82	5	65	90	90	90	90	N-Methyl-4-toluidine
0	27	77	82	5	81	90	90	90	90	N-Ethyl-4-toluidine
0	23	83	82	5	93	90	90	90	90	N-Ethyl-N-methyl-4-toluidine
0	0	0	37	0	0	11	27	38	44	N-Ethyl-N-methyl-4-toluidine-N-oxide
0	10	53	77	3	33	89	88	88	88	4-Ethylaniline
0	27	73	82	7	67	90	90	90	90	N-Methyl-4-ethylaniline
0	27	78	82	5	83	90	90	90	90	N-Ethyl-4-ethylaniline
0	13	50	77	3	19	83	87	87	90	4-Fluoroaniline
2	31	69	82	6	48	90	90	90	90	N-Methyl-4-fluoroaniline
2	35	80	82	7	64	90	90	90	90	N-Ethyl-4-fluoroaniline
3	37	87	82	11	91	90	90	90	90	N-Ethyl-N-methyl-4-fluoroaniline
0	0	0	36	0	0	8	23	35	38	N-Ethyl-N-methyl-4-fluoroaniline-N-oxide
0	20	61	78	4	17	87	87	87	87	4-Chloroaniline
4	50	81	82	11	42	90	90	90	90	N-Methyl-4-chloroaniline
4	56	86	82	13	58	90	90	90	90	N-Ethyl-4-chloroaniline
7	71	94	82	24	90	90	90	90	90	N-Ethyl-N-methyl-4-chloroaniline
0	0	0	37	0	0	9	26	38	42	N-Ethyl-N-methyl-4-chloroaniline-N-oxide
0	22	63	78	4	15	87	87	87	88	4-Bromoaniline
4	53	83	82	13	41	90	90	90	90	N-Methyl-4-bromoaniline
4	59	87	82	15	57	90	90	90	90	N-Ethyl-4-bromoaniline
7	75	94	82	27	91	90	90	90	90	N-Ethyl-N-methyl-4-bromoaniline
0	0	0	35	0	0	13	30	41	49	N-Ethyl-N-methyl-4-bromoaniline-N-oxide
0	24	67	78	4	15	89	88	88	88	4-Iodoaniline
5	57	85	82	15	41	90	90	90	90	N-Methyl-4-iodoaniline
5	65	88	82	17	58	90	90	90	90	N-Ethyl-4-iodoaniline
7	79	94	82	31	91	90	90	90	90	N-Ethyl-N-methyl-4-iodoaniline
0	0	0	37	0	0	11	27	38	43	N-Ethyl-N-methyl-4-iodoaniline-N-oxide
0	3	33	75	0	25	86	87	87	88	4-Anisidine
0	7	43	82	0	51	90	90	90	90	N-Methyl-4-anisidine
0	5	43	82	0	67	90	90	90	90	N-Ethyl-4-anisidine
0	7	49	74	0	88	90	90	90	90	N-Ethyl-N-methyl-4-anisidine
0	0	0	37	0	0	11	24	38	41	N-Ethyl-N-methyl-4-anisidine-N-oxide
0	3	34	75	0	24	83	85	87	87	4-Phenetidine
0	7	43	82	0	59	90	90	90	90	N-Methyl-4-phenetidine
0	5	43	80	0	73	90	90	90	90	N-Ethyl-4-phenetidine
0	4	49	77	0	91	90	90	90	90	N-Ethyl-N-methyl-4-phenetidine
0	0	0	37	0	0	11	25	40	45	N-Ethyl-N-methyl-4-phenetidine-N-oxide
0	16	68	79	3	22	90	90	90	90	4-Aminobiphenyl
0	42	86	82	3	48	90	90	90	90	N-Methyl-4-aminobiphenyl
0	44	86	81	7	67	90	90	90	90	N-Ethyl-4-aminobiphenyl
0	57	93	82	11	91	90	90	90	90	N-Ethyl-N-methyl-4-aminobiphenyl
0	0	0	38	0	0	12	27	42	47	N-Ethyl-N-methyl-4-aminobiphenyl-N-oxide

* Chromatogram heated at 100° for 5 min after spraying.

Detection system

D_1	D_2	D_3	D_4	D_5*	D_6	D_7	D_8	D_9	D_{10}	D_{11}	D_{12}	D_{13}	D_{14}	D_{15}
102B	–	–	173D	156D	118B	5B	44D	49C	6A	–	–	–	–	–
102B	–	189C	118D	118A	118B	4D	41D	28C	158B	43C	–	–	–	–
102B	–	189C	118D	118A	118B	4D	39D	28C	158A	43C	–	–	–	–
102B	–	–	188D	128C	118B	–	–(+)	49A	–(+)	43C	–	88D	–	–
102C	–	–	1D	202C	–	–(+)	87D	–	–	177D	115C	–	173D	111B
102C	–	162D	31D	164C	35D	5B	44D	5B	13B	–	–	–	–	–
102B	–	–	186D	196D	92C	5B	37C	5B	158B	–	–	–	–	–
102B	–	–	189D	196D	92C	4D	39D	5B	158A	–	–	–	–	–
102B	–	–	155C	128C	92C	–	–(+)	4D	–(+)	–	–	–(+)	–	–
102C	–	–	155C	202C	–	–(+)	87D	–	–	144A	115C	–	173D	111B
102B	–	162D	31D	164C	35D	5B	44D	5B	13B	–	–	–	–	–
102B	–	–	73D	196D	92C	4D	37B	5B	158B	–	–	–	–	–
102B	–	–	189D	196D	92C	4D	39D	5B	158A	–	–	–	–	–
102B	–	–	173D	156D	35D	5B	44D	5B	6A	–	–	–	–	–
102B	–	189D	118D	118A	92C	4D	41D	5B	158B	–	–	–	–	–
102B	–	189D	118D	118A	92C	4D	39D	5B	158A	43D	–	–	–	–
102B	–	–	155C	128C	92C	–	–(+)	4D	–(+)	–	–	–(+)	–	–
102C	–	–	–	202C	–	–(+)	87D	–	–	197A	115C	–	173D	111B
102B	86A	162C	173D	164C	35D	5B	47C	5B	6A	–	–	–	–	–
102B	82A	189D	155C	122C	92C	4D	43C	5B	158B	–	–	–	–	–
102B	82A	189D	155C	122C	92C	4D	41D	5B	158A	–	–	–	–	–
102B	82A	162D	155C	128C	92C	–	–(+)	4D	–(+)	–	–	–(+)	–	–
102C	–	–	8D	202C	–	–(+)	87D	–	–	177D	115C	–	173D	111B
102B	86A	162D	173D	164C	35D	5B	47C	5B	6A	–	–	–	–	–
102B	82A	189D	155C	122C	92C	5B	43C	5B	158B	–	–	–	–	–
102B	82A	189D	155C	122C	92C	4D	41D	5B	158A	–	–	–	–	–
102B	82A	162D	155C	128C	92C	–	(+)	4D	(+)	–	–	–	–	–
102C	–	–	8D	202C	–	–(+)	87D	–	–	177D	115C	–	173D	111B
102B	86A	162C	173D	164C	35D	5B	47C	5B	6A	–	–	–	–	–
102B	82A	189D	155C	122C	92C	1A	57D	5B	158B	43C	–	–	–	–
102B	82A	189D	155C	122C	118B	4D	41D	5B	158A	43C	–	–	–	–
102B	82A	162D	155C	128C	92C	–	–(+)	161A	–(+)	–	–	100D	–	–
102C	–	–	–	202C	–	–(+)	87D	–	–	174B	115C	–(+)	173D	111B
102B	–	183D	174C	187C	35D	5B	44C	5B	16A	–	–	–	–	–
102B	–	88D	73D	128C	122C	4D	37B	5B	163B	–	–	–	–	–
102B	–	88D	77D	128C	122C	4D	39D	5B	158A	–	–	–	–	–
102B	–	201D	117B	118C	121D	–	–(+)	161A	(+)	–	–	100D	–	–
102C	–	–	–	202C	–	–(+)	87D	–	–	174B	115C	–(+)	173D	111B
102B	–	183D	174C	187C	35D	5B	44C	5B	16A	–	–	–	–	–
102B	–	88D	73D	128C	122C	4D	37B	5B	163B	–	–	–	–	–
102B	–	88D	77D	128C	122C	4D	39D	5B	158A	–	–	–	–	–
102C	–	201D	117B	118C	121D	–	–(+)	161A	(+)	–	–	100D	–	–
102C	–	–	–	202C	–	–(+)	87D	–	–	174B	115C	–(+)	173D	111B
95A	–	162D	173D	164C	35D	5B	182A	5B	13B	–	–	–	–	–
95A	–	88D	73D	196D	92C	4D	57D	5B	158D	–	–	–	–	–
95A	–	88D	73D	196D	35D	4C	51D	5B	158A	–	–	–	–	–
95A	–	162D	56B	118C	156D	–	–(+)	161A	–(+)	–	–	–(+)	–	–
95A	–	–	–	202C	–	–(+)	87D	–	–	144D	115C	–	173D	111B

TABLE IV

DETECTION OF SOME TERTIARY AMINE-N-OXIDES ON THIN-LAYER CHROMATOGRAMS

For detection systems, see Tables V and VI.

Compound	*Detection system*							
	D_1	D_2	D_8	D_{11}	D_{12}	D_{13}	D_{14}	D_{15}
Aliphatic-N-oxides								
Chlorpromazine	102B	-	-	39C	(+)	39C	-	112A
Chlorpromazine-N-oxide	102B			39C	(+)	39C		112A
Dimethylamphetamine (+-isomer)	-	-	-	-		-	-	112A
Dimethylamphetamine (−-isomer)	-	-	-	-	-	-	-	112A
Dimethylamphetamine-N-oxide	-		-	-(+)	111D	-		153D
Alicyclic N-oxides								
Codeine	(+)	-	-		-	-	-	111B
Codeine-N-oxide	(+)	185A	-	(+)	-		-	-(+)
Tropine	-(+)	-	-		-	-	-	92C
Tropine-N-oxide	-(+)	-	-	(+)	-	-	-	92C
Atropine	-(+)	-	-	-(+)	-		-	111B
Atropine-N-oxide	(+)	-	-		115D	-	-	-
Hyoscine	(+)	-	-	-	-	-	-	112A
Hyoscine-N-oxide	-(+)	-	-	-	115D	-	-	-
Nicotine	102B	86A	-	4B	-	121C	-	92C
Nicotine-1′-N-oxide	95A	185A	-(+)	-(+)	115C	39D		111B
Phendimetrazine	-	-	-		-	-	-	-
Phendimetrazine-N-oxide	102B	155A	-	-(+)	115D		-	111B
Arylamine-N-oxides								
N,N-Dimethylaniline	102B		-	43C		87C	-	-
N,N-Dimethylaniline-N-oxide	102B	-	87D	177D	115C	-(+)	173D	111B
N,N-Diethylaniline	102B	-	-	43C	-	87C	-	-(+)
N,N-Diethylaniline-N-oxide	102B	-	87D	177D	115C	-	(+)	111B
Heterocyclic N-oxides								
2,2′-Bipyridyl	102B	102B	-	-(+)	49B	-	186D	-
2,2′-Bipyridyl-mono-N-oxide	95A	-	-	-(+)		-	-	-
2,2′-Bipyridyl-1,1′-di-N-oxide	95A	102B		-	-	-	-	-
Quinoxaline	-	-	-	-	-	-	-	-
Quinoxaline-N-oxide	102B	185A	-	-(+)	-	-	-	-

give a colour with pyridine-N-oxides[10]. The use of TCNE was first described for the detection of aromatic compounds in paper chromatography[15] and its use as a chromogenic reagent in TLC has subsequently been reported[16,17]. The use of reduced sodium nitroprusside has been described for the detection of aromatic amine N-oxides[4]. The use of these reagents was extended to the detection of a wide range of N-oxidation products which were of specific interest in the biological work carried out in this laboratory.

Where necessary, the use of each detection system is described separately in order to simplify the discussion.

Ultraviolet light, D_1 and D_2

After running the plates they were air dried and examined under UV light of wavelength 254 and 366 nm (using a portable Hanovia UV lamp). As indicated in Table II, at 254 nm the pyridine-N-oxides had a characteristic colour which was

TABLE V

DETECTION SYSTEMS USED FOR THE IDENTIFICATION OF A SERIES OF HETEROCYCLIC AND AROMATIC AMINES AND THEIR N-OXIDES

Detection system	*Components*
D_1	Ultraviolet light, 254 nm
D_2	Ultraviolet light, 366 nm
D_3	Iron(III) chloride (Fe, 2% aqueous solution)
D_4	Potassium permanganate (Mn, 0.1% aqueous solution)
D_5	2,6-Dichloro-*p*-benzoquinone-4-chlorimine (DCQ, 0.2% in ethanol)
D_6	2,6-Dibromo-*p*-benzoquinone-4-chlorimine (DBQ, 0.4% in ethanol)
D_7	4-Dimethylaminobenzaldehyde (DAB, 0.33%) in 50% HCl (Ehrlich reagent)
D_8	4-Dimethylaminocinnamaldehyde (DAC, 0.2% in 1 *M* HCl (50 ml) and ethanol (50 ml)
D_9	Diazotised 4-nitroaniline (DpNA, 0.5% in 2 *M* HCl (5 ml) mixed with 5% aqueous sodium nitrite (0.5 ml)
D_{10}	Picryl chloride (PC, 1%) in ethanol, sprayed and chromatogram placed into a chamber with ammonia
D_{11}	Tetracyanoethylene (TCNE, 0.5% in ethyl acetate)
D_{12}	Reduced sodium nitroprusside (SN), 1.2% aqueous solution (10 ml) reduced with sodium borohydride to give a clear, deep red solution and 0.8 ml of acetic acid (1 *M*) added. After 2 min, 5 ml of water added
D_{13}	3-Methyl-2-benzothiazolone hydrazone hydrochloride (MBTH, 0.35% methanolic solution) and iron(III) chloride solution (1% solution in 0.5 *M* HCl)
D_{14}	Iron(II) thiocyanate (FTC) reagent. Iron fillings (100 mg) were added to 25 ml of a 4% ammonium iron(II) sulphate solution in 0.5 *M* H_2SO_4. After 15 min, the supernatant was added to a 1.33% solution of ammonium thiocyanate in acetone
D_{15}	Cobalt thiocyanate (CTC), 10% aqueous solution

TABLE VI

ROYAL HORTICULTURAL SOCIETY COLOUR CHART (IN ABBREVIATED FORM) USED TO RECORD COLOURS

Code	*Colour*	*Code*	*Colour*
1A– 13D	Yellow	155A–155D	White
14A– 23D	Yellow-orange	156A–156D	Greyed white
24A– 29D	Orange	157A–157D	Green-white
30A– 35D	Orange-red	158A–158D	Yellow-white
36A– 56D	Red	159A–159D	Orange-white
57A– 74D	Red-purple	160A–162D	Greyed yellow
75A– 79D	Purple	163A–177D	Greyed orange
80A– 82D	Purple-violet	178A–182D	Greyed red
83A– 88D	Violet	183A–187D	Greyed purple
89A– 98D	Violet-blue	188A–198D	Greyed green
99A–110D	Blue	199A–199D	Greyed brown
111A–124D	Blue-green	200A–200D	Brown
125A–143D	Green	201A–201D	Grey
144A–154D	Yellow-green	202A–202D	Black

different from that of the parent pyridines. Coupled with the difference in R_F values of these two groups of compounds, examination under UV light at 254 nm can be used for the identification of amounts of the N-oxides as small as 5 μg. However,

it is difficult to use UV light of 254 nm for the identification of the tertiary anilines and their N-oxides because (a) the colour under UV light is not sufficiently different to aid identification and (b) about 50 μg are required for positive detection of the N-oxide. With the arylamine-N-oxide the N–O bond tends to withdraw electrons from the ring, thereby reducing its aromaticity and consequently its UV absorption. Conversely, the N–O bond of the pyridine-N-oxide series tends to increase the aromaticity of the ring, enhancing the UV absorption [log ε (methanol) for pyridine = 3.62; log ε (methanol) for pyridine-N-oxide = 4.15 (refs. 10 and 18)].

Dimethylaminobenzaldehyde (DAB) and dimethylaminocinnamaldehyde (DAC), D_7 and D_8

As expected, DAB was not found to be a particularly useful reagent for the detection of the pyridine series; only those pyridines with amino, acetamido and hydroxy substituents gave a yellow colour. The primary anilines all gave a yellow colour immediately on spraying, while the secondary anilines developed a colour only on heating the plate at 100° for 5 min. This treatment did not, however, develop any coloured products with the tertiary anilines and only a faintly discernible reaction with their N-oxides was evident.

DAC did not give a characteristic colour with the pyridine series using TLC. However, after paper chromatography, spraying with DAC produced a red-violet colour which developed after 24 h with all the pyridine-N-oxides (and the tertiary aniline-N-oxides) but not with the parent bases. A red colour developed immediately after spraying the primary anilines with DAC on aluminium TLC plates*. The secondary anilines and the tertiary aniline-N-oxides gave this colour only on heat treatment (100° for 5 min), whereas the tertiary anilines failed to form coloured products. In this way, DAC proved to be a selective detection reagent for the tertiary aniline-N-oxides.

Both DAB and the vinylogue DAC have aldehyde functional groups which are believed to form coloured condensation products with available hydrogen atoms of the amino group, and tertiary anilines therefore do not react. However, the tertiary aniline-N-oxides do yield coloured complexes after treatment under acidic conditions and it may be that dye formation proceeds following oxidative N-dealkylation.

Tetracyanoethylene (TCNE), D_{11}

TCNE formed yellow complexes with all of the pyridine bases but failed to give any colour with the corresponding N-oxides. The tertiary aniline-N-oxides all gave brilliant colours on spraying with TCNE, which further intensified on standing although the corresponding tertiary anilines (with the exception of N-ethyl-N-methylaniline and N-ethyl-N-methyl-4-iodoaniline) failed to produce any colour until a few hours later. The primary anilines and most of the secondary anilines did not give a colour immediately on spraying with TCNE. It is clear that TCNE is a useful selective chromogenic reagent for detecting tertiary aniline-N-oxides. The red colour that developed with the two tertiary anilines previously mentioned was clearly different from that of their respective N-oxides and did not interfere with identification.

* The colours described for DAC treatment were obtained on commercially available aluminium TLC plates, but not on laboratory-prepared glass TLC plates. It seems that a metal catalyst is necessary for the chromogenic reactions.

Reduced sodium nitroprusside (SN), D_{12}

The sodium nitroprusside spray (according to Ziegler and Pettit[4]) failed to reveal any of the pyridine series or their N-oxides. The primary, secondary and tertiary anilines failed to react with this spray, although the tertiary aniline-N-oxides all gave a characteristic blue colour, indicating its usefulness for this group of N-oxides.

$Fe(D_3)$, $Mn(D_4)$, $DCQ(D_5)$, $DBQ(D_6)$, $DpNA(D_9)$, $PC(D_{10})$, $MBTH(D_{13})$, $FTC(D_{14})$ and $CTC(D_{15})$

With these detection reagents, the most interesting results were as follows. $Fe(D_3)$ produced colours with those pyridines that have amino and hydroxy functional groups. In addition, both quinoline-N-oxide and isoquinoline-N-oxide gave a pale yellow colour. A variety of colours occurred with the primary, secondary and tertiary anilines but none developed with the tertiary aniline-N-oxides. $Mn(D_4)$ produced a yellow colour on a contrasting blue background with most of the pyridine series and their N-oxides. This reagent gave a variety of pale colours with all of the anilines and the tertiary aniline-N-oxides. $DCQ(D_5)$ and $DBQ(D_6)$ proved to be useful chromogenic reagents for differentiating between the primary, secondary and tertiary anilines and tertiary aniline-N-oxides. $PC(D_{10})$ produced positive colours for most of the pyridine bases but no colours with the pyridine-N-oxides. This reagent gave intense yellow colours with the primary anilines, pale yellow colours with the secondary anilines but no positive colours for the tertiary anilines or their N-oxides. $FTC(D_{14})$ and $CTC(D_{15})$ gave greyed orange and blue colours, respectively, with all of the tertiary aniline-N-oxides.

CONCLUSIONS

From Table II, it is apparent that none of the reagents used in this investigation was specific for the detection of the pyridine-N-oxides on TLC plates. However, as discussed previously, examination under UV light at 254 nm coupled with their characteristic R_F values in TLC solvent systems has proved a successful method for the identification of microgram amounts of these N-oxides in biological fluids. DAC, TCNE, SN, FTC and CTC were found to be specific reagents for the detection of the tertiary aniline-N-oxides; DAC and TCNE gave intense colours immediately on treatment with small amounts (5–10 μg) of these N-oxides, whilst SN, FTC and CTC tended to be less sensitive and larger amounts (about 100 μg) on a TLC plate were required for positive detection. These sprays have been found to be useful for the routine detection of these N-oxides in biological fluids. DAC, MBTH and FTC did not detect the aliphatic and alicyclic N-oxides listed in Table IV although CTC was of general use in the detection of both types of amines and their N-oxides. The results obtained with TCNE and SN indicate that they may be of use in the detection of aliphatic and alicyclic N-oxides.

ACKNOWLEDGEMENTS

The authors thank Mr. Maurice Smith for useful discussions during the preparation of this manuscript. They gratefully acknowledge the support of the Science

Research Council, Chelsea College (University of London) and The Council for Tobacco Research, U.S.A. (Grant No. 984).

REFERENCES

1 L. Reio, *J. Chromatogr.*, 88 (1974) 119.
2 M. Kiese, *Methemoglobinemia: A Comprehensive Treatise*, CRC Press, Cleveland, Ohio, 1974.
3 J. H. Ross, *Anal. Chem.*, 40 (1968) 2138.
4 D. M. Ziegler and F. H. Pettit, *Biochem. Biophys. Res. Commun.*, 15 (1964) 188.
5 J. D. Phillipson and S. R. Hemingway, *J. Chromatogr.*, 105 (1975) 163.
6 S. S. Handa, *Ph.D. Thesis*, University of London, 1976.
7 J. W. Bridges, J. W. Gorrod and D. V. Parke (Editors), *Biological Oxidation of Nitrogen in Organic Molecules*, Taylor & Francis, London, 1972.
8 J. W. Gorrod (Editor), *Proceedings of the 2nd International Symposium on the Biological Oxidation of Nitrogen in Organic Molecules*, Elsevier, Amsterdam, 1978.
9 L. H. Patterson, L. A. Damani, M. R. Smith and J. W. Gorrod, in J. W. Gorrod (Editor), *Proceedings of the 2nd International Symposium on the Biological Oxidation of Nitrogen in Organic Molecules*, Elsevier, Amsterdam, 1978, p. 213.
10 L. A. Damani, *Ph.D. Thesis*, University of London, 1977.
11 L. H. Patterson, *Ph.D. Thesis*, University of London, 1978.
12 M. R. Smith, *Ph.D. Thesis*, University of London, 1978.
13 E. Ochiai, *Aromatic Amine Oxides*, Elsevier, Amsterdam, 1967.
14 J. C. Craig and K. K. Purushothaman, *J. Org. Chem.*, 35 (1970) 5.
15 D. S. Tarbell and T. Huang, *J. Org. Chem.*, 24 (1959) 887.
16 G. F. Macke, *J. Chromatogr.*, 36 (1968) 537.
17 J. H. Ross, *Anal. Chem.*, 42 (1970) 564.
18 S. Ghersetti, G. Maccagnani, A. Mangini and F. Montanari, *J. Heterocycl. Chem.*, 6 (1969) 859.

Journal of Chromatography, 155 (1978) 349–353

CHROM. 10,888

DIRECT GAS–LIQUID CHROMATOGRAPHIC ANALYSIS OF SOME PYRIDINE-N-OXIDES

J. W. GORROD and L. A. DAMANI*

Department of Pharmacy, Chelsea College, University of London, Manresa Road, London SW3 6LX (Great Britain)

(Received December 23rd, 1977)

SUMMARY

A method is described for the direct gas–liquid chromatography of some pyridine-N-oxides. A method for the selective extraction of pyridine-N-oxides from biological materials is also described. The use of these techniques has permitted the development of sensitive methods for the detection and quantitation of alkyl- and halogen-substituted pyridine-N-oxides formed as metabolites in *in vitro* or *in vivo* metabolic studies.

INTRODUCTION

Tertiary aliphatic and aromatic N-oxides are thermolabile and have been shown to break down or rearrange during gas–liquid chromatography (GLC). Chlorpromazine-N-oxide is degraded to chlorpromazine, desmonomethylchlorpromazine and N-allyl-2-chlorophenothiazine[1]. Biological samples are usually examined for the presence of thermolabile N-oxides by the method of Beckett *et al.*[2]. This involves exhaustive extraction of the parent tertiary amine using an organic solvent in which the N-oxide is insoluble. The N-oxide, which is left in the aqueous phase, can then be assayed by GLC after reduction to the tertiary amine using titanium(III) chloride.

Heteroaromatic N-oxides are usually more stable than aliphatic or aromatic N-oxides[3]. Metabolic studies in which aromatic heterocyclic N-oxidation has been studied have almost always used radiochemical techniques[4,5]. The N-oxide group in pyridine-N-oxide and related compounds is quantitatively reduced by titanium(III) chloride[6] and the assay method for N-oxides developed by Beckett *et al.*[2] has been used in a study on the *in vivo* metabolism of pyrazines and some alkylpyridines[7]. Because of the inherent stability of aromatic heterocyclic N-oxides, the use of direct analysis by GLC was attempted in this study.

* Present address: Laboratory of Toxicology and Pharmacokinetics, U.C.H. Medical School, London, WC1E 6JJ, Great Britain.

MATERIALS AND METHODS

The substituted pyridines were commercial products from Aldrich (Gillingham, Great Britain) and Koch-Light (Colnbrook, Great Britain). Pyridine-N-oxides were synthesised by methods previously described[8]. Solutions of the compounds were prepared in redistilled ethanol (10 mg per 10 ml of ethanol) and stored at 0° until required. The compounds were stable under these conditions, giving one spot when examined by thin-layer chromatography at intervals.

N-Oxide solutions (1–2 μl) were examined by GLC, at different oven and injection port temperatures, using different nitrogen flow-rates, and using a Hamilton (10-μl) syringe equipped with either a short (5 cm) or a long (7 cm) syringe needle. Peak shapes were noted and the retention times of the peak or peaks were recorded. If more than one peak was obtained after the injection of an N-oxide solution, breakdown of the N-oxide to the parent pyridine was presumed. This was confirmed by injecting the parent pyridine and comparing its retention time (R_T) with the second peak (breakdown peak) obtained during N-oxide chromatography. Attempts were made to reduce breakdown to a minimum by lowering the injection port temperature, using a 7-cm long syringe needle and performing quick on-column injections. These procedures were adopted in order to minimize contact of hot metal with the pyridine-N-oxides.

Gas–liquid chromatography

A Perkin-Elmer F33 gas chromatograph, equipped with a flame-ionization detector and a 0–2.5-mV Perkin-Elmer 56 chart recorder, was used. Pre-coiled glass columns, 0.63 cm O.D., were packed as follows: column A, 1 m, 2% Carbowax 20M + 5% potassium hydroxide on 80–100-mesh AW, DMDCS-treated Chromosorb W; column B, 1 m, 3% OV-17 on 80–100-mesh AW, DMDCS-treated Chromosorb G. The columns were conditioned for 48 h at a temperature 10° higher than the proposed maximal operating temperature. Column B was silanized *in situ* with 3 × 5 μl of hexamethyldisilazane before use. Gas pressures were nitrogen 20 p.s.i. (140 kN/m^2), hydrogen 17 p.s.i. (119 kN/m^2) and air 25 p.s.i. (175 kN/m^2).

Gas–liquid chromatography–mass spectrometry (GLC–MS)

Combined GLC–MS was performed with a VG 12F mass spectrometer linked to a Pye 104 gas–liquid chromatograph, with a glass column (1 m × 0.64 cm O.D.) packed with 2% Carbowax 20M + 5% potassium hydroxide on 80–100-mesh AW, DMDCS-treated Chromosorb W; helium was used as the carrier gas (20–30 ml/min), the oven temperature was 200° and the ionization potential was 70 eV.

Extraction of pyridine-N-oxides from biological materials

Typical microsomal incubates[9] (3.5 ml) or urine samples[13] (3.5-ml aliquots) were rendered alkaline (0.5 ml of 1.0 *N* sodium hydroxide solution) and made up to 5.5 ml by the addition of water (1 ml) and the appropriate internal standard (another N-oxide) (0.5 ml). Aliquots (4 ml) were transferred on to sodium chloride (1 g) in screw-capped tubes (10 ml, Sovirel SVL) and extracted with three 5-ml volumes of freshly double-distilled diethyl ether, the organic phases being discarded. The aqueous phases were extracted with three 5-ml volumes of dichloromethane. The combined dichloro-

methane extracts were collected in evaporating tubes[10] and concentrated to about 10 μl in a water-bath (45°). Aliquots (1–2 μl) were injected on to GLC column A with the oven temperature at 170° and the injection port temperature at 225°, using a syringe equipped with a 7-cm needle. Peak-height ratios of the test compound to the internal standard were measured, and this allowed the determination of pyridine-N-oxide in the original 3.5-ml sample, using previously constructed calibration graphs (see below).

Calibration graphs

Alkyl- or halogen-substituted pyridine-N-oxides (10–100 nmole) in water (1 ml) were added to typical microsomal incubates (3.5 ml) or urine (3.5 ml). Any enzymic activity was terminated by tne addition of 0.5 ml of 1.0 *N* sodium hydroxide solution. The appropriate internal standard (50 nmole of another pyridine-N-oxide in 0.5 ml of water) was then added and 4-ml aliquots were processed as described above. Peak-height ratios of the pyridine-N-oxide to the internal standard were plotted against the concentration of the pyridine-N-oxide.

RESULTS AND DISCUSSION

The pyridine-N-oxides showed partial decomposition to the parent pyridines using column A or B when the oven and injection port temperatures were high (>250°), and when the injection was performed using a syringe equipped with a short (5-cm) needle. The pyridines were eluted very close to the solvent front and their identities were confirmed by comparison of their retentions times with those of authentic compounds, and by combined GLC–MS. However, careful control of the various parameters, for example, a low injection port temperature and performing quick on-column injections on to an all-glass-lined inlet system in the gas–liquid chromatograph, reduced the breakdown of these N-oxides to a minimum, as shown in Fig. 1.

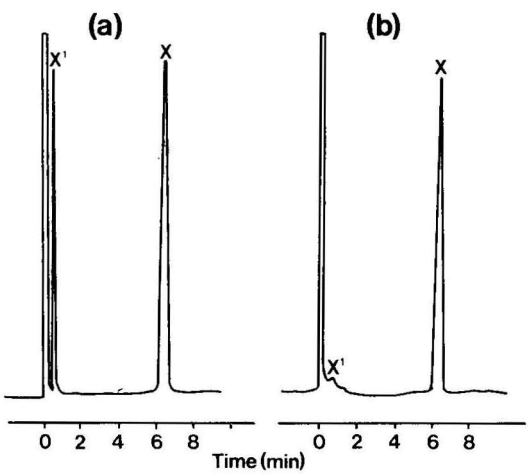


Fig. 1. Chromatograms of 3-ethylpyridine-N-oxide (X) using column A. X^1 represents the thermal breakdown product 3-ethylpyridine. (a) Oven temperature 170°, injection port temperature 300°; (b) oven temperature 170°, injection port temperature 225°.

Column B was found to produce tailing peaks, although it was useful for the direct GLC of several alkyl-, halogen-, acetyl- and cyano-substituted pyridine-N-oxides. This column was also useful for the direct GLC examination of quinoline- and isoquinoline-N-oxides[11]. The identities of the peaks were confirmed by combined GLC–MS (see below).

Column A was found to be of most use in the direct GLC analysis of alkyl- and halogen-substituted pyridine-N-oxides. This column was operated at lower oven and injection port temperatures, and the peaks did not show any significant tailing (see Fig. 2). The identities of the GLC peaks of pyridine-N-oxides were confirmed by GLC–MS, as shown in Fig. 2. For example, GLC–MS of peak 2 (pyridine-N-oxide) gave the molecular ion (*m/e* 95) as the base peak and the diagnostic $M^{+} - 16$ ion at *m/e* 79. Similarly, GLC–MS of peak 5 (3-ethylpyridine-N-oxide) gave the molecular ion (*m/e* 123) as the base peak and the $M^{+} - 16$ ion at *m/e* 107. Details of the MS and GLC–MS of pyridine-N-oxides have been described elsewhere[9,11].

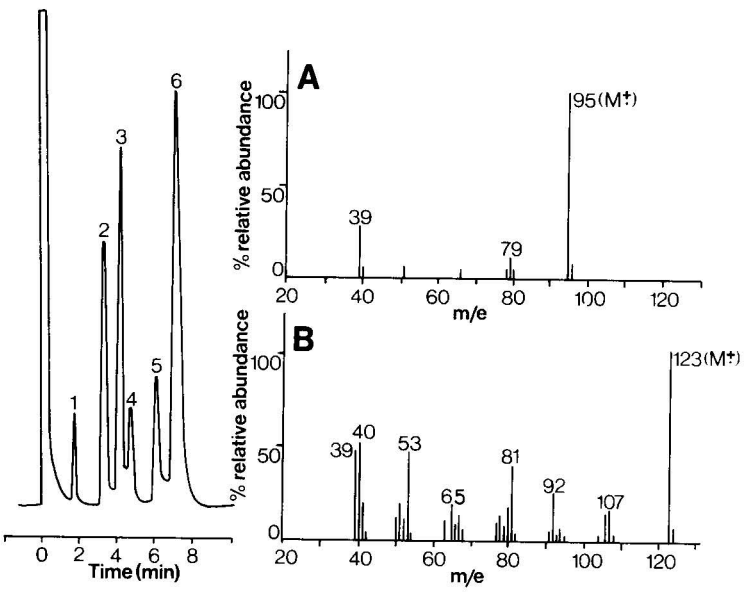


Fig. 2. GLC of 3-fluoropyridine-N-oxide (1), pyridine-N-oxide (2), 3-chloropyridine-N-oxide (3), 3-methylpyridine-N-oxide (4), 3-ethylpyridine-N-oxide (5) and 3-bromopyridine-N-oxide (6). A = GLC–MS of pyridine-N-oxide (peak 2); B = GLC–MS of 3-ethylpyridine-N-oxide (peak 5).

When pyridine-N-oxides were extracted from microsomal incubates or from urine from different animal species and analysed on column A, the normally occurring constituents of microsomes or urine did not interfere in the analysis. However, these constituents interfered with pyridine-N-oxide assay on column B.

Linear and reproducible calibration graphs were obtained in the range 10–500 nmole; regression analysis of the data gave correlation coefficients of not less than 0.999. The retention times of some 3-substituted pyridine-N-oxides are recorded in Table I; column A is also useful for the direct GLC analysis of 2- and 4-alkyl- and halogen-substituted pyridine-N-oxides.

The direct GLC of N-oxides is obviously desirable. This work shows that this

TABLE I

GLC SEPARATION OF SOME 3-SUBSTITUTED PYRIDINES AND THEIR N-OXIDES

Column A: Carbowax 20M–KOH–Chromosorb W (2:5:93); 1-m glass column, N_2 = 20 p.s.i. (140 kN/m²); oven temperature = 170°; injection temperature = 225°. Column B: 3% OV-17 on Chromosorb G (80–100 mesh); 1-m glass column; N_2 = 20 p.s.i. (140 kN/m²); oven temperature = 195°; injection temperature = 250°.

Parent compound	*Retention time (min)*			
	Base		*N-Oxide*	
	Column A	*Column B*	*Column A*	*Column B*
Pyridine	0.3	—	3.5	1
3-Methylpyridine	0.5	—	4.5	1.5
3-Ethylpyridine	0.6	—	6.0	2.0
3-Fluoropyridine	0.2	—	2.0	0.7
3-Chloropyridine	0.5	—	4.0	1.0
3-Bromopyridine	0.7	—	7.5	1.5
3-Acetylpyridine	1.0	—	—	3.5
3-Cyanopyridine	0.6	—	—	2.5

is possible if they are sufficiently thermostable and volatile, and if they can be extracted from aqueous media with a suitable organic solvent. The direct GLC of nicotine-1′-N-oxide using capillary columns has been described[12]. However, this allows only qualitative analysis as some thermal breakdown to nicotine and other products also occurs.

The work reported here has allowed the development of methods for the direct detection and quantitation of pyridine-N-oxides formed in biological systems. Our experience indicates that careful manipulation of instrumental conditions may be necessary to ensure minimal decomposition.

ACKNOWLEDGEMENTS

This work was supported by Grant No. 984 from The Council for Tobacco Research, U.S.A. L.A.D. thanks Chelsea College (University of London) for the award of a scholarship. The GLC–MS data was obtained in collaboration with Dr. D. A. Cowan (Chelsea College).

REFERENCES

1 J. C. Craig, N. Y. Mary and S. K. Roy, *Anal. Chem.*, 36 (1964) 1142.
2 A. H. Beckett, J. W. Gorrod and P. Jenner, *J. Pharm. Pharmacol.*, 23 (1971) 55S.
3 E. Ochiai, *Aromatic Amine Oxides*, Elsevier, Amsterdam, 1967.
4 S. Chaykin and K. Block, *Biochim. Biophys. Acta*, 31 (1959) 213.
5 V. Neuhoff and F. Köhler, *Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmakol.*, 254 (1966) 301.
6 R. T. Brooks and P. D. Sternglanz, *Anal. Chem.*, 31 (1959) 561.
7 G. Hawksworth and R. R. Scheline, *Xenobiotica*, 5 (1975) 389.
8 E. Ochiai, *J. Org. Chem.*, 18 (1953) 534.
9 D. A. Cówan, L. A. Damani and J. W. Gorrod, in preparation.
10 A. H. Beckett, *Dan. Tidsskr. Farm.*, 40 (1966) 197.
11 L. A. Damani, *Ph.D. Thesis*, University of London, 1977.
12 A. Pilotti, C. R. Enzell, H. McKennis, E. R. Bowman, E. Dufra and B. Holmstedt, *Beitr. Tabakforsch.*, 8 (1976) 339.
13 L. A. Damani, L. G. Disley and J. W. Gorrod, in J. W. Gorrod (Editor), *Biological Oxidation of Nitrogen*, Elsevier/North-Holland, Amsterdam, 1978.

Journal of Chromatography, 155 (1978) 355–361

CHROM. 10,959

GAS CHROMATOGRAPHY OF NUCLEOTIDES AND NUCLEOSIDES OF CYTOSINE

KARL H. SCHRAM*, Y. TANIGUCHI and JAMES A. McCLOSKEY**

Departments of Biopharmaceutical Sciences and Biochemistry, University of Utah, Salt Lake City, Utah 84112 (U.S.A.)

(Received December 22nd, 1977)

SUMMARY

3,N^4-Etheno-O-persilyl derivatives of nucleotides and nucleosides of cytosine have been studied as derivatives for gas chromatography and mass spectrometry. Ethenylation blocks further derivatization of the base and therefore precludes chromatographic problems associated with trimethylsilylation at position N^4 of cytosine. The mixed derivatives exhibit satisfactory gas chromatographic properties and produce molecular ions of greater abundance than in the case of the corresponding trimethylsilyl derivatives. Relative retention times and mass spectral data from three nucleotides and three nucleosides are presented. Reaction with chloroacetaldehyde to form etheno derivatives is selective for cytosine and adenine bases, and so the reaction is potentially useful for characterization of nucleotides or nucleosides of unknown structure.

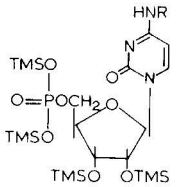
INTRODUCTION

The applications of gas chromatography (GC) and of GC–mass spectrometry (MS) to constituents of nucleic acids have been substantially hampered by the high polarity and chemical instability of nucleotides and nucleosides of cytosine. Although trimethylsilylation generally produces sufficiently volatile derivatives of nucleotides and nucleosides for GC[1,4], previous attempts to obtain a peak from cytidine 5′-monophosphate (as the derivative 1) and related nucleotides have failed[2,5–7]. The trimethylsilyl (TMS) derivative of the corresponding nucleoside cytidine produces a broad, low peak[3,5,8–10], and under some conditions two peaks[7,11]. The trifluoroacetyl-TMS derivative 2 is easily prepared on a micro scale and is suitable for MS but is insufficiently volatile for GC, while the derivative of cytidine is amenable to GC[12]. Likewise, the mixed methoxime–TMS derivative (3) which can be successfully applied to cytidine produced no GC peak from cytidine 2′-monophosphate[6]. Another generally

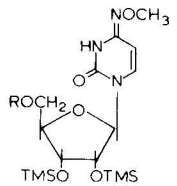
* Present address: Department of Medicinal Chemistry, University of Houston, Houston, Texas 77004, U.S.A.

** To whom correspondence should be addressed.

satisfactory alternative for GC of cytidine is N,O-permethylation[13], which is, however, not applicable to the corresponding nucleotide.

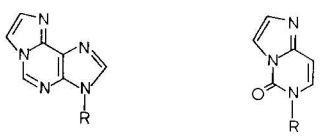


1, R = TMS
2, R = $COCF_3$

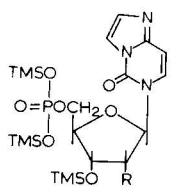


3, R = TMS or $PO(OTMS)_2$

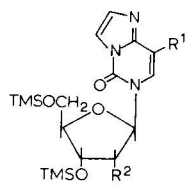
In view of the notable absence of a derivative suitable for the GC of nucleotides of cytosine, we sought a new derivative that can be prepared on a micro scale, exhibits good chromatographic and MS properties, and can be applied to both nucleotides and nucleosides of cytosine. Led by earlier work of Kochetkov *et al.*[14], and by Leonard and co-workers[15,16] on synthesis of etheno derivatives of the general types 4, we have developed a micro scale procedure for preparation of the etheno-TMS derivatives 5–10. These results provide the first successful gas chromatograms of nucleotides of cytosine derivatives (5, 6, 10), and show that etheno-TMS derivatives are useful alternatives for the GC and MS of cytosine-containing nucleosides.



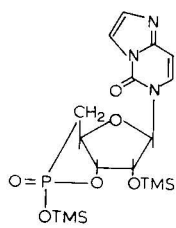
4, R = H or ribosyl



5, R = OTMS
6, R = H



7, R^1 = H, R^2 = OTMS
8, R^1 = R^2 = H
9, R^1 = CH_3, R^2 = H



10

EXPERIMENTAL

Preparation of 3,N^4-etheno derivatives

Experiments were conducted using sample sizes of 20 μg and 1 mg. Chloracetaldehyde (45%, ICN Pharmaceuticals, Irvine, Calif., U.S.A.) was adjusted to pH 4 using sodium hydroxide; 100 μl was added to 1 mg (or 20 μg) of nucleotide or nucleoside and stirred for 12 h at room temperature. The water was removed *in vacuo* and remaining chloroacetaldehyde was removed by trituration with ether. Approximately 50 μg (or the entire product when starting with 20 μg) of the residue was reacted with 40 μl of N,O-bis(trimethylsilyl)trifluoroacetamide (Regis, Chicago, Ill., U.S.A.) and 1% trimethylchlorosilane in 10 μl of dry pyridine at 100° for one hour in a PTFE-lined screw cap vial. Sample equivalent to 0.5–4 μg was submitted to GC or MS.

Gas chromatography

A Varian 2100 gas chromatograph with flame-ionization detector and 6 ft. × 0.25 in. silanized glass columns was used, with 1% OV-17 coated on 100–200 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). All runs were temperature programmed at 4°/min with carrier gas flow-rate of 20 ml/min using sample sizes in the range 0.5–4 μg. GC–MS measurements (see below) were made using a 3 ft. × 0.25 in., 1% OV-17 column.

Methylene unit (MU) values[17] were obtained as described earlier[18] using two *n*-alkanes as internal standards, one earlier and one later-eluting than the component of interest. Sample injections were made at column temperatures 50° lower than the elution temperature of the smaller alkane. MU values were derived by linear interpolation of distances on the recorded chromatogram.

Mass spectrometry

Mass spectra were acquired using an LKB 9000S instrument with ion source temperatures 270°, separator temperature 280°, and ionizing energy 70 eV. Compound 8 was introduced by direct probe after removal of reagents by the probe vacuum system; all others were introduced by gas chromatograph.

Exact mass measurements made on the derivative of cytidine 3′,5′-cyclic monophosphate were done by peak matching at $M/\Delta M = 20{,}000$ using a Varian MAT 731 mass spectrometer, with sample introduction by direct probe.

RESULTS AND DISCUSSION

Gas chromatograms produced by etheno derivatives of the three nucleotides 5, 6, and 10 are shown in Fig. 1, and provide the first successful chromatograms of nucleotides of cytosine. Peak shapes were satisfactory over the range of sample size examined (0.5–4 μg). The greatest tailing observed was that exhibited in Fig. 1a. Low molecular weight artifacts related to the reagent were typically observed to elute shortly after the solvent front, as seen in Fig. 1.

The derivatives were examined by MS to confirm the expected structures as 3,N^4-etheno-O-persilyl derivatives and to examine the utility of MS for characterization of etheno derivatives of cytosine. Mass spectra of the etheno-TMS derivatives

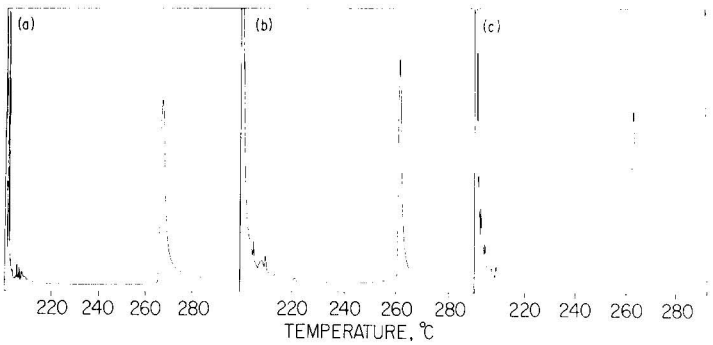


Fig. 1. Gas chromatograms of the 3,N⁴-etheno-TMS derivatives of (a) cytidine 5′-monophosphate, (b) cytidine 3′,5′-cyclic monophosphate, (c) 2′-deoxycytidine 5′-monophosphate.

5 and 10 are presented in Figs. 2 and 3, and principal ions from the spectra of the remaining derivatives are represented in Table I.

Assignments of the major ions largely follow those of trimethylsilylated nucleotides[19] and nucleosides[20]. In addition, Chheda *et al.*[21] have published the mass spectrum of 1,N⁶-ethenoadenosine and of the TMS derivative of a related compound. In every case the molecular ion (M) and $M-CH_3$ values are consistent with the presence of an etheno function in the base, and fully silylated ribose or ribose phosphate. Ions of the base series are of relatively low abundance with the exception of compound 9 (see Table I), while ions of the sugar series predominate in the spectra of ribonucleotides (*m/e* 169), the deoxyribonucleotide 6 (*m/e* 81), and nucleosides (*m/e* 103 from C-5′)[19,20]. The fragment ion at base + 41 a.m.u. that is characteristic

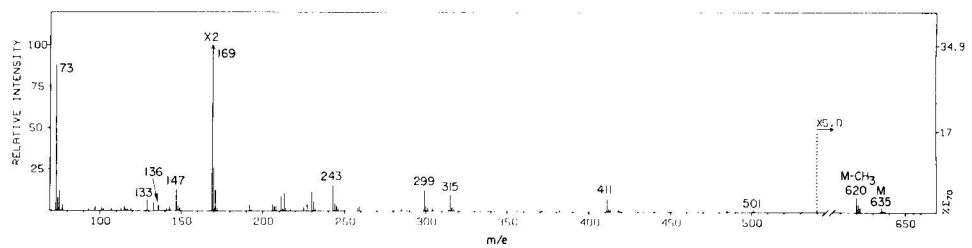


Fig. 2. Mass spectrum of the trimethylsilyl derivative of 3,N⁴-ethenocytidine 5′-monophosphate.

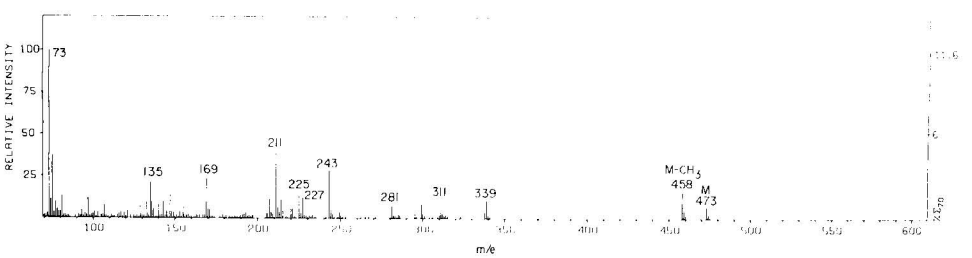


Fig. 3. Mass spectrum of the tris(trimethylsilyl) derivative of 3,N⁴-ethenocytidine 3′,5′-cyclic monophosphate.

TABLE I

SELECTED IONS FROM THE MASS SPECTRA OF ETHENO-TMS DERIVATIVES OF NUCLEOTIDES AND NUCLEOSIDES

Parent compound	*No. TMS groups*	*m/e (relative intensity)*						
		M	*M−CH₃*	*Base + H*	*Base + 2H*	*Other ions*		
Cytidine	3	483 (4.9)	468 (2.6)	135 (3.1)	136 (1.1)	349 (5.0	103 (34)	73 (100)
2′-Deoxycytidine	2	395 (3.5)	380 (0.5)	135 (11.5)	136 (8.8)	261 (4.7)	103 (100)	73 (70)
5-Methyl-2′-deoxy-cytidine	2	409 (16)	394 (1.5)	149 (37)	136 (5.7)	261 (1.9)	103 (100)	73 (75)
2′-Deoxycytidine 5′-monophosphate	3	547 (0.6)	532 (0.3)	135 (1.6)	136 (2.2)	413 (1.6)	299 (3.0)	81 (100)
Cytidine*	0	267 (3.9)		135 (100)	136 (20)	178 (1.6)	148 (2.6)	133 (3.2)
Adenosine	3	507 (6.7)	492 (2.5)	159 (4.4)	160 (6.9)	259 (21)	103 (29)	73 (100)

* Compound 4; R — ribosyl.

of cytidine analogs is absent from all mass spectra, as required by its mechanism of formation and the presence of an imino, rather than amino, group at C-4 (ref. 22). Molecular ion abundances of all etheno-TMS derivatives are markedly greater than in the corresponding TMS derivatives, suggesting their use for selected ion monitoring or quantitative applications of MS. For example the spectrum of cytidine 5′-monophosphate-$(TMS)_5$ exhibits no molecular ion[19] (*vs.* 0.3% in Fig. 1), while that of cytidine-$(TMS)_4$ is 1.3%[22] (*vs.* 6% in Table I).

The mass spectrum of the TMS derivative of 3,N^4-ethenocytidine 3′,5′-cyclic-monophosphate (10) shown in Fig. 2 shares numerous sugar-derived peaks in common with the TMS derivative of adenosine 3′,5′-cyclic monophosphate[19] (*m/e* 169, 211, 225, 227, 243), but exhibits several notable differences. The sugar fragment *m/e* 339 (*s*) predominates over the *s*-H species (*m/e* 338), while the prominent peak at *m/e* 311 in Fig. 3 (*vs. m/e* 310 in the adenosine analog) was shown by measurement of exact mass to result from expulsion of CO from *m/e* 339 (found: 311.0902; calculated: 311.0899 for $C_{10}H_{24}O_5Si_2P$). The significant peak at *m/e* 281, which is somewhat smaller in the case of the adenosine analog, corresponds in exact mass (281.0792) to further loss of CH_2O from the *m/e* 311 ion, presumably from C-5′ (calculated: 281.0795 for $C_9H_{22}O_4Si_2P$). The base + H ion (*m/e* 135) is the only notable member of the base series, in contrast to the adenosine analog in which base-containing ions play a more prominent role.

MU values of the compounds studied are given in Table II, and for comparison some literature values for other derivatives of cytidine and 2′-deoxycytidine are included. The latter data show that the entheno-TMS derivatives are substantially more volatile than the corresponding TMS derivatives and slightly more polar than trifluoroacetyl-TMS derivatives. Success in obtaining a product which is sufficiently stable for GC lies in the inherent stability of the imidazo[1,2-*c*]pyrimidine system and the absence of an active hydrogen or functional group at N^4. By contrast, the N^4-trimethylsilyl function in derivatives of cytidine or cytidylic acid is easily displaced or hydrolyzed, leaving an unprotected and highly polar amino group at C-4 that is detrimental to good chromatographic behavior. In cases when sample quantity is limited and the structure of the component under examination is not known, im-

perfect silylation conditions can often lead to a mixture of derivatives (*e.g.*, ref. 23) in which the base is the site of under-silylation[20]. This problem is reduced or eliminated in the case of etheno derivatives because no labile hydrogen remains on the exocyclic amino group and silylation cannot occur.

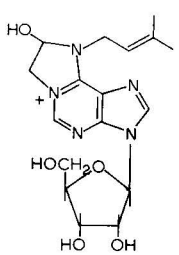
TABLE II

MU VALUES OF DERIVATIVES OF NUCLEOTIDES AND NUCLEOSIDES OF CYTOSINE

Parent compound	*Etheno-TMS*	*TMS**	*Methoxime-TMS***	*Trifluroacetyl-TMS****
Cytidine	27.26	31.73	24.91	26.68
2′-Deoxycytidine	26.97		23.79	26.60
5-Methyl-2′-deoxy-cytidine	27.42			
Cytidine 5′-mono-phosphate	32.57			
Cytidine 3′,5′-cyclic monophosphate	31.35			
2′-Deoxycytidine 5′-monophosphate	32.42			

* 1% OV-17, 4°/min (ref. 18).
** 5% SE-30, program rate not given (ref. 6).
*** 1% OV-17, 4°/min (ref. 12).

In addition to preparation of a derivative which is sufficiently volatile for GC, chemical derivatization, if selective, provides a degree of information in the case of compounds of unknown structure. For example, silylation of 7-methylpurine nucleosides or nucleotides uniquely leads to the 7-methyl-8-oxo derivative[24], and methoxime formation[6] or trifluoroacetylation at N^4 is characteristic of cytidine derivatives[12]. In the present case derivatization is only partly selective in that chloroacetaldehyde reacts to form derivatives of cytidine and adenosine (or their nucleotides), as well as closely related analogs[25,26] but not of the other common nucleosides such as uridine,



11

guanosine, thymidine or inosine[15]. The mass spectrum of the TMS derivative of 1,N^6-ethenoadenosine has been included in Table I, and as expected follows the general behavior of compound 7 and of adenosine-$(TMS)_5$ (ref. 27). In related compounds the ethenylation reaction is blocked by substitution at N-1 of adenine or N-3 of cyosine moieties[15]. A product assigned structure 11 was obtained from N^6-isopentenyladenosine[15], but its polarity presumably precludes GC or MS. Similarly, analogs of

N^4-substituted cytidine presumably do not form derivatives that, after silylation, are detectable by GC or MS. Use of derivatives with specific structural requirements regarding substitution in the base is also potentially useful in establishing the site of substitution when only microgram quantities of material are available.

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Institutes of General Medical Sciences (GM 21584). K.H.S. was recipient of a National Institutes of Health Postdoctoral Fellowship (CA 02466).

REFERENCES

1 R. L. Hancock and D. L. Coleman, *Anal. Biochem.*, 10 (1965) 365.
2 T. Hashizume and Y. Sasaki, *Anal. Biochem.*, 15 (1966) 199.
3 Y. Sasaki and T. Hashizume, *Anal. Biochem.*, 16 (1966) 1.
4 K. H. Schram and J. A. McCloskey, in K. Tsuiji and W. Morowich (Editors), *GLC and HPLC Determination of Therapeutic Agents*, Vol. III, Marcel Dekker, New York, in press.
5 R. L. Hancock, *J. Gas Chromatogr.*, 6 (1968) 431.
7 A. B. Patel and C. W. Gehrke, *J. Chromatogr.*, 130 (1977) 115.
8 V. Miller, V. Pacakova and E. Smolkova, *J. Chromatogr.*, 119 (1976) 355.
9 C. W. Gehrke and A. B. Patel, *J. Chromatogr.*, 123 (1976) 335.
10 J. P. Seiler, *Anal. Biochem.*, 75 (1976) 45.
11 C. W. Gehrke and A. B. Patel, *J. Chromatogr.*, 130 (1977) 103.
12 U. I. Krahmer, J. G. Liehr, K. J. Lyman, E. A. Orr, R. N. Stillwell and J. A. McCloskey, *Anal. Biochem.*, 82 (1977) 217.
13 D. L. von Minden and J. A. McCloskey, *J. Amer. Chem. Soc.*, 95 (1973) 7480.
14 N. K. Kochetkov, V. W. Shibaev and A. A. Kost, *Tetrahedron Lett.*, (1971) 1993.
15 J. R. Barrio, J. A. Secrist, III, and N. J. Leonard, *Biochem. Biophys. Res. Commun.*, 46 (1972) 597.
16 J. A. Secrist, III, J. R. Barrio, N. J. Leonard and G. Weber, *Biochemistry*, 11 (1972) 3499.
17 C. E. Dalgliesh, E. C. Horning, M. G. Horning, K. L. Knox and K. Yarger, *Biochem. J.*, 10 (1966) 792.
18 S. E. Hattox and J. A. McCloskey, *Anal. Chem.*, 46 (1974) 1378.
19 A. M. Lawson, R. N. Stillwell, M. M. Tacker, K. Tsuboyama and J. A. McCloskey, *J. Amer. Chem. Soc.*, 93 (1971) 1014.
20 J. A. McCloskey, A. M. Lawson, K. Tsuboyama, P. M. Krueger and R. N. Stillwell, *J. Amer. Chem. Soc.*, 90 (1968) 4182.
21 G. B. Chheda, S. P. Dutta, A. Mittelman and L. Baczynskyj, *Tetrahedron Lett.*, (1974) 433.
22 J. G. Liehr, D. L. von Minden, S. E. Hattox and J. A. McCloskey, *Biomed. Mass Spectrom.*, 1 (1974) 281.
23 H. Kasai, K. Murao, S. Nishimura, J. G. Liehr, P. F. Crain and J. A. McCloskey *Eur. J. Biochem.*, 69 (1976) 435.
24 D. L. von Minden, R. N. Stillwell, W. A. Koenig, K. J. Lyman and J. A. McCloskey, *Anal. Biochem.*, 50 (1972) 110.
25 K. H. Schram and L. B. Townsend, *Tetrahedron Lett.*, (1974) 1345.
26 K. F. Yip and K. C. Tsou, *Tetrahedron Lett.*, (1973) 3087.
27 J. A. McCloskey, in P. O. P. Ts'o (Editor), *Basic Principles in Nucleic Acid Chemistry*, Vol. I, Academic Press, New York, 1974, Ch. 3.

Journal of Chromatography, 155 (1978) 363–370

CHROM. 10,871

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF POLYCHLORINATED BIPHENYLS

LAURENCE S. KAMINSKY* and MICHAEL J. FASCO

Division of Laboratories and Research, New York State Department of Health, Albany, N.Y. 12201 (U.S.A.)

(First received September 27th, 1977; revised manuscript received January 10th, 1978)

SUMMARY

High-performance liquid chromatography on a reversed-phase, microparticle column (μBondapak C_{18}) employing gradients of water and acetonitrile as the mobile phase has been used to resolve the commercial mixtures of polychlorinated biphenyls (PCBs), Aroclor 1221, 1016 and 1254. Individual PCBs (49) have been chromatographed under similar conditions and used as standards upon which the tentative identification and quantitation of some of the major components of the Aroclors have been based.

INTRODUCTION

The widespread introduction of mixtures of polychlorinated biphenyls (PCBs) into the environment[1] and the subsequent persistence of the more highly chlorinated constituents in animals[2] and man[3] has potentiated numerous investigations into an analytical method for the determination of PCBs. The large number of different PCBs possible (209 + biphenyl), the lack of synthetic samples of all of the PCBs, the presence of other halogenated pesticides as impurities and the low levels of the PCBs in many biological samples combine to hinder development of such an analytical procedure.

Gas chromatographic methods are most frequently utilized for PCB analysis, principally because of the sensitivity of the electron-capture detector (ECD) for chlorinated compounds. Methods have been published on gas chromatographic analysis using single[4] or multiple[5] packed columns and glass capillary columns[6]. The ECD responses of the individual PCBs show great variation and, in the absence of synthetic samples of particular PCBs, quantitation is usually based on predicted responses extrapolated from available PCBs. In many instances the resolution of the components is not sufficient for accurate analysis.

In view of the failure of gas chromatographic methods to yield completely satisfactory analyses of PCBs, the use of high-performance liquid chromatography

* To whom correspondence should be addressed.

(HPLC) presents an alternative procedure worthy of investigation. Only one group has reported on the use of HPLC or high-speed liquid chromatography (HSLC) to separate and quantitate the individual PCB components in commercial mixtures of PCBs[7,8]. A silica gel column which elutes the higher chlorinated PCBs initially was utilized in the normal phase. This system produced a reasonable separation of the lower chlorinated PCBs present predominantly in the commercial mixture Aroclor 1221, but was less efficient in separating the more highly chlorinated PCBs present in Aroclors 1254 and 1260.

The desirability of having an improved analytical procedure for PCBs prompted us to investigate the potential of reversed-phase HPLC. We report here on the analyses of three commercial mixtures of PCBs, Aroclor 1221, 1016 and 1254, and of 49 commercially synthesized individual PCBs.

EXPERIMENTAL

Aroclor 1254, 1016 and 1222 were obtained from Monsanto (St. Louis, Mo., U.S.A.) and were used without further purification or fractionation. Individual PCBs were purchased from Analabs (North Haven, Conn., U.S.A.) or RFR Corp. (Hope, R.I., U.S.A.) and were used as purchased. HPLC analysis of the individual PCBs indicated that the greatest level of impurity was 5% based only on integrated peak areas on the chromatograms. For most of the individual PCBs only a single peak was detected by the HPLC system utilized. The acetonitrile used for eluting the HPLC columns was glass distilled (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.). Water was deionized, glass distilled and filtered through a 0.22-μm membrane (Millipore, Bedford, Mass., U.S.A.) prior to chromatographic use.

A Waters Assoc. Model 244 liquid chromatograph equipped with a Hewlett-Packard Model 3385A automation system (recording integrator) was utilized. All solutions were monitored at 254 nm. The column (30 cm $\times$ 4 mm I.D.) was packed with reversed-phase, microparticle silica (μBondapak C_{18}; Waters Assoc., Milford, Mass., U.S.A.). The solvents used were I, water–acetonitrile (9:1), and II, acetonitrile–water (9:1). All individual PCBs and Aroclors were loaded on to the column in tetrahydrofuran solutions at concentrations of 5 mg/ml. With individual PCBs 1-μl injections were routinely used, and for Aroclor solutions 5-μl injections were used. The elution conditions for the various Aroclor mixtures were varied so as to obtain the best resolution of components with acetonitrile and water as eluents. For Aroclor 1221 elution was initiated with a solvent mixture of 60% of solvent II and 40% of solvent I and the solvent composition was altered in a non-linear gradient (gradient No. 7, Waters Assoc. Model 660 solvent programmer) over a period of 30 min to achieve 100% of solvent II. Elution was continued for a further 6 min. Solvent flow-rates were 2 ml/min throughout. For Aroclor 1016 a solvent mixture of 55% of solvent II and 45% of solvent I (*i.e.*, 54% of acetonitrile) was used isocratically. For Aroclor 1254 the same conditions were used as for Aroclor 1221 but the period of the gradient change was 40 min. A mixture of Aroclor 1254, 1221 and 1016 in the ratio of 1:1:1 (w/w/w) was chromatographed using the conditions developed for Aroclor 1254. The individual PCBs were chromatographed using conditions corresponding to that Aroclor mixture in which their isomeric series was most prominent. The nomenclature used for the individual PCBs is based on that of Brinkman and co-workers[7,8].

Quantitation of all of the Aroclor components that could be so analysed was performed by comparison of integrated peak areas using responses calibrated from weighed samples of individual PCBs.

RESULTS AND DISCUSSION

The absolute retention times and UV detector absorbance responses at 254 nm of 49 individual PCBs are presented in Table I. Detector responses are reported relative to that of biphenyl (arbitrarily set at 1.0). A number of relationships can be derived from the data in Table I and used for predicting the approximate chromatographic behavior of those individual PCBs not yet available synthetically. (i) In general, increasing the chlorine content of PCBs is a major factor in increasing the retention time. Consequently, isomeric groups tend to be eluted at similar times. (ii) Chloro substituents on PCBs in the 2- or 6- (*ortho*) positions decrease retention times relative to PCBs without such substituents, and this effect is enhanced when *o*-chlorosubstituents are on opposite phenyl rings (*e.g.*, the retention times of 2,2′-dichloro- and 2,6-dichlorobiphenyl are 10.24 and 11.09 min, respectively). The effect of two or more *o*-chloro substituents on a PCB is to decrease its retention time to correspond more closely with those of the isomeric series having one lower chlorine number (*e.g.*, 2,2′-dichloro- and 2,6-dichlorobiphenyl have lower retention times than 4-chlorobiphenyl and 2,3,6,2′,3′,6′-hexachlorobiphenyl has a retention time lower than those of some of the pentachlorobiphenyls tested). (iii) A chloro substituent on the 4- (*para*) position increases retention times relative to effects of 3- or 5- (*meta*) substituents (*e.g.*, the retention time of 2,4-dichlorobiphenyl at 14.31 min exceeds those of 2,3-dichloro- and 2,5-dichlorobiphenyl at 12.71 and 13.81 min, respectively). (iv) The UV detector responses were in agreement with previously reported spectral studies[9] and UV detector responses[7,8]. Thus, within an isomeric series of PCBs, the UV detector absorbance responses decrease with increasing retention time, although there are a number of exceptions. (v) 2- or 6-Chloro substituents diminish the responses of PCBs relative to those with no substituents in the *ortho* position, and this effect is again enhanced when the *ortho* substituents are on opposite phenyl rings [*e.g.*, the response of 2,2′-dichlorobiphenyl (0.047) is less than that of 2,6-dichlorobiphenyl (0.075), which in turn is markedly less than that of 2,3-dichlorobiphenyl (0.278) or 2,4-dichlorobiphenyl (0.508)]. (vi) A chloro substituent in the 4-position enhances the response of a PCB relative to that with a 3- or 5-substituent [*e.g.*, compare the responses of 2,4′-dichlorobiphenyl (0.468) with those of 2,3-dichloro-(0.278) and 2,5-dichlorobiphenyl (0.295)].

The HPLC column used in these studies separates on the basis of the relative extents of hydrophobic affinities. It can thus be concluded that *o*-chloro substituents, which force the two phenyl rings of biphenyl out of coplanarity, tend to decrease the hydrophobicity while *p*-chloro substituents increase the hydrophobicity of PCBs relative to those with the same numbers of chloro substituents but in different positions.

The detector responses of the PCBs are based on the κ absorbance band, which has been attributed to the conjugated biphenyl system[9]. *o*-Chloro substituents cause a hypsochromic shift of this band together with a decrease in extinction[9], which results in a diminished response at 254 nm. It is possible that the relatively high responses observed for the nona- and decachlorinated biphenyls (Table I) are a

TABLE I

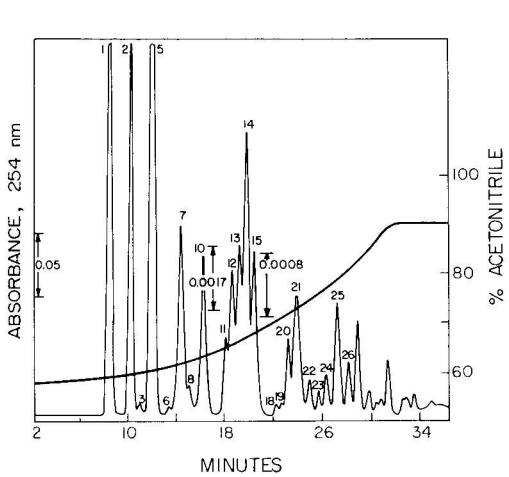
HPLC RETENTION TIMES AND UV SPECTRAL RESPONSES FOR INDIVIDUAL PCBs

UV detector at 254 nm wavelength.

PCB	*Retention time (min)*	*Spectral response (relative to biphenyl = 1000)*	*Analysis conditions*
Biphenyl	7.62	1.000	Aroclor 1221 assay
2-	9.39	0.255	
3-	9.91	0.725	
4-	11.12	0.917	
2,2′-	10.24	0.047	
2,6-	11.09	0.075	
2,3-	12.71	0.278	
2,4′-	13.57	0.465	
2,5-	13.81	0.295	
2,4-	14.31	0.508	
3,3′-	14.80	0.562	
3,4-	15.11	0.917	
4,4′-	15.43	1.087	
3,5-	16.63	0.685	
2,5,2′-	23.59	0.046	Aroclor 1016 assay
2,3,6-	23.82	0.042	
2,4,6-	28.67	0.093	
2,3,4-	30.05	0.394	
3,4,2′-	31.08	0.327	
2,5,3′-	32.12	0.190	
2,5,4′-	33.61	0.506	
2,4,5-	34.02	0.309	
2,4,4′-	36.31	0.674	
2,6,2′,6′-	14.61	0.007	Aroclor 1254 assay
2,3,2′,3′-	18.41	0.173	
2,3,2′,5′-	19.80	0.034	
2,5,2′,5′-	21.10	0.037	
3,4,3′,4′-	21.15	0.086	
2,3,5,6-	21.64	0.092	
2,4,2′,5′-	22.31	0.093	
2,4,2′,4′-	22.80	0.156	
2,3,4,5-	24.95	0.352	
2,5,3′,4′-	24.96	0.364	
2,4,3′,4′-	25.80	0.564	
2,4,5,2′,3′-	24.91	0.088	
2,3,6,2′,5′-	25.48	0.063	
2,3,4,2′,5′-	26.09	0.088	
2,3,4,5,6-	27.21	0.176	
2,4,5,2′,5′-	27.42	0.089	
2,3,6,2′,3′,6′-	25.81	0.007	
2,3,5,6,2′,5′-	28.64	0.019	
2,3,4,2′,3′,4′-	30.83	0.163	
2,4,6,2′,4′,6′-	31.44	0.047	
2,3,4,2′,4′,5′-	31.85	0.245	
2,4,5,2′,4′,5′-	32.88	0.146	
2,3,4,5,6,2′,5′-	34.10	0.096	
2,3,5,6,2′,3′,5′,6′-	37.36	0.024	
2,3,4,5,6,2′,3′,4′,5′-	41.96	0.204	
2,3,4,5,6,2′,3′,4′,5′,6′-	45.12	0.140	

consequence of the enhanced extinction of the "main band"[9] of these compounds. Although the "main bands" for these PCBs are centered at 216 nm, their absorbances tail well past 254 nm[9].

The chromatograms of Aroclor 1221, 1016 and 1254 and a 1:1:1 (w/w/w) mixture of the three Aroclors are depicted in Figs. 1–4. Some of the major constituents of the Aroclors have been tentatively assigned and quantitated, based on comparisons of retention times and responses with those of the available standards (Table I) together with previously reported results[4–8,10]. The sequence of numbering of the



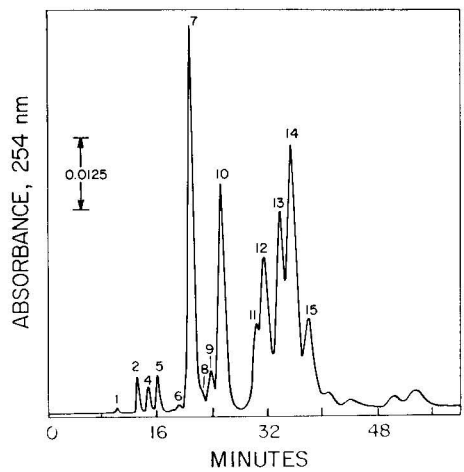


Fig. 1. HPLC separation of Aroclor 1221 (5 mg/ml in tetrahydrofuran) on a μBondapak C_{18} column monitored at 254 nm. Initial conditions, 40% water–acetonitrile (9:1) and 60% water–acetonitrile (1:9); final conditions, 100% water–acetonitrile (1:9); gradient period, 30 min; flow-rate, 2 ml/min; injection volume, 5 μl; amount injected, 25 μg.

Fig. 2. HPLC separation of Aroclor 1016 (5 mg/ml in tetrahydrofuran) on a μBondapak C_{18} column monitored at 254 nm. Conditions, 45% water–acetonitrile (9:1) and 55% water–acetonitrile (1:9); flow-rate, 2 ml/min; injection volume, 5 μl; amount injected, 25 μg.

peaks includes all peaks observed in all the chromatograms and thus some numbers may be absent from a particular chromatogram. Final identification of the components represented by the major peaks can only be made using mass spectrometry and nuclear magnetic resonance spectrometry, and studies are continuing in our laboratories along these lines.

For Aroclor 1221 (21% by weight of chlorine), 31 components were distinguishable chromatographically (Fig. 1). The seven major components have been tentatively identified and quantitated (Table II). 3-Chlorobiphenyl is not completely resolved from an excess of 2-chlorobiphenyl and a trace amount of the former may be present and thus increase our reported value for the latter, which is slightly higher than previously reported values. It is apparent that the patterns of minor components of Aroclor 1221 closely resemble those of the major components of Aroclor 1016 and 1254 (compare Fig. 1 with Figs. 2 and 3).

For Aroclor 1016 (41% by weight of chlorine), 18 components were distinguishable chromatographically (Fig. 2). The resolution of the components was inferior

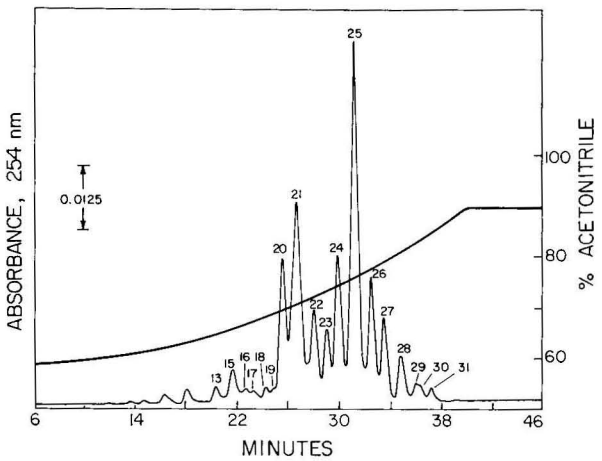


Fig. 3. HPLC separation of Aroclor 1254 (5 mg/ml in tetrahydrofuran) on a μBondapak C_{18} column monitored at 254 nm. Initial conditions, 40% water–acetonitrile (1:9) and 60% water–acetonitrile (1:9); final conditions, 100% water–acetonitrile (1:9); gradient time, 40 min; flow-rate, 2 ml/min; injection volume, 5 μl; amount injected, 25 μg.

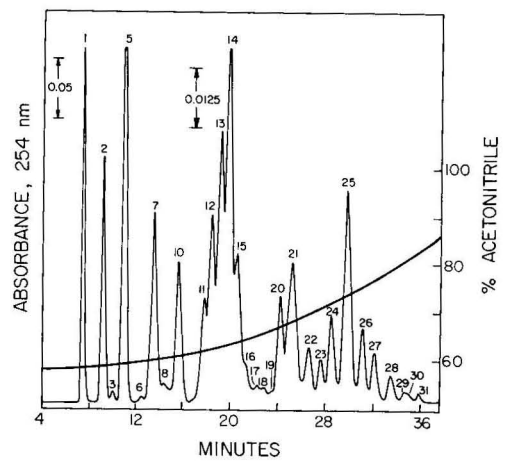


Fig. 4. HPLC separation of Aroclor 1221–Aroclor 1016–Aroclor 1254 (1:1:1, w/w/w) (1.67 mg/ml of each in tetrahydrofuran) on a μBondapak C_{18} column monitored at 254 nm. Initial conditions, 40% water–acetonitrile (9:1) and 60% water–acetonitrile (1:9); final conditions, 100% water–acetonitrile (1:9); gradient time, 40 min; flow-rate, 2 ml/min; injection volume, 10 μl; amount injected, 50 μg total.

to that obtained with Aroclor 1221 and 1254. The peaks up to and including No. 10 have been tentatively identified and together make up approximately 30% by weight of the sample (Table III). The retention time of peak 13 corresponds with that of 2,3,2′,5′-tetrachlorobiphenyl but, based on the known response of this compound, the peak would represent greater than 100% by weight of the sample. It must therefore be assumed that some other PCB co-chromatographs with 2,3,2′,5′-tetrachlorobiphenyl. Peaks 11, 12, 13, 14 and 15, which together represent approximately 70% by

TABLE II
COMPOSITION OF AROCLOR 1221

Peak No.	*PCB*	*Content (wt.-%)*			
		This study	*Ref. 5**	*Ref. 7***	*Ref. 10*
1	Biphenyl	12.6	15.85	13	12.7
2	2-	38.2	32.14	33	28.4
3	2,2′-	5.8	4.81	7.5	9.2
5	4-	21.7	19.07	17	18.7
7	2,4′-	13.4	10.17	15	13.6
8	2,4-	1.6	2.72	1.5	3.5
10	4,4′-	4.7	3.65	5	6.2

* Gas chromatographic method using six columns.
** HPLC method.

weight of Aroclor 1254, have retention times representative of trichlorobiphenyls (but excluding those with high *o*-chloro substitution) and highly *ortho* substituted tetrachlorobiphenyls. The latter, however, are unlikely to be solely represented by any of these peaks for the same reason as discussed previously for excluding 2,3,2′,5′-tetrachlorobiphenyl.

TABLE III
COMPOSITION OF AROCLOR 1016

Peak No.	*PCB*	*Content (wt.-%)*
1	Biphenyl	0.03
2	2-	1.1
5	4-	0.4
7	2,4′-	12.0
9	2,5,2′-	12.7
10	4,4′-	3.4

For Aroclor 1254 (54% by weight of chlorine), 22 components were distinguishable chromatographically (Fig. 3). Those components which have been tentatively identified and quantitated are shown in Table IV. The major peak (based on integrated areas) is No. 25 and probably represents a pentachloro- or hexachlorobiphenyl which has less than two *ortho* substituents. Higher numbers of *ortho* substituents would have responses that would preclude the possibility of a peak of such magnitude.

Although the analysis of PCBs by HPLC using a UV detector at 254 nm has been shown to be a viable method, producing separation of some components that are superior to those of gas chromatographic methods, the relative lack of sensitivity presents a problem. In the present study, 25 μg of the Aroclors were used for each analysis. This could be reduced to 3 μg with the HPLC used in this study without markedly reducing the accuracy of the analysis. The HPLC system permits a much greater volume of sample to be loaded per analysis than is possible with gas chromatographic systems and this, together with the use of higher concentrations of sample

TABLE IV

COMPOSITION OF AROCLOR 1254

Peak No.	*PCB*	*Content (wt.-%)*
13	2,3,2′,5′-	5.3
15	2,5,2′,5′-	10.3
20	2,5,3′,4′-	3.3
22	2,4,5,2′,5′-	11.7
26	2,3,4,2′,4′,5′-	4.9
27	2,4,5,2′,4′,5′-	5.3

solutions, would to a large extent overcome the relatively diminished sensitivity of the HPLC method.

Apart from the potential to analyse environmental samples of PCBs, the HPLC method provides a simple means for purifying individual PCBs, which can then be used for investigations where trace impurities could significantly alter results. The removal of dibenzofuran impurities from PCB samples to be used in induction studies would be particularly significant. The limited nature of the UV detector could also be used to advantage by effectively eliminating the interference of other non-UV-absorbing xenobiotics in environmental samples during PCB analysis. The use of variable-wavelength detectors should facilitate this. Two examples of such xenobiotics are the insecticides mirex and chlordane.

ACKNOWLEDGEMENT

This research was supported in part by grant ES04544 awarded by the National Institutes of Health, PHS/DHEW.

REFERENCES

1 R. W. Risebrough and B. de Lappe, *Environ. Health Perspect.*, 1 (1972) 39.
2 V. W. Burse, R. D. Kimbrough, E. C. Villanueva, R. W. Jennings, R. E. Linder and G. W. Sovocool, *Arch. Environ. Health*, 29 (1974) 301.
3 A. R. Yobs, *Environ. Health Perspect.*, 1 (1972) 79.
4 D. Sissons and D. Welti, *J. Chromatogr.*, 60 (1971) 15.
5 P. W. Albro, J. K. Haseman, T. A. Clemmer and B. J. Corbett, *J. Chromatogr.*, 136 (1977) 147.
6 E. Schulte and L. Acher, *Z. Anal. Chem.*, 268 (1974) 260.
7 U. A. Th. Brinkman, J. W. F. L. Seetz and H. G. M. Reymer, *J. Chromatogr.*, 116 (1976) 353.
8 U. A. Th. Brinkman, A. De Kok, G. De Vries and H. G. M. Reymer, *J. Chromatogr.*, 128 (1976) 101.
9 J. D. MacNeil, S. Safe and O. Hutzinger, *Bull. Environ. Contam. Toxicol.*, 15 (1976) 66.
10 D. E. Willis and R. F. Addison, *J. Fish Res. Board Can.*, 29 (1972) 592.

Journal of Chromatography, 155 (1978) 371–378

CHROM. 10,873

DOSAGE DU KÉTOPROFÈNE* DANS LE SANG PAR CHROMATOGRAPHIE LIQUIDE HAUTE PERFORMANCE

COMPARAISON AVEC LA CHROMATOGRAPHIE EN PHASE GAZEUSE

A. BANNIER, J. L. BRAZIER et B. RIBON

Université Claude Bernard, Faculté de Pharmacie, Département de Chimie Analytique, Chimie Générale et Minérale et Bromatologie, Pr. Cl. Quincy 8, Av. Rockefeller, 69373 Lyon Cédex 2 (France)

(Reçu le 27 décembre 1977)

SUMMARY

Determination of ketoprofen in plasma using high-performance liquid chromatography. Comparison with gas–liquid chromatography

A new method of determination of ketoprofen 2-(3-benzoyl phenyl) propionic acid in plasma using high-performance liquid chromatography (HPLC) is described. After extraction by diethyl ether in acidic medium, ketoprofen and the internal standard, 2-(4-benzoyl phenyl) butyric acid, are methylated with gaseous diazomethane and their concentrations measured by HPLC using a LiChrosorb Si 60 (5 μm) column and dichloromethane–hexane (60:40) as the mobile phase. The absolute retention times of the internal standard and ketoprofen are 11.6 and 12.8 min, respectively. The precision of the method is $\pm 4\%$ and the lower detection limit ranges from 0.06 to 0.10 μg/ml. The results obtained by HPLC show a very good correlation with those obtained by gas–liquid chromatography.

The proposed method is sensitive, reproducible and rapid and very suitable for ketoprofen determination in pharmacokinetic studies.

INTRODUCTION

Le kétoprofène ou acide (benzoyl-3 phényl)-2 propionique (Fig. 1) est un dérivé de la benzophénone doué d'une importante activité antiinflammatoire[1]. Les

Fig. 1. Structure chimique de (a) kétoprofène et de (b) l'étalon interne.

* Profénid (ND, Spécia, Paris, France).

premiers résultats cliniques ont confirmé son intérêt thérapeutique[2], si bien qu largement utilisé en rhumatologie, il a fait l'objet de nombreuses études cliniques[3–] et pharmacocinétiques[6–9].

Trois méthodes de dosage du kétoprofène dans les milieux biologiques utili sant les diverses fonctions réactives de la molécule ont été décrites[10]: la colorimétrie la polarographie et la chromatographie en phase gazeuse (CPG). Seule cette dernièr est applicable au sérum et contitue en raison de sa spécificité et de sa sensibilité l méthode de référence. Toutefois les nombreuses phases de la technique ne permetten pas une précision supérieure à $\pm 10\%$ et imposent un temps d'analyse assez long seul un petit nombre de prélèvements peuvent être analysés chaque jour.

Aussi nous proposons une nouvelle méthode de dosage du kétoprofène dan le sang par chromatographie liquide haute performance (HPLC). Cette méthode es spécifique, sensible, reproductible et rapide. L'étalon interne utilisé est un compos de structure voisine l'acide (benzoyl-4 phényl)-2 butyrique (Fig. 1).

MATÉRIEL ET MÉTHODES

Réactifs

Kétoprofène et acide (benzoyl-4 phényl)-2 butyrique utilisé comme étalon interne (Spécia, Paris, France). Tous les réactifs et solvants sont de pureté pour analyses: acétone, sulfate de sodium anhydre, héxane, propanol-2, dichlorométhane, N-méthyl N-nitroso *p*-toluène sulfonamide (Prolabo, Paris, France); méthanol, hydroxyde de potassium, acide chlorhydrique, éther diéthylique, (E. Merck, Darmstadt, R.F.A.); plaques de chromatographie sur couche mince F 1500 LS 254 (Schleicher & Schüll, Dassel, R.F.A.).

Appareils

L'appareil utilisé pour la CPG est un chromatographe en phase gazeuse Carlo Erba Fractovap 2200 équipé d'un détecteur à ionisation de flamme. La colonne en verre pyrex (longueur 2 m et diamètre interne 3 mm) est remplie d'OV-17 à 3% sur du Chromosorb W AW DMCS.

Les séparations par HPLC ont été effectuées sur un chromatographe en phase liquide Chromatem 38 (Touzart & Matignon, Paris, France) équipé d'un détecteur UV (254 nm) modèle 153 (Altex, Berkeley, Calif., U.S.A.) et d'un calculateur intégrateur modèle 3385 A (Hewlett-Packard, Orsay, France). La colonne (longueur 250 mm et diamètre interne 4.7 mm) est remplie avec du LiChrosorb Si 60 (5 μm; E. Merck) selon une méthode par voie humide[11]. Le nombre de plateaux théoriques calculé sur le kétoprofène est de 53,000 plateaux par mètre.

Solutions étalons

L'acide (benzoyl-4phényl)-2 butyrique (100 mg/l dans l'acétone) est diluée au 1/10 dans un mélange eau–éthanol (50:50) au moment de l'emploi. Solution de kétoprofène et d'étalon interne méthylés à 10 mg/l dans l'acétone; cette solution sert à déterminer le coefficient de réponse du détecteur en CPG. Pour la HPLC, 1 ml de la solution est évaporé et le résidu est repris par 1 ml de phase mobile.

Méthodes

CPG. Les dosages sanguins du kétoprofène ont été réalisés selon la technique publiée par Populaire *et al.*[10] modifiée par Brazier *et al.*[12]. Dans une ampoule à décantation de 150 ml, sont ajoutés 1 ml de sérum, 1 ml de solution d'étalon interne à 10 mg/l et 1 ml d'acide chlorhydrique 1 *N*. L'ensemble est extrait par deux fois 30 ml d'éther diéthylique. Les extraits rassemblés sont lavés successivement par 10 ml d'HCl 1 *N*, 10 ml d'HCl 0.1 *N* et 10 ml d'eau distillée. Après filtration sur sulfate de sodium anhydre, l'extrait est évaporé sous courant d'azote à 40°. Le résidu repris par 3 ml d'acétone est transféré dans un tube de verre à fond conique et à bouchon rodé pour être méthylé par le diazométhane gazeux[13]. L'extrait méthylé est évaporé, repris par 150 μl d'acétone et les différents constituants sont fractionnés par chromatographie sur couche mince sur film de silice dans le système hexane–acétone (85:15). Les bandes correspondantes au kétoprofène et à l'étalon interne sont éluées par l'acétone. Après évaporation, le résidu repris par 50 μl de propanol-2 est analysé par CPG. La séparation s'effectue en régime isotherme à 240°. La température de l'injecteur et du détecteur est de 280°. Le débit du gaz vecteur (azote) est de 30 ml/min.

HPLC. L'extraction est réalisée dans les mêmes conditions. Les extraits méthylés sont évaporés et repris par 50 μl de phase mobile. 3 μl de la solution sont alors injectés dans le chromatographe. La phase mobile, dichlorométhane–hexane (60:40), est pompée à travers la colonne à un débit de 1.3 ml/min sous une pression de 35 bar.

RÉSULTATS

Paramètres chromatographiques

CPG. La Fig. 2 représente le chromatogramme d'un extrait plasmatique. Les temps de rétention absolus du kétoprofène et de l'étalon interne sont respectivement de 4.8 et de 6.6 min. Les concentrations correspondantes sont de 7.90 mg/l en kétoprofène et de 5 mg/l en étalon interne.

HPLC. La Fig. 3a montre le chromatogramme d'un extrait plasmatique contenant 8.6 mg/l de kétoprofène et 5 mg/l d'étalon interne. La Fig. 3b montre le chromatogramme d'un blanc sérum du même sujet. Dans les conditions adoptées précédemment, les temps de rétention absolus de l'étalon interne et du kétoprofène sont respectivement de 11.6 et de 12.8 min. Les facteurs de capacité de l'étalon interne (k'_1) et du kétoprofène (k'_2) calculés par rapport au pic de l'air (t_R = 3.8 min) sont les suivants: k'_1 = 2.05, k'_2 = 2.37. La résolution, R_S est 1.25.

Calcul de la concentration en kétoprofène

La méthode de standardisation utilisée dans les deux cas est la méthode d'étalonnage interne.

CPG. La solution étalon de kétoprofène et d'étalon interne méthylés à 10 mg/l dans l'acétone sert à déterminer le coefficient de proportionnalité par la mesure du rapport surface étalon sur surface de kétoprofène. Dix injections ont été effectuées pour ce calcul et le coefficient de proportionnalité obtenu est égale à 0.99 $\pm$ 0.016. La reproductibilité de la méthode est de $\pm 10\%$ et la limite inférieure de sensibilité est comprise entre 0.02 et 0.04 mg/l de kétoprofène[10].

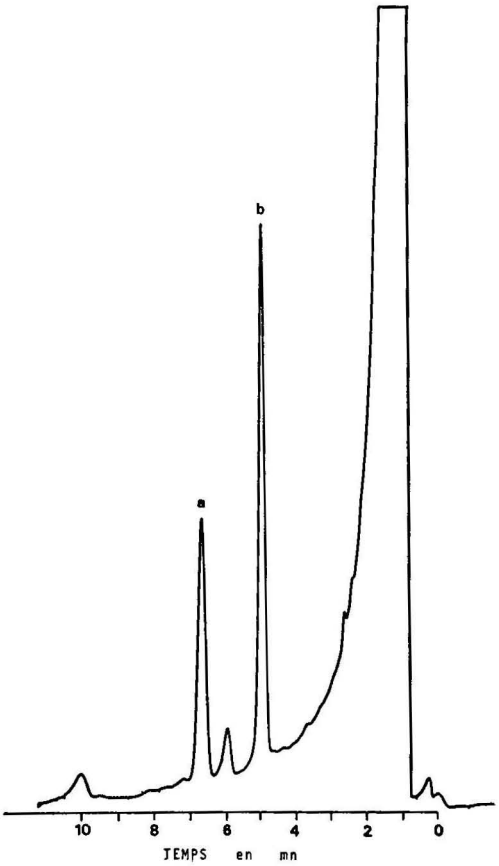


Fig. 2. Chromatogramme d'un extrait plasmatique obtenu par CPG contenant 7.90 mg/l de kétoprofène (b) et 5 mg/l d'étalon interne (a).

HPLC. Le coefficient de proportionnalité mesuré avec la solution étalon de kétoprofène et d'étalon interne à 10 mg/l dans la phase mobile est égal à 1.04 ± 0.013 ($n = 10$). Avant chaque série de dosage le coefficient de proportionnalité est vérifié.

Linéarité de la méthode HPLC

La courbe d'étalonnage a été réalisée sur des extractions de sérum contenant respectivement 2, 4, 6, 8 et 10 mg/l de kétoprofène. Les résultats obtenus sont portés sur la Fig. 4. Chaque point porté sur le graphe correspond à la moyenne de 3 injections. Les paramètres de la droite d'étalonnage (ordonnée à l'origine 0.026, pente 0.153 et coefficient de regression 0.999) montrent que la linéarité est très bonne dans la gamme des taux thérapeutiques.

Réproductibilité et précision

La reproductibilité de la méthode HPLC a été étudiée en mesurant la concentration en kétoprofène dans des échantillons sanguins chargés respectivement à 5 mg/l et à 10 mg/l en kétoprofène. Les résultats obtenus (Tableau I) montrent que

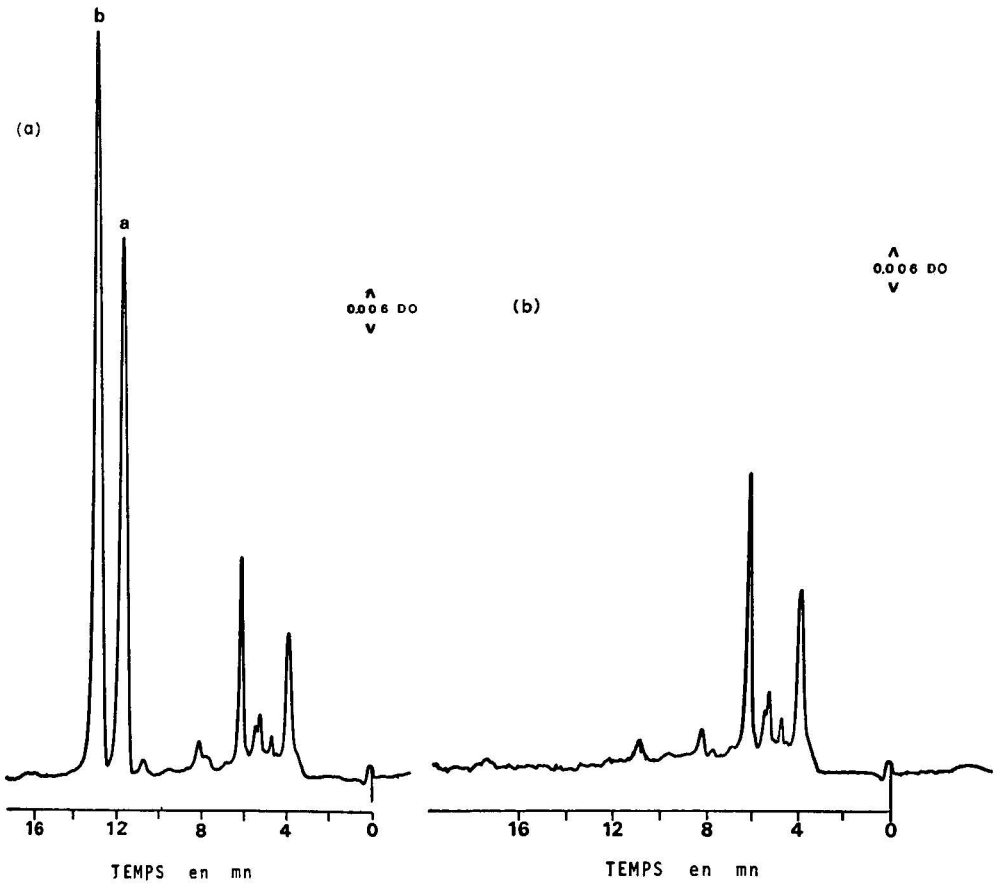


Fig. 3. Chromatogramme d'un extrait plasmatique obtenu par HPLC. Extrait contenant 8.6 mg/l de kétoprofène (b) et 5 mg/l d'étalon interne (a). Extrait d'un blanc plasma; colonne, 250 × 4.7 mm, LiChrosorb Si 60 (5 μm); phase mobile, dichlorométhane–hexane (60:40), débit, 1.3 ml/min; détection, UV (254 nm).

les taux moyens retrouvés sont respectivement de 4.91 mg/l $\pm$ 0.221 (n = 10) et de 9.81 mg/l $\pm$ 0.391 (n = 10). La précision de la méthode est de $\pm$4%.

Limite de détection quantitative

La méthode HPLC permet encore de mesurer avec une bonne précision des concentrations de kétoprofène comprises entre 0.06 et 0.10 mg/l.

Exactitude

L'exactitude de la méthode HPLC a été testée en mesurant les concentrations sériques de kétoprofène par les deux méthodes (HPLC et CPG) chez 4 sujets ayant reçu une dose orale de kétoprofène de 150 mg. 44 Dosages ont été ainsi effectués parallèlement. La Fig. 5 montre la corrélation existant entre les résultats obtenus. En ordonnée sont portés les valeurs trouvées par CPG et en abscisse les valeurs trouvées par HPLC.

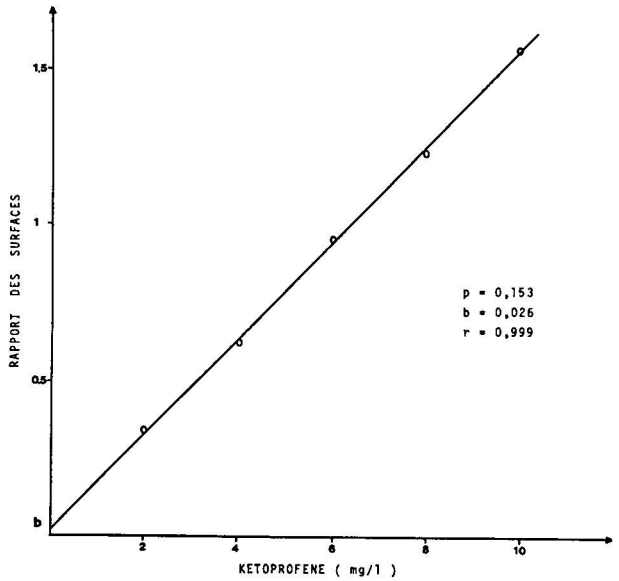


Fig. 4. Courbe d'étalonnage. En ordonnées sont portés les rapports surface kétoprofène sur surface étalon interne et en abscisses les concentrations de kétoprofène en mg/l. r, Coefficient de régression; b, ordonnée à l'origine; p, pente.

TABLEAU I

RÉPRODUCTIBILITÉ ET PRÉCISION DE LA MÉTHODE HPLC

Nombre d'essais	*Quantité ajoutée (µg/ml)*	*Quantité retrouvée (µg/ml)*	*Écart type (µg/ml)*	*C.V. (%)*
10	5	4.91	0.221	4.5
10	10	9.81	0.331	3.98

Les paramètres de la droite de régression sont les suivants: pente, 1.041 ± 0.022; ordonnée à l'origine, -0.240 ± 0.102; coefficient de corrélation, 0.991. Le test t pour les couples de valeurs donnent les résultats suivants: moyenne des différences (HPLC − CPG), 0.0725; n, 44; erreur standard, 0.0514.

Pour l'hypothèse d'une différence nulle, la valeur de t montre qu'il n'y a pas de différence significative entre la dispersion des résultats selon les deux méthodes.

DISCUSSION

Dans la méthode proposée, l'étalon interne, acide (benzoyl-4 phényl)-2 butyrique, utilisé par Populaire *et al.*[6] a été conservée pour doser le kétoprofène. En effet ces deux composés, de structure voisine, possédent le même comportement à l'extraction et cela nous a permis une comparaison des résultats valable entre les deux méthodes de dosage par HPLC et par CPG à partir du même prélèvement. 3 µl de l'extrait méthylé repris par la phase mobile ont été injectés en HPLC et une aliquot du même extrait a été dosée par CPG.

La précision obtenue par HPLC ($\pm 4\%$) est meilleure que celle obtenue par

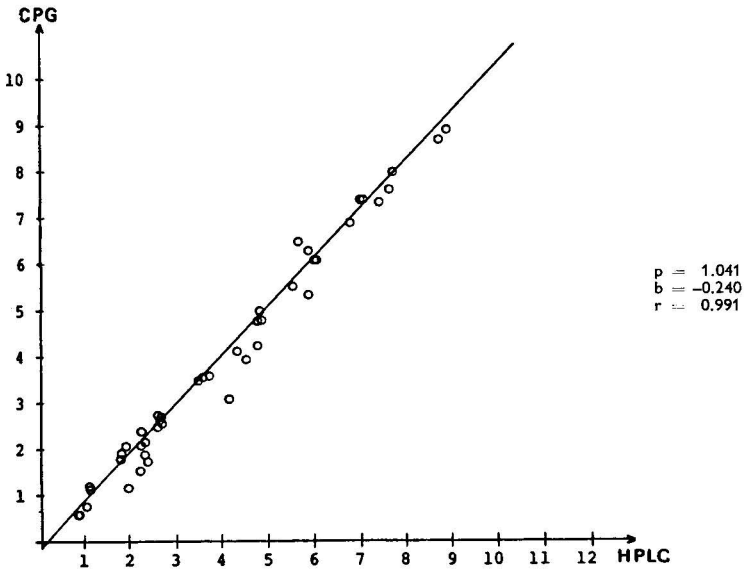


Fig. 5. Corrélation entre les résultats obtenus par CPG et par HPLC.

CPG ($\pm 10\%$), vraisemblablement grâce à la diminution des étapes intermédiaires: en effet la chromatographie sur couche mince, étape particulièrement longue, peut être responsable de pertes importantes pouvant affecter indifféremment le kétoprofène ou l'étalon.

La sensibilité de la méthode proposée est légèrement inférieure à celle obtenue en CPG. La limite de détection quantitative est respectivement de 0.100 mg/l et de 0.04 mg/l de kétoprofène. Toutefois comme le montre la Fig. 3b, aucun pic parasite n'est suspectible d'interférer au cours du dosage et il est toujours possible d'augmenter la sensibilité en reprenant les extraits par 30 μl de phase mobile et en augmentant le volume injecté. D'autre part, la méthode est suffisamment sensible pour suivre l'évolution des taux sanguins au cours des 12 h suivant la prise de 150 mg de kétoprofène.

Enfin le gain de temps est important: d'une part, l'étape intermédiaire la plus longue a été supprimée et d'autre part nous avons observé lors de l'injection des extraits en CPG des pics dont les temps de rétention sont voisins de 45 min. Ces pics de nature inconnue et présents dans tous les sérum limitent à 3 le nombre d'injections pouvant être effectuées consécutivement alors qu'en HPLC ce problème n'est pas rencontré.

La méthode que nous proposons est reproductible, sensible et rapide. Elle est parfaitement adaptée au dosage du kétoprofène dans le sang pour des études de pharmacocinétiques et de biodisponibilité.

RÉSUMÉ

Nous décrivons une méthode de dosage du kétoprofène ou acide (benzoyl-3 phényl)-2 propionique dans le sang par chromatographie liquide haute performance.

Après extraction par l'éther en milieu acide, le kétoprofène et l'acide (benzoyl-4 phényl)-2 butyrique utilisé comme étalon interne sont méthylés par le diazométhane gazeux et analysés par chromatographie liquide haute performance [colonne de Li Chrosorb Si 60, 5 μm; phase mobile, dichloro méthane–hexane (60:40)]. Les temps de rétention absolus de l'étalon interne et du kétoprofène sont respectivement de 11.6 et 12.8 min. La précision de la méthode est de $\pm 4\%$ et la limite de détection quantitative est comprise entre 0.06 et 0.100 μg/ml de kétoprofène. Les résultats comparés avec ceux obtenus en chromatographie en phase gazeuse montrent une excellente corrélation entre les deux méthodes.

La méthode proposée est sensible, reproductible et rapide. Elle est parfaitement adaptée au dosage du kétoprofène dans le sang en vue d'études pharmacocinétiques.

BIBLIOGRAPHIE

1 L. Julou, J. C. Guyonnet, R. Ducrot, C. Ducrot, C. Garret, M. C. Bardone, G. Maignan et J Pasquet, *J. Pharmacol. (Paris)*, 2 (1971) 259.
2 B. Amor, A. de Géry et F. Delbarre, *Rev. Rhum.*, 40 (1973) 451.
3 J. Fossgreen, *Scand. J. Rheum.*, suppl. 14 (1976) 7.
4 J. Sany et H. Serré, *Rheum. Rehab.*, (1976) 67.
5 P. Coupron, J. L. Brazier, P. Meunier, B. Ribon et A. Bannier, *XIV Int. Congr. Rheum., San Francisco, Calif., 26 juin – 1 juillet 1977*, J. R. Rice, Bethesda, Md., 1977, p. 72.
6 P. Populaire, B. Terlain, S. Pascal, B. Decoulevaere, A. Renard et J. P. Thomas, *Ann. Pharm. Fr.*, 31 (1973) 735.
7 A. Castegnaro, F. Annotta et C. Pollini, *Farmaco. Sci.*, 29 (1974) 520.
8 O. R. W. Lewellen et R. Templeton, *Scand. J. Rheum.*, suppl. 14 (1976) 53.
9 P. Meunier, R. Coupron, J. L. Brazier, B. Ribon et A. Bannier, *Rev. Rhum.*, 44 (1977) 519.
10 P. Populaire, B. Terlain, S. Pascal, B. Decoulevaere, G. Lebreton, A. Renard et J. P. Thomas, *Ann. Pharm. Fr.*, 31 (1973) 679.
11 B. Coq, C. Gonnet et J. L. Rocca, *J. Chromatogr.*, 106 (1975) 249.
12 J. L. Brazier, A. Bannier et B. Ribon, Rapport Interne, Spécia, Paris, 1976.
13 A. I. Vogel, *Practical Organic Chemistry*, Longmans, London, 1951, p. 971.

Journal of Chromatography, 155 (1978) 379–387

CHROM. 10,868

SEPARATION AND IDENTIFICATION OF GEOMETRIC ISOMERS OF RETINOIC ACID AND METHYL RETINOATE*

R. M. McKENZIE, D. M. HELLWEGE, M. L. McGREGOR, N. L. ROCKLEY, P. J. RIQUETTI and E. C. NELSON**

Department of Biochemistry, Oklahoma Agricultural Experiment Station, Oklahoma State University, Stillwater, Okla. 74074 (U.S.A.)

(Received January 10th, 1978)

SUMMARY

Geometric isomers of retinoic acid and methyl retinoate were separated by high-performance liquid chromatography on bonded, reversed-phase, octadecylsilane columns using methanol–water mixtures as solvents. The isomers have been identified by nuclear magnetic resonance, infrared, ultraviolet and mass spectral analyses as 9,11,13-tri-*cis*-, 11,13-di-*cis*-, 13-*cis*-, 9,13-di-*cis*-, 11-*cis*-, 9-*cis*- and all-*trans*-methyl retinoate. Seven isomers were observed simultaneously upon chromatography of retinoic acid isomerates, while eight isomers were observed when methyl retinoate was chromatographed.

INTRODUCTION

The metabolism of retinoic acid has been studied in this laboratory for several years. The vitamins A are sensitive to light, thermally unstable, readily oxidized and easily isomerized, and their separation and handling is difficult. Thus, a need exists for routine methods of purification and analysis of the starting materials as well as unknown metabolites.

The advent of high-performance liquid chromatography (HPLC) and the development of high-performance columns have made such analytical procedures more readily available. Examples of this particular use of HPLC have been provided by Vecchi *et al.*[1], who demonstrated the separation of some of the geometric isomers of retinyl acetate using both adsorption and normal-phase columns, and by Tsukida *et al.*[2], who separated geometric isomers of retinaldehyde. The present paper is concerned with the separation of the geometric isomers of retinoic acid and methyl retinoate on reversed-phase octadecylsilane (ODS) columns.

* Presented in part at the Annual Meeting of the Federation of American Societies for Experimental Biology, April 11, 1977, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 36 (1977) 1103.

** To whom correspondence should be addressed.

EXPERIMENTAL

Glass-distilled residue-free solvents (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.) were used throughout. The water used was deionized, filtered over charcoal and distilled from glass.

All-*trans*-retinoic acid was a gift from Dr. W. E. Scott, Hoffmann-La Roche Inc. (Nutley, N.J., U.S.A.). Isomerates of retinoic acid or methyl retinoate, in solution, were prepared by irradiation using a fluorescent lamp (GE F-15 T8 CW). The irradiations were carried out in either 4-ml specimen vials (flint glass) or 22-ml liquid scintillation vials (borosilicate glass) held at a distance of *ca.* 5 cm from the lamp. Methyl esters were prepared using diazomethane in methanol–diethyl ether solutions, essentially as described by Schlenk and Gellerman[3].

Separations were performed on 0.46 × 25 cm, bonded, octadecylsilane (ODS) columns (Partisil 10-ODS; Whatman, Clifton, N.J., U.S.A.). Two high-pressure pumping systems were used: a DuPont Model 830 liquid chromatograph (E. I. Du Pont de Nemours & Co., Wilmington, Del., U.S.A.) equipped with a single-beam 254-nm photometer, and a Model 314 pump (Isco, Lincoln, Nebr., U.S.A.) operated through a DuPont gradient-elution accessory. The second pumping system was coupled to a Model 25 spectrophotometer (Beckman, Fullerton, Calif., U.S.A.) fitted with a set of Model LC-25 microcells (Waters Assoc., Milford, Mass., U.S.A.). The detectors were readily interchangeable to serve with either pumping system or, if desired, could be placed in series to operate simultaneously with a single pumping system. Sample application in both systems was via six-port sampling valves (Valco, Houston, Texas, U.S.A.). If not already in an appropriate solvent, samples were dried under a gentle stream of nitrogen with the aid of a warm water-bath. The samples were then redissolved in methanol and diluted with water until the solvent strength was less than or equal to that being used for elution at that time.

Nuclear magnetic resonance (NMR) spectral analyses were performed on a Varian XL-100(15) instrument (Varian, Palo Alto, Calif., U.S.A.) using tetramethylsilane as the internal standard, and infrared (IR) spectra were obtained on a Model 457 grating spectrophotometer (Perkin-Elmer, Norwalk, Conn., U.S.A.). Low-resolution mass spectral analyses were obtained via the direct inlet probe as described by Lin *et al.*[4] and Reid *et al.*[5] for vitamin A and related compounds.

RESULTS AND DISCUSSION

The separation of an isomerate of methyl retinoate by HPLC on a reversed-phase column is shown in Fig. 1. The separation of eight peaks between 35 and 70 min is readily apparent in the trace of the 254-nm spectra. Peak 5 was only a shoulder on the trace made at 350 nm, and peak 0 was not seen at all at this wavelength. The resolution of peaks 3–5 was the most difficult part of this separation. The observation of the three isomers depended upon their relative abundance, the detector wavelength utilized and the maintenance of peak column performance.

The separation of the free acid forms of these isomers is shown in Fig. 2. This separation was more demanding due to the broader peaks obtained. The situation was further complicated by an inversion in the elution order of two of the isomers.

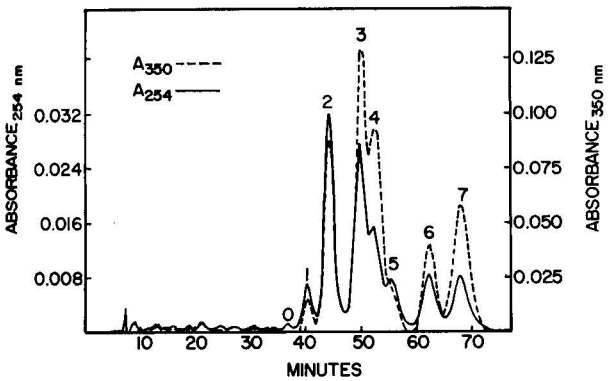


Fig. 1. The HPLC elution profile of an isomerate resulting from irradiation for 3 h of all-*trans*-methyl retinoate in heptane–diethyl ether (9:1). Elution was with methanol–water (70:30) at 20° and a flow-rate of 0.55 ml/min. Approximately 100 μg of methyl retinoate were applied to the column. The identity of peak 0 has not been obtained. Peaks 1–7 have been identified as 9,11,13-tri-*cis*-, 11,13-di-*cis*-, 13-*cis*-, 9,13-di-*cis*-, 11-*cis*-, 9-*cis*- and all-*trans*-methyl retinoate, respectively.

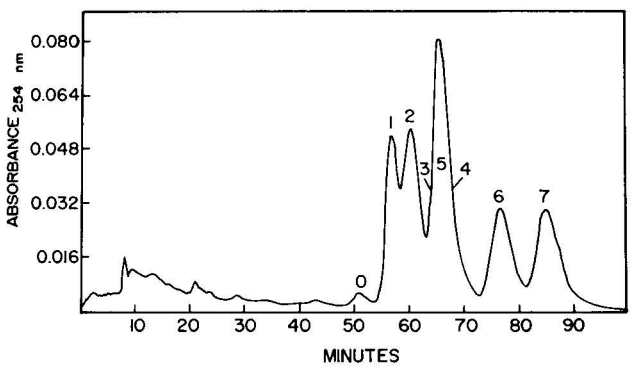


Fig. 2. The elution profile of an isomerate resulting from the irradiation for 1 h of all-*trans*-retinoic acid in methanol–water (70:30). Elution was with methanol–water (65:35) at 20° and a flow-rate of 0.4-ml/min. Approximately 250 μg of retinoic acid were applied to the column. Isomer identification is as in Fig. 1.

Retaining the peak identifications used in Fig. 1, peaks 4 and 5 were found to be reversed when chromatographed as the corresponding acids.

The minimum number of peaks observed in an isomerate of retinoic acid containing all of the eight isomers was six, as represented in Fig. 2. It was possible, given the appropriate relative isomer balance, to observe seven peaks. In such instances, peaks 3 and 5 or peaks 3 and 4 were resolved, while either 4 or 5 seemed to be absent. The resolution of peaks 4 and 5 in this system was insufficient to permit their simultaneous demonstration. However, the actual presence of all the eight isomers in acid isomerates could be demonstrated by methylation of the isomerates and chromatography of the resulting methyl retinoate isomers.

Initially, peak 0 was ignored. It was thought to be a minor contaminant generated during the isomerization process since its spectral characteristics ($\lambda_{max.} \approx$

263 nm, ratio of absorbances at 263 and 350 nm $\approx$ 10:1) were not typical of known methyl retinoate isomers. However, this peak, more so than any of the other unidentified, faster eluting, material, became even more prominent with increasing irradiation time (Fig. 3). A small quantity of this material was isolated and analyzed by mass spectroscopy. As was the case with all of the other isomers of methyl retinoate, the spectrum contained the molecular ion, *m/e* 314, and all of the fragment ions found in the mass spectrum of all-*trans*-methyl retinoate[4]. The geometry of this isomer has not been elucidated.

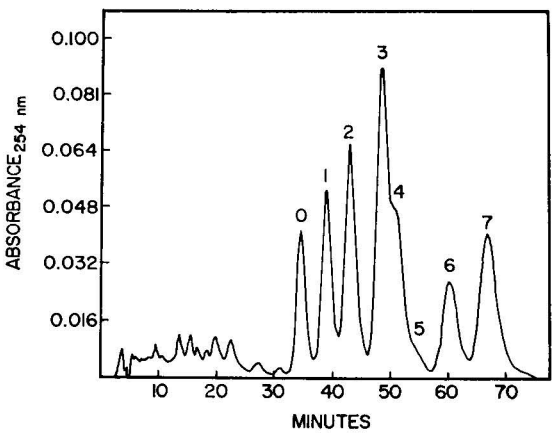


Fig. 3. The elution profile of a methyl retinoate isomerate resulting from irradiation for 72 h in heptane–diethyl ether (9:1). Elution was with methanol–water (70:30) at 25° and a flow-rate of *ca.* 0.6 ml/min. Approximately 250 μg of methyl retinoate were applied. Isomer identification is as in Fig. 1.

The vinyl regions of the NMR spectra of methylated retinoic acid isomers are shown in Fig. 4. The varying quality of the spectra was due to the quantity of isomers collected from the HPLC separations. Peaks 3 and 7 were obtained by single scans while the other peaks required 200-scan time-averaged spectra. Chemical shifts (δ) for singlets further upfield are listed in Table I.

In the high-field region of the NMR spectra (Table I) the C-20 singlets of peaks 1 and 2 are between the usual positions for the 13-*cis* and 13-*trans* isomers. Considering the assignments made to peaks 3–7 below, the most reasonable explanation for this anomalous behavior is that it is a peculiarity of the combination of 11-*cis* and 13-*cis* geometry. It does not happen in 11,13-di-*cis*-retinaldehyde[6], where the C-20 methyl resonates at δ 2.07 in $CDCl_3$. However, the additional steric requirements of the $-OCH_3$ group in the ester could cause this region of the molecule to twist so that the C-20 methyl is positioned closer to a deshielding region. IR spectroscopy (see below) seems to support the 11-*cis* geometry. The slightly higher δ value for C-18 (Table I) in peak 1 suggests 9-*cis* geometry by analogy with the other isomers (only the 9-*cis* isomers give $\delta \geqslant 1.70$), but these differences are marginal. In the vinyl regions of the spectra there is more evidence of a downfield shift for the C-8 doublet (characteristic of 9-*cis*) in peak 1. The small absorption near δ 6.1 could be the C-10 doublet shifted slightly upfield (also characteristic of 9-*cis*). The UV spectra of peak

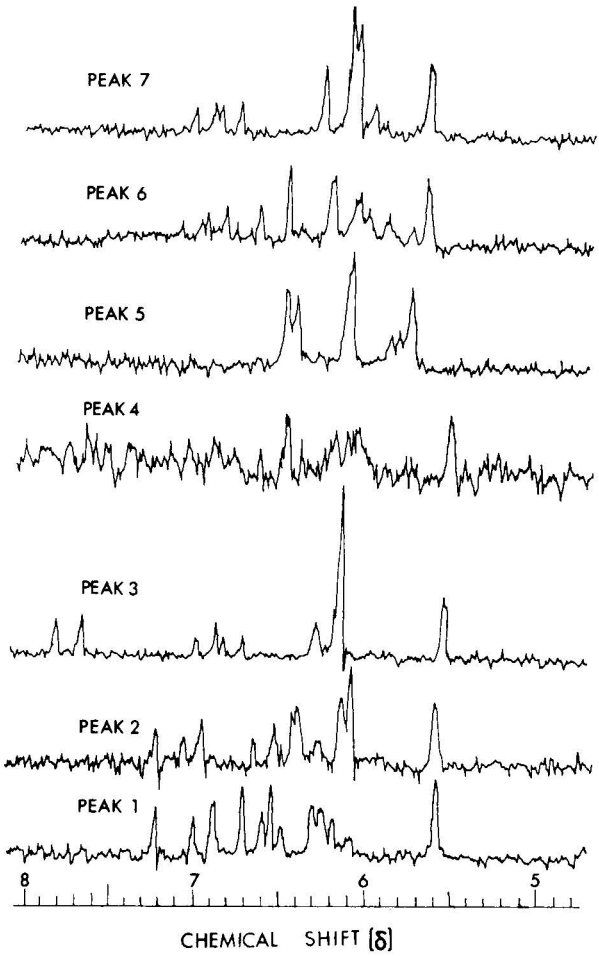


Fig. 4. The vinyl regions of the NMR spectra of geometric isomers of methyl retinoate. Peak numbers refer to compounds collected from the HPLC separations as shown in Fig. 1.

TABLE I

CHEMICAL SHIFT (δ) VALUES FOR THE VARIOUS METHYL SINGLETS IN THE NMR SPECTRA OF THE HPLC PEAKS OBTAINED FROM LIGHT ISOMERATES OF METHYL RETINOATE

Peak	*C-16,17*	*C-18*	*C-19*	*C-20*	$-OCH_3$
1	1.01	1.70	1.96	2.15	3.59
2	1.00	1.67	1.95	2.16	3.60
3	1.01	1.69	1.97	2.05	3.63
4	1.02	1.71	1.98	2.04	3.62
5	1.00	1.68	1.94	2.30	3.64
6	1.03	1.72	1.98	2.30	3.63
7	1.00	1.68	1.97	2.31	3.63

1 displayed a $\lambda_{max.}$ of 333 nm, about 10 nm less than the $\lambda_{max.}$ values of peaks 2–7 (342–353 nm). Hence, the evidence is consistent with peak 1 being probably 9,11,13-tri-*cis*- and peak 2 being 11,13-di-*cis*-methyl retinoate.

The NMR spectra of peaks 3 and 7 (Fig. 4) were obtained on samples collected by preparative HPLC of a mixture produced by isomerization of all-*trans*-methyl retinoate in the presence of iodine in the dark. Peak 7 is the starting material, all-*trans*-methyl retinoate, and peak 3 is 13-*cis*-methyl retinoate[7]. Notable changes from the all-*trans* spectrum for peak 3 include a shift in the C-12 doublet down to δ *ca.* 7.7, and the expected upfield shift of the C-20 singlet caused by the removal of that methyl group from the deshielding region of the carbonyl group in the 13-*cis* configuration.

The NMR absorption for the C-20 methyl group in peak 4 indicates 13-*cis* geometry. The vinyl region (Fig. 4) of the spectrum is very weak because of the small sample size, but there is a strong suggestion of a doublet ($J = 16$ Hz) centered between δ 6.5 and δ 6.6, which could be C-8 in a 9-*cis* isomer. Therefore, peak 4 is the 9,13-di-*cis* isomer.

Peaks 5 and 6 were isolated by preparative HPLC of a 30-min light isomerate in 70% methanol, in which these isomers were at their highest concentrations. The vinyl region of the NMR spectrum for peak 5 corresponds to a 100-MHz theoretical spectrum of 11-*cis*-retinoic acid, drawn from data at 220 MHz[6]. The absence of any significant absorption downfield from δ 6.5 together with the C-12 absorption at $\delta < 6.0$ seems adequate to enable peak 5 to be assigned as the 11-*cis* isomer. Peak 6 is the 9-*cis* isomer since the δ value for C-20 (Table I) shows 13-*trans* geometry and the double doublet for C-11 in the vinyl region (Fig. 4) appears in its usual position, just below δ 7.0, indicating 11-*trans* geometry. In addition, the doublet centered at δ 6.54 ($J = 16$ Hz) is highly characteristic of 9-*cis* geometry. A 100-MHz theoretical spectrum was drawn, based again on the 220-MHz data for 9-*cis*-retinoic acid found in Schwieter *et al.*[6], and it revealed a vinyl pattern very similar to that in Fig. 4. Thus, there is little doubt that peak 6 is the 9-*cis* isomer.

The NMR spectrum of peak 7 (Fig. 4) clearly indicates that it is the all-*trans* isomer and compares well with the spectrum of this isomer given by Korver *et al.*[7]. The primary differences in our spectrum are a slight downfield shift due to the use of CCl_4 instead of $CDCl_3$ as the solvent, and an increased resolution due to the use of a 100-MHz (instead of a 60-MHz) instrument.

In IR spectra of the retinaldehydes it has been demonstrated that the absorption at 10.3–10.4 μm (*ca.* 960–975 cm^{-1}, C–H deformation in a *trans*-$R_1CH{=}CHR_2$ system) decreases in intensity for the 11-*cis* isomers[8]. In our spectra (Fig. 5) this band is much more intense than the nearby bands in isomers 3, 6 and 7 (11-*trans* isomers), but not in peak 5 which was previously assigned as 11-*cis*. Thus, the IR spectra are consistent with a 11-*cis* geometry in peaks 1 and 2 as well as in other isomers. Isomers 6 and 7 exhibit spectra so nearly alike that it may be argued that geometric isomerism at $C_9{=}C_{10}$ has practically no effect on the IR absorption.

Based on these data, peaks 1–7 have been identified as 9,11,13-tri-*cis*-, 11,13-di-*cis*-, 13-*cis*-, 9,13-di-*cis*-, 11-*cis*-, 9-*cis*- and all-*trans*-methyl retinoate, respectively.

The present study has not been fully extended to other vitamin A compounds. However, some observations on the behavior of the all-*trans* isomers of the vitamin A group are perhaps of some interest. Using methanol–water isocrats (70:30), the

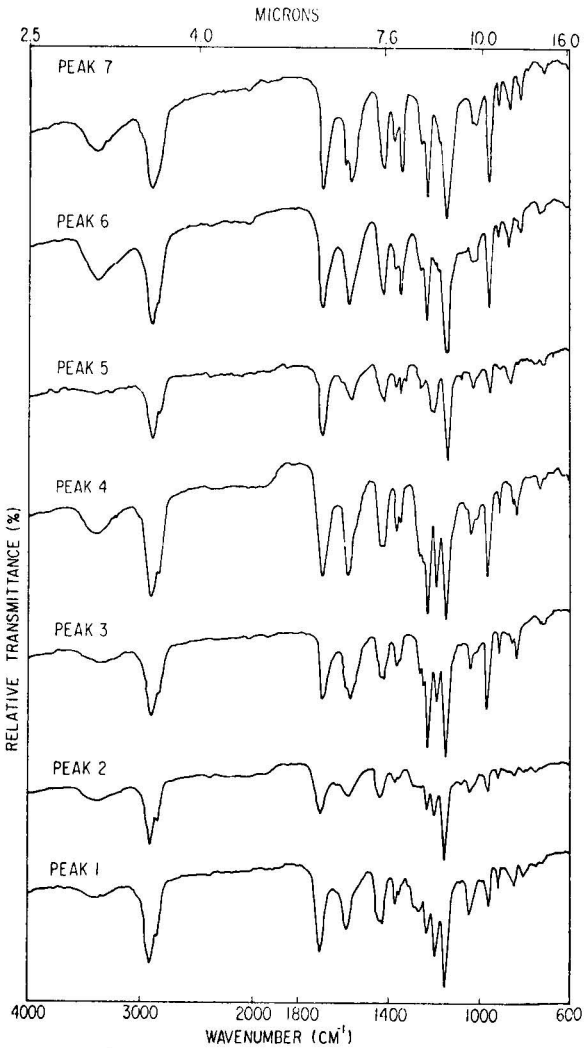


Fig. 5. IR spectra of geometric isomers of methyl retinoate. Peak numbers refer to compounds collected from the HPLC separation as shown in Fig. 1.

retention time of all-*trans*-retinyl acetate was found to be essentially the same as that of all-*trans*-methyl retinoate. All-*trans*-retinaldehyde and all-*trans*-retinol had retention times intermediate between those of all-*trans*-retinoic acid and all-*trans*-methyl retinoate. The approximate relative retention times of these three compounds with respect to the all-*trans*-methyl ester were retinoic acid 0.5, retinol 0.55 and retinaldehyde 0.6.

All-*trans*-retinyl palmitate cannot be recovered from ODS columns when methanol–water solvents are used. Since 100% methanol does not elute high-molecular-weight non-polar compounds, retinyl palmitate would probably go unobserved if these separation methods were carelessly applied to unknown mixtures. With re-

spect to the possible loss of compounds on ODS columns, it is important to emphasize that such columns can be readily fouled. The performance of a highly efficient column can be significantly impaired by a single injection of an inappropriately screened sample. The recommended procedure for regenerating the reversed-phase columns was to elute with a solvent such as a chloroform–methanol mixture. In the present study the compounds having the most dramatic adverse effect on column performance were not readily removed by use of chloroform–methanol. Excellent results were obtained by regenerating fouled columns with 5% aqueous acetic acid.

Owing to differences in the biopotencies of the isomers of retinol and related compounds, the isomeric purity as well as the general chemical purity of these compounds is of importance in biological studies. The procedures outlined here provide a rapid and accurate means of determining the isomeric and chemical purity of retinoic acid and methyl retinoate as well as a procedure for purifying most of the isomers in question. If desired, the procedures permit quantitation on a relative basis. Absolute quantitation will not be possible until all of the required spectral values are acquired. The time required for the analysis is entirely dependent upon the composition of the samples and the degree of separation desired. If only qualitative results are required, the analysis time can be reduced by increasing the methanol concentration and/or flow-rate.

An effort was made to decrease the analysis time, while retaining resolution, through operation at elevated temperatures. However, as the column efficiency improved, the gains were nullified by declines in the relative differences between the partition coefficients. Using the same isomers and operating conditions reported in Fig. 1, but at a temperature of 54°, the column operating efficiency (number of theoretical plates) was approximately doubled. All-*trans*-methyl retinoate eluted with a retention time of 30–35 min. The isomerate separation was similar to that in Fig. 3 except for a compressed time scale. However, the separation of peaks 3–5 was borderline on a column which was operating at maximal efficiency. An even more efficient column might permit the total separation of the isomerate in 30 min on a routine basis. However, with the columns described, those adjustments in the flow-rate and solvent strength that were necessary to assure the separation seen in Fig. 1 increased the retention times to the extent that any advantage gained by operating at elevated temperature was insufficient to justify the inconvenience of doing so. An equally effective, decreased analysis time could be more conveniently obtained by an increase in methanol concentration and operation at ambient temperature.

ACKNOWLEDGEMENTS

Journal article 3331 of the Oklahoma Agricultural Experiment Station.

This investigation was supported in part by Public Health Service Research Grant AM-09191 from the National Institute of Arthritis, Metabolism and Digestive Diseases and NSF No. CHE76-05571. We gratefully acknowledge the technical assistance of W. Dobson and M. Burger.

REFERENCES

1 M. Vecchi, J. Vesely and G. Oesterhelt, *J. Chromatogr.*, 83 (1973) 447.

2 K. Tsukida, A. Kodama and M. Ito, *J. Chromatogr.*, 134 (1977) 331.
3 H. Schlenk and J. L. Gellerman, *Anal. Chem.*, 32 (1960) 1412.
4 R. L. Lin, G. R. Waller, E. D. Mitchell, K. S. Yang and E. C. Nelson, *Anal. Biochem.*, 35 (1970) 435.
5 R. Reid, E. C. Nelson, E. D. Mitchell, M. L. McGregor, G. R. Waller and K. V. John, *Lipids*, 8 (1973) 558.
6 U. Schwieter, G. Englert, N. Rigassi and W. Vetter, *Pure Appl. Chem.*, 20 (1969) 365.
7 P. K. Korver, C. Kruk, P. J. van der Haak, J. L. Baas and H. O. Huisman, *Tetrahedron*, 22 (1966) 277.
8 C. D. Robeson, W. P. Blum, J. M. Dieterle, J. D. Cawley and J. G. Baxter, *J. Amer. Chem. Soc.*, 77 (1955) 4120.

Journal of Chromatography, 155 (1978) 389–398

CHROM. 10,843

HIGH-PRESSURE LIQUID CHROMATOGRAPHIC SEPARATION OF 3-GLUCOSIDES, 3,5-DIGLUCOSIDES, 3-(6-O-*p*-COUMARYL)GLUCOSIDES AND 3-(6-O-*p*-COUMARYLGLUCOSIDE)-5-GLUCOSIDES OF ANTHOCYANIDINS

MARTIN WILLIAMS and GEZA HRAZDINA*

Department of Food Science and Technology, Cornell University, New York State Agricultural Experiment Station, Geneva, N.Y. 14456 (U.S.A.)

and

MARGUERITE M. WILKINSON, JAMES G. SWEENY and GUILLERMO A. IACOBUCCI

Corporate Research and Development Department, The Coca Cola Company, P.O. Drawer 1734, Atlanta, Ga. 30301 (U.S.A.)

(Received December 27th, 1977)

SUMMARY

A high-pressure liquid chromatographic method has been developed for separation of anthocyanins from fruits and their products on a μBondapak C_{18} analytical column. Separation of 3- and 3,5-diglucosides of anthocyanidins can be achieved with aqueous acetic acid. That of the *p*-coumaryl-3 and 3,5-diglucosides requires an aqueous methanolic acetic acid solution. For the separation of complex mixtures of anthocyanins containing members of all four above mentioned pigment groups, a programmed non-linear-gradient elution between aqueous acetic acid and aqueous methanolic acetic acid solution is required. This technique enables a relatively fast separation and identification of twenty anthocyanins in one run without prior treatment of the plant extract, or derivatization of the pigments.

INTRODUCTION

Paper and thin-layer chromatographic methods are commonly used in the process of identification of anthocyanins and other flavonoid compounds[1–3]. Because of the time-consuming development for both paper (up to 36 h) and thin-layer chromatograms (2–5 h), a speedy analysis of a larger number of samples were cumbersome. Recent developments in high-pressure liquid chromatography, instrumentation and column support materials, allowed the analysis of a number of natural products whose properties did not permit derivatization and gas chromatographic detection.

Successful high-pressure liquid chromatographic separation of anthocyanidins

* To whom correspondence should be addressed.

was reported recently[4] on a μBondapak C_{18} column with the aid of a Schoeffel variable-wavelength UV-VIS detector. Using this, or similar instrumentation, anthocyanins and anthocyanidins can be monitored selectively at their $\lambda_{max.}$ without any interference from other compounds.

The availability of a relatively large number of anthocyanins in our laboratory (M.W. and G.H) and the lack of a speedy analytical method for detection of anthocyanins prompted us to investigate the properties of the μBondapak C_{18} column support material toward separation of anthocyanins. The data obtained for the twenty anthocyanins commonly found in *Vitis* sp. are presented here.

EXPERIMENTAL

Materials and methods

Grape juice was obtained from ripe Concord, Ives and De Chaunac (S-9549) grapes grown in the Experiment Station vineyards. Chromatograms were run on a Waters liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.) using a 300 × 4 mm I.D. μBondapak C_{18} column (Waters Assoc.) and a Schoeffel SF770 UV-VIS detector. The anthocyanins were monitored at 520 nm.

Solvent systems

The following solvent systems were used for the separation of anthocyanins: A, 0.1% H_3PO_4 in acetic acid–water (10:90); B, acetic acid–water (15:85); C, water–acetic acid–methanol (65:15:20).

Authentic samples of anthocyanin pigments were obtained from our laboratory collections. Samples for chromatographic analysis were prepared by dissolving approximately 1 mg of the compound in 1 ml of distilled water. Because of differences in purity of the various pigment preparations the exact concentration of each pigment in the complex mixture was purposely varied in order to obtain chromatograms showing peaks of similar heights.

Analysis

Analysis of anthocyanidin-3,5-diglucosides. A sample of 5 μl of a mixture (containing approximately 0.1% each of the 3,5-diglucosides of cyanidin, petunidin, peonidin and malvidin) was injected into the column and eluted with solvent A at a flow-rate of 1.0 ml/min.

Analysis of anthocyanidin-3-glucosides. A sample of 5 μl of a mixture (containing approximately 0.1% each of the 3-glucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin) was injected into the column and eluted with solvent B at a flow-rate of 1.5 ml/min.

Analysis of a mixture of 3- and 3,5-diglucosides of anthocyanidins. A sample of 10 μl containing 5 μl each of a mixture of 3-monoglucosides and that of 3,5-diglucosides was injected into the column and subjected to gradient elution starting with 99% of solvent B and 1% of solvent C. Solvent C was increased from 1 to 100% during a period of 40 min on program 9 of the solvent programmer. The flow-rate was 0.2 ml/min for the first 50 min of the elution and then increased to 0.3 ml/min.

Separation of anthocyanidin-3-(6-O-p-coumarylglucoside)-5-glucosides. A sample of 5 μl of a mixture (containing approximately 0.2% each of the 3-(6-O-*p*-

coumarylglucoside)-5-glucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin) was injected into the column and eluted with solvent C at a flow-rate of 0.3 ml/min.

Separation of anthocyanidin-3-(6-O-p-coumarylglucoside)s. A sample of 7.5 μl of a mixture (containing approximately 0.2% each of the 6-O-*p*-coumarylglucosides of delphinidin, petunidin and malvidin) was injected into the column and eluted with solvent C at a flow-rate of 0.3 ml/min.

Analysis of a mixture of anthocyanidin-3-(6-O-p-coumarylglucoside)s and -3-(6-O-p-coumarylglucoside)-5-glucosides. A sample of 10 μl containing 5 μl of a mixture of acylated monoglucosides and 5 μl of a mixture of acylated diglucosides was injected into the column and eluted with solvent C at a flow-rate of 0.3 ml/min.

Analysis of a complex mixture of anthocyanins. A sample of 20 μl of a complex mixture of anthocyanins [containing 5 μl each of a mixture of 3-glucosides, 3,5-glucosides, 3-*p*-coumarylglucosides and 3-(6-O-*p*-coumarylglucoside)-5-glucosides] as described above was injected into the column and subjected to non-linear-gradient elution starting with 95% of solvent B and 5% of solvent C. The percentage of solvent C was increased from 5 to 100 during a period of 20 min at a flow-rate of 0.2 ml/min on program 9 of the solvent programmer.

Analysis of anthocyanins in grape juices. A 20-μl sample of the grape juice was injected into the column and subjected to non-linear-gradient elution starting with 95% of solvent B and 5% of solvent C and increasing the percentage of solvent C from 5 to 100 during a period of 20 min at a flow-rate of 0.2 ml/min on program 9 of the solvent programmer.

RESULTS

Separation of the anthocyanidin-3,5-diglucosides

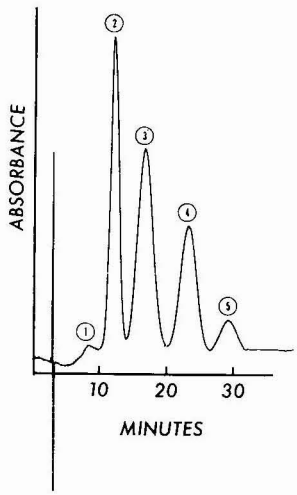
Solvent system A, consisting of 0.1% of H_3PO_4 in 10% acetic acid gave the optimal elution for the anthocyanidin-3,5-diglucosides. The separation of the five *Vitis* anthocyanidin-3,5-diglucosides occurred in the reverse order of that reported earlier on conventional columns of polyvinylpyrrollidone[5]. As shown in Fig. 1, the delphinidin derivative was the first pigment eluted (peak 1), followed by derivatives of cyanidin, petunidin, peonidin, and malvidin, showing the order of elution to be in decreasing polarity of the compounds (peaks 2, 3, 4 and 5, resp.).

Separation of the anthocyanidin-3-glucosides

Solvent A showed a slow and inferior separation of the anthocyanidin-3-glucosides. Therefore, the acidity of the solvent was increased to 15% acetic acid and H_3PO_4 was omitted. With this solvent system (solvent B) the 3-glucosides of the *Vitis* anthocyanidins separated well, as shown in Fig. 2. As is the case of the 3,5-diglucosides of anthocyanidins, the 3-glucosides gave the same elution order, delphinidin-3-glucoside (peak 6) being the first pigment eluted from the column, followed by the 3-glucosides of cyanidin (peak 7), petunidin (peak 8), peonidin (peak 9) and malvidin (peak 10).

Separation of mixtures of anthocyanidin-3-, and -3,5-glucosides

For the separation of the individual pigments from the mixture a non-linear-



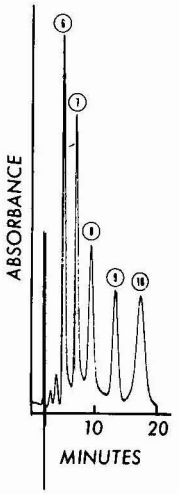


Fig. 1. Separation of the anthocyanidin-3,5-diglucoside on a μBondapak C_{18} column. Solvent A. 1 = Delphinidin-3,5-diglucoside; 2 = cyanidin-3,5-diglucoside; 3 = petunidin-3,5-diglucoside; 4 = peonidin-3,5-diglucoside; 5 = malvidin-3,5-diglucoside. Flow-rate: 1.0 ml/min.

Fig. 2. Separation of anthocyanidin-3-glucosides on a μBondapak C_{18} column. Solvent B. 6 = Delphinidin-3-glucoside; 7 = cyanidin-3-glucoside; 8 = petunidin-3-glucoside; 9 = peonidin-3-glucoside; 10 = malvidin-3-glucoside. Flow-rate: 1.5 ml/min.

gradient (program 9) elution was required. Fig. 3 shows such a separation. With the exception of malvidin-3,5-diglucoside (peak 5) and cyanidin-3-glucoside (peak 7) which overlapped in the above, and also in other solvent systems tried, all anthocyanins were separated. The separation of the individual components of anthocyanins required 70 min.

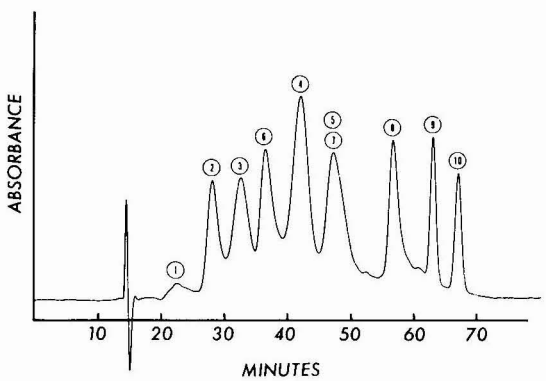


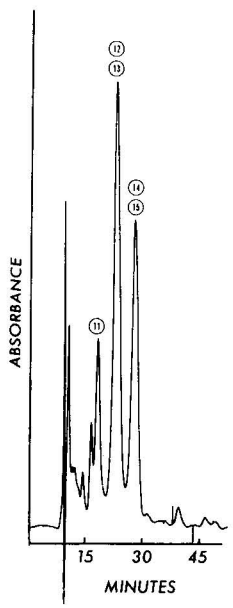
Fig. 3. Separation of mixtures of anthocyanidin-3- and -3,5-glucosides on a μBondapak C_{18} column. Solvent: non-linear gradient between solvent B and solvent C on program 9 of the Waters solvent programmer. Flow-rate 0.2 ml/min for 50 min; then increased to 0.3 ml/min. Peak numbers correspond to those in Figs. 1 and 2.

Separation of anthocyanidin-3-(6-O-p-coumarylglucoside)-5-glucosides

Purely aqueous acetic acid solutions did not separate the anthocyanidin-3-(6-O-*p*-coumarylglucoside)-5-glucosides. A good separation of these pigments could be obtained however when methanol was introduced (at 20% concentration) into the solvent system used for the separation of the 3-glucosides. The so obtained solvent system (solvent C) gave three peaks for the five anthocyanin pigments injected into the column. While delphinidin-3-(6-O-*p*-coumarylglucoside)-5-glucoside separated from the other pigments as a single peak (peak 11), the same derivatives of cyanidin or petunidin (peaks 12 and 13) and peonidin and malvidin (peaks 14 and 15) were eluted as two pairs, shown in Fig. 4.

Separation of anthocyanidin-3-(6-O-p-coumarylglucoside)s

Solvent C at a flow-rate of 0.3 ml/min gave a good separation of all five 3-(6-O-*p*-coumarylglucoside)s of the *Vitis* anthocyanidins. The order of elution was the same as in previous separations (Fig. 5). Delphinidin derivative (peak 16) was eluted first, followed by derivatives of cyanidin (peak 17), petunidin (peak 18), peonidin (peak 19) and malvidin (peak 20).



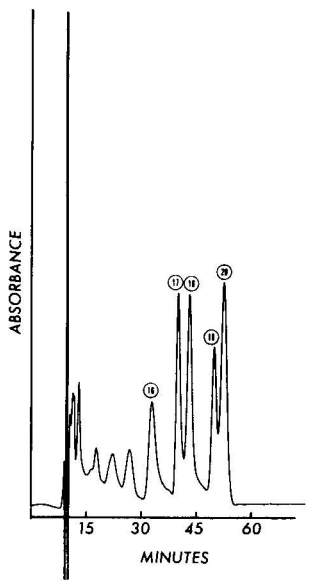


Fig. 4. Separation of anthyocyanidin-3-(6-O-*p*-coumarylglucoside)-5-glucosides on a μBondapak C_{18} column. Solvent C. 11 = Delphinidin-3-(6-O-*p*-coumarylglucoside)-5-glucoside; 12 = cyanidin-3-(6-O-*p*-coumarylglucoside)-5-glucoside; 13 = peninidin-3-(6-O-*p*-coumarylglucoside)-5-glucoside; 14 = peonidin-3-(6-O-*p*-coumarylglucoside)-5-glucoside; 15 = malvidin-3-(6-O-*p*-coumarylglucoside)-5-glucoside. Flow-rate: 0.3 ml/min.

Fig. 5. Separation of the anthocyanidin-3-(6-O-*p*-coumarylglucoside)s on a μBondapak C_{18} column. Solvent C. 16 = Delphinidin-3-(6-O-*p*-coumarylglucoside); 17 = cyanidin-3-(6-O-*p*-coumarylglucoside); 18 = petunidin-3-(6-O-*p*-coumarylglucoside); 19 = peonidin-3-(6-O-*p*-coumarylglucoside); 20 = malvidin-3-(6-O-*p*-coumarylglucoside). Flow-rate: 0.3 ml/min.

Separation of mixtures of anthocyanidin-3-(6-O-p-coumarylglucoside)s and -3-(6-O-p-coumarylglucoside)-5-glucosides

The separation of mixtures of the acylated anthocyanidin-3 and -3,5-diglucosides appeared in the same order as that reported for the individual groups (Fig. 6) using a single solvent system (solvent C). Delphinidin-3-(6-O-*p*-coumarylglucoside)-5-glucoside was eluted first and malvidin-3-(6-O-*p*-coumarylglucoside) was eluted last. The time required for the separation was 55 min at 0.3 ml/min flow-rate. Peaks preceding the acylated anthocyanins on the chromatogram were contaminations in the preparates by non-acylated anthocyanidin-3- and -3,5-diglucosides.

Separation of mixtures containing the 3-glucosides, 3,5-diglucosides, 3-(6-O-p-coumarylglucoside)s and 3-(6-O-p-coumarylglucoside)-5-glucosides of anthocyanidin

A complex mixture containing the above derivatives of the 5 *Vitis* anthocyanidins could not be separated by the use of one single solvent system. Therefore, a non-linear gradient between solvent systems B and C was used. As shown in Fig. 7 seventeen peaks for the twenty anthocyanins were obtained. The overlapping pigments were malvidin-3,5-diglucoside and cyanidin-3-glucoside (peaks 5 and 7), the 3-(6-O-*p*-coumarylglucoside)-5-glucosides of cyanidin and petunidin (peaks 12 and 13) and those of peonidin and malvidin (peaks 14 and 15). All other anthocyanins separated clearly. The structural relationship and retention times of the investigated anthocyanins obtained by the above described non-linear-gradient elution between solvent systems B and C are shown in Fig. 8.

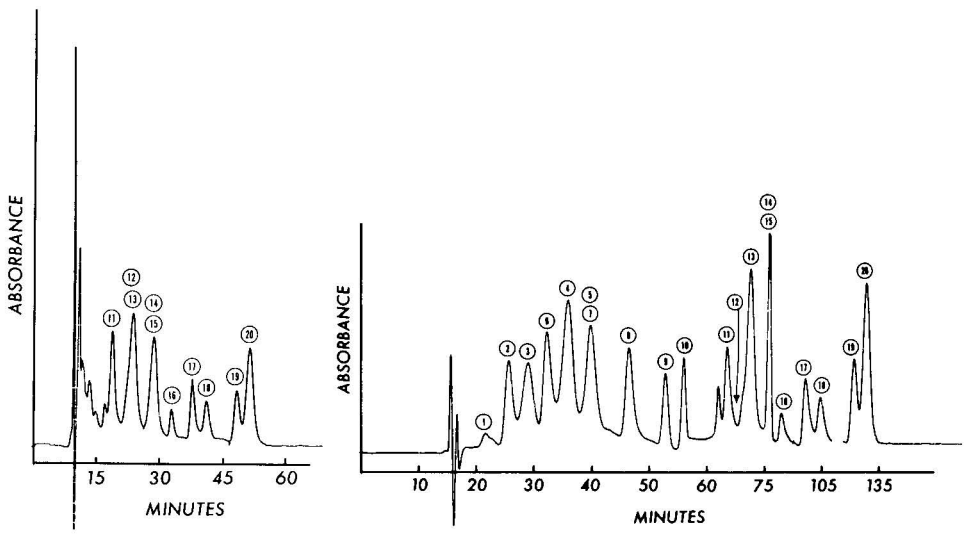
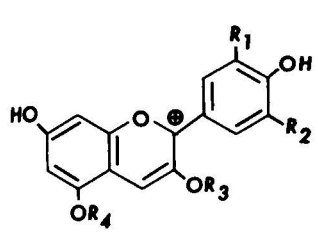
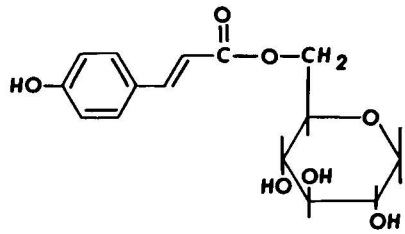


Fig. 6. Separation of mixtures of anthocyanidin-3-(6-O-*p*-coumarylglucoside)s and anthocyanidin-3-(6-O-*p*-coumarylglucoside)-5-glucosides on a μBondapak C_{18} column. Solvent C. Peak numbers correspond to those in Figs. 4 and 5. Flow-rate: 0.3 ml/min.

Fig. 7. Separation of anthocyanidin-3-glucosides, 3,5-diglucosides, 3-(6-O-*p*-coumarylglucoside)s and 3-(6-O-*p*-coumarylglucoside)-5-glucosides. Solvent: non-linear gradient between solvents B and C on program 9 of the Waters solvent programmer. Flow-rate: 0.2 ml/min. Peak numbers correspond to those in Figs. 1–5.



Anthocyanin



(6-O-p-coumarylglucoside) = (PCG)

Order of elution	Compound	Substitution R_1	R_2	R_3	R_4	Retention times t_R (min)
Diglucosides						
1	Delphinidin 3,5-diglucoside	OH	OH	Glu	Glu	22.0
2	Cyanidin 3,5-diglucoside	OH	H	Glu	Glu	26.0
3	Pentunidin 3,5-diglucoside	OCH_3	OH	Glu	Glu	30.1
4	Peonidin 3,5-diglucoside	OCH_3	H	Glu	Glu	37.8
5	Malvidin 3,5-diglucoside	OCH_3	OCH_3	Glu	Glu	42.1
Monoglucosides						
6	Delphinidin 3-glucoside	OH	OH	Glu	H	33.2
7	Cyanidin 3-glucoside	OH	H	Glu	H	42.1
8	Petunidin 3-glucoside	OCH_3	OH	Glu	H	49.3
9	Peonidin 3-glucoside	OCH_3	H	Glu	H	53.2
10	Malvidin 3-glucoside	OCH_3	OCH_3	Glu	H	55.1
Acylated diglucosides						
11	Delphinidin 3(6-O-p coumarylglucoside)-5 glucoside	OH	OH	PCG	Glu	64.5
12	Cyanidin 3(6-O-p-coumarylglucoside)-5 glucoside	OH	H	PCG	Glu	70.0
13	Petunidin 3(6-O-p-coumarylglucoside)-5 glucoside	OCH_3	OH	PCG	Glu	72.0
14	Peonidin 3(6-O-p-coumarylglucoside)-5 glucoside	OCH_3	H	PCG	Glu	79.1
15	Malvidin 3(6-O-p-coumarylglucoside)-5 glucoside	OCH_3	OCH_3	PCG	Glu	79.1
Acylated monoglucosides						
16	Delphinidin 3(6-O-p-coumarylglucoside)	OH	OH	PCG	H	87.0
17	Cyanidin 3(6-O-p-coumarylglucoside)	OH	H	PCG	H	100.9
18	Petunidin 3(6-O-p-coumarylglucoside)	OCH_3	OH	PCG	H	111.0
19	Peonidin 3(6-O-p-coumarylglucoside)	OCH_3	H	PCG	H	129.9
20	Malvidin 3(6-O-p-coumarylglucoside)	OCH_3	OCH_3	PCG	H	140.2

Fig. 8. Structure and retention times of anthocyanins on a μBondapak C_{18} column.

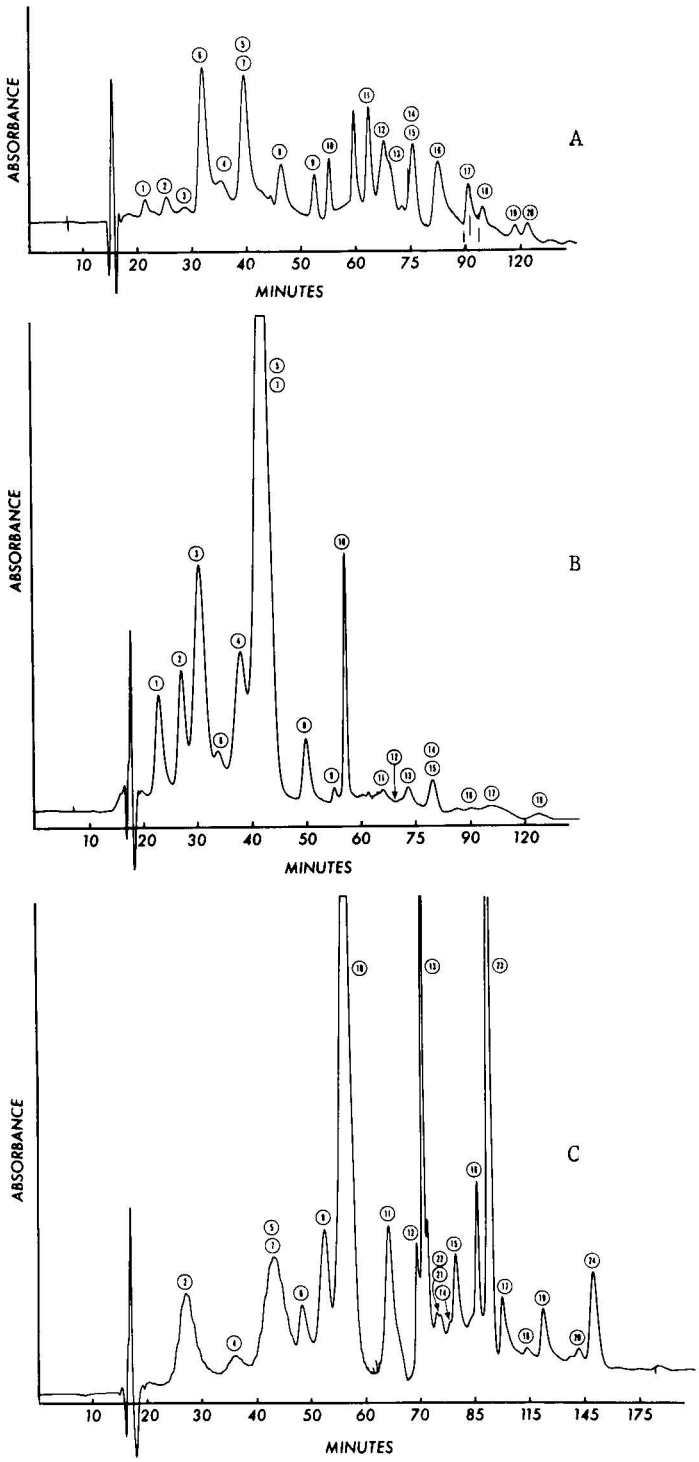


Fig. 9. Anthocyanin profile of Concord (A), De Chaunac (Seibel-9549) (B) and Ives (C) grapes. Solvent: non-linear gradient between solvents B and C on program 9 of Waters solvent programmer. Peak numbers correspond to those in Figs. 1–5. Flow-rate: 0.2 ml/min. Peaks 21–24 were not identified.

Qualitative analysis of grape juices for anthocyanin content

The following figures show the separation of anthocyanins in the untreated grape-juice samples. The pigment profile of Concord grape juice (Fig. 9A) was obtained by injection of 20 μl sample into the column. It has been reported earlier that this grape contains the derivatives of delphinidin and cyanidin in the largest amount[3]. Clearly, the separation shows that the 3-glucosides (peaks 6 and 7) and 3-(6-O-*p*-coumarylglucoside)s (peaks 16 and 17) of delphinidin and cyanidin are the major pigments present. Peak 5/7 is caused by about a 20:1 ratio of cyanidin–malvidin-3,5-diglucoside and not by large amounts of malvidin-3,5-diglucoside. In Fig. 9B the individual anthocyanins of De Chaunac (S-9549) grapes are shown. As is the case with the Concord sample, 20 μl untreated grape juice was directly injected into the column. This grape variety contains the 3,5-diglucosides of the five *Vitis* anthocyanins in large amounts[5]. The third grape variety investigated was that of Ives (Fig. 9C). This grape contains in accord with earlier reports[2] larger amounts of anthocyanins acylated with *p*-coumaric acid. Major anthocyanins present in this grape sample were the 3,5-diglucosides of cyanidin (peak 2) and malvidin (peak 5), the 3-glucoside of malvidin (peak 10), the 3-(6-O-*p*-coumarylglucoside)-5-glucosides of delphinidin (peak 11), petunidin (peak 13) and malvidin (peak 15), and also the 3-(6-O-*p*-coumarylglucoside)s of delphinidin (peak 16), cyanidin (peak 17) and peonidin (peak 19). In addition to the known anthocyanins, two other unknown major pigment peaks (peaks 23 and 24) and some minor unidentified anthocyanins (peaks 21 and 22) were observed.

DISCUSSION

The result shows that with a μBondapak C_{18} column separation and analysis of anthocyanin pigments can be achieved by using acetic acid solution for the non-acylated glucosides and an acetic acid solution containing methanol for the acylated glucosides. The reproducibility of the chromatographic data largely depends upon the pH of the eluting solvent system, the operating pressure and the temperature at which the chromatography is performed. Thus chromatograms obtained for the same sample, containing non-acylated and acylated pigments, run on different days at a slightly different temperature and a very slight variation in the pH of two eluting solvent systems, resulted in minor differences in the retention times. Therefore, a reference mixture should be analyzed first or an appropriate internal standard must be incorporated in the sample.

The results also show that the polarity of the compounds play a vital role in the determination of the retention times. On this basis the non-acylated diglucosides, being the most polar, are the least retained by the column, followed by the non-acylated monoglucosides, acylated diglucosides and finally the acylated monoglucosides. Within the individual groups the substitution in the B-ring is a key factor in the determination of the retention times. With increasing hydroxylation in the B-ring the retention time of the compounds decrease. Thus delphinidin with three hydroxy groups in the B-ring is less retained than cyanidin with its two hydroxy groups. Methylation of the hydroxy groups in the B-ring increases the retention time of the anthocyanins. Among the methylated anthocyanins, derivatives of petunidin with two hydroxy groups and one methoxy group are retained for a shorter time than

derivatives of peonidin which has one hydroxy and one methoxy group. Malvidin derivatives in all groups of the investigated anthocyanins showed the longest retention times.

Since anthocyanins can be selectively detected in the region of 520 nm, where few other compounds absorb, this method does not require a preliminary-treatment of samples. The samples (aqueous plant extracts) can be directly injected into the column, and their pigment constituents so determined. During preliminary investigation with anthocyanidin-3-bioside-5-glucosides and their acylated derivatives we found that these compounds gave an unsatisfactory separation in the solvent systems described above. Column and solvent conditions for these latter compounds apparently will have to be modified.

ACKNOWLEDGEMENT

One of us (M.W.) thanks the Welch Food Company (Westfield, N.Y., U.S.A.) for a post-doctoral fellowship.

REFERENCES

1 J. B. Harborne, *Comparative Biochemistry of the Flavonoids*, Academic Press, London, New York, 1967, Ch. 1, p. 12–15.
2 G. Hrazdina and A. J. Franzese, *Phytochemistry*, 13 (1974) 225.
3 G. Hrazdina, *Lebensm.-Wiss. Technol.*, 8 (1975) 111.
4 M. Wilkinson, J. G. Sweeny and G. A. Iacobucci, *J. Chromatogr.*, 132 (1977) 349.
5 G. Hrazdina, *J. Agr. Food Chem.*, 18 (1970) 243.

Journal of Chromatography, 155 (1978) 399–406

CHROM. 10,856

NACHWEIS VON SULFAMETHOXAZOL UND N^4-ACETYL-SULFAMETHOXAZOL IN BIOLOGISCHEN FLÜSSIGKEITEN DURCH HOCHDRUCKFLÜSSIGKEITSCHROMATOGRAPHIE IN UMGEKEHRTER PHASE*

KLAUS HARZER

Chemisches Untersuchungsamt der Landeshauptstadt Stuttgart, Stafflenbergstrasse, 7000 Stuttgart 1 (B.R.D.)

(Eingegangen am 30. September 1977; geänderte Fassung eingegangen am 6. Januar 1978)

SUMMARY

Detection of sulfamethoxazol and N^4-acetylsulfamethoxazol in biological fluids by reversed-phase high-pressure liquid chromatography

Sulfonamide drugs can be detected by reversed-phase high-pressure liquid chromatography. The possibilities of this method in the direct qualitative and quantitative analysis in blood or in urine without enrichment and derivatization are illustrated by the analysis of sulfamethoxazol and its metabolite N^4-acetylsulfamethoxazol.

EINFÜHRUNG

Eine Untersuchung auf Sulfonamide in Blut oder Urin kann nötig sein, um z.B. bei Kindern, die unbekannte Medikamente eingenommen haben, eine Vergiftung zu bestätigen oder auszuschliessen. Eine quantitative Bestimmung wird notwendig zur Kontrolle einer Therapie oder zur Bestimmung pharmokologischer Daten. Der Nachweis von Sulfonamiden kann qualitativ dünnschichtchromatographisch[1] oder gaschromatographisch[2] geführt werden. Eine quantitative Untersuchung ist UV-spektrophotometrisch nach Bratton und Marshall[3] und wiederum gaschromatographisch[2] möglich. Ausser bei der Dünnschichtchromatographie müssen bei den beiden anderen Methoden Derivatisierungsschritte durchgeführt werden. Dies ist bei der Hochdruckflüssigkeitschromatographie nicht nötig. Von den verschiedenen Arten der Hochdruckflüssigkeitschromatographie wurden für die Untersuchung auf Sulfonamide verwendet die Adsorptionschromatographie[4,5], die Ionenaustauschchromatographie[6–8], die Ionen-Paar-Chromatographie[9] sowie die Chromatographie in umgekehrter Phase[10,11].

Für den Nachweis von Sulfonamiden, insbesondere von Sulfamethoxazol und

* Auszugsweise vorgetragen auf der Hauptversammlung der Gesellschaft deutscher Chemiker in München 1977.

dessen bei der Körperpassage entstehenden Metaboliten N^4-Acetyl-Sulfamethoxazol, verwendeten wir ebenfalls die Hochdruckflüssigkeitschromatographie in umgekehrter Phase, wobei zwei Säulen und zwei Laufmittel zur Anwendung kamen.

EXPERIMENTELLES

Dünnschichtchromatographie

Die Dünnschichtchromatographie wurde auf Fertigfolien Polygram Sil G/UV_{254} der Fa. Macherey, Nagel & Co. (Düren, B.R.D.) durchgeführt. Die Entwicklung erfolgte mit Chloroform–Methanol (95:5) (modifiziert nach Gänshirt[1]). Sulfamethoxazol: R_F-Wert 0.6; Detektion mit Ehrlichs Reagenz (1 g *p*-Dimethylaminobenzaldehyd in 100 ml 6 *M* HCl)[12]. N^4-Acetyl-Sulfamethoxazol: R_F-Wert 0.4; Detektion unter UV-Licht bei 254 nm.

UV-Spektrophotometrie

Die Spektren zur Bestimmung der Maxima wurden mit einem Beckman Spektralphotometer DK-2A in Äthanol aufgenommen.

Hochdruckflüssigkeitschromatographie

Gerät: Perkin-Elmer Hochdruckflüssigkeitschromatograph 1250 mit variablem UV-Detektor Perkin-Elmer LC 55. Säulen: (1) Stahlsäule 25 cm × 4 mm mit C_{18}-Reversed-Phase-Material auf LiChrosorb Si 100 (Korngrösse 10 μm) (Merck, Darmstadt, B.R.D.); (2) Hibar®-Fertigstahlsäule 25 cm × 3 mm (Merck) mit C_8-Reversed-Phase Material LiChrosorb (Korngrösse 7 μm). Einspritzteil: Rheodyne Modell 7105. Laufmittel: (1) Methanol–Wasser (3:7). (2) Acetonitril–Wasser (1:3).

Die Untersuchungen wurden bei Zimmertemperatur durchgeführt; der Druck war 1200–1500 p.s.i.; die Durchflussgeschwindigkeit 0.4–0.6 ml/min; der Papiervorschub betrug 5 mm/min.

Untersuchungsmaterial

Die Proben stammten von Personen, denen therapeutisch Bactrim® verabreicht worden war.

Zusatzversuche

Zu 1 ml Urin wurden 150 μg Sulfamethoxazol und 190 μg N^4-Acetyl-Sulfamethoxazol gelöst in 200 μl Äthanol gegeben.

ERGEBNISSE

Die Retentionszeiten von Sulfamethoxazol und N^4-Acetyl-Sulfamethoxazol neben anderen Sulfonamiden sind in Tabelle I angegeben. Es wurden dabei eine C_8 und eine C_{18} Reversed-Phase-Säule und zwei verschiedene Laufmittel verwendet. Gleichzeitig sind die UV-Maxima in Äthanol angegeben. Wenn nur auf eine Substanz untersucht werden soll, kann deren Maximum eingestellt werden. Sonst empfiehlt es sich, die Detektion bei 270 nm durchzuführen. Diese Wellenlänge stellt etwa den Mittelwert dar und bei 270 nm wurden auch die weiteren Untersuchungen durchgeführt.

Die Figs. 1 und 2 zeigen die Auftrennung einiger ausgewählter Sulfonamied

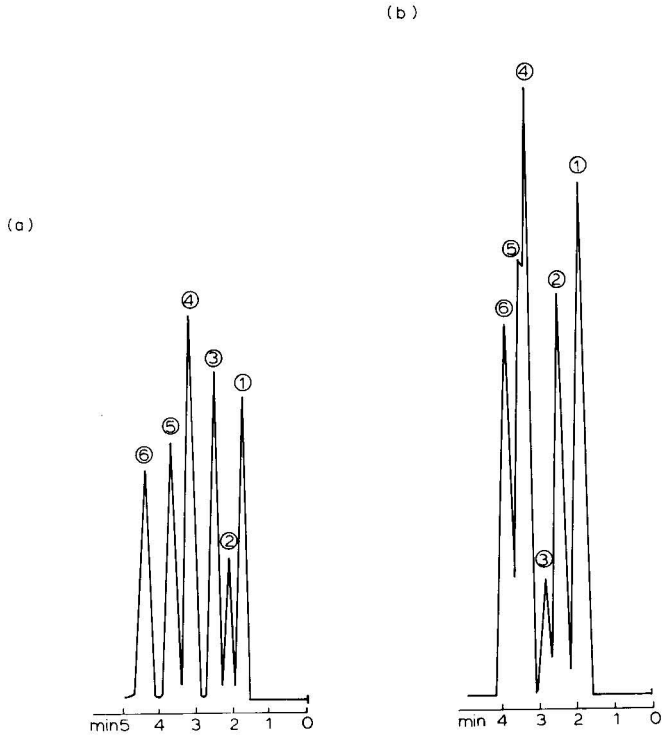


Fig. 1. Trennung von Sulfonamiden mit der C_{18}-Säule, Laufmittel: (a) Methanol–Wasser (3:7); (b) Acetonitril–Wasser (1:3). 1 = Sulfanilthiocarbamid; 2 = N^4-Acetyl-Sulfamethoxazol; 3 = Sulfamethoxazol; 4 = Sulfanilamidothiazol; 5 = Sulfamethoxydiazin; 6 = Sulfamoxal.

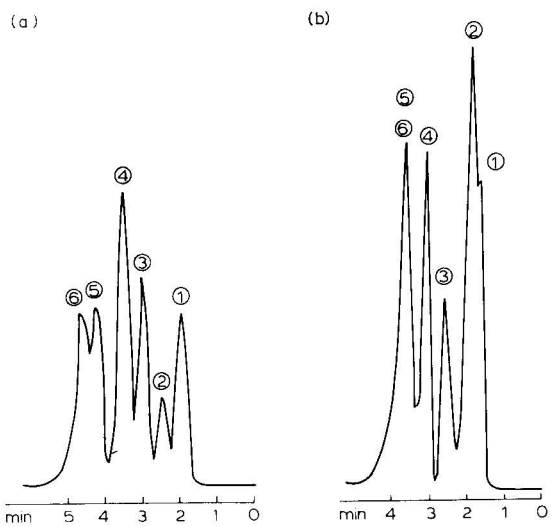


Fig. 2. Trennung von Sulfonamiden mit der C_8-Säule. Laufmittel: (a) Methanol–Wasser (3:7); (b) Acetonitril–Wasser (1:3). 1 = Sulfanilthiocarbamid; 2 = N^4-Acetyl-Sulfamethoxazol; 3 = Sulfamethoxazol; 4 = Sulfanilamidothiazol; 5 = Sulfamethoxydiazin; 6 = Sulfamoxal.

TABELLE I

RETENTIONSZEITEN UND UV-MAXIMA VERSCHIEDENER SULFONAMIDE

Substanz	*Handelsname*	*UV-Maxima in Äthanol (nm)*	*Retentionszeiten (min)*			
			Säule C_{18}		*Säule C_8*	
			Methanol–Wasser (3:7) 0.6 ml/min	*Acetonitril–Wasser (1:3) 0.5 ml/min*	*Methanol–Wasser (3:7) 0.4 ml/min*	*Acetonitril–Wasser (1:3) 0.4 ml/min*
Phthalylsulfathiazol	Talendron	257/282	3.2	3.4	3.8	3.2
Sulfacetamid	Albucid	272	3.4	3.6	3.8	3.6
Sulfanilthiocarbamid	Badional	260	3.5	3.6	3.8	3.5
Sulfafurazol	Gantrisin	261/282	3.5	3.8	4.2	3.6
N^4-Acetyl-Sulfamethoxazol	—	263	4.1	4.2	4.4	3.8
Sulfamethoxazol	Gantanol	270	4.8	5.5	5.8	5.7
Sulfanilguanidin	Resulfon	262	5.0	5.8	5.4	5.6
Sulfanilamid	Prontalbin	262	5.4	6.6	5.6	6.2
Sulfanilamidothiazol	Eleudron	257/282	6.2	6.2	7.0	6.0
Sulfaphenazol	Orisul	267	6.4	6.2	8.8	6.2
Sulfamethoxydiazin	Durenat	270/230	7.2	7.0	8.4	7.2
Sulfamoxal	Sulfuno	268	8.8	7.8	9.2	7.4

bei den verschiedenen Bedingungen. Für den Nachweis von Sulfamethoxazol und N^4-Acetyl-Sulfamethoxazol wird am besten das Laufmittel Acetonitril–Wasser (1:3) und die C_8-Säule verwendet, da damit beide Substanzen am besten getrennt werden.

Erfassungsgrenze

Unter den zuletzt genannten Bedingungen ergab sich eine Erfassungsgrenze von 2 μg/ml, wenn 1 μl eingespritzt wird.

Linearität

Im Bereich von 50 μg–850 μg/ml ist die Korrelation zwischen Peakhöhe und Konzentration von N^4-Acetyl-Sulfamethoxazol und Sulfamethoxazol linear.

Standardabweichung

Die Standardabweichung betrug für N^4-Acetyl-Sulfamethoxazol 0.27 und für Sulfamethoxazol 0.18.

Wiederfindungsrate

Bei Zusatzversuchen zu Urin ergaben sich beim direkten Einspritzen für Sulfamethoxazol und N^4-Acetyl-Sulfamethoxazol Wiederfindungsraten von $100 \pm 3\%$ ($n = 7$).

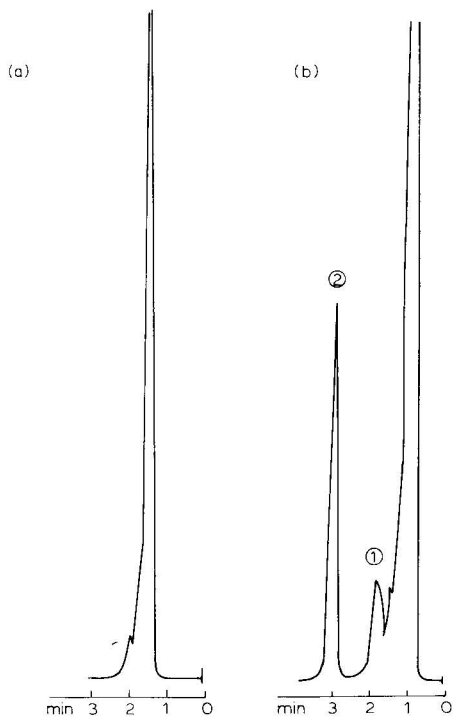


Fig. 3. Nachweis von Sulfamethoxazol und N^4-Acetyl-Sulfamethoxazol im Blut durch Direkteinspritzung mit der C_8-Säule. (a) Leerblut; (b) Blut A. Laufmittel: Acetonitril–Wasser (1:3). 1 = 18 μg/ml N^4-Acetyl-Sulfamethoxazol; 2 = 95.7 μg/ml Sulfamethoxazol.

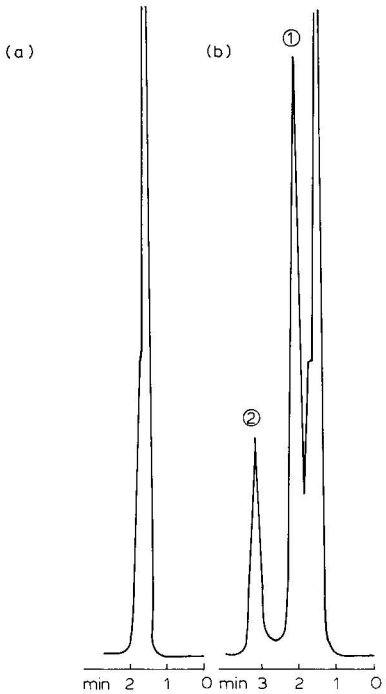


Fig. 4. Nachweis von Sulfamethoxazol und N^4-Acetyl-Sulfamethoxazol durch Direkteinspritzung mit der C_8-Säule. (a) Leerurin; (b) Urin A. Laufmittel: Acetonitril–Wasser (1:3). 1 = 855.6 µg/ml N^4-Acetyl-Sulfamethoxazol; 2 = 400.2 µg/ml Sulfamethoxazol.

Die Übertragung der Methode auf die Untersuchung von Blut und Urin zeigen die Fig. 3 und 4. Es können Sulfamethoxazol und N^4-Acetyl-Sulfamethoxazol nebeneinander nachgewiesen werden, wenn man 1 µl Urin oder 1–3 µl Blut direkt einspritzt. Die Befunde wurden dünnschichtchromatographisch nach Extraktion[13] abgesichert.

Die Werte der quantitativen Bestimmungen in verschiedenen Blut- und Urinproben zeigt Tabelle II. Die gleichen Buchstaben der Blut- und Urinproben gehören

TABELLE II

WERTE VON SULFAMETHOXAZOL UND N^4-ACETYL-SULFAMETHOXAZOL NACH THERAPEUTISCHER DOSIERUNG

Patient	*Sulfamethoxazol (µg/ml)*		*N^4-Acetyl-Sulfamethoxazol (µg/ml)*	
	Blut	*Urin*	*Blut*	*Urin*
A	95.7	400.2	18.0	855.6
B	64.1	80.6	41.5	1003.7
C	66.2	113.4	28.1	668.6
D	120.8	70.5	105.5	627.3
E		243.6		829.0
F		25.1		525.9
G		97.4		328.0

zu einem Patienten. Die Dosierung und der Einnahmezeitraum waren ausser beim Urinwert G nicht bekannt. Dieser Wert wurde erhalten nach Einnahme von 1 Dragee Bactrim forte® (Inhaltsstoffe 800 mg Sulfamethoxazol und 160 mg Trimethoprim) und Entnahme des Urins 14 h später.

DISKUSSION

Die geschilderte Methode liefert schnell quantitative Werte, ohne dass Extraktions- oder Derivatisierungsschritte durchgeführt werden müssen, da Blut, Plasma, Serum oder Urin direkt eingespritzt werden können. Hierbei besteht die Gefahr, dass die Trennleistung der Säule nachlässt, und es empfiehlt sich deshalb nach fünf bis zehn Untersuchungen mit *ca.* 50 ml Methanol die Säule zu spülen. Bei den wenigen Untersuchungen auf Sulfonamide, die im toxikologischen Bereich anfallen, hat sich die Direkteinspritzung der Proben ohne weitere Aufarbeitung bewährt und innerhalb eines halben Jahres trat dabei keine Verschlechterung der Säulencharakteristik auf.

Bei häufigen Untersuchungen könnte ein Schutz der Trennsäule durch eine Vorsäule[14] erreicht werden. Eine weitere Möglichkeit unerwünschte Begleitstoffe aus dem biologischen Material zu entfernen besteht darin, dass man die Blut- oder Urinproben im Verhältnis 1:1 mit Acetonitril schüttelt, anschliessend zentrifugiert und dann die überstehende Lösung einspritzt[15].

Die Erfassungsgrenze reicht aus, um sicher therapeutische Dosierungen von Sulfamethoxazol nachweisen zu können. Sollte der Nachweis geringer Konzentrationen nötig sein, können grössere Mengen Urin oder Blut eingespritzt werden (z.B. 10–20 μl). Der Nachweis kann gestört werden durch Diuretika, die ebenfalls eine Sulfonamidstruktur besitzen, wie z.B. Hydrochlorothiazid (Esidrix®) und Furosemid (Lasix®).

Barbiturate, Antiepileptika und Benzodiazepine[16] stören den Nachweis nicht.

DANK

Wir danken dem Katharinenhospital Stuttgart, Urologische Klinik (Leiter Prof. Dr. Arnholdt) sowie Herrn Dr. Klas, Stuttgart für die Überlassung von Proben, der Fa. Hoffmann-La Roche für Vergleichssubstanzen.

ZUSAMMENFASSUNG

Sulfonamide können durch Hochdruckflüssigkeitschromatographie in umgekehrter Phase nachgewiesen werden. Am Beispiel von Sulfamethoxazol und dessen Metaboliten N^4-Acetyl-Sulfamethoxazol wird gezeigt, dass eine qualitative und quantitative Bestimmung direkt in Blut und Urin ohne Anreicherung und Derivatisierung möglich ist.

LITERATUR

1 H. Gänshirt, in E. Stahl (Herausgeber), *Synthetische Arzneistoffe in Dünnschichtchromatographie*, Springer, Berlin, Heidelberg, New York, 1967, S. 517.
2 E. Röder und W. Stuthe, *Z. Anal. Chem.*, 271 (1974) 281.
3 A. C. Bratton und E. K. Marshall, *J. Biol. Chem.*, 128 (1939) 573.

4 P. H. Cobb und G. T. Hill, *J. Chromatogr.*, 123 (1976) 444.
5 F. M. Rabel, *Anal. Chem.*, 45 (1973) 957.
6 G. R. Gordon und D. C. Ghoul, *J. Pharm. Sci.*, 12 (1975) 1205.
7 T. Inaba, M. E. Besley und E. J. Chow, *J. Chromatogr.*, 104 (1975) 165.
8 B. C. Karger und S. C. Su, *J. Chromatogr. Sci.*, 12 (1974) 678.
9 S. C. Su, A. V. Hartkopf und B. L. Karger, *J. Chromatogr.*, 119 (1976) 523.
10 K. L. Johnson, D. T. Jeter und R. C. Claiborne, *J. Pharm. Sci.*, 64 (1975) 1657.
11 B. Fransson, K.-G. Wahlund, I. M. Johansson und G. Schill, *J. Chromatogr.*, 125 (1976) 327.
12 M. Geldmacher-von Mallinkrodt, in H. Breuer, H. Büttner, G. Hillmann und D. Stamm (Herausgeber), *Einfache Untersuchungen auf Gifte im klinisch-chemischen Laboratorium in Klinische Chemie in Einzeldarstellungen*, Georg Thieme, Stuttgart, 1976, S. 85,
13 E. G. C. Clarke, *Isolation and Identification of Drugs in Pharmaceuticals and Post Mortem Material*, Pharmaceutical Press, London, 1971, S. 552.
14 J. J. Kirkland, *Analyst (London)*, 99 (1974) 859.
15 P. M. Kabra, B. E. Stafford und L. J. Marton, *Clin. Chem.*, 23 (1977) 1284.
16 K. Harzer und R. Barchet, *J. Chromatogr.*, 132 (1977) 83.

Journal of Chromatography, 155 (1978) 407–413

CHROM. 10,939

IMPROVED SEPARATION OF NUCLEOSIDES, NUCLEOTIDES, AND AMINOACYL tRNA ON A STRONG ANION-EXCHANGE RESIN

M. KRATOVICH and B. A. ROE
Department of Chemistry, Kent State University, Kent, Ohio 44242 (U.S.A.)
(Received December 22nd, 1977)

SUMMARY

Nucleosides and nucleotides can be separated on a polystyrene–divinylbenzene anion-exchange resin, Chromex DA-X8-11 at 50° using isocratic elution with 1.0 *M* ammonium carbonate pH 8.8 or 0.05 *M* formic acid pH 2.4. The resin can also resolve aminoacyl tRNA on either the analytical or preparative scale.

INTRODUCTION

In recent years several chromatographic procedures have been developed for the separation and detection of nucleic acid components. Two-dimensional thin-layer chromatography (TLC) on cellulose or silica gel, coupled with tritium post labeling[1] is an extremely sensitive method, which has recently been applied to the complete nucleotide sequence analysis of tRNA[2,3]. Since this TLC procedure requires several days before the results can be analyzed, high-performance liquid chromatography (HPLC) has often been employed as an alternative when rapid analysis is desired. By using small particle diameter ion-exchange resins such as Aminex[4–8] and reversed-phase resins (RPC-5)[9,10] the complete analysis of nucleic acid components can be obtained within a few hours. We now wish to report the isocratic separation of nucleosides and nucleotides on a new polystyrene–divinylbenzene 8% cross-linked quaternary amine anion exchanger, Chromex DA-X8-11, and its application to RNA sequence analysis. In addition, we have also investigated the elution profiles of Walker 256 mammary tumor and rat liver $tRNA^{Tyr}$ on Chromex DA-X8-11 to demonstrate the feasibility of this resin for determining alterations in the chromatographic behavior between normal and tumor aminoacyl tRNAs[5,11–14].

EXPERIMENTAL

The anion-exchange resin, DA-X8-11 (Lot No.: 71-81), was obtained from Durrum (Sunnyvale, Calif., U.S.A.). Nucleosides and nucleotide monophosphates, obtained from Calbiochem (La Jolla, Calif., U.S.A.), were dissolved in deionized distilled water. In some cases dilute HCl was added to enhance the solubility of purine nucleosides.

Chromatographic methods

Column chromatography was carried out in a stainless-steel jacketed column (30 × 0.5 cm I.D.) which was prepared by joining Swagelock end fittings (Akron Valve and Fitting, Akron, Ohio, U.S.A.) onto 316 stainless tubing (Tube Sales, Rocky River, Ohio, U.S.A.). A stainless-steel fritted end (Swagelock reducing union with an SR-17 snubber) served as the bed support. The column temperature was maintained at 50° by a Haake circulating water bath.

A Milton Roy pump (Mil-Roy-D) with a maximum pressure rating of 1000 p.s.i. was employed to obtain flow-rates of 0.5–1.0 ml/min. Typical eluent pump pressures measured with a WIKA pressure gauge (O. W. Heyman, Cleveland, Ohio, U.S.A.) ranged from 500 to 1000 p.s.i. Samples (25–50 μl, 0.2–0.4 A_{260} units* for nucleosides and nucleotides) were introduced using an off-column septum sample injector by means of a Hamilton syringe. The effluent was monitored at 254 nm with an Altex UV detector equipped with a 20-μl flow cell. Full-scale detector output ranged from 0.01 to 2.54 absorbance units and was displayed on a Laboratory Data Control-Servographic (Riviera Beach, Fla., U.S.A.) strip-chart recorder (10 mV) at a chart speed of 12 in./h.

The resin was slurried in 1.0 *M* ammonium carbonate, poured into the column, and packed by pumping with the same buffer for 60 min at a flow-rate of 1.0 ml/min. Equilibration was with 0.05 *M* formic acid (pH 2.4) for nucleic acid analysis under acidic conditions, 1.0 *M* ammonium carbonate (pH 8.8) for analysis under basic conditions or 0.1 *M* NaCl, 0.01 *M* sodium acetate (pH 4.5), 0.01 *M* $MgCl_2$, and 0.001 *M* sodium thiosulfate for aminoacyl tRNA analysis. All buffers were throughly degassed before use.

Analysis conditions

tRNA hydrolysis. E. coli $tRNA^{Met}$ (0.3 mg) was hydrolyzed to nucleosides using a mixture of pancreatic ribonuclease, snake venom phosphodiesterase, and alkaline phosphatase[7]. Nucleotide 3′ isomers were obtained from a ribonuclease T_2 digest of $tRNA^{Met}$ (0.3 mg)[15].

Eluted solutes were identified by comparing observed retention times with those of standard nucleosides or nucleotides. The identification of 3′ nucleotide isomers was verified by examination of both the elution positions and retention times of a 3′ nucleotide mixture obtained from a ribonuclease T_2 digestion of $tRNA^{Met}$ as well as by observing the UV absorption curve of each eluted peak on a Cary Model 116C recording spectrophotometer.

Aminoacylation and chromatography of tRNA. Preparation, aminoacylation, and chromatography of tRNA isolated from rat liver and Walker 256 mammary carcinosarcoma tissue was accomplished as previously described[5,16,17]. Preparative aminoacylation[16] employed [^{14}C]tyrosine (50 μCi/μmole from Schwarz/Mann, Orangeburg, N.Y., U.S.A.) for tumor tRNA and [^{3}H]tyrosine (250 μCi/μmole from New England Nuclear, Boston, Mass., U.S.A) for liver tRNA. After incubation at 37° for 30 min, the aminoacyl tRNA was isolated by phenol extraction and ethanol precipitation[14].

* A_{260} unit is defined as that amount of material per ml of solution which produces an absorbance of 1 in a 1-cm light path at 260 nm.

Approximately 20,000 cpm ^{3}H-labeled rat liver $tRNA^{Tyr}$ and 10,000 cpm ^{14}C-labeled Walker $tRNA^{Tyr}$ were combined by dissolving in 250 μl of buffer solution B (0.01 *M* sodium acetate pH 4.5, 0.01 *M* $MgCl_2$, and 0.001 *M* sodium thiosulfate) containing 0.1 *M* NaCl. After application of the sample, elution was performed at room temperature with a 73 ml concave gradient (42 ml buffer solution B containing 0.1 *M* NaCl and 31 ml of buffer solution B containing 1.2 *M* NaCl) and the flow-rate was maintained at 0.5 ml/min with a Milton-Roy pump. Fractions (0.5 ml) were collected in 2-ml (half-dram) vials (Rochester Scientific, Rochester, N.Y., U.S.A.). After the addition of 1.5 ml of Aquasol (New England Nuclear), radioactivity (^{14}C and ^{3}H) was determined by dual-label liquid scintillation counting in a refrigerated Nuclear-Chicago Isocap 300.

RESULTS AND DISCUSSION

Separation of nucleosides and nucleotides under basic conditions by anion-exchange chromatography

Chromatographic separations of nucleosides and nucleotides at pH 8.8 are shown in Figs. 1a and b. With the exception of the overlapping 2′,3′ CMP–UMP pair, all components are sufficiently resolved to permit identification and quantitation. Theoretically, an improved resolution of the overlapping CMP–UMP pair could be

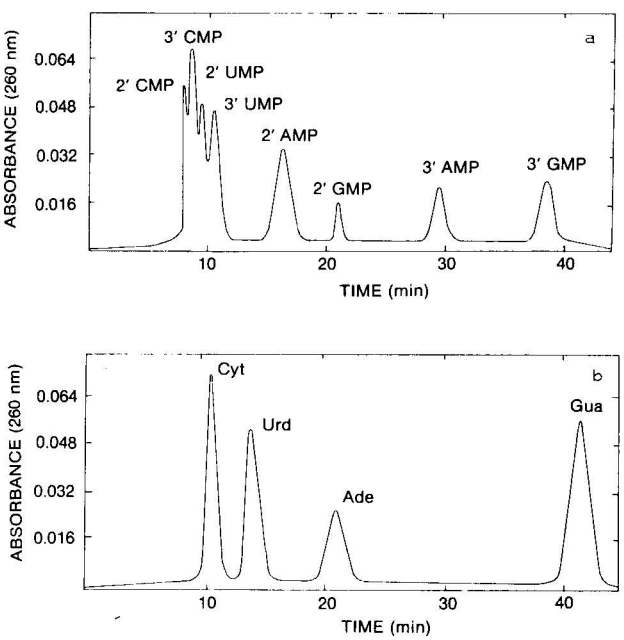


Fig. 1. (a) Separation of a mixture of 2′ and 3′ isomers of CMP, UMP, AMP and GMP on Chromex DA-X8-11. The resin bed was maintained at 50° during elution with 1.2 *M* ammonium carbonate pH 8.8 at a flow-rate of 1.0 ml/min. (b) Separation of cytidine, adenosine, uridine and guanosine nucleosides on Chromex DA-X8-11. Conditions were identical with those described in (a) except that 1.0 *M* ammonium carbonate pH 8.8 was the eluent.

achieved by choosing a higher eluent pH, which would increase the extent of the enolized form of UMP (pK_a 9.43)[18] and increase its retention time. Experiments aimed at improving the resolution of the CMP–UMP pair are presently in progress. Alternatively, by varing the ionic strength an improved CMP–UMP separation could be obtained since formation of the negatively charged enolized uracil base in the nucleotide is substantially favored at a higher ionic strength[18].

The elution order of the nucleosides is Cyt, Urd, Ade, Gua; this result is consistent with their ionic charges at pH 8.8. Cyt and Ade exist as non-ionized species (pK_a 4.15 and 3.6)[18] and therefore elute earlier. Urd (pK_a 9.2) and Gua (pK_a 9.25) exhibit a partial negative charge due to ionization of the oxo groups at pH 8.8 and therefore longer retention times. Non-polar interaction between the purine rings of Gua and Ade and the divinylbenzene cross-linked polystyrene matrix may contribute to the increased retention times of those solutes in comparison with their pyrimidine counterparts[7,19]. The same rationale can explain the similar elution order of nucleotide monophosphates.

Separation of nucleosides and nucleotides under acidic conditions by cation-exclusion chromatography

In ion-exclusion chromatography[7] solutes are repelled from the similarly charged exchange groups. In the pH range 2.4–2.8, the nucleoside bases Cyt and Ade are essentially fully protonated, they would be repelled by the resin's quaternary amine exchange groups and should therefore elute earlier. Such a result was obtained in the chromatographic separations for nucleosides and nucleotides at pH 2.8 and 2.4 shown in Figs. 2a and b. By changing the pH of the eluent, the charges on solutes can be altered, thereby optimizing the exclusion of selected species as shown in Fig. 3.

Application of this method to the separation of nucleosides in an enzymatic hydrolyzate of $tRNA^{Met}$ is shown in Fig. 4. Here the modified nucleosides, pseudouridine and ribothymidine are resolved from the four unmodified nucleosides and can easily be quantitated. We are currently using this HPLC analytical technique to rapidly locate specific fragments from a ribonuclease T_1 digest of crude *E. coli* and mammalian tRNA for studies on the role of modified nucleotides in tRNA.

Both cation-exclusion and anion-exchange chromatography on Chromex DA-X8-11 can be used to separate nucleic acid components. Isocratic elution does not require the re-equilibration needed when gradient elution is used. In addition, the four nucleotides or nucleosides can be resolved more rapidly and at lower eluent pump pressures (500–1000 p.s.i.) than other ion-exchange procedures[7,20,21]. Our separations (Figs. 1, 2, and 4) are comparable to those obtained when similar solutes are chromatographed on Aminex resins. Shorter retention times are observed for nucleoside separations (25 min) using Chromex DA-X8-11, significantly enhancing the ease of analysis compared to chromatography on Aminex A-25[7], A-14[19], and A-6[22] under similar conditions.

Chromatography on aminoacyl-$tRNA^{Tyr}$

Fig. 5 shows the elution profiles obtained by co-chromatography of Walker 256 rat liver tRNA aminoacylated with [^{14}C]- and [^{3}H]tyrosine, respectively. As reported earlier[5], chromatography on Aminex A-28 resolves at least two isoaccepting species of $tRNA^{Tyr}$. As shown in Fig. 5 the two isoaccepting species of $tRNA^{Tyr}$

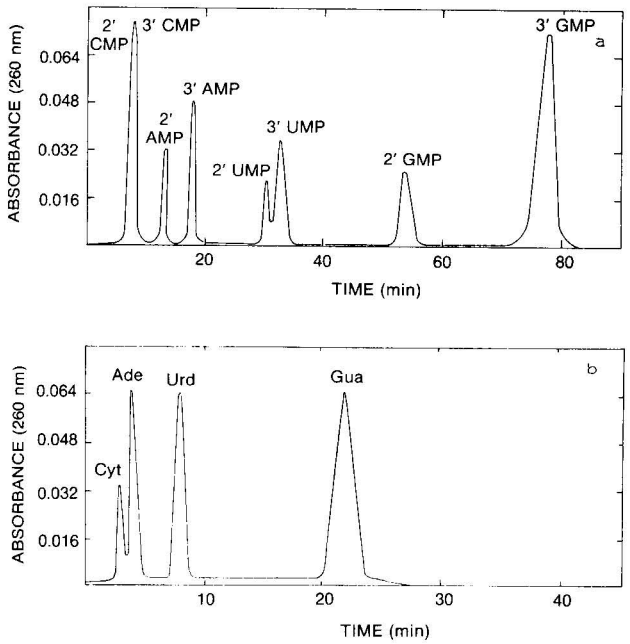


Fig. 2. (a) Separation of a mixture of 2′ and 3′ isomers of CMP, UMP, AMP and GMP on Chromex DA-X8-11. The resin bed was maintained at 50° during elution with 0.3 *M* ammonium formate pH 2.8 at a flow-rate of 1.0 ml/min. (b) Separation of a mixture of cytidine, adenosine, uridine and guanosine nucleosides on Chromex DA-X8-11. The resin bed was maintained at 50° during elution with 0.05 *M* formic acid pH 2.4, at a flow-rate of 0.5 ml/min.

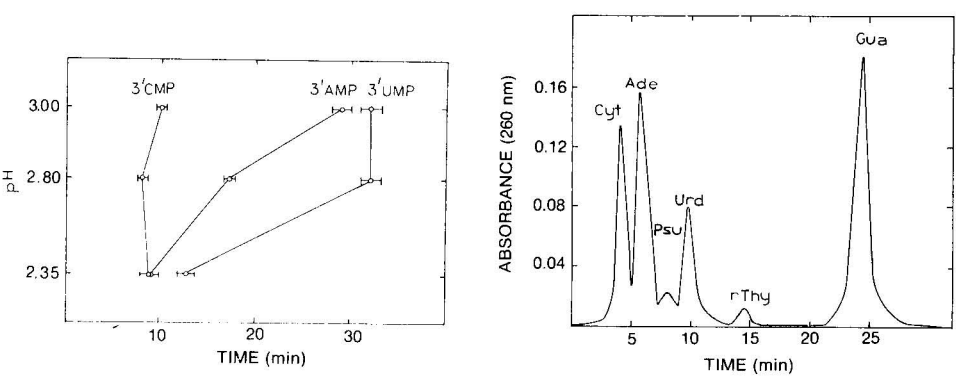


Fig. 3. Effect of pH on retention times for 3′ CMP, AMP and UMP. Bar indicates peak width at half height. Conditions are the same as those reported in Fig. 2.

Fig. 4. Separation of a nucleoside mixture obtained from the enzymatic hydrolysis of 0.6 A_{260} units of $tRNA^{Met}$ on Chromex DA-X8-11. The resin bed was maintained at 50° during elution with 0.025 *M* formic acid pH 2.6 at a flow-rate of 0.5 ml/min.

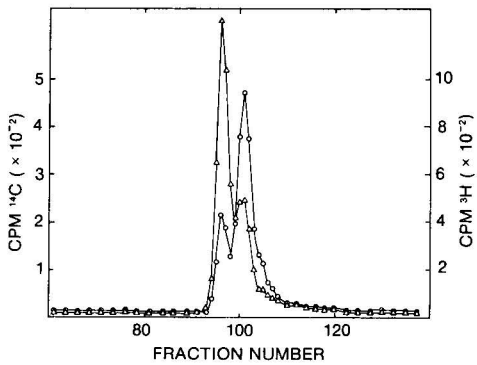


Fig. 5. Chromatography of [^{14}C]Tyr-tRNA^{Tyr} from Walker 256 tumor (○–○) and [^{3}H]Tyr-tRNA^{Tyr} (△–△). 35 A_{260} units of each aminoacyl tRNA were applied to a Chromex column, eluted, and the radioactivity determined as described in Experimental.

are also resolved on Chromex DA-X8-11 but the Chromex resin has the advantage that the required operating pressures (500 p.s.i.) are much less than the 4000 p.s.i. required for Aminex A-28.

In conclusion, the resolution obtained by Chromex DA-X8-11 chromatography under both ion-exchange and ion-exclusion conditions is comparable to previously reported methods[7,19,22]. However, the major advantage of this particular resin is that the running pressure is greatly reduced at faster flow-rates and thereby, the total analysis time is considerably shortened. In addition this resin can also be employed as a sensitive analytical tool for the determination of alterations in tRNA from normal and tumor tissues with all the advantages mentioned above.

ACKNOWLEDGEMENT

This work was supported in part by grants from the National Institutes of Health (GM-21405) and the American Cancer Society (NP-240). B.A.R. is a recipient of a National Institutes of Health Research Career Development Award (KO-4-GM-00178).

REFERENCES

1 K. Randerath, E. Randerath, L. S. U. Chia and B. J. Nowack, *Anal. Biochem.*, 59 (1974) 263.
2 E. Y. Chen and B. A. Roe, *Biochem. Biophys. Res. Commun.*, 78 (1977) 632.
3 B. A. Roe, M. P. J. S. Anandaraj, L. S. Y. Chia, E. Randerath, R. C. Gupta and K. Randerath, *Biochem. Biophys. Res. Commun.*, 66 (1975) 1097.
4 M. P. J. S. Anandaraj and B. A. Roe, *Biochemistry*, 14 (1975) 5068.
5 B. A. Roe, A. F. Stankiewicz and C. Y. Chen, *Nucleic Acids Res.*, 2 (1977) 853.
6 R. P. Singhal, *Eur. J. Biochem.*, 43 (1974) 245.
7 R. P. Singhal and W. E. Cohn, *Biochemistry*,12 (1973) 1532.
8 J. X. Khym, *J. Chromatogr.*, 97 (1974) 277.
9 B. A. Roe, K. Marcu and B. Dudock, *Biochem. Biophys. Acta*, 319 (1973) 25.
10 F. S. Anderson and R. Murphy, *J. Chromatogr.*, 121 (1976) 251.
11 B. N. White, *Biochem. Biophys. Acta*, 353 (1974) 282.
12 R. P. Singhal, G. D. Griffin and D. G. Novelli, *Biochemistry*, 15 (1976) 5083.

13 Y. Kuchino and E. Borek, *Cancer Res.*, 36 (1976) 2932.
14 B. J. Ortwerth, G. R. Yonvschot and J. V. Carlson, *Biochemistry*, 12 (1973) 3985.
15 F. M. Harada, F. Kimura and S. Nishimura, *Biochemistry*, 10 (1971) 3269.
16 B. A. Roe, *Nucleic Acids Res.*, 2 (1975) 21.
17 K. Marcu, R. Mignary, R. Reszelback, B. Roe, M. Sirover and B. Dudock, *Biochem. Biophys. Res. Commun.*, 55 (1973) 477.
18 P. O. T'so, *Basic Principles in Nucleic Acid Chemistry*, Vol. 1, Academic Press, New York, 1974 p. 461.
19 A. Floridi, C. A. Palmerini and C. Fini, *J. Chromatogr.*, 138 (1977) 213.
20 R. P. Singhal and W. E. Cohn, *Anal. Biochem.*, 45 (1972) 585.
21 M. Uziel, C. K. Koh and W. E. Cohn, *Anal. Biochem.*, 25 (1968) 77.
22 R. P. Singhal, *Arch. Biochem. Biophys.*, 152 (1972) 800.

Journal of Chromatography, 155 (1978) 415–420

CHROM. 10,859

MODIFIED ETHYLENEDIAMINE CONDENSATION METHOD AND ITS APPLICATION IN THE ANALYSIS OF CATECHOLAMINES BY ION-EXCHANGE CHROMATOGRAPHY

TOKUICHIRO SEKI

Department of Genetics, Osaka University School of Medicine, 3–57, 4 Chome, Nakanoshima, Kita-ku, Osaka 530 (Japan)

(Received December 28th, 1977)

SUMMARY

A modified ethylenediamine condensation method is described in which catecholamines dissolved in a borate buffer are converted into fluorescent products by oxidation with hexacyanoferrate(III) in the presence of ethylenediamine under alkaline conditions. The method was applied successfully in the fluorimetric determination of catecholamines eluted from a column of weakly acidic ion-exchange resin with a mixed buffer of pH 6.3 containing 0.35 *M* boric acid, 0.12 *M* succinic acid and 0.002 *M* disodium ethylenediaminetetraacetate. An analysis of human urine for catecholamines is described.

INTRODUCTION

The ethylenediamine condensation method has been widely used for the determination of catecholamines[1]. The method has been automated[2], and coupled with a high-speed liquid chromatograph[3–5].

On the other hand, an ion-exchange chromatographic method for the separation of catecholamines was developed in which a boric acid-containing buffer was used as the eluent and a weakly acidic ion-exchange resin as the stationary phase[6]. An attempt was made to measure fluorimetrically catecholamines in an eluate from the ion-exchange column using the ethylenediamine condensation method, but dopamine and norepinephrine could be determined only with low sensitivity.

Since this failure seemed to be due to a low rate of oxidation of dopamine and norepinephrine by air oxygen in the presence of borate ion, oxidation of catecholamines with a solution of hexacyanoferrate(III) in the presence of ethylenediamine was tried with success. The modified ethylenediamine condensation method could be automated and used to measure catecholamines eluted from a column of Amberlite IRC-50 with a mixed buffer of pH 6.3 containing 0.35 *M* boric acid, 0.12 *M* succinic acid and 0.002 *M* disodium ethylenediaminetetraacetate. The determination of catecholamines extracted from human urine is also described in this paper.

EXPERIMENTAL

Reagents

Epinephrine hydrogen tartrate was purchased from Nakarai Pharmaceutica Co. (Kyoto, Japan) and norepinephrine hydrogen tartrate, dopamine hydrochlorid and ethylenediamine dihydrochloride from Yashima Pharmaceutical Co. (Osaka Japan). Other chemicals used were of reagent grade. Stock solutions of catecholamin base were prepared in 0.01 *M* hydrochloric acid. Fluorescent impurities contained i ethylenediamine dihydrochloride were removed by passing a 0.5 *M* solution of ethyl enediamine dihydrochloride through a column of activated charcoal (15 × 1.5 cm).

Equipment

A syringe-type pump (Jasco, Model FLC-150) was used to pump buffe solution through the chromatographic columns. Peristaltic pumps (Mitsumi Scien tific Industry, Models SJ-1211 H and L) were used to pump air and reagents into a gas-segmented-flow reaction detector. A spectrofluorimeter (Jasco, Model FP-4 equipped with a flow cell prepared from a quartz tube (4 mm I.D.) was used t measure fluorescence. pH was measured at 20° using a TOA Electronics (Tokyo, Japan) Model HM-5A glass electrode pH meter.

Human urine

Daytime specimens of urine were acidified immediately after collection by adding 1% (v/v) of 6 *M* hydrochloric acid and stored in a refrigerator. Just before analysis, they were filtered.

Preparation of Amberlite CG-50 column

Amberlite CG-50 (type II) was converted into the Na^+ form and graded according to size by the sedimentation method[7]. The fraction of size range 85–120 μm was collected and washed on a glass filter with ten volumes of the following reagents in turn: 4 *M* hydrochloric acid, water, 1 *M* sodium hydroxide solution, water and 0.4 *M* phosphate buffer (pH 6.5). The buffered resin was poured into a tube with phosphate buffer of pH 6.5 (0.4 *M*) and allowed to settle under gravity to a height of 12 cm (the tube was 20 × 0.5 cm I.D. with a 10-ml reservoir); the column was washed with 2 ml of water before use[8].

Preparation of Amberlite IRC-50 column

Amberlite IRC-50 (40–55 μm and 50–60 μm in the Na^+ form) was prepared and washed as described previously[9], and suspensions of the washed resins (Na^+ form) were buffered at pH 6.3 with a succinic acid solution (0.5 *M*) and then washed with eluent. The eluent was a mixed buffer of pH 6.3 containing 0.12 *M* succinic acid, 0.35 *M* boric acid and 0.002 *M* disodium ethylenediaminetetraacetate. The washed resin was suspended in 2 volumes of the eluent. The suspension of the finer resin was poured into a short chromatographic tube (15 × 0.8 cm), and a suspension of the coarse resin was poured into a longer tube (24 × 0.8 cm) and allowed to settle under gravity. Then both chromatographic tubes were fitted with a column adjuster and the bottom of the short tube was connected to the longer one. The temperature of the column was kept at 42° and, after the eluent had been pumped through the

column at a rate of 0.75 ml/min for several hours, the height of the resin column in the top tube was 9 cm and in the bottom tube 19 cm.

Separation of catecholamine fraction from human urine

A 4-ml portion of filtered urine was mixed with 0.5 ml of a 5% solution of disodium ethylenediaminetetraacetate and 0.5 ml of a 1% solution of ascorbic acid, and the pH of the mixture was adjusted to 6.1–6.2 with 1 *M* sodium hydrogen carbonate solution. The mixture was then applied to an Amberlite CG-50 column, the column was washed with 6 ml of deionized water and then with 2 ml of 2/3 *M* boric acid solution, then a further 2 ml of the boric acid solution were used to elute catecholamines from the column, the eluate being collected in a test-tube containing 0.07 ml of 0.5 *M* sodium dihydrogen phosphate solution in 1 *M* hydrochloric acid. The eluate in the test-tube was adjusted to pH 6.3 with a solution of 0.5 *M* sodium dihydrogen phosphate in 1 *M* hydrochloric acid and diluted to 4 ml with a succinate buffer of pH 6.3 (succinic acid 0.12 *M*, disodium ethylenediaminetetraacetate 0.002 *M*). The catecholamine fraction was stored in a refrigerator.

Chromatographic separation of samples

A 1-ml volume of a solution of the amines in the eluent to be used for chromatography or 1.0 ml of a catecholamine fraction prepared as described above was added to the column of Amberlite IRC-50. The sample was forced into the column by an air pressure of 1 kg/cm^2, then elution was carried out with the eluent at a flow-rate of 0.75 ml/min.

Fluorimetric determination of catecholamines

A gas-segmented-flow reaction detector was assembled from commercial parts and Pyrex coils. The Pyrex coils were made by winding 4-mm Pyrex tubing around a brass tube of 14 mm O.D. As shown in Fig. 1, eluate from a column was fed to the detector and segmented by air, mixed with 0.5 *M* ethylenediamine dihydrochloride (mixing coil, 10 turns), 1.5 *M* sodium hydroxide (mixing coil, 8 turns), 0.3% (w/v) hexacyanoferrate(III) (mixing coil, 10 turns), heated at 75° (heating coil,

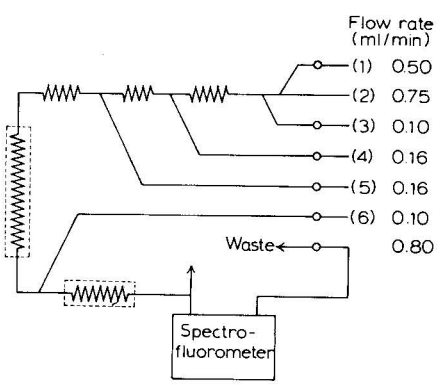


Fig. 1. Schematic diagram of the gas-segmented-flow reaction detector. The pH of the waste was 9.5. (1) Air; (2) eluate; (3) 0.5 *M* ethylenediamine dihydrochloride; (4) 1.5 *M* NaOH–0.1% (w/v) Triton X-405; (5) 0.3% (w/v) hexacyanoferrate(III); (6) 10% (w/v) $NaSO_3 \cdot 7H_2O$.

30 turns) and finally mixed with a 10% (w/v) solution of sodium sulphite heptahydrate and warmed at 42° (heating coil, 15 turns). Then bubbles were removed from the stream and the fluorescence was measured at 510 nm, with excitation at 400 nm.

RESULTS AND DISCUSSION

Three catecholamines could be separated from each other and determined fluorimetrically within 70 min (Fig. 2). The elution pattern was reproducible and the peak heights of 120 ng each of epinephrine, norepinephrine and dopamine were 71.8 ± 0.76, 72.4 ± 0.74 and 48.5 ± 0.89, respectively (five determinations). As shown in Fig. 3, the catecholamine fraction prepared from human urine gave three

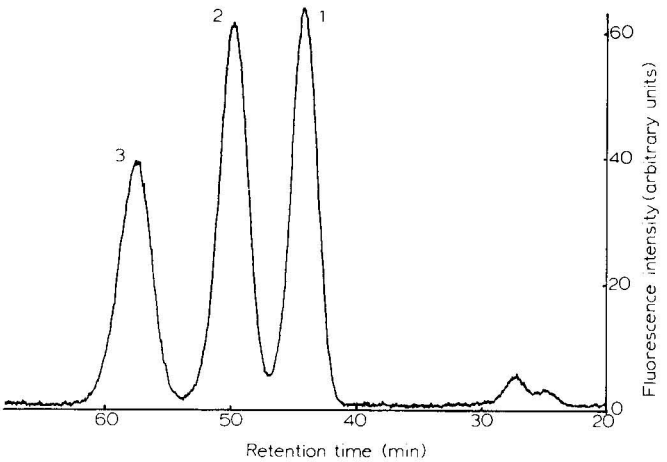


Fig. 2. Elution and fluorimetric determination of catecholamines. A mixture of 100 ng each of epinephrine (1), norepinephrine (2) and dopamine (3) was separated on the column of Amberlite IRC-50 and determined using the detector described in Fig. 1.

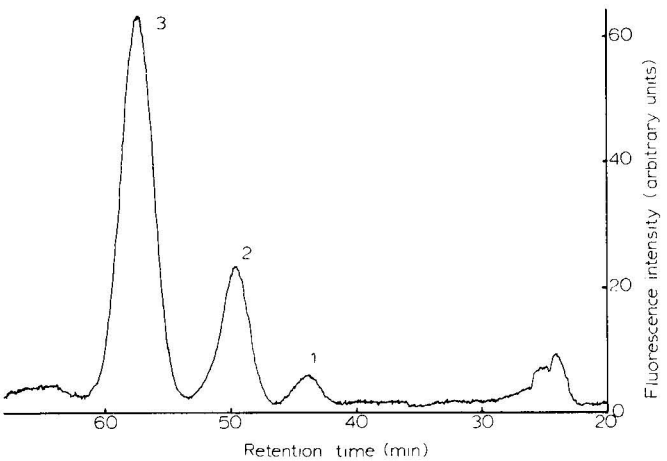
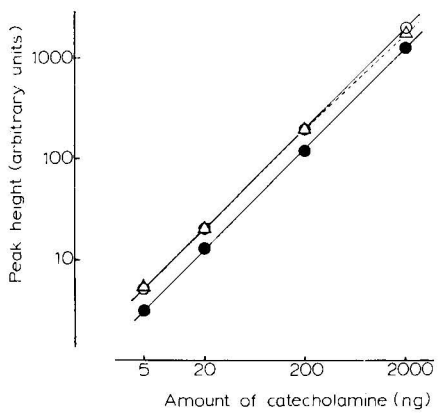


Fig. 3. Elution and fluorimetric determination of catecholamine fraction prepared from human urine. Retention times of peaks (1), (2) and (3) corresponded to those of epinephrine, norepinephrine and dopamine, respectively.

peaks corresponding to epinephrine, norepinephrine and dopamine. Based on six determinations of 4-ml aliquots of the same human urine sample, the mean amounts of epinephrine, norepinephrine and dopamine per millilitre of urine were calculated to be 6.9 ng ± 0.42, 34.1 ng ± 1.6 and 151 ng ± 6.3, respectively. When 100 ng of epinephrine, 200 ng of norepinephrine and 400 ng of dopamine were added to a urine sample before the procedure described above and one quarter of the catecholamine fraction of each sample was analysed, the amounts recovered were 23.5 ng ± 2, 46.4 ng ± 2.3 and 90.8 ng ± 10.2, respectively (five determinations).

A mixed buffer containing a lower concentration of boric acid (0.35 *M*) than the boric acid solution (2/3 *M*) was used as the eluent, because catecholamines are specifically retarded with an eluent containing lower concentration of boric acid, and their separation from impurities will be improved with such an eluent.

A linear relationship between peak height and the amount of amines added to the column was obtained over the range 5–2000 ng for norepinephrine and dopamine and 5–200 ng for epinephrine, when the amines were oxidized and subjected to reaction with ethylenediamine under alkaline condition (pH 9.5, Fig. 4). When catecholamines were treated under less alkaline or neutral conditions, the relative peak heights of these amines changed, as shown in Fig. 5. Although the peak heights of catecholamines were higher at pH 8.65 than at pH 9.5, the dynamic range of norepinephrine was narrower at pH 8.65.



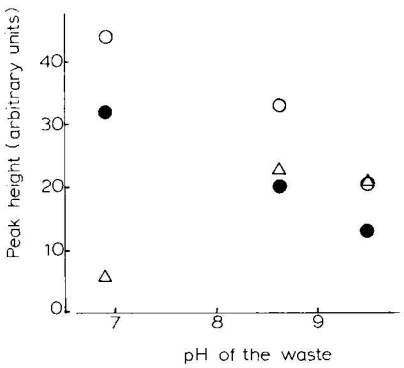


Fig. 4. Relationship between peak height and amount of catecholamines. Catecholamines were separated on the column of Amberlite IRC-50 and determined by using the detector described in Fig. 1. △, Epinephrine; ○, norepinephrine; ●, dopamine.

Fig. 5. Relationship between pH of the reaction mixture and the peak height of catecholamines. Catecholamines (20 ng of each) were separated and detected as in Fig. 4; 0.05, 0.75 or 1.5 *M* sodium hydroxide solution was used to keep the pH of the waste at 6.9, 8.65 or 9.5. Other conditions as in Fig. 1. △, Epinephrine; ○, norepinephrine; ●, dopamine.

Oxidation of catecholamines with hexacyanoferrate(III) in the presence of ethylenediamine under alkaline condition was preferable to oxidizing them at neutral pH prior to the addition of ethylenediamine and sodium hydroxide solution, because the latter reaction sequence yielded narrower dynamic ranges for dopamine and norepinephrine. With a different batch of ethylenediamine dihydrochloride, the

relationship of peak height to the amount of catecholamines added to the column changed to some extent, but the dynamic range of catecholamines remained unchanged. The concentration of hexacyanoferrate(III) used to oxidize catecholamines was 0.3%, but epinephrine and norepinephrine could be determined with higher sensitivity by oxidizing them with a more concentrated solution of hexacyanoferrate(III) (Fig. 6). Excess of hexacyanoferrate(III) had to be reduced, because it absorbs the exciting light, and the time for reduction was 2.5 min. The time for the reaction of ethylenediamine with the oxidation products of catecholamines could be reduced to 5 min by keeping the temperature of the reaction coil at 75° and the total time of mixing and reaction was 15 min. Therefore, the modified ethylenediamine condensation method described here can be conveniently utilized for the on-line determination of catecholamines in an eluate from a chromatographic column.

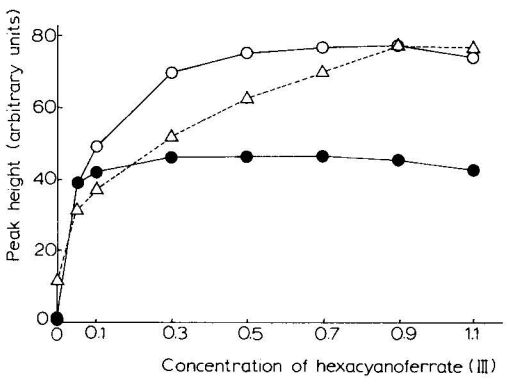


Fig. 6. Relationship between peak height and concentration of hexacyanoferrate(III) (%, w/v) used to oxidize catecholamines. Catecholamines (100 ng of each) were separated and detected as in Fig. 4. A different batch of ethylenediamine was used in this experiment, and except for the concentration of hexacyanoferrate(III), the reaction conditions were as described in Fig. 1. △, Epinephrine; ○, norepinephrine; ●, dopamine.

ACKNOWLEDGEMENTS

The author thanks Japan Spectroscopic Co. Ltd. and Mitsumi Scientific Industry Co. Ltd. for the loan of equipment, and Prof. H. Wada of the Department of Pharmacology II who made the peristaltic pumps available.

REFERENCES

1 H. Weil-Malherbe and A. D. Bone, *Biochem. J.*, 51 (1952) 311.
2 J. K. Viktora, A. Baukal and F. W. Wolff, *Anal. Biochem.*, 23 (1968) 513.
3 A. Kojima-Sudo, *Ind. Health*, 12 (1974) 153.
4 K. Mori, *Jap. J. Ind. Health*, 17 (1975) 170.
5 G. Schwedt, *Chromatographia*, 10 (1977) 92.
6 T. Seki, *J. Chromatogr.*, 124 (1976) 511.
7 P. Decker and H. Höller, *J. Chromatogr.*, 7 (1962) 392.
8 T. Seki and H. Wada, *J. Chromatogr.*, 114 (1975) 227.
9 T. Seki and K. Matsumoto, *J. Chromatogr.*, 27 (1967) 423.

Journal of Chromatography, 155 (1978) 421–426

CHROM. 10,854

RAPID ENZYMATIC DETERMINATION OF 3-OXO-BILE ACIDS SEPARATED BY THIN-LAYER CHROMATOGRAPHY

W. T. BEHER, J. SANFIELD, S. STRADNIEKS and G. J. LIN
Henry Ford Hospital, Detroit, Mich. 48202 (U.S.A.)
(First received August 29th, 1977; revised manuscript received December 27th, 1977)

SUMMARY

A method for the rapid quantification of 3-oxo-5β-cholan-24-oic acids has been developed. The acids are separated on silica gel G and located using a water spray or iodine vapor. Each oxo acid is eluted from the gel and reduced with sodium borohydride. The resulting α- and β-hydroxy acids are then oxidized in a reaction catalyzed by 3-hydroxysteroid dehydrogenase during which NAD is reduced to NADH. The absorbance of the reaction mixture is determined at 340 nm and is directly proportional to the amount of 3-oxo acid originally present on the thin-layer plate.

INTRODUCTION

The 3-oxo-5β-cholan-24-oic acids (3-oxo bile acids) are a group of substances which (1) are products of bile acid metabolism in the gastrointestinal tract and are found in feces and the portal blood[1], (2) are intermediates in the organic synthesis of bile acids, and (3) are sometimes used as choleretics, *viz.* dehydrocholic acid. In the past, the only practical approach to quantification of small amounts of these substances was gas–liquid chromatography[2]. This method, although accurate, is time-consuming and involves the preparation of derivatives prior to assay. We have developed a rapid new method which can be applied to large numbers of samples. The principal of the method is: 3-oxo-5β-cholan-24-oic acids, separated by thin-layer chromatography (TLC), are reduced to 3α- and 3β-hydroxy-5β-cholan-24-oic acids using sodium borohydride. The hydroxy bile acids are then oxidized to 3-oxo acids in a reaction catalyzed by a mixture of 3α- and 3β-hydroxysteroid dehydrogenases[3,4]. β-Nicotinamide-adenine dinucleotide (NAD) accepts hydrogen and is reduced to NADH, while hydrazine traps 3-oxo acids and forces the reaction to completion. The amount of NADH formed is directly proportional, on a one to one molar basis, to the amount of 3-oxo-5β-cholan-24-oic acids originally present. The concentration of NADH is determined by reading the absorbance of the reaction mixture at 340 nm.

MATERIALS

All of the chemicals used were reagent grade and all solvents were distilled prior to use. 3-Oxo-5β-cholan-24-oic, 3,6-dioxo-5β-cholan-24-oic, 3,7,12-trioxo-5β-cholan-24-oic, 3,12-dioxo-5β-cholan-24-oic, 3α,7α,12α-trihydroxy-5β-cholan-24-oic, 3α,7α-dihydroxy-5β-cholan-24-oic, and 3α,12α-dihydroxy-5β-cholan-24-oic acids were purchased from Steraloids (Wilton, N.H., U.S.A.). All were checked for purity by TLC and purified when necessary using preparative TLC (see below). 7α-Hydroxy-3-oxo-5β-cholan-24-oic, 6α-hydroxy-3-oxo-5β-cholan-24-oic, 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic, and 12α-hydroxy-3-oxo-5β-cholan-24-oic acids were synthesized as outlined below. NAD, sodium borohydride and 3-hydroxysteroid dehydrogenase (3HSD; desiccated *Pseudomonas testosteroni* cells) were all purchased from Sigma (St. Louis, Mo., U.S.A.). Hydrazine hydrate (64% in water) was purchased from Eastman-Kodak (Rochester, N.Y., U.S.A.) and silica gel G from E. Merck, (Darmstadt, G.F.R.).

Pyrophosphate buffer (PPB), pH 10, 0.1 *M*, is prepared by dissolving 44.61 g of sodium pyrophosphate decahydrate in 900 ml of distilled water. The pH of the solution is adjusted to 10 with 0.1 *M* hydrochloric acid. The buffer is then diluted to 1 l with water. NAD solution is made by dissolving 45 mg of NAD in 100 ml of distilled water. Hydrazine hydrate solution is made by diluting 12.5 g of commercial hydrazine hydrate (64% in water) to 90 ml with PPB. The pH of the resulting solution is adjusted to 10 with 6 *M* hydrochloric acid and the final volume adjusted to 100 ml with PPB. 3HSD solution is prepared by homogenizing 100 mg of dried *Pseudomonas testosteroni* cells together with 10 ml of PPB for 5 min in an ice bath. The content of the homogenizer is centrifuged at 50,000 *g* for 20 min. The supernatant solution containing 3HSD is decanted and stored in an ice-bath until used. Alternately, dried extracts of *Pseudomonas testosteroni* cells are available (Sigma) and are simply dissolved in PPB prior to use.

METHODS

Synthesis of 3-oxo-5β-cholan-24-oic acids

One gram of the 3α-hydroxy-5β-cholan-24-oic acid corresponding to the desired 3-oxo-5β-cholan-24-oic acid dissolved in 50 ml of PPB, 2.5 g of NAD dissolved in 100 ml of PPB and 5 g of hydrazine hydrate dissolved in 40 ml of PPB are placed in a liter erlenmeyer flask. Crude desiccated *Pseudomonas testosteroni* cells (700 mg) are homogenized in 70 ml of PPB. The resulting suspension is centrifuged for 45 min at 20,000 *g*. The supernatant containing 3HSD is then added to the flask, 140 ml of PPB are added and the contents of the flask mixed. The flask and its contents are then incubated with shaking at 37° in a water-bath. Small aliquots of the solution are removed at 15-min intervals and their absorbance determined at 340 nm to check for NADH which results from the oxidation of the 3α-hydroxyl group of the bile acid. When there is no further increase in the absorbance, the flask is removed from the incubator and the pH of its content adjusted to 1 using 12 *M* hydrochloric acid. The solution is then transferred to a separatory funnel and extracted four times with two volumes of diethyl ether. The extract which contains the hydrazone of the 3-oxo-5β-cholan-24-oic acid is evaporated to dryness. The hydrazone is suspended in

100 ml of 2 *M* hydrochloric acid and refluxed for 30 min. The suspension is cooled, transferred to a separatory funnel and extracted four times with three volumes of ethyl ether. The ether extract containing the 3-oxo-5β-cholan-24-oic acid is evaporated to dryness. The crude acid is dissolved in chloroform–methanol (2:1, v/v) and purified via preparative TLC using plates coated with silica gel G and developed with ethyl acetate–isooctane–acetic acid (10:10:2, v/v/v). The final products were chromatographically pure; overall yields averaged from 50 to 60%.

Thin-layer chromatography

Preparation of plates. Silica gel G soft-coated plates (200 μm) were used in all quantitative and qualitative studies. To prepare them, 30 g of silica gel G are mixed with 70 ml of distilled water acidified with five drops of glacial acetic acid. The mixture is stirred until it just begins to thicken (about 8 min) and is then rapidly spread on 20 $\times$ 20 cm glass plates. After spreading, the plates are allowed to air-dry and are then stored in desiccators over silica gel. Prior to use, the plates are channeled so that each plate has 12 to 14 separate channels. The plates are then activated in a drying oven at 100° for 1 h.

R_F and relative mobility determinations. A 2-μl portion (10 μg) of each of the bile acid solutions (methanolic) is applied to the individual channels of the plate and the methanol is allowed to evaporate. The plates are developed in equilibrated (4 h) filter paper-lined glass tanks using ethyl acetate–isooctane–acetic acid (10:10:2). When development is complete, the plates are removed from the tanks and air-dried. The plates are then lightly sprayed with 0.05% of pyrene dissolved in hexane[5]. The bile acids are visualized using short-wave ultraviolet radiation. The positions of the centers of the spots are marked and the necessary measurements made.

Quantitative determination of 3-oxo-5β-cholan-24-oic acids. Aliquots (1–6 μl; 5–120 μg) of methanolic bile acid solutions are applied to the individual channels of TLC plates. The methanol is allowed to evaporate and the plates are developed in equilibrated (4 h) filter paper-lined glass tanks using ethyl acetate–isooctane–acetic acid (10:10:2). When developed, the plates are removed from the tanks and allowed to dry in a filtered stream of air at room temperature. The dry plates are sprayed with water[6] and the positions of the acids outlined with a sharp stylus. The plates are then allowed to dry thoroughly and the silica gel G in the marked areas is transferred to centrifuge tubes by scraping the gel from the plates with the squared end of a stainless-steel spatula. Silica gel from a blank plate developed and dried in the same way is transferred to centrifuge tubes for use in Blanks and Standards. Two ml of chloroform–methanol (2:1) are added to each centrifuge tube. The gel is suspended in the solvent by swirling the tubes with a vortex mixer; they are then warmed and swirled again. The tubes are now centrifuged for 5 min at 800 *g*. The supernatant fluid is quantitatively transferred to 10 $\times$ 100 mm screw-capped or glass-stoppered test tubes. The extraction is repeated three times. When extraction is complete, the fluid in the tubes is evaporated to dryness using a stream of air or nitrogen and a hot water-bath. The content of each tube, except the standards, is then dissolved in 0.5 ml of PPB. A 0.5-ml portion of a standard solution containing 90 μg of 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid per 0.5 ml is added to the blank tubes to be used as standards. A 0.2-ml portion of sodium borohydride solution (18 mg per 10 ml of PPB) is next added to each tube. The tubes are allowed to stand for

1 h at room temperature to effect complete reduction of 3-oxo-5β-cholan-24-oic acids to 3-hydroxy-5β-cholan-24-oic acids. A 0.2-ml portion of 12 *M* hydrochloric acid is now added to each tube and the tubes are placed in a hot water-bath to destroy excess sodium borohydride. After 10–15 min, 0.4 ml of 6 *M* sodium hydroxide is added to each tube to neutralize hydrochloric acid. It is well to test the pH of the first few tubes after adding alkali to make sure that they are slightly basic. If not, a small adjustment of the volume of alkali may be necessary. Exact pH adjustment is unnecessary at this point since PPB is added at a later stage in the procedure. Next, 1 ml of NAD solution, 1.2 ml of hydrazine hydrate solution and 0.3 ml of 3HSD solution are added. The tubes are thoroughly mixed and placed in a water-bath maintained at 37° for 30 min. Exact timing of the samples is unnecessary since the reaction is essentially complete in 30 min and after that time the absorbance of the solutions does not change appreciably for over 1 h. After incubation the absorbance of the solutions is determined at 340 nm.

RESULTS AND DISCUSSION

The μ*M* absorbance figures in the right hand columns of Table I show two things. First, in the range from 20 to 60 μg, absorbance is directly proportional to concentration, *i.e.*, Beer's law is followed. Second, since the standard deviations of the μ*M* absorbance values are small, the method has precision. The range of the method is greater than the figures in Table I imply since the relationship between mass and absorbance is linear from 5 μg to at least 120 μg.

A number of solvent systems were investigated to find a mixture which would

TABLE I

THIN-LAYER CHROMATOGRAPHY AND QUANTITATIVE DETERMINATION OF 3-OXO-5β-CHOLAN-24-OIC ACIDS

Solvent system: isooctane–ethyl acetate–acetic acid (10:10:2); TLC data are the average of four determinations.

-5β-Cholan-24-oic acid	R_F	*Relative mobility**	*μM Absorbance*** ± *S.D.*		
			μg 3-oxo-5β-cholan-24-oic acid applied to plate		
			20	*40*	*60*
3-Oxo-	0.64	9.25	1.46 ± 0.02	1.47 ± 0.01	1.44 ± 0.03
3,6-Dioxo-	0.48	6.92	1.51 ± 0.04	1.41 ± 0.03	1.43 ± 0.02
3,12-Dioxo-	0.44	6.41	1.44 ± 0.03	1.42 ± 0.01	1.38 ± 0.02
3,7,12-Trioxo-	0.24	3.50	1.43 ± 0.02	1.43 ± 0.05	1.46 ± 0.01
6α-Hydroxy-3-oxo-	0.32	4.58	1.38 ± 0.04	1.40 ± 0.02	1.43 ± 0.03
7α-Hydroxy-3-oxo-	0.42	6.00	1.43 ± 0.03	1.42 ± 0.05	1.47 ± 0.07
12α-Hydroxy-3-oxo-	0.39	5.58	1.47 ± 0.02	1.42 ± 0.05	1.44 ± 0.06
7α, 12α-Dihydroxy-3-oxo-	0.16	2.33	1.44 ± 0.04	1.45 ± 0.05	1.38 ± 0.03
3α,7α-Dihydroxy-	0.24	3.64	1.48 ± 0.05	1.40 ± 0.01	1.43 ± 0.04
3α,12α-dihydroxy-	0.29	4.25	1.40 ± 0.02	1.44 ± 0.03	1.39 ± 0.02
3α,7α,12α-Trihydroxy-	0.07	1.00	1.43 ± 0.04	1.44 ± 0.02	1.42 ± 0.01

* With respect to 3α, 7α,12α-trihydroxy-5β-cholan-24-oic acid.

** The absorbance at 340 nm (1-cm path) of the reaction products of 1 μ*M* of acid, 3HSD, NAD and hydrazine hydrate in a 3.8 ml total volume.

successfully separate the 3-oxo acids. Ethyl acetate–isooctane–acetic acid (10:10:2) was the most satisfactory. The R_F and relative mobility data in Table I show that this solvent system effectively separates the acids. Very small samples of some of the acids, however, must be applied to the plates to achieve complete separation because of similarities in some R_F values.

Several types of substances were tested before a detection reagent was found that was compatible in the quantitative procedure. Iodine vapor was tried and was capable of detecting from 5 to 20 μg of oxo acid. Iodine could be removed from the plates by allowing them to stand in a stream of air at room temperature. Although it would appear that iodine would be satisfactory for locating the acids prior to quantification, we have encountered cases (fecal extracts) where it is very difficult or impossible to remove the last traces of this substance. A spray reagent containing 0.05% pyrene dissolved in hexane can be used to detect very small amounts of the oxo acids (<0.5 μg) under ultraviolet radiation[5]. Unfortunately, pyrene interferes during quantification. Although it is possible to remove pyrene from the plates by redeveloping with ethyl ether–light petroleum (b.p. 36–54°) (2:3, v/v) prior to quantification of bile acids, this cannot be done in the case of oxo acids since many of them migrate to some extent during redevelopment. A water spray[6] appears to be a satisfactory detecting agent since it is easily and completely removable. Although it is not as sensitive as pyrene, it is capable of detecting from 1 to 2 μg of the oxo acids which is somewhat below the practical limits of the quantitative procedure. Care must be taken to observe the plates during drying since various oxo acids show up most clearly at different stages of this process.

It might appear that the method could be simplified by carrying out borohydride reduction and quantification with 3HSD without prior removal of silica gel. In fact, it has been shown[7] that it is unnecessary to elute bile acids from silica gel during their quantification with 3HSD. We therefore attempted this modification of the procedure. The results were entirely unsatisfactory with losses ranging up to 55%. Interestingly, if silica gel was added to a solution containing 3-oxo acids and reduction and quantification effected, recoveries were satisfactory. This probably means that borohydride reduction does not take place if the acids are absorbed on silica gel. 3HSD, on the other hand, is capable of catalyzing the oxidation of absorbed bile acids.

The reduction of the 3-oxo group is very rapid in the presence of sodium borohydride: 0.3 mg of borohydride is able to reduce 60 μg of oxo acid in less than 30 min; 6- and 7-oxo groups are also easily reduced. 12-Oxo groups are slowly reduced and as much as 24 h are required to effect complete reduction of the 12-oxo group of 3,7,12-trioxo-5β-cholan-24-oic acid.

ACKNOWLEDGEMENT

This work was supported in part under Project R58820 from funds by the Henry Ford Hospital via a Ford Foundation bequest and partially from funds derived from an NIH grant, No. AM 20522.

REFERENCES

1 W. T. Beher, *Monogr. Atheroscler.*, 6 (1976) 34.
2 P. Eneroth, K. Hellström and J. Sjövall, *Acta Chem. Scand.*, 22 (1968) 1729.
3 P. Talalay, *Methods Biochem. Anal.*, 8 (1960) 119.
4 T. Iwata and K. Yamasaki, *J. Biochem. (Tokyo)*, 56 (1964) 424.
5 M. A. Eastwood and D. Hamilton, *Biochem., J.* 105 (1967) 37c.
6 H. Gänshirt, F. W. Koss and K. Morianz, *Arzneim.-Forsch.*, 10 (1960) 943.
7 W. T. Beher, G. J. Lin and S. Stradnieks, *H. Ford Hosp. Med. J.*, 23 (1975) 87.

Journal of Chromatography, 155 (1978) 427–431

CHROM. 11,020

Note

Gas chromatographic analysis of bile acid methyl esters as partial trimethylsilyl ether derivatives using N,O-bis(trimethylsilyl)trifluoroacetamide as silylating reagent

ROBERT L. CAMPBELL, J. STEPHEN GANTT and NORMAN D. NIGRO
Wayne State University, School of Medicine, Department of Surgery, Detroit, Mich. 48201 (U.S.A.)
(First received November 18th, 1977; revised manuscript received March 21st, 1978)

Trimethylsilyl (TMS) ethers of methyl esters are routinely used derivatives for the gas–liquid chromatographic (GLC) analysis of fecal bile acids. A variety of liquid phases such as SE-30, QF-1, or HiEFF 8BP have been reported to be useful to achieve separation[1,2]. A major problem, particularly in the analysis of rodent feces, is that no one liquid phase can effectively separate the wide spectrum of primary and secondary bile acids as their TMS derivatives. Additional purification of the fecal extracts by thin-layer chromatography and subsequent GLC analysis is often required to detect certain bile acids such as hyodeoxycholic acid (HDC)[3]. This bile acid is found in substantial quantities in the feces of several strains of rats but its detection is not always reported[2].

In this investigation we have used N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), a compound previously used in steroid analysis[4], as the silylating reagent to form partial TMS ethers of bile acids. The derivatives formed allow effective separation on 3% QF-1 of six important fecal bile acids including HDC found in rat feces. We have used this procedure in several studies relating fecal bile acids to intestinal carcinogenesis[5,6].

MATERIALS

Cholic, deoxycholic, chenodeoxycholic, hyodeoxycholic and lithocholic acids were purchased as methyl esters from Applied Science Labs. (State College, Pa., U.S.A.) The β-muricholic acid sample purified from mouse bile by Dr. H. Eyssen, Rega Institute (Louvain, Belgium), was kindly provided by Dr. David Madsen, University of Notre Dame (Notre Dame, Ind., U.S.A.). The 12-ketolitholic acid was synthesized in this laboratory by the method of Bergstrom and Haslewood[7] and methylated with diazomethane.

The silylating reagents BSTFA and the mixture of hexamethyldisilazane (HMDS), chlorotrimethylsilane (CTMS) and pyridine (3:1:9) were obtained from Applied Science Labs. HMDS and N,N-dimethylformamide were from Sigma (St. Louis, Mo., U.S.A.).

METHODS

Full TMS ethers of bile methyl esters were prepared by reacting at room temperature 100 μg of bile acid methyl ester and 25 μg of 5α-cholestane (Applied Science Labs.) as internal standard with 0.2 ml of HMDS–CTMS–pyridine (3:1:9) for 30 min according to the method of Grundy *et al.*[1]. The partial TMS derivatives were formed by reacting the bile acid methyl esters and 5α-cholestane in sealed tubes with either 0.2 ml of BSTFA as solvent and reagent at 37° overnight or by the method of Eneroth *et al.*[8] using 0.06 ml dry N,N-dimethylformamide and 0.03 ml HMDS at 50° for 3 h. Excess reagents were removed under a stream of dry nitrogen and the residue dissolved in 0.1 ml of carbon disulfide.

GLC analyses were performed on a Hewlett-Packard Model 5831A gas chromatograph equipped with a 6-ft. glass column (2 mm I.D. × 0.25 in. O.D.) packed with 3% QF-1 on Gas-Chrom Q (100–120 mesh; Applied Science Labs.). Temperatures were maintained at 230° for the column, 250° for the injector, and 275° for the detector. The nitrogen carrier gas flow-rate was maintained at 20 ml/min. The flame-ionization detector gases were held at 40 ml of hydrogen per min and 300 ml of air per min. Retention times and peak areas were reported directly by the instrument.

Bile acids from rat feces were extracted and purified as previously described[5] and partial TMS derivatives were prepared with BSTFA for comparison to standard bile acid chromatograms.

RESULTS

Table I summarizes the relative retention time values for bile acid methyl ester TMS ethers prepared by the three methods described. A mixture of lithocholic,

TABLE I

RELATIVE RETENTION TIMES ON 3% OF-1 OF DIFFERENT BILE ACID METHYL ESTERS AS TRIMETHYLSILYL ETHER DERIVATIVES FORMED WITH DIFFERENT SILYLATING REAGENTS*

sh = shoulder on the peak.

Bile acid methyl ester	*Hydroxyl positions*	*Relative retention times*		
		*HMDS–TMCS–pyridine*** *(3:1:9)*	*HMDS****	*BSTFA*
Lithocholic	3α	3.28	3.29	3.27
Hyodeocholic	3α,6α	3.94	4.00	3.95
Deoxycholic	3α,12α	3.54	6.33	6.29
Chenodeoxycholic	3α,7α	3.77	7.08	7.04
12-Ketolithocholic	3α	10.79	10.84	10.78
Cholic	3α,7α,12α	3.72	13.95	13.87
β-Muricholic	3α,6β,7β	4.89	-	7.90sh

* Retention times relative to 5α-cholestane at 230° at carrier gas flow-rate of 20 ml/min.

** Full TMS derivatives formed[1].

*** Hydroxyls derivatizing are 3α, 6α and 7β according to Eneroth *et al.*[8] and Briggs and Lipsky[9].

hyodeoxycholic, deoxycholic, chenodeoxycholic and cholic acids as full TMS ethers were poorly resolved and appeared as two merged peaks under the conditions of analysis described. As full TMS derivatives β-muricholic and 12-ketolithocholic acids were separated from the other bile acids. The relatively long retention time of the keto acid is characteristic of the QF-1 liquid phase. In contrast, bile acid TMS derivatives formed with BSTFA or HMDS were well separated. Fig. 1 shows a typical standard GLC chromatogram without β-muricholic acid for derivatives formed using BSTFA.

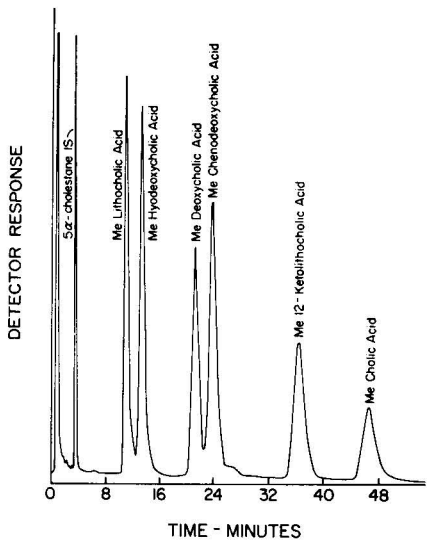


Fig. 1. Chromatogram for mixture of standard bile acid methyl esters derivatized with BSTFA as described under Methods. Amounts of bile acids were 1 μg with 0.25 μg of 5α-cholestane as internal standard injected in a 1.0-μl volume of CS_2. The small shoulder after Me chenodeoxycholic acid is an unidentified contaminant.

TMS ethers only on the unhindered hydroxyls (3α, 6α and 7β) are reportedly formed using HMDS[8]. The nearly identical relative times for derivatives formed with either HMDS or BSTFA suggest that partial derivatives are formed with each reagent. Such a result would seem logical since BSTFA is a large molecule and thus would be unable to react with sterically hindered hydroxyls. Further, the retention times for HDC derivatives prepared by all three reagents are the same while bile acids with 7α and/or 12α hydroxyls appear at longer retention times when HMDS or BSTFA is used as silylating reagent. The shoulder on the β-muricholic acid peak appearing at a longer retention time is most likely an incomplete ether formation at the 7β hydroxyl with BSTFA. Thus β-muricholic acid, appearing in small amounts in the feces of most rat strains, is best detected as a full TMS derivative.

Fig. 2 shows a GLC chromatogram of bile acids derivatized with BSTFA that were extracted from the feces of Sprague–Dawley rats[5]. Tentative identification of the peaks was made by comparison to the relative retention times of the standard bile acid chromatogram. Only one peak of considerable size, at 32 min retention, remains unidentified.

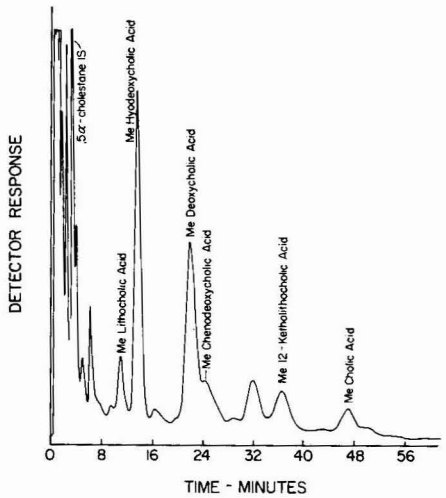


Fig. 2. Chromatogram of fecal bile acids excreted by Sprague–Dawley rats using BSTFA as silylating reagent, GLC conditions as described under Methods. Peaks as labeled were identified by comparison of relative retention times.

DISCUSSION

The results of this study indicate that the GLC analysis of fecal bile acids from rats may be simplified by using QF-1 as a liquid phase and a combination of full and partial TMS derivatives. Lithocholic, hyodeoxycholic, deoxycholic, chenodeoxycholic, 12-ketolithocholic, and cholic acids which constitute a large part of the bile acids excreted by conventional rats can be conveniently determined as partial TMS derivatives by using BSTFA as silylating reagent. The resulting derivatives have retention times identical to those formed with HMDS by a method previously documented for the formation of partial derivatives[8]. The use of BSTFA, however, is advantageous since it can serve as reaction solvent, it is highly reactive, and its by-products appear in the solvent front. Further, if BSTFA is used as injection solvent the transient appearance of HF in the flame-ionization detector helps prevent excessive silicon dioxide deposits.

The method described may also be used quantitatively. Standard response curves have been found to be linear from less than 0.3 to 5 μg or more of each of the six bile acids examined. Reproducibility appears excellent if conditions and reagents are kept constant. However, we have found in further work (data not presented here) that if N,N-bis(trimethylsilyl)trifluoroacetamide is used in place of the usual reagent N,O-compound, hyodeoxycholic acid appears as two peaks, the new peak appearing between chenodeoxycholic and 12-ketolithocholic acids. The reason for this change is not known.

In conclusion, the use of BSTFA to form partial TMS derivatives allows the GLC separation on 3% QF-1 of six important bile acids found in rat feces. Subsequent conversion to full TMS derivatives allows the detection of β-muricholic acid as well as keto acids that are greatly retained by the QF-1 liquid phase. This method is useful for structural determinations by peak shift analysis and for quantitation.

Extension of this method to less common bile acids such as α-muricholic and ω-muricholic acids where available coupled with GLC and mass spectrometry studies will more clearly define the silylating specificity of this reagent toward bile acids.

ACKNOWLEDGEMENTS

This work was supported by the Matilda R. Wilson Fund, Detroit, Mich. We thank Miss Kathleen Smith for superior secretarial assistance.

REFERENCES

1 S. M. Grundy, E. H. Ahrens and T. A. Miettinen, *J. Lipid Res.*, 6 (1965) 397.
2 B. I. Cohen, R. F. Raicht, G. Salen and E. H. Mosbach, *Anal. Biochem.*, 64 (1975) 567.
3 D. Madsen, M. Beaver, L. Chang, E. Bruckner-Kardoss and B. Wostmann, *J. Lipid Res.*, 17 (1976) 107.
4 H. Gleispach, *J. Chromatogr.*, 91 (1974) 407.
5 N. D. Nigro, R. L. Campbell, D. V. Singh and Y. N. Lin, *J. Nat. Cancer Inst.*, 57 (1976) 883.
6 N. D. Nigro, R. L. Campbell, J. S. Gantt, Y. N. Lin and D. V. Singh, *Cancer Res.*, 37 (1977) 3198.
7 S. Bergstrom and G. A. Haslewood, *J, Chem. Soc., Suppl.*, 36 (1939) 450.
8 P. Eneroth, B. Gordon, R. Ryhage and J. Sjovall, *J. Lipid Res.*, 1 (1976) 511.
9 T. Briggs and S. R. Lipsky, *Biochim. Biophys. Acta*, 97 (1965) 579.

Journal of Chromatography, 155 (1978) 432–435

CHROM. 11,021

Note

Gas–liquid chromatographic separation of monomethylguanines as their trimethylsilyl derivatives*

GARY M. MUSCHIK** and WAYNE B. MANNING

Chemical Carcinogenesis Program, NCI Frederick Cancer Research Center, Frederick, Md. 21701 (U.S.A.)

(First received January 19th, 1978; revised manuscript received March 20th, 1978)

A number of workers[1–10] have reported gas–liquid chromatography (GLC) data for purine and pyrimidine bases, nucleosides and nucleotides but data for only a few monomethylated derivatives have been reported. This note reports a method employing GLC for the separation of silyl derivatives of mono-methylated guanines, and describes this separation.

EXPERIMENTAL

A Varian Model 1440 gas–liquid chromatograph equipped with a flame-ionization detector and a linear-temperature programmer was used for this study. Helium carrier-gas flow-rate was 48 ml/min, air flow-rate was maintained at 200 ml/min and the hydrogen flow-rate was 20 ml/min as measured by a soap-bubble flow-meter. Sample injection volumes were 1–2 μl. The chromatographic columns were either 5.7% (w/w) SE-30 on Chromosorb W HP (100–120 mesh) or 5% (w/w) OV-3 on Chromosorb W HP (100–120 mesh), packed in glass columns 6 ft. $\times$ 2 mm I.D. The column temperatures were programmed from 150 to 275° at 6°/min.

A Finnigan series 1015C CI-EI GLC–mass spectrometry (MS) apparatus equipped with a chemical ionization (CI) source and interfaced with a Finnigan 6000 MS data system was used to collect the MS data. All spectra were collected at an ionization potential of 130 eV with methane as the CI gas.

The monomethylguanines were obtained commercially except for O^6-methylguanine that was prepared by the method of Balsiger and Montgomery[11] and 8-methylguanine which was prepared by the method of Daves *et al.*[12]. N,O-Bis-(trimethylsilyl)acetamide (BSA) was obtained from Sigma (St. Louis, Mo., U.S.A.).

The trimethylsilyl (TMS) derivatives of the methylguanines were prepared as follows. A stock solution containing 2.7 mg of phenanthrene (internal standard), 4 ml of acetonitrile and 0.1 ml of BSA was prepared as the silylating reagent. Known amounts (0.1–0.3 mg) of each of all monomethylguanines were placed in a micro

* Presented in part at the Federation of Analytical Chemistry and Spectroscopy Societies Conference, Philadelphia, Pa., U.S.A.

** To whom correspondence should be addressed.

reaction vessel (Supelco, Bellefonte, Pa., U.S.A.), along with known volumes (0.1–0.15 ml) of the above silylating reagent. The micro reaction vessels were placed into a 130° oil bath for 90 min and then analyzed by GLC.

RESULTS AND DISCUSSION

The conversion of the monomethylguanine isomers to their respective TMS derivatives resulted in a volatile and thermally-stable derivative for GLC analysis and gave a fast and sensitive method for the analysis of these compounds.

The advantages and limitations of this GLC method have been examined using SE-30 and OV-3 liquid phases. A comparison of relative retention values on the SE-30 liquid phase with those obtained on the OV-3 liquid phase was made and appears in Table I. The SE-30 liquid phase had the distinct advantage of separating all monomethylguanine-TMS derivatives (Fig. 1). Lakings *et al.*[7] reported that the separations of a broad spectrum of commercially-available methylated bases were best achieved on OV-3. However, our findings indicate that SE-30 gave better resolution for the separation of monomethylguanines. Neither SE-30 nor OV-3 could separate guanine from 1-methylguanine-TMS derivatives. A disadvantage of OV-3 was its inability to resolve the O^6-methylguanine, 1-methylguanine and guanine-TMS derivatives. In contrast, SE-30 resolved O^6-methylguanine from either 1-methylguanine or guanine TMS derivatives. The mixture of 7-methylguanine and 8-methylguanine TMS derivatives was also unresolved on OV-3. However, a mixture consisting of the TMS derivatives of N^2-methylguanine, O^6-methylguanine, 7-methylguanine, and 9-methylguanine was better resolved on OV-3 than on SE-30. We found that satisfactory resolution of all volatile monomethylguanine derivatives could be achieved on SE-30.

The number of TMS groups per monomethylguanine molecule (Table I) was determined from the GLC–CI–MS quasi-molecular ion values of the product formed under silylating conditions which resulted in complete derivatization. Guanine and 8-methylguanine gave tri-TMS derivatives. Di-TMS derivatives were observed for 1-

TABLE I

RETENTION TIMES AND NUMBER OF SILYL GROUPS FOR THE RESPECTIVE MONO-METHYLGUANINE-TMS DERIVATIVES

α — Relative retention time (phenanthrene — 1.00).

Compound	*TMS retention times*				*Number of silyl groups*
	OV-3		*SE-30*		
	Time (min)	α	*Time (min)*	α	
Guanine	22.1	1.75	17.9	1.53	3
1-Methylguanine	22.1	1.75	17.4	1.49	2
N^2-Methylguanine	20.0	1.59	16.4	1.40	2
3-Methylguanine					
O^6-Methylguanine	22.1	1.75	17.3	1.48	2
7-Methylguanine	23.3	1.85	18.1	1.55	2
8-Methylguanine	23.6	1.87	18.7	1.60	3
9-Methylguanine	19.0	1.51	15.2	1.30	2
Phenanthrene	12.6	1.00	11.7	1.00	

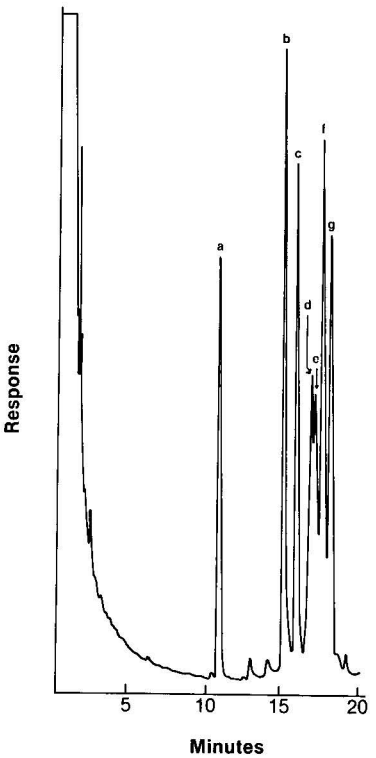


Fig. 1. Separation of monomethylguanine-TMS derivatives on SE-30. a = phenanthrene (internal standard); b = 9-methylguanine; c = N^2-methylguanine; d = O^6-methylguanine; e = 1-methylguanine; f = 7-methylguanine; g = 8-methylguanine.

methylguanine, N^2-methylguanine, O^6-methylguanine, 7-methylguanine, and 9-methylguanine, as might be anticipated from their respective structures. The di-TMS structure for 7-methylguanine and the tri-TMS structure for guanine were observed previously and reported by electron impact MS by Hattox and McCloskey[13]. The GLC profiles and GLC–CI–MS molecular ion data on samples prepared under incomplete reaction conditions showed the presence of both di-TMS and tri-TMS derivatives for guanine and 8-methylguanine; both mono-TMS and di-TMS derivatives for 9-methylguanine. These respective mono-, di- and tri-TMS derivatives were observed to have different GLC relative retention (α) values. It is therefore necessary in qualitative and quantitative analysis of these compounds, to generate the completely silylated products. For example, the di-TMS derivatives of guanine and 9-methylguanine had approximately the same relative retention values on the SE-30 column. The GLC lower detection limit, expressed in nanograms of purine, providing discernible and useful peaks for quantitative measurements ranged from about 2 ng for guanine to 20 ng for 1-methylguanine.

This method has been useful in separating standard mixtures of monomethylguanines, the monomethylguanines obtained from hydrolysis of methylated guanosine, and the monomethylguanines from hydrolysis of methylated polyguanylic acid (Fig. 2).

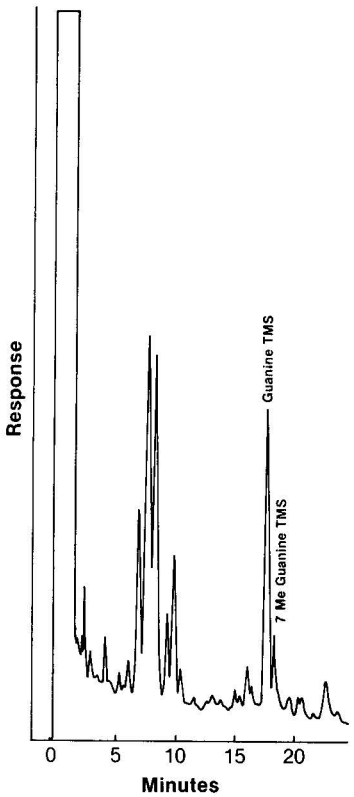


Fig. 2. Analysis of TMS derivatives from the hydrolysis of methylated polyguanylic acid. Methylation conditions: polyguanylic acid, dimethylsulfate, 37°, 18 h, phosphate buffer pH = 7.0. Hydrolysis conditions: hydrochloride, 100°, 1 h. Silylation conditions: see Experimental.

ACKNOWLEDGEMENT

This work was sponsored by the National Cancer Institute under Contract No. NO1-CO-25423 with Litton Bionetics.

REFERENCES

1 I. A. Muni, C. H. Altschuler and J. C. Neicheril, *Anal. Biochem.*, 50 (1972) 354.
2 W. C. Butts, *Anal. Biochem.*, 46 (1972) 187.
3 W. C. Butts, *J. Chromatogr. Sci.*, 8 (1970) 474.
4 R. L. Hancock, *J. Chromatogr. Sci.*, 7 (1969) 366.
5 R. L. Hancock, *J. Gas Chromatogr.*, 4 (1966) 363.
6 D. B. Lakings and C. W. Gehrke, *Clin. Chem.*, 18 (1972) 810.
7 D. B. Lakings, C. W. Gehrke and T. P. Waalkes, *J. Chromatogr.*, 116 (1976) 69.
8 S. Y. Chang, D. B. Lakings, R. W. Zumwalt and C. W. Gehrke, *J. Lab. Clin. Med.*, 83 (1974) 816.
9 C. W. Gehrke, D. L. Stalling and C. D. Ruyle, *Biochem. Biophys. Res. Commun.*, 28 (1967) 869.
10 C. W. Gehrke and D. B. Lakings, *J. Chromatogr.*, 61 (1971) 45.
11 R. W. Balsiger and J. A. Montgomery, *J. Org. Chem.*, 25 (1960) 1573.
12 G.12 G. D. Daves, Jr., C. W. Noell, R. K. Robins, H. C. Koppel and A. G. Beaman, *J. Amer. Soc.*, 82 (1960) 2633.
13 S. E. Hattox and J. A. McCloskey, *Anal. Chem.*, 46 (1974) 1378.

Journal of Chromatography, 155 (1978) 436–438

CHROM. 11,008

Note

Improved high-pressure liquid chromatographic separation of amino acid phenylthiohydantoins

JACQUES ELION
Institut de Pathologie Moleculaire, 24, rue du Fb. Saint Jacques, 75014 Paris (France)
MICHAEL DOWNING
Cardiovascular Research Unit, Mayo Clinic, Rochester, Minn. (U.S.A.)
and
KENNETH MANN*
Hematology Research Unit, Mayo Clinic, Rochester, Minn. (U.S.A.)
(Received March 2nd, 1978)

The determination of NH_2-terminal sequences of peptides or proteins by the Edman degradation involves the identification of the residue removed from the peptide at each cycle (for a recent review, see ref. 1). The phenylthiohydantoin (PTH) derivatives of the amino acids are commonly identified by thin-layer chromatography (TLC) or by gas–liquid chromatography (GLC) both before and after silylation. High-pressure liquid chromatography (HPLC) has been more recently used for this purpose and presents several advantages over other procedures, including identification and quantitation of all the residues, including histidine and arginine, without derivatization. Several HPLC procedures have been described[2–6] and we have reported the separation of most of the PTH amino acid derivatives in 40 min on a μBondapak C_{18} column utilizing an acetonitrile–acetate solvent system[7]. We describe here a new procedure utilizing the same column and a methanol–acetate solvent system. This procedure results in a shorter time of analysis and better resolution. All of the PTH derivatives are resolved in 32 min with the exception of the pair PTH-valine–PTH-methionine.

MATERIAL AND METHODS

Standard PTH derivatives were obtained from Pierce (Rockford, Ill., U.S.A.). Methanol was purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.) and used without further purification. Water used in the preparation of the buffers was purified on a Milli-Q four place Millipore system (Millipore, Bedford, Mass., U.S.A.). Prior to use on the high pressure liquid chromatograph, the buffers were filtered through a 0.45-μm Millipore filter.

Analysis of the PTH derivatives was carried out on a Waters Assoc. ALC/GPC 202 high-pressure chromatograph equipped with a second Model 6000

* To whom correspondence should be addressed.

pump and a Model 660 programmer. Samples (1 to 20 μl) were injected without solvent interruption through a Waters Assoc. U6K injector. Separations were carried out at room temperature on a μBondapak C_{18} column (30 cm $\times$ 4 mm). Amino acid PTHs were detected by absorption at 254 nm utilizing the UV detector of the liquid chromatograph.

Solvents were pumped at a total flow-rate of 3 ml/min, resulting in a pressure of 4000 p.s.i. Solvent A was 0.01 *M* sodium acetate pH 4.0–methanol (9:1) and solvent B 0.01 *M* sodium acetate pH 4.0–methanol (1:9). The column was equilibrated with solvent B contributing 5% to the total volume of the eluent. PTH derivatives were eluted from the column by a concave gradient (curve 7 on the programmer) from initial conditions (5% solvent B) to 40% of solvent B over 22 min, followed by an isocratic elution with 40% solvent B for 10 min. Both solvents A and B were stirred continuously while being supplied to the solvent delivery pump.

RESULTS AND DISCUSSION

HPLC is the technique of choice for the quantitative identification of all the PTH amino acids without derivatization. The limitations of this technique up to now have been the time of analysis, incomplete resolution of PTH derivatives, cost of the instrumentation and of solvents.

Fig. 1 shows the elution profile of a standard mixture of PTH derivatives of ten amino acids. The elution positions of the twenty usual PTH amino acid derivatives

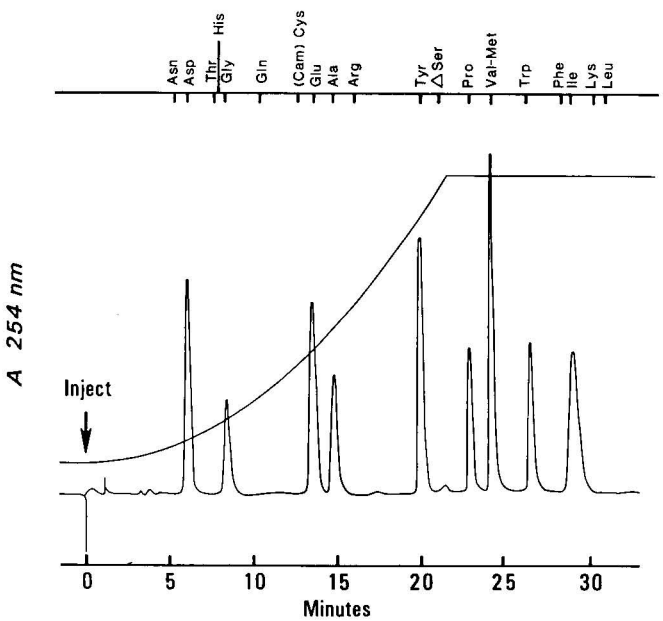


Fig. 1. Elution profile of a mixture of 10 PTH derivatives of amino acids by HPLC. Elution conditions are described in the text. The profile of the elution gradient is shown by the concave line running through the chromatogram, and followed by an isocratic elution for 10 min. The full-scale deflection represents 0.32 absorbance units for 3–7 nmoles of each PTH amino acid derivative.

are illustrated on the upper part of the figure. The PTH amino acids are resolved one from the other in 32 min, with the exception of PTH-valine and PTH-methionine which are eluted as a single peak. The PTH derivatives of phenylalanine, tryptophan and isoleucine which were eluted in a single peak in the acetonitrile–acetate system[7] are all resolved in the present system. Similarly proline is clearly separated from the valine–methionine peak.

Several hundred residues obtained from an automatic liquid-phase sequencer have been positively identified utilizing this HPLC program, with very high reproducibility. Reproducibility is enhanced if the column is washed with the second solvent (solvent B) for 10 min after every 5 identifications. Standard PTH serine is eluted between PTH-aspartic acid and PTH-threonine. However, the PTH serine derivatives produced by the sequencer are always found as the derivative dehydroserine (PTH Δ Ser). This derivative gives a characteristic double peak eluting after PTH-tyrosine. Since PTH-histidine and PTH-arginine are resolved from the other PTH amino acids, the extraction of the organosoluble PTH derivatives from the aqueous conversion medium is theoretically no longer necessary. Good results are obtained when N,N,N′,N′,-tetrakis (2-hydroxypropyl)ethylenediamine (Quadrol) is used as the coupling buffer in the sequencer. However, when the N,N′-dimethylallylamine (DMAA) buffer is used, a DMAA peak is present in the void volume which may interfere with the identification of PTH-asparagine, and extraction is therefore preferable. Utilization of a DMAA sequencer program often results in an additional peak eluting near the valine–methionine peak which occasionally interferes with the identification of these two residues.

In addition to improved resolution and shorter analysis time, which allows identification to keep pace with the automatic sequencer, the use of methanol instead of acetonitrile represents a substantial decrease in the running cost of the liquid chromatograph.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the research support provided by the Mayo Clinic and Grants HL-16150, CA-15083 and HL-17430 D. J. Elion was supported in part by a fellowship from the Philippe Foundation; and M. Downing was supported by Blood Banking and Hemostasis Training Grant HL-07069. K. Mann is an American Heart Established Investigator.

REFERENCES

1 Z. Deyl, *J. Chromatogr.*, 127 (1976) 91.
2 G. Frank and W. Strubert, *Chromatographia*, 6 (1973) 522.
3 C. L. Zimmerman, J. J. Pisano and E. Appella, *Biochem. Biophys. Res. Commun.*, 55 (1973) 1220.
4 A. P. Graffeo, A. Haag and B. L. Karger, *Anal. Lett.*, 6 (1973) 505.
5 E. W. Mathews, P. G. H. Byfield and I. Mac Intyre, *J. Chromatogr.*, 110 (1975) 369.
6 C. L. Zimmerman, E. Appella and J. J. Pisano, *Anal. Biochem.*, 77 (1977) 569.
7 M. R. Downing and K. G. Mann, *Anal. Biochem.*, 74 (1976) 298.

Journal of Chromatography, 155 (1978) 439–442

CHROM. 11,007

Note

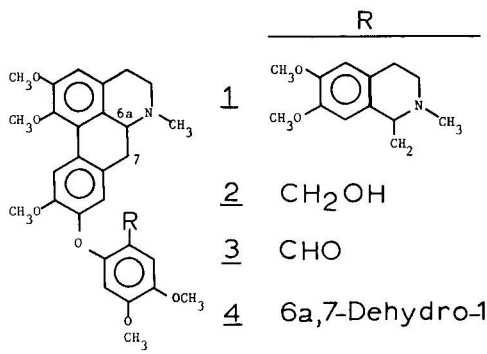
High-performance liquid chromatography of thalicarpine, hernandaline, hernandalinol and dehydrothalicarpine

MARK SMELLIE, MICHAEL CORDER and JOHN P. ROSAZZA*

College of Pharmacy and The College of Medicine, The University of Iowa, Iowa City, Iowa, 52242 (U.S.A.)

(Received March 13th, 1978)

Thalicarpine (1) is a benzyltetrahydroisoquinoline–aporphine alkaloid which was first isolated from *Thalictrum*[1,2] and *Hernandia*[3] species, and its structure was proven by total synthesis[4]. Thalicarpine is of interest as an antitumor compound[5,6], and hernandalinol (2) was obtained as a microbial metabolite of thalicarpine, presumably by reduction of the intermediate hernandaline (3)[7]. Dehydrothalicarpine (4) is commonly formed by air oxidation when (1) is incubated in aqueous medium.



Further microbial and mammalian metabolism studies with thalicarpine would be greatly facilitated by the availability of a sensitive, simple and rapid analytical technique. This report describes the development of a high-performance liquid chromatographic (HPLC) system useful in the detection of compounds 1–4.

EXPERIMENTAL

Thalicarpine was obtained from the National Cancer Institute (Bethesda, Md., U.S.A.)[7]. Hernandalinol was produced by $NaBH_4$ reduction of hernandaline, a product of the $KMnO_4$ oxidation of thalicarpine[7]. Dehydrothalicarpine was prepared

* To whom correspondence should be addressed.

by palladium on carbon treatment of I according to the procedure of Cava *et al.*[8]. All compounds were fully characterized and gave single spots on thin-layer chromatograms.

HPLC experiments were performed using a Waters Assoc. (Milford, Mass., U.S.A.) ALC/GPC 202 instrument equipped with an M6000 solvent delivery system, a U6K universal injector, and a 254-nm differential UV detector. Alkaloids were best separated from one another on a Waters Assoc. μPorasil column (0.4 × 30 cm) with a solvent system of cyclohexane–chloroform–diethylamine (25:150:0.3). Nominal operating conditions employed were 1900 p.s.i. at a flow-rate of 2.4 ml/min. Samples of alkaloids and extracts were dissolved in chloroform.

Thin-layer chromatography (TLC) was performed on 0.25-mm thick layers of silica gel GF_{254} (Merck, Darmstadt, G.F.R.), and all plates were activated for 30 min at 120° before use. Solvent systems used in TLC analysis were (a) benzene–methanol–58% NH_4OH (80:30:0.1), and (b) acetone–100% ethanol (50:1). Visualization of developed TLC plates was accomplished with 254-nm UV light, and by spraying plates with Dragendorff's reagent, or with 2,4-dinitrophenylhydrazine reagent[7].

Urine specimens were collected from patients being treated with thalicarpine at an average dose of 1100 mg/m^2 of body area administered intravenously. The times of collecting of urines varied from 4 to 24 h following administration of the drug. Urines were pooled, and stored under toluene in the cold until required for analysis. All urine specimens were divided into portions which received one of the following treatments.

(A) *Untreated.* Samples of 50 ml of urine were adjusted to pH 8.0 with 1.0 *N* NaOH and were exhaustively extracted with four equal volumes of chloroform. The chloroform extracts were dried over anhydrous Na_2SO_4 before being concentrated to dryness. The dried extracts were redissolved in 1.0 ml of chloroform for use in HPLC and TLC analyses.

(B) *Glusulase treated.* Sufficient 2 *M* sodium acetate buffer was added to a volume of urine resulting in a final buffer concentration of 0.1 *M*. The urine specimens were adjusted to pH 5.0 exactly and were reacted with a combination β-glucuoronidase and sulfatase enzyme preparation (1000 Fishman units and 500 sulfatase units/ml of urine; Endo Labs., Richmond Hill, N.Y., U.S.A.) at 37° for 24 h. Incubation mixtures were then adjusted to pH 8.0 and extracted as described before. The efficiency of the enzyme preparation was determined by measuring the rate of hydrolysis of phenolphthalein-β-glucuronidase added to urine samples.

RESULTS AND DISCUSSION

Several types of columns and solvent systems were initially examined for their abilities to separate the alkaloids. Phenyl-Bondapak (Waters Assoc.) and μPorasil columns with acetonitrile–ethanol or chloroform–methanol mixtures were tried without success. Hernandaline and hernandalinol were well resolved with acetonitrile–0.1% ammonium carbonate (1:1) on a Phenyl-Bondapak column, while a 7:1 mixture of these solvents could be used to separate 1 and 2 from one another. None of these chromatographic systems could be used to resolve all four of the alkaloids.

Excellent separations were obtained with a μPorasil column using a cyclohexane–chloroform–diethyl amine (25:150:0.3) solvent system. All of the alkaloids

TABLE I

HPLC AND TLC PROPERTIES OF THALICARPINE, DEHYDROTHALICARPINE, HERNANDALINE AND HERNANDALINOL

Compound	*HPLC**		*TLC R_F values*	
	Retention volume (ml)	*Limits of detection (ng)*	*Benzene–methanol–NH_4OH (80:30:0.1)*	*Acetone–ethanol (50:1)*
Dehydrothalicarpine	5.4	40	0.7	0.33
Hernandaline	6.7	40	0.75	0.45
Thalicarpine	17.7	400	0.65	0.05
Hernandalinol	33.1	400	0.65	0.32

* Analyses were performed with a μPorasil column at 1900 p.s.i., 2.4 ml/min using cyclohexane–chloroform–diethylamine (25:150:0.3).

** Silica gel GF_{254} TLC plates, 0.25 mm, visualized with 254 nm light and with Dragendorff's reagent.

were well separated from one another within 15 min. Average retention volumes, and limits of detectability (signal to noise ratio of 2:1) of the alkaloids are presented in Table I, and a typical HPLC chromatogram is shown in Fig. 1. Although peak sharpness and resolution could be improved by the addition of more diethylamine to the solvent system, this would also require the use of a detector at higher wavelength (280 nm).

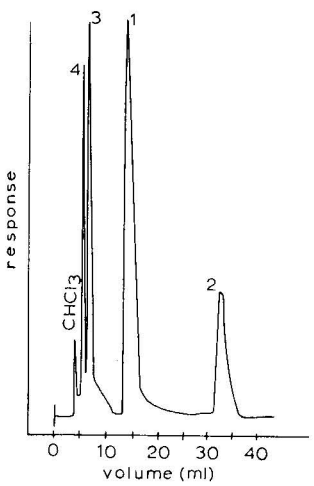


Fig. 1. HPLC Separation of thalicarpine (1), hernandalinol (2), hernandaline (3) and dehydrothalicarpine (4) achieved at 25° under the following conditions: cyclohexane–chloroform–diethyl amine (25:150:0.3) at a flow-rate of 2.3 ml/min, 1900 p.s.i. using a 254-nm UV detector.

The HPLC and TLC systems were used to detect thalicarpine and potential thalicarpine metabolites 2–4 in urine specimens collected from patients receiving 1 as a drug. The presence or absence of each compound was confirmed by spiking extracts with the known alkaloids, and by examining the extracts by TLC. A typical HPLC chromatogram of a chloroform extract of a urine specimen is shown in Fig.

2. All of the urine extracts examined contained thalicarpine and dehydrothalicarpine. Dehydrothalicarpine was probably present as an artifact formed during urine sample workup. The amounts of 4 ranged from 1.4–4% of the amounts of thalicarpine also found in samples. The total amount of thalicarpine recovered ranged from 0.25%–0.64% of the doses administered to the patients. These results are consistent with those of Palm *et al.*[9], who reported that little urinary or biliary excretion of thalicarpine occurs in test animals. Neither 3 nor 2 could be conclusively demonstrated to occur in any of the urine extracts.

Chromatographic systems described in this report will be useful in performing further microbial and mammalian metabolism studies with thalicarpine.

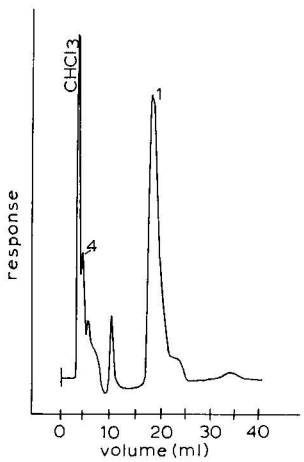


Fig. 2. HPLC analysis of a chloroform extract of urine performed on μPorasil using cyclohexane–chloroform–diethyl amine (25:150:0.3) at a flow-rate of 2.4 ml/min at 1900 p.s.i. using a 254-nm UV detector.

ACKNOWLEDGEMENTS

We wish to acknowledge financial support for this work through NIH Grant CA-13786, and from the Division of Undergraduate Education in Science, National Science Foundation, Grant No. SM176-82927.

REFERENCES

1 S. M. Kupchan, K. K. Chakravarti and N. Yodoyama, *J. Pharm. Sci.*, 52 (1963) 985.
2 P. L. Schiff, Jr. and R. W. Doskotch, *Lloydia* 33 (1970) 403.
3 M. Tomita, H. Furukawa, S. T. Lu and S. M. Kupchan, *Tetrahedron Lett.*, (1965) 4309.
4 S. M. Kupchan, A. J. Liepa, V. Kameswaran and K. Sempuka, *J. Amer. Chem. Soc.*, 95 (1973) 2995.
5 J. L. Hartwell and B. J. Abbott, *Advan. Pharm. Chemother.*, 7 (1968) 117.
6 N. M. Mollov, K. B. Duchevska, K. Silyanovska and S. Stoicev, *C. R. Acad. Sci.*, 21 (1968) 605; *C.A.*, 69 (1968) 58087.
7 T. Nabih, P. J. Davis, J. F. Caputo and J. P. Rosazza, *J. Med. Chem.*, 20 (1977) 914.
8 M. P. Cava, D. L. Edie and J. J. Saa, *J. Org. Chem.*, 40 (1975) 3602.
9 P. E. Palm, M. S. Mick, E. P. Arnold, D. W. Yesair and M. M. Callahan, *U.S. Nat. Techn. Inform. Serv., PB Report No. 201914*, 1971, 164 pp.; *C.A.* 76 (1972) 68093g.

Journal of Chromatography, 155 (1978) 443–445

CHROM. 11,061

Note

New chromatographic method for the preparation of DNA–adriamycin complexes

A. HUOT and M. PAGE

Centre de Recherches, l'Hôtel-Dieu de Québec, 11 Côte du Palais, Québec G1R 2J6 (Canada)

(Received March 9th, 1978)

The use of adriamycin as an antineoplastic agent is very often limited to patients with advanced disease because of its side effects including its cardiotoxicity[1]. Complexes of adriamycin with DNA have been prepared for injection in order to reduce this toxicity[2,3]. These complexes are active as antineoplastic agents but they still have a certain toxicity for normal cells. It is very well established that adriamycin is bound to DNA by intercalation in the flat base pairs of the double helix[4,5], but other types of binding have also been proposed[6,7].

Amberlite XAD-2 is a non-ionic resin which is used to isolate a large variety of drugs including alkaloids, barbiturates, amphetamines, phenothiazines and methadone[8]. These molecules are retained on the column by hydrophobic binding. We describe a rapid method for the preparation of adriamycin–DNA complexes using this resin, and we compare the complexes thus obtained to the ones obtained by the usual method[3,9].

MATERIALS AND METHODS

Deoxyribonucleic acid type I (calf thymus DNA) was purchased from Sigma (St. Louis, Mo., U.S.A.). Adriamycin was a gift from Adria Labs. (Toronto, Canada). Amberlite XAD-2 resin was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.). All solvents and other reagents were reagent grade or of a better quality.

Preparation of Amberlite XAD-2 resin

The resin was washed extensively on a buchner funnel with methanol followed by at least 5 volumes of 0.5% sodium chloride and with 1% sodium carbonate. The resin was then washed extensively with distillated water, packed in a 1 × 10 cm column and equilibrated with 0.2 *M* Tris–HCl buffer at pH 7.5.

Chromatographic method

A 1-mg amount of adriamycin was dissolved in 1 ml of 0.2 *M* Tris–HCl buffer at pH 7.5. This solution was then added to an equivalent volume of a DNA solution (2 mg/ml) prepared in the same buffer. The mixture was stirred until the complex was dissolved. The complex was then passed through an Amberlite XAD-2 resin column (1 × 10 cm) and eluted with two column volumes of buffer. The free drug was

eluted from the resin with the following solvent: carbon tetrachloride–*tert.*-butyl alcohol–methanol–ethanolamine (1:1:1:0.5). The amount of adriamycin and DNA in the complex were measured at 480 and 260 nm, respectively[10].

Spontaneous mixture and dialysis

In order to compare the complexes prepared by the above method with the ones prepared by other procedures, complexes were prepared by the method described by Trouet *et al.*[3] utilising a 0.2 *M* Tris–HCl buffer at pH 7.5 and by dialysis[9] with a 0.01 *M* Tris–HCl, 0.01 NaCl buffer at pH 7.0. These complexes were either analysed directly or separated by chromatography as described above.

RESULTS

Table I shows that after passage on XAD-2 Amberlite resin the adriamycin–DNA ratio ($\mu g/\mu g$) was 0.088 in our assay conditions, 0.053 for the complex prepared by Trouet's method[3] and 0.026 for the complex prepared by simple dialysis. The drug–DNA ratio obtained with the last two methods correspond to the already reported ratios[3,10]. However when complexes prepared by Trouet's method were separated on the Amberlite XAD-2 column, 36% of the drug could be removed from the complex showing that the hydrophobic portion of the drug was still available for binding to the resin. This portion probably represents free drug or adriamycin bound by ionic interaction or hydrogen binding. According to the model of Pigram *et al.*[4], the hydrophobic portion of the intercalated anthracycline molecule is hidden between the base pairs of the DNA molecule, these would then be unavailable to the XAD-2 resin and the drug bound to DNA by intercalation would not be retarded by the hydrophobic resin. Most of the adriamycin bound to DNA after simple dialysis remained attached to DNA after passage on the resin, thus showing that the intercalation type of binding is favored by this procedure.

TABLE I

COMPARATIVE RATIOS OF ADRIAMYCIN–DNA

Method of purification	*μg Adriamycin / μg DNA (initial)*	*μg Adriamycin / μg DNA (after passage on Amberlite XAD-2)*	*Adriamycin bound to DNA after passage on Amberlite XAD-2 (%)*
Chromatography	–	0.088	
Trouet *et al.*[3]	0.083	0.053	64
Dialysis	0.029	0.026	90

DISCUSSION

As shown in Table I, complexes prepared by hydrophobic chromatography have a higher adriamycin to DNA ratio. This is explainable by the higher drug–DNA ratios that could be used in the initial incubation since the loosely bound drug could be easily removed by the procedure described above. The intercalated molecules are

much more stable and susceptible to penetrate into the tumor cells by lysosomotropism as shown by Trouet *et al.*[3].

The described procedure being rapid offers another advantage over the dialysis method since adriamycin loses its activity within 48 h in aqueous solutions.

ACKNOWLEDGEMENTS

This work was supported by the NCI and the CRSQ. We are also indebted to Adria Labs. for a gift of adriamycin and to Dr. L. P. Joly for his useful suggestion concerning the solvent extraction of the drug.

REFERENCES

1 F. S. Philips, A. Gilladoga, H. Marquardt, S. S. Stenberg and P. M. Vidal, *Cancer Chemother. Rep.*, Part 3, 6 (1975) 177.
2 M. Rozencweig, Y. Kenis, G. Atassi, M. Staquet and M. Karim, *Cancer Chemother. Rep.*, Part 3, 6 (1975) 131.
3 A. Trouet, D. Deprez-De Campeneere, M. DeSmedt-Malengraux and G. Atassi, *Eur. J. Cancer*, 10 (1974) 405.
4 W. J. Pigram, W. Fuller and L. D. Hamilton, *Nature (London)*, 235 (1972) 17.
5 N. Waring, in R. E. Hahn (Editor), *Progress in Molecular and Subcellular Biology*, Vol. 2, Springer, Berlin, 1971, p. 216.
6 N. Waring, *J. Mol. Biol.* ,54 (1970) 247.
7 N. F. Goodman, G. M. Lee and N. R. Bachur, *J. Biol. Chem.*, 252, 8 (1977) 2670.
8 J. M. Fujimoto and J. H. Wang, *Toxicol. Appl. Pharmacol.*, 16 (1970) 186.
9 F. Zunino and A. Zaccara, personal communication.
10 F. Zunino, R. Gambetta, A. Di Marco and A. Zaccara, *Biochim. Biophys. Acta*, 277 (1972) 489.

Journal of Chromatography, 155 (1978) 446–449

CHROM. 11,011

Note

Simultane Bestimmung von reduzierenden Zuckern und Zuckeralkoholen

M. H. SIMATUPANG, M. SINNER und H. H. DIETRICHS

Institut für Holzchemie und chemische Technologie des Holzes der Bundesforschungsanstalt für Forst und Holzwirtschaft, Reinbek/Hamburg (B.R.D.)

(Eingegangen am 19. August 1977; geänderte Fassung eingegangen am 15. März 1978)

Hydrolysate von verholzten Pflanzenmaterialien enthalten grössere Mengen a Glucose neben Mannose und/oder Xylose sowie kleinere Anteile Arabinose un Galactose. Durch katalytische Hydrierung dieser Zucker entstehen Zuckeralkohole Zuckeralkohole haben eine gewisse technische Bedeutung und werden zum Teil auc als Süsstoff in Lebensmitteln verwendet. Die simultane Bestimmung eines Gemische aus den obengenannten Zuckern und die durch katalytische Hydrierung daraus ge wonnenen Alkohole wurde bereits beschrieben[1]. Diese verteilungschromatographisch Methode mit einem Äthanol–Wassergemisch als mobile Phase ergab eine gute Tren nung, ist jedoch zeitraubend. Die ionenaustauschchromatographische Trennun einiger Zuckeralkohole mit einem Stufengradienten[2] unter Verwendung eines Borat puffers wurde ebenfalls in der Literatur beschrieben[3,4]. Nachfolgend wird eine ein fache Methode zur simultanen Trennung von reduzierenden Zuckern und Zucke alkoholen dargestellt.

EXPERIMENTELLES

Die verwendete Apparatur ist eine NC II P (Technicon, Frankfurt/Mai B.R.D.), die mit einer Glastrennsäule (250 × 5 mm) und einer Probenschleife aus gerüstet ist. Die Trennsäule war mit einem in der Boratform vorliegenden Anione austauscher (Typ Durrum DAX 4, Korngrösse 20 μm) gefüllt. Die mobile Phas wurde durch Auflösen von 0.11 *M* Kaliumtetraborat und 0.17 *M* Borsäure in 1 l hergestellt. Für die angegebenen Trennungen wurde der Puffer verdünnt (4 Teil Boratpuffer und 1 Teil Wasser)[6]. In frischem Zustand hat der Boratpuffer einen pH Wert von 8.8, wie es auch von Floridi[5] angegeben wird. Nach kurzer Zeit steigt de pH-Wert, bis er konstant auf 9.2 bleibt. Die hier angegebenen chromatographische Trennungen wurden mit solch einem Puffer durchgeführt. Das Säuleneluat wurd in drei Teile zerlegt, zwei dienten der Bestimmung von Zuckeralkoholen un Zuckern. Der dritte Teil wurde in diesem Fall verworfen, kann aber gegebenenfall für den Nachweis anderer Verbindungen verwendet oder in einem Fraktionssammle aufgefangen werden. Eine schematische Darstellung des Analysensystems zeigt Fig. 1

Zum Nachweis reduzierender Zucker und Ketozucker diente Neocuproin[7] ode Orcin-Schwefelsäure[5]. Zuckeralkohole wurden zunächst mit 0.01 *M* Natriumperjoda in 0.1 *N* Schwefelsäure oxidiert[2]. Nach Zersetzung des überschüssigen Perjodats m 0.5 *M* Natriumarsenit[1] wurde der gebildete Formaldehyd mit einem Gemisch aus

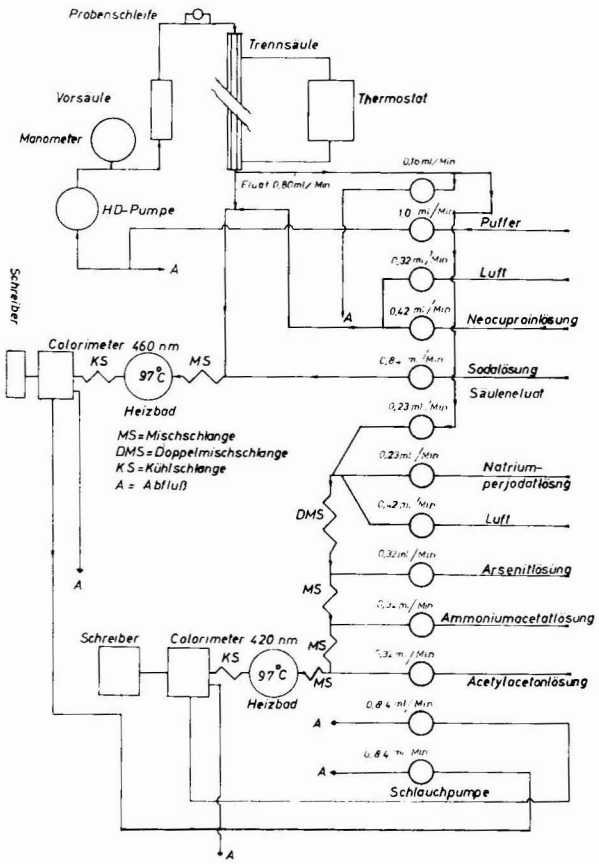


Fig. 1. Schema des Analysensystems zur simultanen Trennung von reduzierenden Zuckern und Zuckeralhokolen.

M Ammoniumacetat, 0.06 *M* Acetylaceton und 0.07 *M* Essigsäure[8] bei 420 nm nachgewiesen. Um das Fliessverhalten des Flüssigkeitsstroms in dem Nachweissystem zu verbessern, ist der Zusatz eines Detergenzes (Brij-35) zu der Arsenitlösung erforderlich. Die verwendeten Chemikalien und Vergleichssubstanzen lieferten Merck (Darmstadt, B.R.D.), Riedel de Haen (Seelze-Hannover, B.R.D.) und Serva (Heidelberg, B.R.D.).

ERGEBNISSE UND DISKUSSION

Ein Chromatogramm eines Gemisches aus reduzierenden Zuckern, das etwa der Zusammensetzung eines Fichtenholzhydrolysates entspricht, sowie von Zuckeralkoholen, die aus solch einem Hydrolysat gewonnen werden könnten, ist in Fig. 2 dargestellt. Für die reduzierenden Zucker wird eine ausreichende Trennung erzielt. Mannit und Galactit sind dagegen bei dieser Analyse nicht voneinander getrennt. Durch eine reduzierte Durchflussgeschwindigkeit des Boratpuffers von 0.8 ml auf 0.5 ml pro min sind diese beiden Zuckeralkohole zu trennen (Fig. 3). Einfacher ist es,

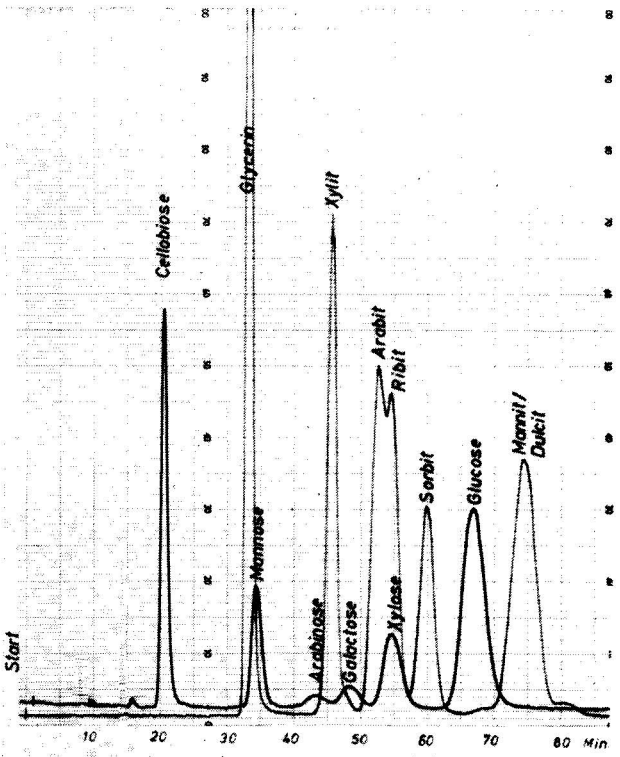


Fig. 2. Chromatographische Trennung eines Gemisches aus reduzierenden Zuckern und Zuckeralkoholen. Trennsäule: 250 × 5 mm. Temperatur 55°. Mobile Phase: 0.488 *M* Boratpuffer, pH 9.2, 0.8 ml/min. Nachweissystem wie im Fig. 1 mit Neocuproin. Aufgetragene Menge Cellobiose: 20 γ, Mannose 18 γ, Arabinose 6 γ, Galactose 12 γ, Xylose 14 γ und Glucose 50 γ. Zuckeralkohole je 20 γ; Schreiber: Vollausschlag: 0.5 E.

wenn die Temperatur der Trennsäule erhöht wird. Eine Temperatur von 70° erlaubt bereits eine ausreichende Trennung, um orientierungsweise entscheiden zu können, ob eine Probe Mannit oder Galactit enthält. Zur Orientierung wurde Lactose bei 55°, 60° und 70° chromatographisch getrennt. Unter diesen Bedingungen kann keine Isomerisierung von Lactose, wie sie von Carubelli[9] beobachtet wurde, festgestellt werden. Der Grund dürfte die kürzere Verweilzeit des Zuckers auf der Säule sein.

Das hier beschriebene Einpuffersystem ermöglicht die Trennung von Saccharose, Maltose, Ribose, Mannose, Arabinose, Galactose, Xylose und Glucose sowie die der Zuckeralkohole Glycerin, Xylit, Arabit, Ribit, Glucit (Sorbit), Mannit und Galactit (Dulcit). Die Nachweisgrenze mit der Neocuproinmethode für Arabinose ist 0.3 γ und mit der Perjodatmethode für Arabit 0.15 γ. Lactose kommt zusammen mit Maltose, während Rhamnose nicht von Ribose getrennt werden kann. Fructose kommt vor Arabinose und zeigt eine einigermassen gute Trennung. Während die reduzierenden Zucker mit Neocuproin nachgewiesen werden können, ist für Saccharose nur Orcin-Schwefelsäure brauchbar.

Ein Einpufferverfahren hat den Vorteil, dass die Säule vor jeder Analyse nicht konditioniert zu werden braucht. Somit kann kontinuierlich analysiert werden, wie

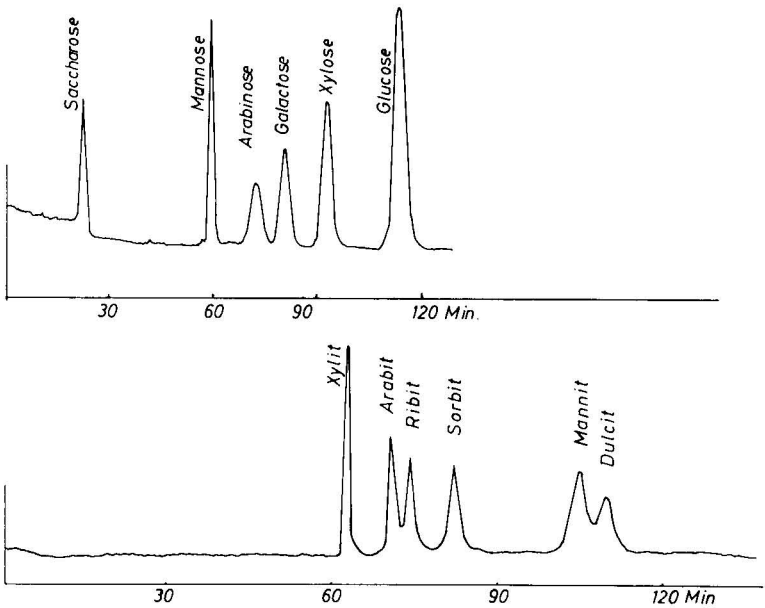


Fig. 3. Chromatographische Trennung eines Gemisches aus Zuckern und Zuckeralkoholen. Trennbedingungen ähnlich wie im Fig. 2, nur Durchflussgeschwindigkeit 0.5 ml/min. Nachweissystem: Zuckeralkohole ähnlich wie im Fig. 1 und Zucker mit Orcin-Schwefelsäure. Auftragsmenge: Saccharose 5 γ und von allen anderen Verbindungen die Hälfte der Menge auf Fig. 2. Schreiber: Vollausschlag 0.5 E.

es bereits für Hydrolysate mit Erfolg angewendet wird[6]. Bei einer genauen Kenntnis der in der Probe vorhandenen Kohlenhydrate ist das Einpufferverfahren eine brauchbare Methode, Zucker und Zuckeralkohole zu bestimmen.

LITERATUR

1 O. Samuelson und H. Strömberg, *Carbohyd. Res.*, 3 (1966) 89.
2 N. Spencer, *J. Chromatogr.*, 30 (1967) 566.
3 L. Hough, A. M. Y. Ko und P. Wustemann, *Carbohyd. Res.*, 44 (1975) 97.
4 K. Larsson und O. Samuelson, *Carbohyd. Res.*, 50 (1976) 1.
5 A. Floridi, *J. Chromatogr.*, 59 (1971) 61.
6 M. Sinner, M. H. Simatupang und H. H. Dietrichs, *Wood Sci. Technol.*, 9 (1975) 307.
7 M. H. Simatupang und H. H. Dietrichs, *Chromatographia*, 11 (1978) 89.
8 H. Cho Tun, J. F. Kennedy, M. Stacey und R. R. Woodbury, *Carbohyd. Res.*, 11 (1969) 225.
9 R. Carubelli, *Carbohyd. Res.*, 2 (1966) 480.

Journal of Chromatography, 155 (1978) 450–453

CHROM. 10,993

Note

Separation of mixtures of atranorin and chloroatranorin by thin-layer chromatography

J. L. RAMAUT*, M. BROUERS, E. SERUSIAUX** and M. CORVISIER

Laboratoire de Botanique pharmaceutique et de Chimiotaxonomie et Laboratoire de Botanique systématique et de Phytogéographie, Université de Liège, Sart Tilman, 4000 Liège (Belgium)

(First received December 12th, 1977; revised manuscript received March 9th, 1978)

Atranorin (Fig. 1) is the most frequently encountered para-depside in lichens. It occurs frequently together with chloroatranorin (Fig. 1). It seems possible that the joint occurrence of these two "lichen acids" is dependent upon environmental factors.

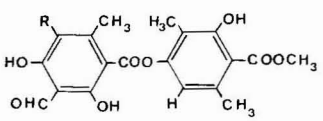


Fig. 1. Structural formula of atranorin (R = H) and chloroatranorin (R = Cl).

Atranorin and chloroatranorin have been effectively separated by column chromatography on alumina by Seshadri and Subramanian[1].

Although several authors have tested and improved the thin-layer chromatography (TLC) of lichen substances, especially depsides and depsidones[2–4] (see Santesson[5] for a review), separation by TLC and identification of chloroatranorin mixed with atranorin remain very difficult (see Table I).

In this paper, we describe a new method that allows a good separation by TLC of mixtures of chloroatranorin and atranorin. The absorption of pure isolated chloroatranorin is measured in the UV region of the spectrum.

MATERIALS AND METHODS

Preparation of the lichen extracts

Two specimens of *Everniopsis trulla* containing atranorin, chloratranorin and usnic acids were analysed: the first one was collected in Africa, Zaire, Khuzi massif, S.W. versant, 2250 m by Lambinon (71/1272, LG); the second one was collected in 1926 in South America, Peru, Apurimac Valley, Cuesta de Limatambo, 3450 m, by Herrera (FH).

* To whom requests for reprints should be sent.
** Aspirant of the Fonds National de la Recherche Scientifique (F.N.R.S.), Belgium.

The extraction procedure was identical for both specimens. Dry lichen powder (1 g) was first extracted with 5 ml of light petroleum (b.p. 40–60°). The filtered residue was then extracted with 15 ml of anhydrous redistilled diethyl ether.

After filtration, the ethereal solution was concentrated under vacuum. The yellow precipitate was filtered and redissolved in 2 ml of acetone. The ethereal and the acetone extracts were purified by TLC next to an ethereal solution of reference chloroatranorin (supplied by Dr S. Huneck).

Preparation of the TLC plates

Merck precoated F_{254}, laboratory-made Merck HF_{254} and laboratory-made NaAc HF_{254} TLC plates were used. The sodium acetate plates were prepared from a suspension of Merck silica gel HF_{254} in 0.2 *M* sodium acetate solution (30 g for 65 ml, layer thickness 0.25 mm). This adsorbent was proposed by Ramaut[6] for TLC of lichen acids revealed by Echtblausalz reagents. The plates were dried in air at room temperature for 2 h, activated at 105° for 1 h and stored over anhydrous calcium chloride.

TLC conditions

The extracts were applied 1.0 cm above the lower edge and developed, using the ascending method, in chromatotanks saturated with the developing mixture benzene–dioxan–acetic acid (90:25:4; Pastuska phase, as proposed by Bachmann[7]). Filter paper wetted with the developing solvent was placed in the chromatotank to improve saturation.

Various conditions of temperature and light during the chromatography were tested.

Identification of the spots

The air-dried chromatograms were viewed in short- and long-wavelength UV light. Afterwards the plates were sprayed either with 10% H_2SO_4 and heated to 110° for 30 min, or with a 1% ethanolic solution of *p*-phenylenediamine (PD test).

Registration of UV absorption spectra

Identification of chloroatranorin was achieved by comparing the UV absorption spectrum, in 95% ethanol, of chloroatranorin isolated from the lichen with that of the reference chloroatranorin.

The chromatographic spots were first eluted for 2 h in redistilled diethyl ether for analysis. The ethereal solutions were then filtered and evaporated to dryness under vacuum for 2 h before addition of 5 ml of 95% ethanol (Merck spectrograde). UV absorption spectra of ethanolic solutions were registered on a Cary 17-R spectrophotometer.

RESULTS AND DISCUSSION

The best separation of atranorin and chloroatranorin was obtained under the following conditions: TLC plates, 20 × 20 cm prepared from suspension of silica gel HF_{254} (Merck) in 0.2 *M* sodium acetate solution; developing solvent, benzene–dioxan–acetic acid (90:25:4); temperature, 15°; light conditions, complete darkness.

The results are summarized in Fig. 2 and Table I. Traces of atranorin remaining in the reference chloroatranorin are separated by the TLC.

Identical results were obtained from both specimens of *Everniopsis trulla*.

TLC of the lichen extracts on silica gel HF_{254} plates (whether prepared with 0.2 *M* sodium acetate or not) in normal conditions of light and temperature leads to bad separation of chloroatranorin from atranorin. Moreover, the usnic acid spot sometimes interferes with the chloroatranorin spot.

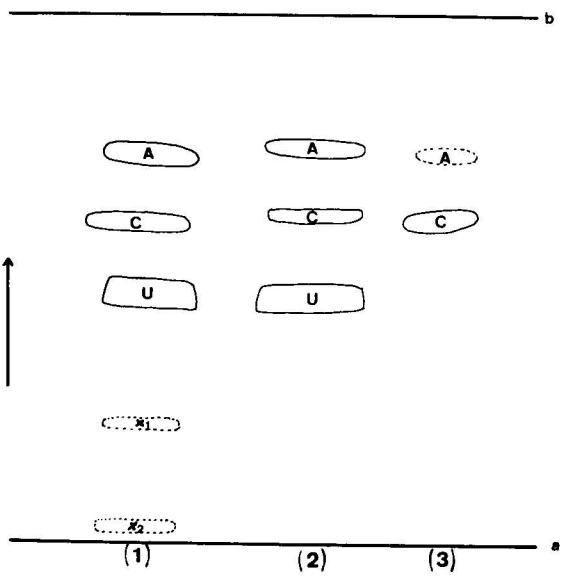


Fig. 2. A diagram of a chromatographic plate run in Pastuska solvent mixture at 15°, in the dark. 1, Extract of *Everniopsis trulla* in diethyl ether; 2, extract of *Everniopsis trulla* in acetone; 3, reference chloroatranorin extract. A, Atranorin; C, chloroatranorin; U, usnic acid; X_1 and X_2, traces of unknown substances. a, Deposit line; b, solvent front.

TABLE I

R_F VALUES OF ATRANORIN AND CHLOROATRANORIN IN VARIOUS CONDITIONS OF TLC

Lichen acid	*R_F values* × *100*					
	*1a**	*1b**	*2a***	*2b***	*2c***	*3****
Atranorin	82	94	62	53	65	85
Chloroatranorin	81	94	62	53	65	61

* (1) Santesson[2], Eastman chromatogram sheets type K 301R2; developing mixtures (a) toluene–acetic acid (9:1), (b) toluene–diethyl ether–acetic acid (3:6:1).

** (2) Culberson and Kristinsson[3], Merck silicagel F_{254} plates; developing mixtures: (a) benzene–dioxan–acetic acid (90:25:4), (b) hexane–diethyl ether–formic acid (5:4:1), (c) toluene–acetic acid (85:15).

*** (3) This work: Merck silicagel F_{254} TLC plates prepared in 0.2-*M* NaAc; developing mixture: benzene–dioxan–acetic acid (90:25:4), 15° in the dark.

TLC of lichen extracts previously kept for 2 days (or more) at room temperature and in the light shows the presence of denaturation or degradation products (photodecomposition?).

Fig. 3 shows the UV absorption spectra in 95% ethanol of chloroatranorin purified by TLC from the extracts of *Everniopsis* and from extract of the reference product. The two spectra are similar and differ from the spectrum of atranorin (see for example Klee and Steubing[8]). This, together with the identical R_F values and chemical tests for both chromatographic spots, proves the occurrence of chloroatranorin in both analysed specimens of *Everniopsis trulla*.

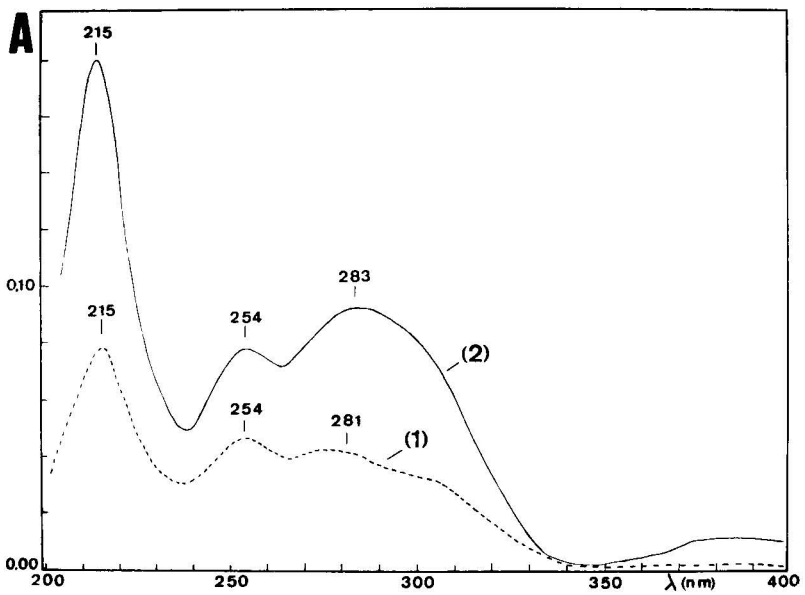


Fig. 3. UV absorption spectra in 95% ethanol of (1) chloroatranorin purified from *Everniopsis trulla*, (2) reference chloroatranorin.

ACKNOWLEDGEMENT

We thank Dr S. Huneck for supplying the reference chloroatranorin sample.

REFERENCES

1 T. R. Seshadri and G. B. V. Subramanian, *J. Indian Chem. Soc.*, 40 (1963) 7.
2 J. Santesson, *Acta Chem. Scand.*, 21 (1967) 1162.
3 C. F. Culberson and H. Kristinsson, *J. Chromatogr.*, 46 (1970) 85.
4 C. F. Culberson, *J. Chromatogr.*, 72 (1972) 113.
5 J. Santesson, in V. Ahmadjian and M. E. Hale (Editors), *The Lichens*, Academic Press, New York, London, 1973, p. 641.
6 J. L. Ramaut, *J. Chromatogr.*, 31 (1967) 243.
7 O. Bachmann, *Österr. Botan. Z.*, 110 (1963) 103.
8 R. Klee and L. Steubing, *J. Chromatogr.*, 129 (1976) 478.

Journal of Chromatography, 155 (1978) 454

CHROM. 11,024

Note

Simplified o-phtalaldehyde urea spray for the detection of taurine on thin-layer chromatographic plates

JEFFREY LEFKOWITZ and ARTHUR SOHLER*

Brain Bio Center, Princeton, N.J. 08540 (U.S.A.)

(First received December 1st, 1977; revised manuscript received March 21st, 1978)

Taurine (2-aminoethanesulfonic acid) is a compound whose physiological significance has not been completely elucidated. Several important roles have been proposed for this compound including neurotransmitter function, anti-epileptic, regulation of absorption and digestion of lipids, effects on eating and drinking behavior, and a role in depressive illness[1].

Recently we have studied taurine in mixtures of amino acids and in plasma using thin-layer chromatography (TLC). We adapted the *o*-phthalaldehyde (OPT)–urea reaction originally described by Curzon and Giltrow[2] and subsequently modified by Gaitonde and Short[3] to the detection of taurine on TLC plates.

Curzon and Giltrow[2] used OPT to detect taurine and other amino acids on paper chromatograms. Subsequently Gaitonde and Short[3] developed a quantitative spectrophotometric assay for taurine involving the reaction of taurine with OPT in the presence of urea. We have modified the reagents employed by Gaitonde and Short so that the reaction can be conveniently run on TLC plates by employing a single reagent incorporating the OPT and urea.

The reagent was modified as follows. An amount of 30 g of urea was dissolved in 90 ml of 0.01 *M* sodium phosphate buffer pH 6.8 to which was added 10 ml of a 4% (w/v) OPT solution in methanol. The chromatograms were sprayed with this reagent. The plate was stored at 4° for 5 min. It was then sprayed with glacial acetic acid. Taurine under these conditions produces a brown spot which changes to purple over a period of 10 min. The only other amino acid producing a purple reaction is glycine. Glycine, however, can be readily separated from taurine.

Chromatography was carried out on Quantum LQD silica gel plates using 95% ethanol–water (63:37, v/v) as the solvent system. Volumes of 5 μl of amino acid solutions (5 μmoles/ml) were spotted. Under these conditions, glycine moves with an R_F value of 0.55 while taurine has an R_F value of 0.71.

The limit of detection for taurine using the modified spray is $6.25 \cdot 10^{-7}$ g (5 nmoles).

We believe the modified OPT–urea spray adapted to silica gel plate may be of value in studies of taurine metabolism.

REFERENCES

1 K. H. Tachiki, H. C. Hendric, J. Kellams and M. H. Aprison, *Clin. Chim. Acta*, 75 (1977) 455.
2 O. Curzon and J. Giltow, *Nature (London)*, 173 (1954) 314.
3 M. K. Gaitonde and R. A. Short, *Analyst (London)*, 96 (1971) 274.

* To whom correspondence should be addressed.

Journal of Chromatography, 155 (1978) 455–456

CHROM. 11,016

Book Review

Pyrolysis–gas chromatography, by R. W. May, E. F. Pearson and D. Scothern, Chemical Society, London, 1977, VII + 109 pp., price £ 7.20; ISBN 0-85186-767-7.

This is the third volume in the Analytical Sciences Monographs Series presently being produced by the Chemical Society of London.

One-fifth of the content is devoted to the now all too familiar "brief" introduction to gas chromatography which is almost woefully lacking in report on recent thought or development. It would be a relief if authors were to accept the validity of the technique in a few well-chosen words and then merely refer to one of the excellent treatises on the subject rather than attempt to condense so much into so little, however basic it may appear to be.

The second chapter neatly summarises the major contributions to the design, development and operation of a variety of pyrolysis devices but again is scant in the many significant contributions made to the literature over the last six or seven years. Even more surprising is the omission of the system due to Shulman and Simmonds in 1968 for total on-column pyrolysis specifically intended for use with capillary columns.

The section concerned with applications of the technique is fairly indicative of the scope of the method and groups sample types under the generic headings Synthetic polymers, Involatile non-polymeric organics, Volatiles (an as yet vastly under-rated sector of interest) and Biochemical and biological materials. This is, without doubt, that part of the book that could have most benefitted by considerable expansion for the extent of the analytical usefulness of this, in the past, much-maligned approach to the identification and even elucidation of large molecules is still barely appreciated and a more detailed appraisal would have given greater and truer perspective as well as resulting in an improved textual balance.

The ensuing notes on Peak identification also suffer from the paucity of recent reference with the consequence that the benefits of the modern fast-scanning quadrupole mass spectrometer in conjunction with a matched high-speed data system pass unsung, such is, of course, not commensurate with the importance of this most powerful adjunct to the assignment of a positive identity to an emergent peak.

Finally the vexing question of Standardisation is examined but the accent is very much biassed toward "in-house" practice and takes no cogniscance of the Correlation Trials conducted respectively by the Pyrolysis Sub-Group of the Chromatography Discussion Group and the American Society for Testing and Materials (that have sought to establish a general code of practice designed to achieve an average situation that allows free choice of mode and method within declared limits, such recommendations have a statistical validity but must be the subject of constant review that they might gain in certainty).

There is gentle irony in the fact that pyrolysis–gas chromatography–mass

spectrometry was the very method that revealed batch-to-batch variation in the polymeric partitioning medium that the authors themselves choose as their "standard" column packing!

An extensive appendix includes a handsome collection of pyrograms produced under very carefully controlled conditions that demonstrably argue the power of the technique and afford the would-be practitioner with a ready-made library should he or she suitably reproduce the declared conditions.

The absence of indices, either subject or author, is to be deprecated; there is no greater aggravation than to seek through a text fruitlessly even though in this case it be no more than 109 pages.

Nonetheless the book is the only one extant devoted to the subject and thereby will serve as an introduction to pyrolysis–gas chromatography.

Redhill (Great Britain) C. E. ROLAND JONES

Author Index

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

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

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

Title. The title of the paper should be concise and informative. Since titles are widely used in information retrieval systems, care should be taken to include the key words. The title should be followed by the authors' full names, academic or professional affiliations, and the address of the laboratory where the work was carried out. If the present address of an author is different from that mentioned, it should be given in a footnote. Acknowledgements of financial support are *not* to be made in a footnote to the title or name of the author, but should be included in the Acknowledgements at the end of the paper.

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

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. One original and two photocopies are required. Attention should be given to any lettering (which should be kept to a minimum) and to spacing on axes of graphs in order to ensure that numbers etc. remain legible after reduction. Axes of a graph should be clearly labelled. The figures should preferably be of such a size that the same degree of reduction can be applied to all of them. Photographs should have good contrast and intensity. Sharp, glossy photographs are required to obtain good halftones. References to the illustrations should be included in appropriate places in the text using arabic numerals. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the authors' expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. The numbers should appear in the text at the appropriate places in square brackets. In the reference list, periodicals [1], books [2], multi-author books [3] and proceedings [4] should be cited in accordance with the following examples:

1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication". The *Journal of Chromatography; Journal of Chromatography, Biomedical Applications* and *Chromatographic Reviews* should be cited as *J. Chromatogr.*

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints of Full-length papers, Short communications and Notes will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

News. News releases of new products and developments, and information leaflets of meetings should be addressed to: The Editor of the News Section, Journal of Chromatography/Journal of Chromatography, Biomedical Applications, Elsevier Scientific Publishing Company, P.O. Box 330, 1000 AH Amsterdam, The Netherlands.

Advertisements. Advertisement rates are available from the publisher on request. The Editors of the journal accept no responsibility for the contents of the advertisements.