ISSN 0021-9673

BLISHED
EKLY

VOL. 212 NO. 3 AUGUST 7, 1981

THIS ISSUE COMPLETES VOL. 212

RNAL OF

CHROMATOGRAPHY

ERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHOD

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

JOURNAL OF CHROMATOGRAPHY

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. Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere. For copyright regulations, see below.

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* and the *Biomedical Applications* section can be subscribed to separately.

Publication. The *Journal of Chromatography* (incl. *Biomedical Applications, Chromatographic Reviews* and *Cumulative Author and Subject Indexes, Vols. 201–210* and *211–220*) has 24 volumes in 1981. The subscription prices for 1981 are:

J. Chromatogr. (incl. *Chromatogr. Rev.* and *Cum. Indexes*) + *Biomed. Appl.* (Vols. 203–226):
Dfl. 3240.00 plus Dfl. 432.00 (postage) (total *ca.* US$ 1708.00)

J. Chromatogr. (incl. *Chromatogr. Rev.* and *Cum. Indexes*) only (Vols. 203–220):
Dfl. 2556.00 plus Dfl. 324.00 (postage) (total *ca.* US$ 1339.50).

Biomed. Appl. only (Vols. 221–226):
Dfl. 852.00 plus Dfl. 108.00 (postage) (total *ca.* US$ 446.50).

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 202) are available at Dfl. 156.00 (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 Elsevier/North-Holland Inc., Journal Information Centre, 52 Vanderbilt Avenue, New York, NY 10164. Tel: (212) 867-9040.

Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Index Medicus, and Science Citation Index.

See page 3 of cover for Publication Schedule, Information for Authors, and information on the News Section and Advertisements.

Printed in The Netherlands

CONTENTS

(Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Index Medicus, and Science Citation Index)

(Continued overleaf)

Contents (continued)

from ELSEVIER your partner in successful CHEMICAL ANALYSIS

Structural Analysis of Organic Compounds
by Combined Application of Spectroscopic Methods

J. T. Clerc, E. Pretsch and J. Seibl, Zürich, Switzerland.

Studies in Analytical Chemistry, 1

Spectroscopic methods have certainly captured the lion's share of organic analysis with at least one such method in current use in all chemical laboratories. Now at last a concise and logically structured reference work details how their combined application substantially increases overall effectiveness. By giving examples which demonstrate different methods of approach and reasoning, and supplementing these with comments and hints on previously neglected analytical aspects, the authors have produced a work to cover the widest possible variety of chemical structures and spectroscopic capabilities.

1981 280 pages
0-444-99748-2
US $66.00/Dfl. 135.00

Evaluation and Optimization of Laboratory Methods and Analytical Procedures

D. L. Massart, L. Kaufman and A. Dijkstra

Techniques and Instrumentation in Analytical Chemistry, 1

Acclaimed as ... "a valuable addition to the analytical literature" by *Analytical Chemistry*, "a comprehensive and practical handbook... all aspects of optimization are discussed" by *Laboratory Equipment* and "... a real aid to postgraduate students following taught courses" by *Chromatographia*. The authors provide in a single volume, a discussion of all aspects of optimization from the simple evaluation of procedures to the organization of laboratories and the selection of optimal complex analytical programmes. No laboratory or library can afford to be without this book.

1978. 1st reprint 1980
612 pages 0-444-41743-5
US $68.25/Dfl. 140.00

Chemical Derivatization in Gas Chromatography

J. Drozd, Brno, Czechoslovakia.

Journal of Chromatography Library, 19.

The novice will find here explanatory coverage of the entire range of problems, will become acquainted with all types of derivatives and methods, and will be able to apply the information without recourse to original sources. For proficient workers it will be a valuable information source, a guide to the most recent research results and an indication of future trends.

1981 244 pages
0-444-41917-9
US $58.50/Dfl. 120.00

Analytical Isotachophoresis

Proceedings of the 2nd International Symposium on Isotachophoresis, Eindhoven, September 9-11, 1980.

F. M. Everaerts, Eindhoven The Netherlands (Editor).

Analytical Chemistry Symposia Series, 6

"A new twig on the tree of differential separation methods": Thus has isotachophoresis been described. It is proving a valuable technique in clinical (bio)chemistry, pharmacy, physical and inorganic chemistry, as well as in the industrial and environmental field.

Theoretical and practical aspects of the method, new developments in isotachophoretic equipment and applications are presented clearly.

1981 246 pages
0-444-41957-8
US $58.50/Dfl. 120.00

Send your order with your payment to one of the addresses below and receive the book(s) ordered postfree.
For further details on these titles and our Spring list of analytical chemistry publications check number 3 on the reader enquiry card.

ELSEVIER

P.O. Box 211
1000 AE Amsterdam
The Netherlands

52 Vanderbilt Ave
New York, N.Y. 10017
U.S.A.

Journal of Chromatography, 212 (1981) 251–260
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,792

PREPARATION AND EVALUATION OF N-(2-AMINOETHYL)-γ-AMINOPROPYLTRIMETHOXYSILANE-TREATED SILICA COLUMNS FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF SOME AROMATIC AMINES

MITSUYOSHI OKAMOTO*
Gifu Prefectural Institute of Public Health, 6-3, Noishiki 4 Chome, Gifu 500 (Japan)
and
HIROSHI KISHIMOTO
Faculty of Pharmaceutical Sciences, Nagoya City University, 3-1, Tanabe-dori, Mizuho-ku, Nagoya 467 (Japan)
(Received March 13th, 1981)

SUMMARY

The retention and selectivity behaviour of some aromatic amines were studied by high-performance liquid chromatography using amino-chemically bonded stationary phases (column gel), prepared from silica gel treated with benzene solution containing 5–50% of N-(2-aminoethyl)-γ-aminopropyltrimethoxysilane (N2AAPTS). The N2AAPTS gel was compared with the 3-aminopropyltriethoxysilane (3APTS)-treated silica gel. From elemental analysis data for nitrogen and carbon, the maximum number of accessible amino surface groups per 100 Å^2 of silica gel (mean pore diameter 85 Å, particle size distribution 5.8 μm) in N2AAPTS gel was estimated to be 1.81. The N2AAPTS gel provided better resolution of some amines than the 3APTS gel. The effect of the kind of component in the mobile phase on the capacity factor was studied using various alkanes (basic component) and normal alcohols (additive component).

INTRODUCTION

Because of the carcinogenic properties of certain aromatic amines, their analysis in the environment has received a great deal of attention. Amino chemically bonded stationary phases were selected for the high-performance liquid chromatographic (HPLC) analysis of aromatic amines owing to their weakly basic properties. Although separations were obtained using various reversed-phase chemically bonded chain lengths[1–13], such as C_2, C_3, C_8 or C_{18}, there have been few reports on the HPLC analysis of aromatic amines using amino chemically bonded stationary phases or the influence of the length of the amino chemically bonded chain. Therefore, the preparation and evaluation of amino chemically bonded stationary phases and the separation of aromatic amines by HPLC using these columns were studied.

0021-9673/81/0000–0000/$02.50

In a previous paper[14] we considered the use of several 3-aminopropyltriethoxysilane (3APTS) stationary phases in HPLC. Therefore, in this work we compared the retention behaviour of aromatic amines on the length of the aminomethylene bonded chain using N-(2-aminoethyl)-γ-aminopropyltrimethoxysilane (N2AAPTS) and 3APTS[14], and considered the number of accessible N2AAPTS surface groups per 100 Å^2 of silica gels with several pore sizes and particle sizes, *i.e.*, 85 Å, 5.8 μm; 70 Å, 10 μm; 153 Å, 8.4 μm; and 180 Å, 8.4 μm. We also studied the effect of the mobile phase composition on the chromatographic behaviour of some aromatic amines.

EXPERIMENTAL

Reagents

o-, *m*- and *p*-nitroanilines and 3,4-, 2,6- and 2,4-dinitrotoluenes were obtained from Wako (Osaka, Japan). N2AAPTS was purchased from Tokyo Kasei (Tokyo, Japan) and four kinds of highly microporous spherical silica gels differing in mean particle size and mean pore diameter were purchased from Fuji-Davison (Nagoya, Aichi, Japan) (Table I). *n*-Pentane, *n*-hexane, *n*-heptane, methanol, ethanol, 1-propanol, 1-butanol and 1-pentanol from Wako were used after distillation. All chemicals were of analytical-reagent grade.

TABLE I

SILICA GELS USED

*Silica gel**	*Mean particle size (μm)*	*Mean pore diameter (Å)*	*Specific surface area (m^2/g)*	*Pore volume (ml/g)*
Fuji-Davison 1	10.0	70	490	0.87
Fuji-Davison 2	8.4	153	204	0.78
Fuji-Davison 3	8.4	180	167	0.75
Fuji-Davison 4	5.8	85	400	0.85

* These names and serial numbers were assigned by the authors for convenience and are not commercial names.

Apparatus

The HPLC measurements were carried out using a KHU 16 Kyowa Seimitsu Mini Pump equipped with a Uvidec 100-II Jasco variable-wavelength detector.

Stationary phase and elemental analysis

According to the previous method[14], 5 g of dried Fuji-Davison 4 silica gel were added to 50 ml of a 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 or 5.0% benzene solution of N2AAPTS. After stirring for 24 h at room temperature, the silica gel suspension was filtered with a glass filter (1 μm), washed several times with benzene and methanol and then dried *in vacuo* at 70°C for 2 days, finally producing the silica gels for HPLC, which are listed as N2AAPTS 4–0 to 4–50, respectively, in Table II. Also, after 5 g of dried Fuji-Davison 1, 2 or 3 had been added to 50 ml of a 5.0% benzene solution of N2AAPTS, the same procedure as with the N2AAPTS 4 series in Table II was carried

TABLE II

SURFACE TREATMENTS AND ELEMENTAL ANALYSES FOR GELS PREPARED FROM FUJI-DAVIDSON SILICA GEL USING DIFFERENT CONCENTRATIONS OF N2AAPTS IN BENZENE

Mono = Monofunctional, Bi = bifunctional (see text).

Treated gel	*Concentration of N2AAPTS in benzene (%)*	*Specific surface area (m^2/g)*	*N calculated (%)*		*N found (%)*	*C calculated (%)*		*C found (%)*
			Mono	*Bi*		*Mono*	*Bi*	
N2AAPTS 4-0	0	400	0	0	0	0	0	0
N2AAPTS 4-5	0.5	357	0.51	0.51	0.62	1.53	1.32	2.23
N2AAPTS 4-10	1.0	324	0.98	0.99	1.13	2.93	2.54	3.84
N2AAPTS 4-15	1.5	302	1.41	1.43	1.59	4.23	3.68	5.86
N2AAPTS 4-20	2.0	296	1.81	1.84	2.04	5.43	4.75	6.75
N2AAPTS 4-25	2.5	292	2.18	2.23	2.34	6.54	5.74	7.72
N2AAPTS 4-30	3.0	274	2.52	2.59	2.42	7.57	6.68	7.38
N2AAPTS 4-50	5.0	287	3.69	3.85	2.61	11.07	9.90	7.81

out, producing N2AAPTS 1–50, 2–50 and 3–50, which are shown in Table III together with N2AAPTS 4–50. Hereafter, N2AAPTS 4–0 to 4–50 and 1–50 to 3–50 will be abbreviated to "column gel".

The nitrogen and carbon contents of each column gel were determined by elemental analysis using an MT-3 Yanagimoto CHN elemental analyser, giving the data indicated as "Found" in Tables II and III. The specific surface areas of the column gels were determined with an SA-1000 Shibata surface area pore volume analyser, giving the data in Tables II and III.

TABLE III

SURFACE TREATMENTS AND ELEMENTAL ANALYSES FOR GELS PREPARED FROM FUJI-DAVISON 1, 2, 3 AND 4 SILICA GELS

Concentration of N2AAPTS in benzene: 5.0%.

Treated gel	*Specific surface area (m^2/g)*	*N found (%)*	*C found (%)*
N2AAPTS 1-50	330	2.85	7.98
N2AAPTS 2-50	166	1.55	4.21
N2AAPTS 3-50	125	1.31	3.35
N2AAPTS 4-50	287	2.61	7.81

Column preparation

The column gels were packed into stainless-steel columns (250 × 4 mm I.D.) using a balanced density method through a 10-ml stainless-steel packer at a rate of 500 kg/cm^2 with a Kyowa Seimitsu KHW-20 ultra-high-pressure pump.

RESULTS AND DISCUSSION

Figs. 1A and 2A show the correlations between the capacity factors (k') of nitroaniline or diaminotoluene and the concentration of N2AAPTS solution with

which the silica gel was treated. Saturation of log k' was observed at least from N2AAPTS 4–20 to 4–30, almost all of the N2AAPTS-reactive hydroxyl groups on the silica gel surface having been replaced with N2AAPTS.

Figs. 1B and 2B show the corresponding curves obtained with 3APTS stationary phase, which has a shorter aminomethylene bonded chain than N2AAPTS.

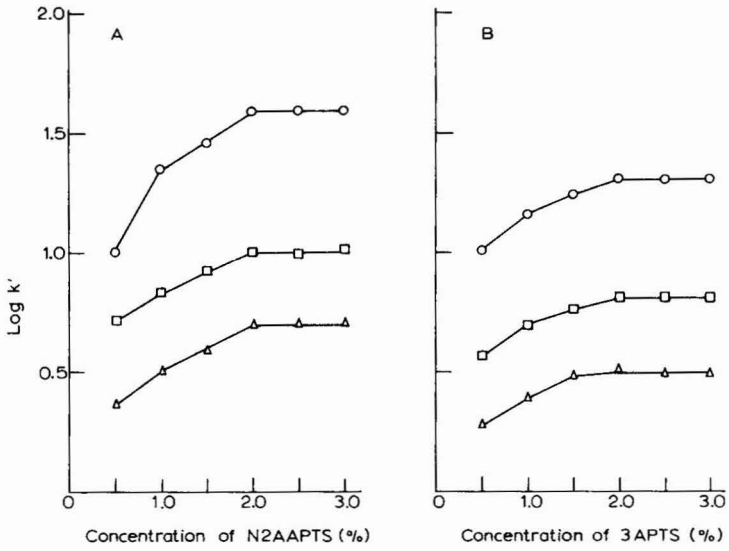


Fig. 1. Relationships between capacity factors (log k') of nitroanilines and the concentrations of (A) N2AAPTS and (B) 3APTS in benzene solution. Column: 250 × 4 mm I.D. Mobile phase: *n*-hexane–ethanol (25:1). Flow-rate: 2.0 ml/min. Detection: UV, 254 nm. ○, *p*-Nitroaniline; □, *m*-nitroaniline; △, *o*-nitroaniline.

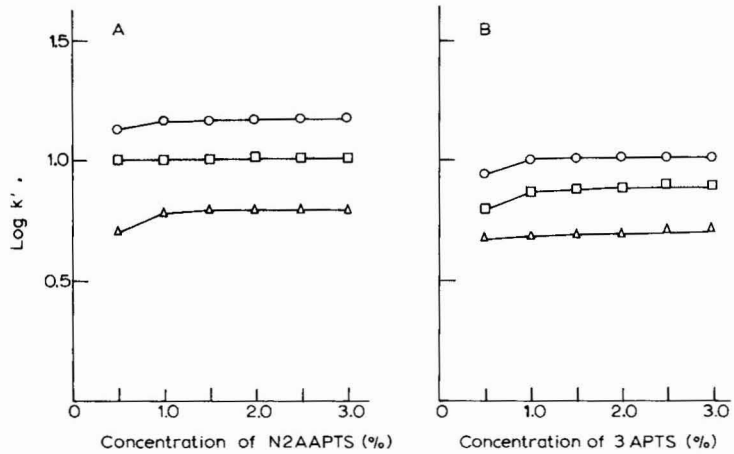
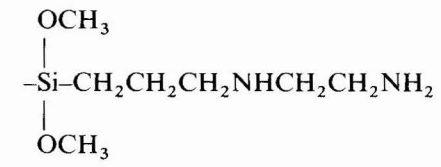


Fig. 2. Relationships between capacity factors (log k') of diaminotoluenes and the concentrations of (A) N2AAPTS and (B) 3APTS in benzene solution. Conditions as in Fig. 1. ○, 2,4-Diaminotoluene; □, 2,6-diaminotoluene; △, 3,4-diaminotoluene.

From the elemental analysis of silica gel treated with various concentrations of N2AAPTS, the number of accessible amino surface groups per 100 Å^2 of silica gel surface can be estimated as follows.

If N2AAPTS is substituted monofunctionally on silica gel, the surface structure of the saturated column gel can be written as



The number of accessible amino surface groups per 100 Å^2 of silica gel surface is then given by

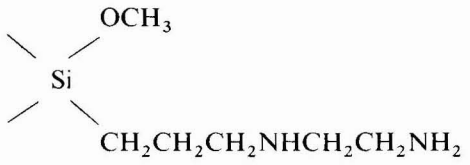
$$[(N/100)/14.0067 \cdot 2] \cdot 6.022 \cdot 10^{23}/S \cdot 10^{18} \tag{1}$$

or

$$[(C/100)/12.011 \cdot 7] \cdot 6.022 \cdot 10^{23}/S \cdot 10^{18} \tag{2}$$

where N = weight percentage of nitrogen, C = weight percentage of carbon, $6.022 \cdot 10^{23}$ = Avogadro's number and S = specific surface area (m^2/g) of treated silica gel.

If N2AAPTS is substituted bifunctionally on silica gel, the surface structure of saturated column gel can be written as



The number of accessible amino surface groups per 100 Å^2 of silica gel surface is then given by

$$[(N/100)/14.0067 \cdot 2] \cdot 6.022 \cdot 10^{23}/S \cdot 10^{18} \tag{3}$$

or

$$[(C/100)/12.011 \cdot 6] \cdot 6.022 \cdot 10^{23}/S \cdot 10^{18} \tag{4}$$

Substitution of the values of N and C found by elemental analysis into eqns. 1–4 gives the number of accessible amino surface groups per 100 Å^2 of silica gel surface, indicated as "Found" in Table IV. As can be seen from the "Found" data in Tables II and Table IV, an increase in the N2AAPTS concentration in benzene increases the surface modification of the silica gel, but approaches saturation over about 2.0% of

N2AAPTS. This tendency explains well the saturated curves of log k' from N2AAPTS 4–20 to 4–30 in Fig. 1A. The log k' curves obtained with 3APTS, reported previously[14], show the same pattern as those obtained with N2AAPTS in this work. As the agreement between the values ("Found") for accessible amino surface groups in Table IV according to the monofunctional reaction mechanism (eqns. 1 and 2) seems to be better than that between the values according to the bifunctional reaction mechanism (eqns. 3 and 4), it is suggested that the reaction between silica gel and N2AAPTS, and also 3APTS[14], takes place monofunctionally. We calculated the nitrogen and carbon percentages and the number of accessible amino surface groups according to eqn. 1, 2, 3 or 4 with the assumption that all N2AAPTS molecules react with silica gel. Both the former and the latter values are shown as "Calculated" in Tables II and IV, respectively.

TABLE IV

SURFACE TREATMENTS AND NUMBER OF ACCESSIBILE AMINO SURFACE GROUPS PER 100 Å^2

Mono = Monofunctional; Bi = bifunctional (see text).

Column gel	*No. of accessible NH_2 surface groups per 100 Å^2*							
	Calc. Mono		*Found Mono*		*Calc. Bi*		*Found Bi*	
	Eqn. 1	*Eqn. 2*	*Eqn. 1*	*Eqn. 2*	*Eqn. 3*	*Eqn. 4*	*Eqn. 3*	*Eqn. 4*
N2AAPTS 4-0	0	0	0	0	0	0	0	0
N2AAPTS 4-5	0.31	0.31	0.37	0.44	0.31	0.31	0.37	0.52
N2AAPTS 4-10	0.65	0.64	0.75	0.84	0.66	0.66	0.75	0.99
N2AAPTS 4-15	1.00	1.00	1.13	1.38	1.02	1.02	1.13	1.62
N2AAPTS 4-20	1.31	1.31	1.48	1.62	1.33	1.34	1.48	1.90
N2AAPTS 4-25	1.60	1.60	1.72	1.89	1.64	1.64	1.72	2.21
N2AAPTS 4-30	1.98	1.98	1.95	1.93	2.03	2.04	1.95	2.15
N2AAPTS 4-50	2.75	2.75	1.95	1.95	2.87	2.87	1.95	2.27

The comparison between these calculated values and the corresponding "Found" values shows again that saturation of N2AAPTS on silica gel taken place at about 2.0% of N2AAPTS in benzene. Using the data from N2AAPTS 4–20 to 4–50 in Table IV, the number of accessible amino surface groups per 100 Å^2 of silica gel was calculated to be 1.81.

On the other hand, from the data given in Tables I and III, the important parameters of silica gel with respect to the number of accessible N2AAPTS groups per 100 Å^2 of silica gel were considered to be the pore diameter and the surface area.

n-Pentane, *n*-hexane and *n*-heptane were used as basic components of the mobile phase. Taking into consideration that lower viscosities lead to higher column efficiencies, *n*-pentane was the best component (the viscosities of *n*-pentane, *n*-hexane and *n*-heptane are 0.22, 0.31 and 0.40 cP, respectively, at 22°C). However, the low boiling point of *n*-pentane[15] may cause bubble formation in the detector, so *n*-hexane seemed to be the optimal basic component. The addition of an alcohol to a mobile phase can be used to adjust the retention volumes of aromatic amines, because of its

competitive hydrogen bonding with amines against the amino-bonded stationary phase on the column gel. The k' values of nitroaniline and diaminotoluene were measured using various kinds of mobile phase containing an *n*-alkanol and a basic component. As can be seen in Fig. 3, the k' values generally increased with increasing carbon numbers or decreasing polarity[16] of the *n*-alkanols, reflecting the decreasing solubility in the mobile phase.

Based on the relationship between k' and the carbon number of *n*-alkanols, methanol is the most suitable. However, methanol has a very low solubility in *n*-

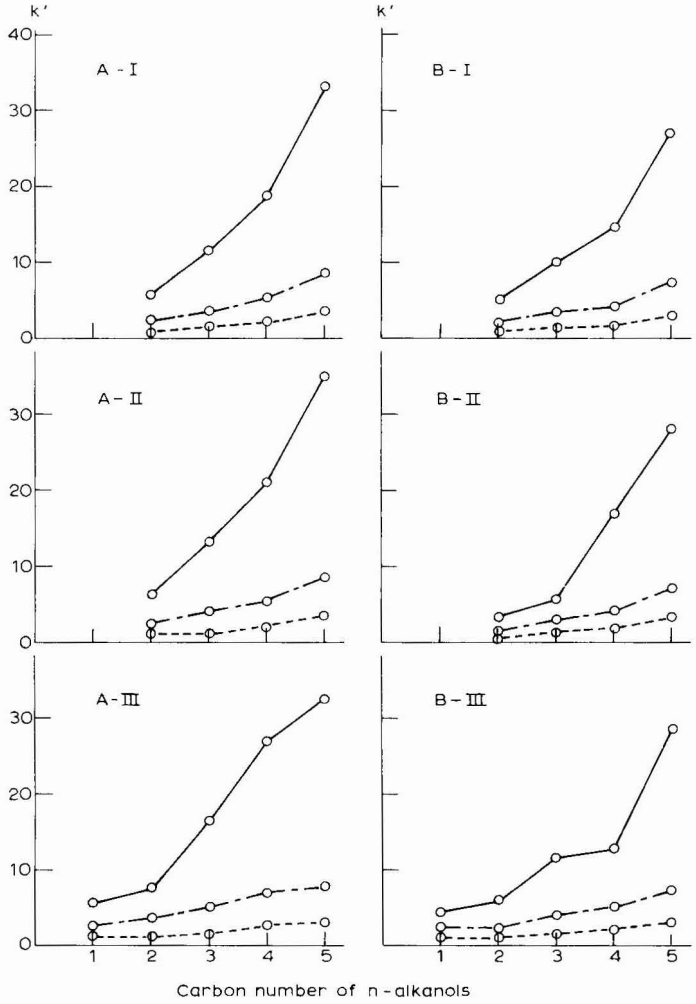


Fig. 3. Relationships between capacity factors (k') of nitroaniline and the *n*-alkanol added to the mobile phase. Column: 250 × 4 mm I.D. Stationary phase: A, silica gel treated with 2.5% N2AAPTS; B, silica gel treated with 2.5% 3APTS. Mobile phase: basic components–aliphatic alcohols (9:1) (basic components: I = *n*-heptane, II = *n*-hexane, III = *n*-pentane; aliphatic alcohols: 1 = methanol, 2 = ethanol, 3 = 1-propanol, 4 = 1-butanol, 5 = 1-pentanol). Flow-rate: 1.49 ml/min. Detection: UV, 254 nm. O--- ··· O, *p*-Nitroaniline; O · O, *m*-nitroaniline; O O, *o*-nitroaniline.

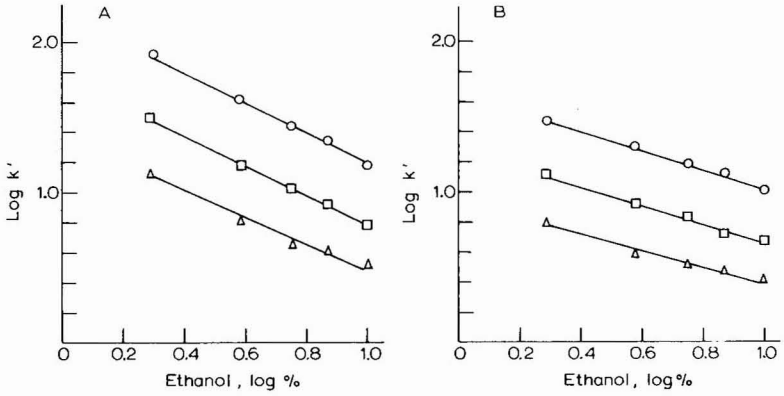


Fig. 4. Relationship between capacity factors (log k') of nitroanilines and concentration of ethanol in *n*-hexane. Column: 250 × 4 mm I.D. Stationary phase: A, silica gel treated with 2.5% N2AAPTS; B, silica gel treated with 2.5% 3APTS. Flow-rate: 1.49 ml/min. Detection: UV, 254 nm. ○, *p*-Nitroaniline; □, *m*-nitroaniline; △, *o*-nitroaniline.

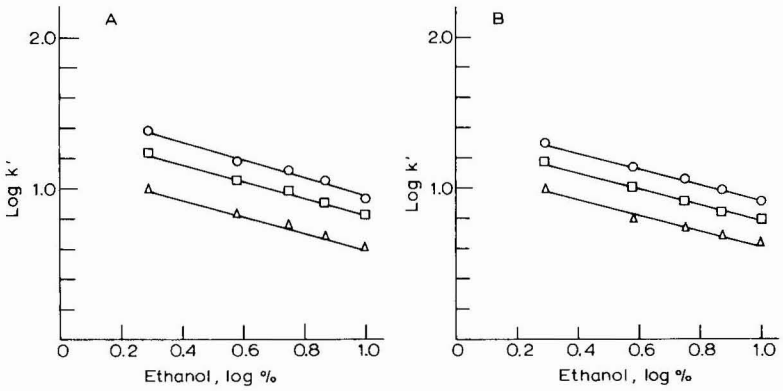


Fig. 5. Relationship between capacity factors (log k') of diaminotoluenes and concentration of ethanol in *n*-hexane. Conditions as in Fig. 4. ○, 2,4-Diaminotoluene; □, 2,6-diaminotoluene; △, 3,4-diaminotoluene.

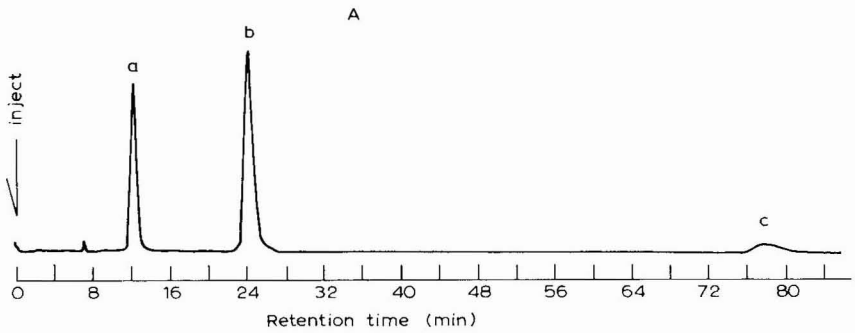


Fig. 6.

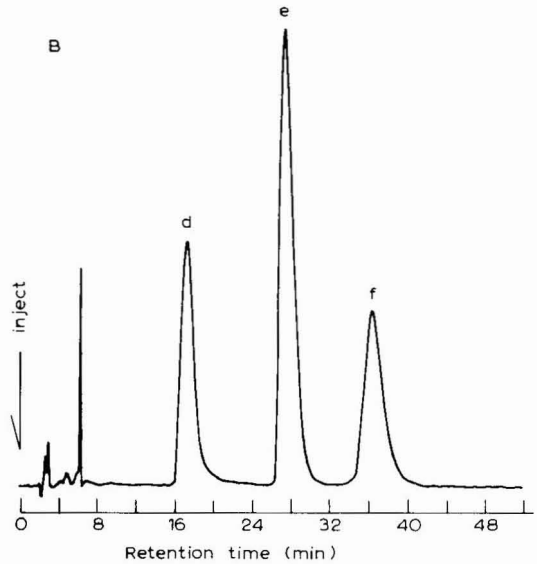


Fig. 6. Separation behaviour of nitroanilines (A) and diaminotoluenes (B) on N2AAPTS-treated silica gel column. Stationary phase: silica gel treated with 2.5% N2AAPTS. Mobile phase: *n*-hexane–ethanol (25:1); flow-rate, 2.0 ml/min. Detection: UV, 254 nm, 0.32 a.u.f.s. Peaks: a = *o*-nitroaniline (3.7 μg); b = *m*-nitroaniline (4.4 μg); c = *p*-nitroaniline (3.4 μg); d = 3,4-diaminotoluene (21.4 μg); e = 2,6-diaminotoluene (22.4 μg); f = 2,4-diaminotoluene (17.1 μg).

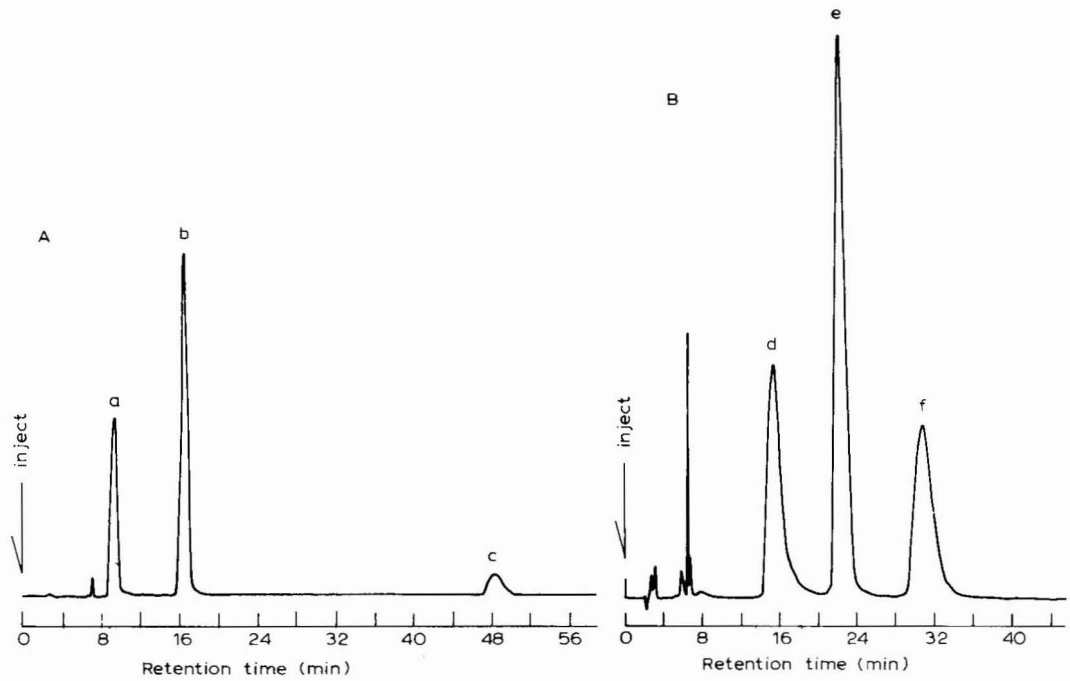


Fig. 7. Separation behaviour of nitroanilines (A) and diaminotoluenes (B) on 3APTS-treated silica gel column. Stationary phase: silica gel treated with 2.5% 3APTS. Other conditions and peaks as in Fig. 6.

hexane, so that ethanol seems to be optimal from the point of view of the separation. The dependence of k' on the alcohol concentration was studied with ethanol, and optimal separations of nitroanilines and diaminotoluenes were achieved with 25:1 *n*-hexane–ethanol (Figs. 4 and 5). Figs. 6 and 7 show typical liquid chromatograms obtained with nitroanilines and diaminotoluenes on N2AAPTS and 3APTS stationary phases.

ACKNOWLEDGEMENTS

We are indebted Dr. F. Yamada, Dr. A. Ohtsuka, Dr. S. Kawai, Dr. S. Jinnouchi and Dr. S. Nishijima for technical assistance.

REFERENCES

1 H. Colin and G. Guiochon, *J. Chromatogr.*, 141 (1977) 289.
2 K. Karch, I. Sebestian and I. Halász, *J. Chromatogr.*, 122 (1976) 3.
3 K. K. Unger, N. Becker and P. Roumeliotis, *J. Chromatogr.*, 125 (1976) 115.
4 J. J. Kirkland, *Chromatographia*, 12 (1975) 661.
5 R. K. Gilpin and M. F. Burke, *Anal. Chem.*, 45 (1973) 1383.
6 H. Hemetsberger, W. Maasfeld and H. Ricken, *Chromatographia*, 9 (1976) 303.
7 B. B. Wheals, *J. Chromatogr.*, 187 (1980) 65.
8 N. Tanaka, K. Sakagami and M. Araki, *J. Chromatogr.*, 199 (1980) 327.
9 K. Unger, *Kontakte*, 2 (1979) 32.
10 F. M. Rabel, E. T. Butts, M. L. Hellman and D. J. Popovich, *J. Liq. Chromatogr.*, 1 (1978) 631.
11 V. Řehák and E. Smolková, *J. Chromatogr.*, 191 (1980) 71.
12 R. E. Majors and M. J. Hopper, *J. Chromatogr. Sci.*, 12 (1974) 767.
13 C. F. Poole and A. Zlatkis, *J. Chromatogr. Sci.*, 17 (1979) 115.
14 M. Okamoto, *J. Chromatogr.*, 202 (1980) 55.
15 A. Zlatkis and R. E. Kaiser, *HPTLC —High Performance Thin-Layer Chromatography*, Elsevier, Amsterdam, 1977, p. 127.
16 L. R. Snyder, *J. Chromatogr.*, 92 (1974) 223.

Journal of Chromatography, 212 (1981) 261–269
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,828

CHARACTERIZATION OF CHROMOSORB POROUS POLYMER BEAD COLUMNS BY GAS CHROMATOGRAPHIC RETENTION VALUES OF LIGHT HYDROCARBONS AND CARBON DIOXIDE

GIANRICO CASTELLO* and GIUSEPPINA D'AMATO
Istituto di Chimica Industriale, Università di Genova, Corso Europa 30, I-16132 Genoa (Italy)
(First received December 29th, 1980; revised manuscript received April 1st, 1981)

SUMMARY

The gas chromatographic behaviour of Chromosorb "century series" porous polymer beads was investigated by analysing mixtures of gases (H_2, CO_2, CH_4, C_2H_2, C_2H_4 and C_2H_6) at different temperatures on columns filled with various types of beads (Chromosorb 101, 102, 103, 104 and 105). The adsorption enthalpies of these compounds were calculated in order to characterize the various Chromosorb types. The relative retention with respect to ethylene was used for the rapid identification of the type of Chromosorb and to compare their gas chromatographic behaviour with that of Porapak polymer beads.

INTRODUCTION

The reproducibility of gas chromatographic (GC) retention data obtained by using porous polymer beads (PPBs) has been widely discussed. In the analysis of inorganic gases, light hydrocarbons and small organic molecules, different authors have reported wide variations of absolute and relative retention times, depending on the performance of various types and batches of polymer[1].

Although many of the reported discrepancies can be explained on the basis of the temperature dependence of the retention times[2], other parameters that influence the column behaviour remain to be investigated: mesh size of the beads, porosity, composition and physical structure, etc. Some of these parameters are given by the producer, while others are not available to the GC user, making the choice of PPB for a given separation an art rather than a science, depending mainly on previous experience and sometimes requiring troublesome and expensive trial-and-error tests.

A general classification of the GC performance of the types of commercially available PPBs, sold by various producers under different trade names, would be very useful. A knowledge of both similarity (*i.e.*, the possibility of replacing a given stationary phase with another readily available) and difference (*i.e.*, the choice of a stationary phase having separation properties intermediate between those of different trade names) are important for the user of these packings.

Comparison of PPBs of different trade names have previously been reported.

0021-9673/81/0000–0000/$02.50

The widest range of types was investigated by Dave[3] (Chromosorb 101, 102 and 103; Porapak N, P, Q, R, S, T and Q-S; Par 1 and 2) and by Supina and Rose[4] (Chromosorb 101, 102 and 103; Porapak N, P, R, S and T). Other papers[5–9] report comparisons between two or three different types of Chromosorb "century series" and Porapak.

The characterization of Porapak® by analysis of light hydrocarbons was previously described[10]. The use of the retention time relative to ethylene, $\alpha_{C_2H_4}$, of various gases (CH_4, CO_2, C_2H_6), whose measurement is simple with respect to the determination of the thermodynamic functions (ΔH^0, ΔG^0 and ΔS^0), permitted an evaluation of the batch-to-batch reproducibility of the column behaviour, and a semiquantitative classification of the "polarity" of the various types of Porapak. Here we report the results of a similar investigation on Chromosorb "century series"® polymer beads.

EXPERIMENTAL

Stainless-steel columns (3 m × 2.4 mm I.D.) were filled with carefully weighed amounts of Chromosorb 101, 102, 103, 104 and 105 (80–100 mesh), from different batches. A thermal conductivity detector with semi-diffusion cells (Aerograph A 350) was used at high sensitivity (filament current 250 mA) with helium carrier gas (22 cm^3 min^{-1}) in order to permit analysis of the smallest possible amount of sample and to avoid peak asymmetry[2].

The column temperature was measured with an accuracy of ±0.1°C. The flow was measured with a bubble flow meter, and the pressure at the head and at the end of the column was monitored with a mercury manometer, with an accuracy of ±1 Torr, in order to calculate the pressure gradient correction factor, J, of James and Martin[11]. Adjusted retention volumes, V'_R, net retention volumes, V_N, retention relative to ethylene, $\alpha_{C_2H_4}$, ΔH^0, ΔS^0 and ΔG^0 were measured or calculated as previously described[10]. In the equations and discussion below, the $\alpha_{C_2H_4}$ values will be indicated, for sake of brevity, by the symbol α.

RESULTS AND DISCUSSION

As previously observed in the case of Porapak columns[10], plots of ln V_N as a function of $1/T$ (reciprocal of the column's absolute temperature) were linear in the interval examined (Fig. 1). The values of the parameters p and q of the equation

$$\ln V_N = p\,(1/T) + q \qquad (1)$$

are shown in Table I and the intersection temperatures, where the given compounds have the same retention time, calculated by solving a system of equations of the type 1, are shown in Table II, together with the temperature limits (isothermal)[12].

The α values obtained on the various Chromosorbs and typical peak shapes on 3-m (80–100 mesh) columns are shown in Fig. 2. As the experimental conditions were exactly the same as in the investigations on Porapak columns[10], both the p and q values and the α values can be directly compared. Table III shows the classification of the various Chromosorb types on the basis of the α values at 30 and 50°C. The comparison of these results and of Figs. 1 and 2 with the corresponding results for the

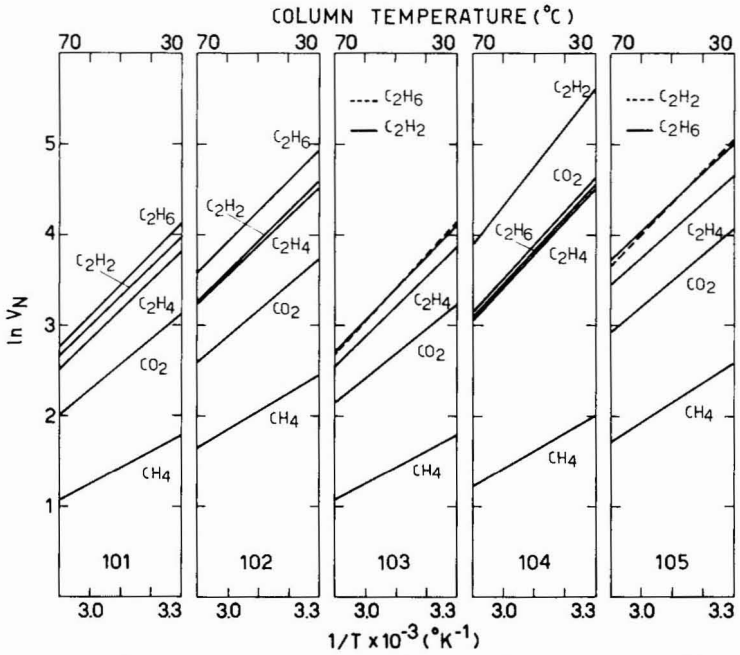


Fig. 1. Plot of ln V_N against $1/T$ on various Chromosorb "century series" (80–100 mesh). Helium flow-rate: 22 cm^3 min^{-1}.

TABLE I

VALUES OF THE SLOPE (p) AND INTERCEPT (q) OF EQN. 1 FOR LIGHT HYDROCARBONS AND CO_2 ON CHROMOSORB COLUMNS

Columns: 3 m long, 80–100 mesh. Carrier gas: helium, 22 cm^3 min^{-1}.

	p				
	101	*102*	*103*	*104*	*105*
CH_4	1862	2063	1835	1956	1844
CO_2	2797	2760	2680	3779	2901
C_2H_6	3227	3322	3568	3625	3023
C_2H_4	3307	3222	3304	3722	3231
C_2H_2	3413	3285	3476	4333	3500
	q				
	101	*102*	*103*	*104*	*105*
CH_4	−4.35	−4.32	−4.22	−4.41	−3.57
CO_2	−6.10	−5.37	−5.59	−7.81	−5.49
C_2H_6	−6.84	−6.02	−7.62	−7.45	−5.32
C_2H_4	−6.93	−6.08	−7.05	−7.71	−5.65
C_2H_2	−7.13	−6.22	−7.34	−8.68	−6.49

TABLE II

INTERSECTION TEMPERATURES AT WHICH TWO COMPOUNDS SHOW THE SAME RETENTION TIME ON VARIOUS CHROMOSORB "CENTURY SERIES"

Values marked with asterisks are of practical interest due to the temperature limit of the stationary phase (T_1) given by the producer[12].

Chromosorb	T_1 (°C)	*Compound*	*Intersection temperature (°C)*			
			CH_4	CO_2	C_2H_4	C_2H_2
101	300	C_2H_6	285*	324	377	252*
		C_2H_2	288*	342	679	
		C_2H_4	275*	304		
		CO_2	262*			
102	250	C_2H_6	467	586	–	–
		C_2H_2	369	340	173*	
		C_2H_4	385	372		
		CO_2	393			
103	275	C_2H_6	273*	165*	191*	55*
		C_2H_2	253*	182*	322	
		C_2H_4	246*	154*		
		CO_2	344			
104	250	C_2H_6	263	–	107*	357
		C_2H_2	284	362	305	
		C_2H_4	276	163*		
		CO_2	264			
105	250	C_2H_6	394	1842	361	49*
		C_2H_2	295	331	137*	
		C_2H_4	400	–		
		CO_2	277			

Porapak columns previously reported[10] shows that the behaviour of Chromosorb 101 and 102 is similar (but not identical) to that of Porapak P and Q respectively. No intersection point of ethylene and acetylene retention times was observed on Chromosorb 102 in the considered temperature interval, while for Porapak Q the intersection point of these compounds was observed at 44°C. Resolution of ethylene and acetylene is difficult with these columns. The behaviour of Chromosorb 103 and 105 can be compared with that of Porapak S and R respectively. As shown by the parallel α plots for C_2H_2 and C_2H_6 on Chromosorb 103 and Porapak S, and by the intersection point at 49°C for Chromosorb 105, no increase in resolution is found by changing the temperature of analysis, while a satisfactory increase in the resolution between C_2H_2 and C_2H_6 is obtained by decreasing the temperature from 70 to 30°C on Porapak R. Chromosorb 104 has no analogous PPB in the Porapak series. Both Porapak N and T show a very long retention time for C_2H_2, as Chromosorb 104 does, but the peculiar feature of 104 is the long retention of CO_2 compared with hydrocarbons. This is clearly shown by the α values of Fig. 2. Porapak N and T also retain CO_2 more strongly than other Porapaks and Chromosorbs, but this compound is always eluted before C_2H_4.

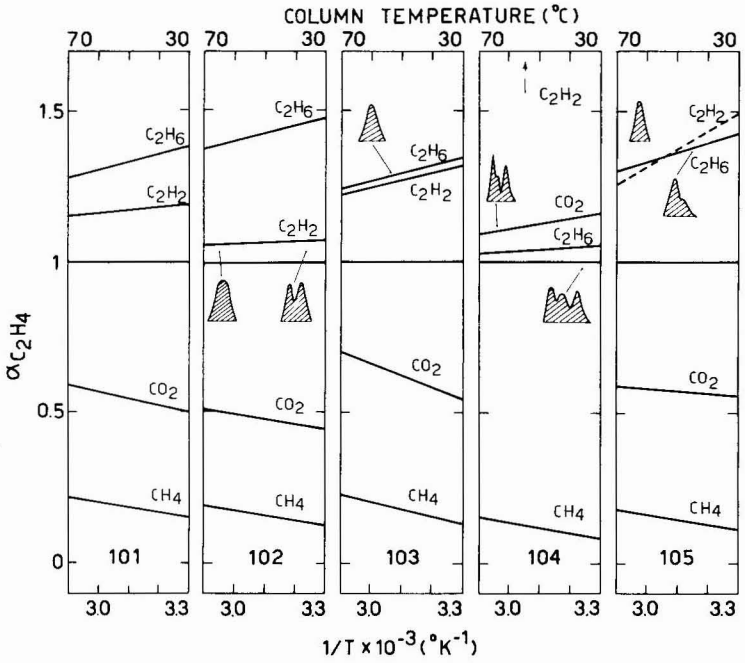


Fig. 2. Values of $\alpha_{C_2H_4}$ and peak resolution as a function of temperature on various Chromosorbs. Conditions as in Fig. 1. Acetylene off-scale on Chromosorb 104.

TABLE III

VALUES OF $\alpha_{C_2H_4}$ AT 30 AND 50°C ON VARIOUS CHROMOSORBS (80–100 MESH), USED AS A QUANTITATIVE EVALUATION OF THE "POLARITY" OF THE PHASE

Compound	*Temperature (°C)*	*Chromosorb*				
		101	*102*	*103*	*104*	*105*
CO_2	30	0.504	0.45	0.55	1.16	0.56
	50	0.55	0.48	0.62	1.13	0.57
C_2H_2	30	1.19	1.075	1.33	3.01	1.50
	50	1.17	1.07	1.27	2.62	1.37
C_2H_6	30	1.39	1.48	1.35	1.05	1.42
	50	1.33	1.43	1.29	1.04	1.36

The differences and similarities between Porapak and Chromosorb obviously depend on the composition and surface area of the various beads. Unfortunately, little information is available on the chemical structure of some of the bead types. Table IV summarizes data taken from bulletins or catalogues.

The effect of the carrier gas flow-rate and of the columns length on the α values is very small, as previously observed with Porapak columns.

The linear dependence of the α values on the reciprocal of the absolute tem-

TABLE IV

CHEMICAL COMPOSITION AND SURFACE AREA OF PORAPAK AND CHROMOSORB POLYMER BEADS, TAKEN FROM CATALOGUES (WATERS AND JOHNS-MANVILLE) AND FROM REF. 16

Type	*Surface area* (m^2/g)	*Chemical composition*
Chromosorb 101	< 50	Styrene–divinylbenzene
Chromosorb 102	300–400	Styrene-divinylbenzene
Chromosorb 103	15–25	Cross-linked polystyrene
Chromosorb 104	100–200	Acrylonitrile–divinylbenzene
Chromosorb 105	600–700	Acrylic esters
Porapak N	225–350	A* + vinyl pyrrolidone
Porapak P	100–200	Styrene–divinylbenzene
Porapak Q	500–600	Ethylvinylbenzene + divinylbenzene
Porapak R	450–600	A* + vinylpyrrolidone
Porapak S	300–450	A* + vinylpyridine
Porapak T	250–350	A*

* A = Styrene–divinylbenzene or ethylvinylbenzene–divinylbenzene polymers modified with polar monomers.

perature, as a consequence of eqn. 1, permits calculation of the $\alpha(C)_i^T$ value of a compound C on the stationary phase i at a given absolute temperature T. The values of the constants m and n

$$\alpha(C)_i^T = m_i\,(1/T) + n_i \tag{2}$$

for the various types of Chromosorb are shown in Table V. Moreover, the behaviour of mixed columns made from various amounts of different Chromosorb types in order to obtain a change in the elution order and resolution of the various compounds can be calculated. It has been shown[13] that the α values for a given C at a constant temperature T on a column prepared by mixing various amounts of two different types, i and j, of Porapak

$$\alpha(C)_{i+j}^T = (\alpha(C)_j^T - \alpha(C)_i^T)\,x_j + \alpha(C)_i^T \tag{3}$$

TABLE V

VALUES OF THE COEFFICIENTS OF EQNS. 2 AND 4 FOR CHROMOSORB COLUMNS

Chromosorb	CH_4		CO_2		C_2H_2		C_2H_6	
	m_i	n_i	m_i	n_i	m_i	n_i	m_i	n_i
101	−247	0.947	−231	1.267	105	0.848	262	0.523
102	−154	0.637	−168	1.003	46	0.923	268	0.601
103	−244	0.937	−377	1.791	254	0.488	276	0.437
104	−182	0.684	165	0.617	1898	−3.252	67	0.832
105	−169	0.674	−75	0.807	609	−0.513	314	0.388

where x_j is the weight fraction of the phase j and $x_i + x_j = 1$. The same behaviour, with the limits of the experimental determination of the α values for homogeneously mixed or multilayer packings[13], has been found for Chromosorb "century series" PPBs (Fig. 3). Therefore, the α of a given compound C on a mixed column $i + j$ can be found at any temperature by a combination of eqns. 2 and 3:

$$\alpha(\mathrm{C})^{T}_{i+j} = \left[(m_j - m_i)\cdot\frac{1}{T} + n_j - n_i\right]x_j + m_i\cdot\frac{1}{T} + n_i \qquad (4)$$

The preparation of mixed columns is of practical interest when PPBs of different "polarity" are used (Fig. 3). Compatibility tests must be done by mixing small amounts of the two packings in order to determine whether electrostatic effects due to the surface groups and composition of the various types of Chromosorb cause adhesion of the beads, which impairs the filling by gravity of the columns with homogeneously mixed packings. A mixed column with two non-compatible Chromosorb types can be prepared by alternately pouring small amounts of the two phases in the column, giving a multilayer packing. It has been found that such columns are similar to homogeneous columns when the number of layers increases. The behaviour of mixed Porapak–Chromosorb columns can also be calculated by using the previously published α values[10]. Little or no advantages are obtained by mixing packings of similar "polarity", while mixed columns of PPBs giving very different elution orders (*e.g.*, Chromosorb 104 with Chromosorb 101, 102 or 103, see Fig. 3) can replace any column having intermediate polarity. Coupled or series columns can also be used, but

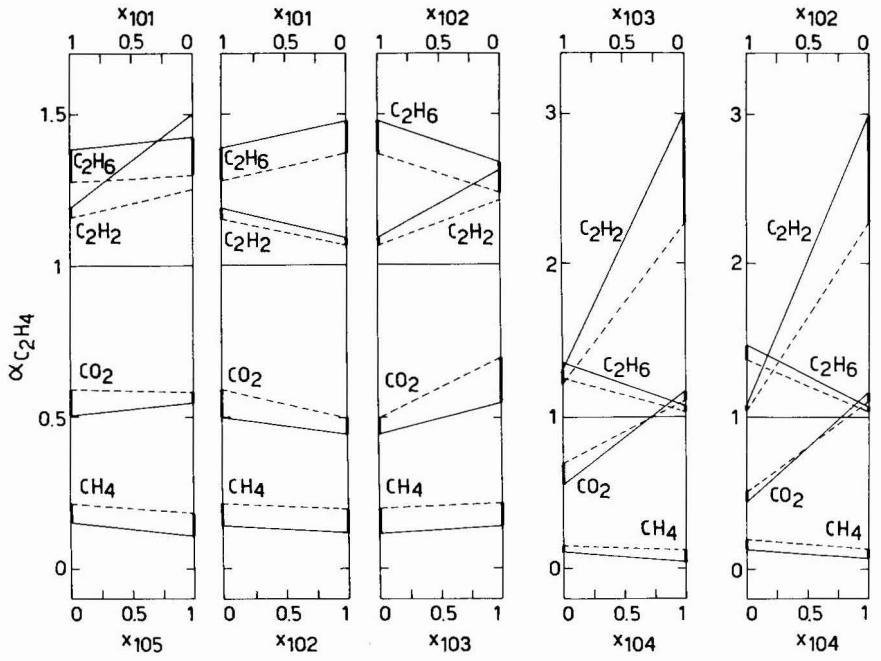


Fig. 3. Behaviour of mixed columns of various Chromosorb types at 30°C (— · —) and 70°C (— — —). Abscissa values: above, x_i; below, x_j.

TABLE VI

VALUES OF $-\Delta H^0$ (kcal mol^{-1}) FOR LIGHT HYDROCARBONS AND CO_2 ON VARIOUS CHROMOSORBS

Conditions as in Table I.

Compound	*Chromosorb*				
	101	*102*	*103*	*104*	*105*
CH_4	3.70	4.10	3.65	3.89	3.66
CO_2	5.54	5.48	5.32	7.51	5.76
C_2H_6	6.41	6.60	7.09	7.20	6.00
C_2H_4	6.57	6.40	6.56	7.39	6.42
C_2H_2	6.78	6.53	6.91	8.61	6.95

in this case the prevailing influence of the up-stream column working at higher pressure must be taken into account.

The thermodynamic functions were calculated as described elsewhere[10,14,15] in order to obtain more information on the partition equilibrium and on the "polarity" of the various PPBs. Table VI shows the values of the adsorption entalphies ($-\Delta H^0$), whose values for acetylene increase in the order Chromosorb 102, 101, 103, 105, 104, in accordance with the $\alpha_{C_2H_4}$ values of Table III, with the polarity order given by the producer[11] and with that obtained by analyzing a polarity mixture of acetonitrile, benzene, methyl ethyl ketone and *tert.*-butanol[12]. It can be concluded that Chromosorb 102 and 104 have respectively the lowest and the highest polarity in the Chromosorb "century series" packings. The linear correlation between $-\Delta H^0$ and $\alpha_{C_2H_4}$ values (see Fig. 4) is better for the Chromosorb than for the Porapak series where

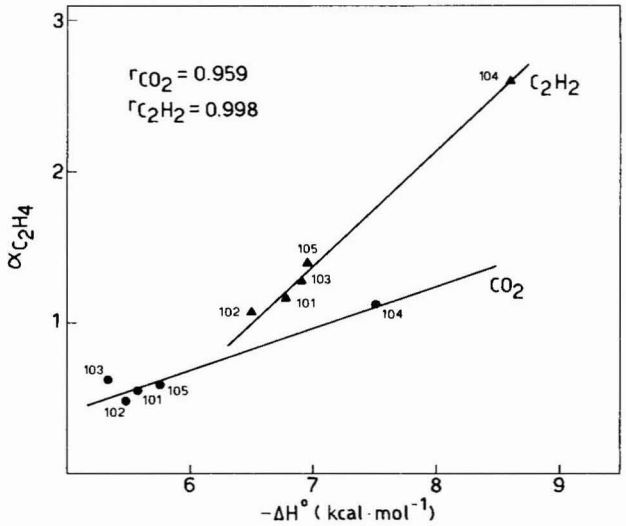


Fig. 4. Linear correlation between $\alpha_{C_2H_4}$ and $-\Delta H^0$ values of CO_2 and C_2H_2 on various Chromosorbs at 50°C. Correlation coefficients, r, are also shown.

inversion between R and S, T and N types was observed[10]. This is shown by the correlation coefficients, those at 50°C on Porapak type columns being 0.789 for CO_2 and 0.724 for C_2H_2, much smaller than the corresponding values for the Chromosorb "century series", shown in Fig. 4.

The dependence of the α values of C_2H_6 on the $-\Delta H^0$ values is almost random both on Porapak ($r = 0.141$) and on Chromosorb ($r = 0.678$), while a satisfactory linear correlation is found between the α and $-\Delta H^0$ values for CO_2 and C_2H_2.

CONCLUSIONS

The measured values of $\alpha_{C_2H_4}$ refer to a restricted number of batches of Chromosorb "century series" of different types. It is possible that single batches would give $\alpha_{C_2H_4}$ and $-\Delta H^0$ values which are slightly different from the values reported. In the case of the Porapaks we investigated the effect of mesh size on the $\alpha_{C_2H_4}$ values and found a variation of about 5%, that is probably of the same order of magnitude as the fluctuations due to change in mesh size of the Chromosorb beads.

This notwithstanding, the $\alpha_{C_2H_4}$ values can be used as a mean for the rough classification of the polarity of Chromosorb and Porapak PPBs and for the choice of packing or of mixed column that can replace another type of PPB not readily available for a given separation. Moreover, both for Chromosorbs and Porapaks, the measurement of the $\alpha_{C_2H_4}$ values of CO_2 and C_2H_2 at room temperature on a freshly prepared Chromosorb column permits a rapid evaluation of the reproducibility of this column with respect to that of a column previously used for the same analysis but which it is desired to replace.

REFERENCES

1 O. L. Hollis, *J. Chromatogr. Sci.*, 11 (1973) 335.
2 G. Castello, G. D'Amato and G. Canciani, *Ann. Chim. (Rome)*, 68 (1978) 255.
3 S. B. Dave, *J. Chromatogr. Sci.*, 7 (1969) 389.
4 W. R. Supina and L. P. Rose, *J. Chromatogr. Sci.*, 7 (1967) 192.
5 T. N. Gvosdovich and Ja. I. Jashin, *J. Chromatogr.*, 49 (1970) 36.
6 T. A. Gough and C. F. Simpson, *J. Chromatogr.*, 51 (1970) 129.
7 K. Sakodynsky and L. Panina, *Chromatographia*, 4 (1971) 113.
8 R. G. Ackman, *J. Chromatogr. Sci.*, 10 (1972) 506.
9 H. L. Gearhart and M. F. Burke, *J. Chromatogr. Sci.*, 15 (1977) 1.
10 G. Castello and G. D'Amato, *J. Chromatogr.*, 196 (1980) 245.
11 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
12 *Technical bulletin FF-194A*, Johns-Manville, Denver, CO, 1967.
13 G. Castello and G. D'Amato, *Ann. Chim. (Rome)*, 69 (1979) 541.
14 F. M. Zado and J. Fabecic, *J. Chromatogr.*, 51 (1970) 37.
15 J. Czubryt and H. D. Gesser, *J. Chromatogr.*, 59 (1971) 1.
16 S. B. Dave, *Ind. Eng. Chem., Prod. Res. Develop.*, 14 (1975) 85.

Journal of Chromatography, 212 (1981) 271–281
Elsevier Scientific Publishing Company, Amsterdam Printed in The Netherlands

CHROM. 13,877

HYDROPHOBIC CHROMATOGRAPHY WITH DYNAMICALLY COATED STATIONARY PHASES

V. "DUPLEX SOAP CHROMATOGRAPHY"

YOUSSEF GHAEMI and RICHARD A. WALL*
Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ (Great Britain)
(Received April 9th, 1981)

SUMMARY

The retention of a wide range of ionised and non-ionised analytes on silica gel liquid chromatographic columns modified by dynamic interaction with solutions of mixtures of ionic and nonionic surfactants has been examined systematically using both aqueous and aqueous methanolic eluent systems. It is shown that efficient separations of mixtures of several classes of organic compounds can be achieved by these "duplex soap chromatography" procedures.

INTRODUCTION

Knox and Laird[1], Gilbert and Wall[2], and the present authors[3–5] have shown that aqueous alcoholic solutions of surfactants interact with oxide gel liquid chromatographic column packing materials to generate a liquid–solid interface that facilitates separations of charged and uncharged solutes by a combination of ion-exchange and solvophobic processes. An essentially similar technique was used by Armstrong and Terrill[6] for separations by chromatography on thin alumina layers without examination of the mechanism of separation. It was clear from the study[7] of effects of addition of anionic surfactants (which do not interact in aqueous or aqueous alcoholic solutions with acidic oxides like silica) to retentive silica–non-ionic surfactant systems that this "duplex soap chromatography" was of considerable potential interest.

The present study describes the effects of additions of either a quaternary ammonium salt soap, or an ammonium salt soap, or alkyl sulphate salt soaps to chromatographic column eluents containing polyoxyethylene sorbitan ester (Tween®) non-ionic surfactants. Retentions of several groups of analytes were studied as functions of non-ionic soap concentration and (independently) of ionic soap concentration.

* Present address: Department of Pharmacology, University of British Columbia, 2176 Health Sciences Mall, Vancouver, BC, V6T 1W5, Canada.

0021-9673/81/0000–0000/$02.50

EXPERIMENTAL

Instrumentation

Chromatographic systems were assembled from components as outlined in earlier reports in this series[3–5,7]. Columns were packed by the "upward slurry" techniques described by Bristow *et al.*[8] at 300–500 bar constant pressure, using methanol both for packing and suspension of the spherical silica gel (Hypersil, $d_p \approx 5$ μm, $S_{BET} \approx 170$ m^2 g^{-1}, Shandon Southern Instruments, Runcorn, Great Britain) used in all the reported experiments.

Solvents and reagents

Solvent methanol was HPLC grade (Rathburn Chemicals, Walkerburn, Great Britain) and water was doubly distilled from glass and stored in glass containers. Care with preparation and storage of solvent water largely eliminated the problems of column contamination observed in the earlier study[7] on peptide separations by these techniques.

Hexadecyltrimethyl ammonium bromide (CTAB), sodium lauryl sulphate (SDS), puriss. grade, and Tergitol 7 (T.7) were purchased from Fluka, Buchs, Switzerland. Tweens 20 and 40, stated to be of "industrial quality" were obtained from Sigma (London) (Poole, Great Britain). All other reagents used were of reagent grade and were used as received from the various suppliers.

Procedures

In all experiments except the aqueous solution series, the measurement of retention with a given set of surfactants in the eluent was carried out on columns packed with silica which had been in contact with only those unique soaps. The columns were brought to equilibrium by washing with at least 0.5 l of Tween solution (2–5 g dm^{-3}) before initiation of duplex soap experiments, and in all cases columns were washed with the chosen eluent until constant retention was observed for the test substances.

In practice, this equilibration procedure (carried out at ambient temperature, 15–21°C) usually consisted of passage of some 0.2 l of eluent through a column before any measurement of retentive power. More often than not work timetabling led to partial equilibration on one working day followed by data collection on the following or later days. No particular significance attached to this timing when aqueous organic eluents were used, since achievement of a constant retaining surface appeared to be solely a function of passage of sufficient eluent through the column. However, when purely aqueous eluent systems were examined carefully, it was observed that retention of neutral analytes often increased after overnight storage of a column already in apparent equilibrium with the eluent. Fortunately, this very slow reorganisation of the retaining surface molecular architecture was found to be complete after 16–20 h storage. All aqueous soap chromatographic data reported below were collected only after an overnight storage period following passage of at least 0.2 l of eluent through the column. Studies on the time and temperature dependence of the retentive surface characteristics are now in hand.

RESULTS AND DISCUSSION

Dynamic anion-exchange systems

It was clearly of interest to see whether or not the interactions between non-ionic and anionic surfactants found earlier[7] to be so useful could also be demonstrated when a *cationic* soap was used in place of the SDS or T.7 of the peptide cation-exchange experiments. Fig. 1 shows that mixed cationic–non-ionic soap systems exhibit properties different from those of single surfactant–oxide gel chromatographic procedures. This figure outlines the large variations in selectivity attainable by changing the concentration of the ionic soap component of the eluent. Comparison of the present Fig. 1 with the data expressed as Fig. 1 of the earlier study on CTAB–silica interactions[3] shows clearly that a slightly lower maximum retention of charged sulphonic acid analytes is obtained in the duplex soap system, and that this maximum k' [$k' = (t_{\text{sample retention}} - t_{\text{void volume clearance}})/t_{\text{void volume clearance}}$] is achieved at much lower ionic surfactant concentrations than in the single surfactant system ($k' \approx 20$ at $2 \cdot 10^{-2}$ $\overline{M}$ [CTAB] *versus* $k' \approx 13$ at $6 \cdot 10^{-3}$ $\overline{M}$ [CTAB].

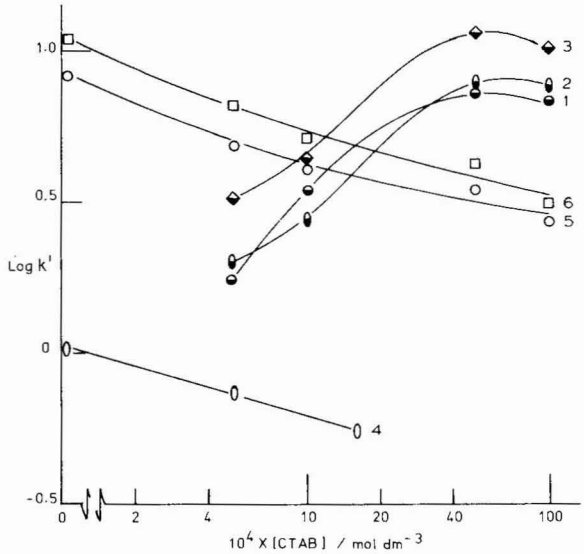


Fig. 1. Effect of variation of quaternary ammonium salt concentration on sample retention. Water–methanol (1:1, v/v) solution of Tween 40 (2 g dm^{-3}), pH 5 (with H_3PO_4). Analytes: 1 = di-J-acid; 2 = Schäffer's acid; 3 = dioxy-J-acid; 4 = acetophenone; 5 = fluorenone; 6 = naphthalene. For structures of sulphonic acids *cf.* Knox and Laird[1].

A second, potentially useful difference between the single soap and duplex soap eluents is apparent in the very different relationship of retention to CTAB concentration for unionised analytes. There is a steady increase to maximum k' for all analytes in the single soap system, whereas retention of non-polar eluites is steadily *reduced* by addition of CTAB to the Tween–SiO_2 column system. That this difference, which is probably an exaggerated case of the phenomena detailed by Graham and Rogers[9] in their investigation of surfactant effects on retention of neutral analytes on a column of octadecyl silica, is general will be shown later in this present work.

Apparently the analogy used in the peptide study[7] of a "brush" type of induced ion-exchanging surface might also account for the observed chromatographic properties of the duplex cationic–non-ionic soap-modified silica surface. As the ionic soap concentration increases, so also retention of anionic eluites increases through a maximum and thereafter decreases as the solvating power of the surfactant in the mobile phase begins to dominate the distribution of solute between stationary and mobile phases.

Determination of the uptake of CTAB from a water–methanol (1:1, v/v) solution containing Tween 40 (2 g dm^{-3}) showed $0.06 \cdot 10^{-6}$ mol m^{-2} at [CTAB] = $5 \cdot 10^{-3}$ mol dm^{-3}. This coverage of the silica surface should be compared to the binding capacity for alkylbenzyldimethyl ammonium salts on the ODS-Spherosil surface, which was reported by Tomlinson *et al.*[10] to be $0.13 \cdot 10^{-6}$ mol m^{-2} from a 10^{-3} mol dm^{-3} solution of the surfactant in water–methanol (1:1, v/v).

The apparent success of the above experiments in generation of what might be described as a "strong" anion exchanger led to attempts to combine the effects of a non-ionic surfactant and the surface active dodecylammonium ion. Fig. 2 shows that the retention *vs.* [dodecylammonium] relationship is similar in form to that observed above with the fully quaternised CTAB. The eluent systems were buffered to pH 5.5 in this latter series of experiments to ensure protonation of the amine soap as well as (*ca.* 80% complete) ionisation of the carboxylic acid analytes.

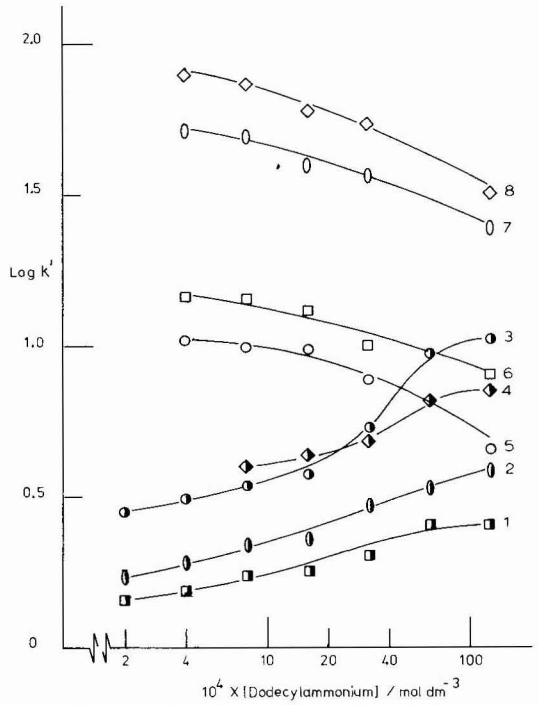


Fig. 2. Changes in eluite retention with variation in dodecylammonium ion concentration. Water–methanol (1:1, v/v) solution of Tween 40 (2 g dm^{-3}), pH 5.5 (with H_3PO_4). Analytes: 1 = 3-phenylbutanoic acid; 2 = *trans*-2-phenylcyclopropane-1-carboxylic acid; 3 = 3,3-diphenylpropenoic acid; 4 = 1-naphthyl acetic acid; 5 = fluorenone; 6 = naphthalene; 7 = anthracene; 8 = pyrene.

It can be seen that maximum retention of the carboxylate anions occurs at significantly higher cationic soap concentrations than did the corresponding maximum retention of arylsulphonate anions on the Tween 40–CTAB–silica columns. This finding is consistent with the greater surfactant activity of CTAB as opposed to dodecylammonium – the critical micelle concentration (CMC) of the quaternary ion is slightly less than a tenth that of the primary ammonium ion in water[11]. Interestingly, retention of non-polar analytes appears to be significantly greater (at equimolar cationic soap concentrations) in the dodecylammonium system than in the CTAB eluent, possibly reflecting a greater induced polarity of the surface from the quaternary salt.

Dynamic cation-exchange systems

A more complete investigation of the relationship of anionic surfactant (T.7) concentration to ionised and non-polar analyte retention on a silica column equilibrated with Tween 40 (2 g dm^{-3} in water–methanol, 1:1) than was illustrated in the early study[7] of this system is shown in Fig. 3. Comparison of the data represented in Fig. 3 with those showing the equivalent relationship for the shorter chain alkyl sulphate, SDS, reveals that retention in the duplex system does not appear to be as direct a function of surfactant chain length as with the single soap eluent–oxide column (*cf.* Fig. 3 of ref. 4 and Figs. 2 and 3 of ref. 5). It would appear that the lipophilicity of the dynamically generated stationary phase in duplex soap systems is

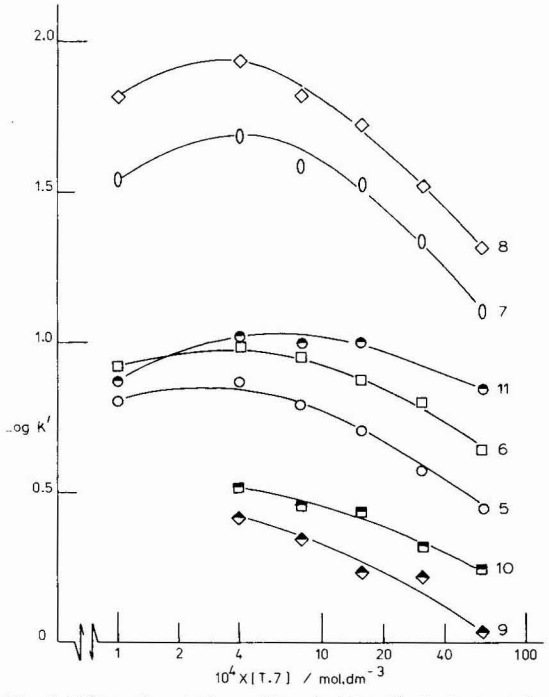


Fig. 3. Effect of variation of Tergitol 7 ("C_{17}" alkyl sulphate) concentration on sample retention. Water–methanol (1:1, v/v) solution of Tween 40 (2 g dm^{-3}), pH 4.0 (with H_3PO_4). Analytes 5–8 as in Fig. 2, and: 9 = 2-nitroaniline; 10 = 1-amino-1-phenyl ethane; 11 = 1-amino-1-(1-naphthyl)ethane.

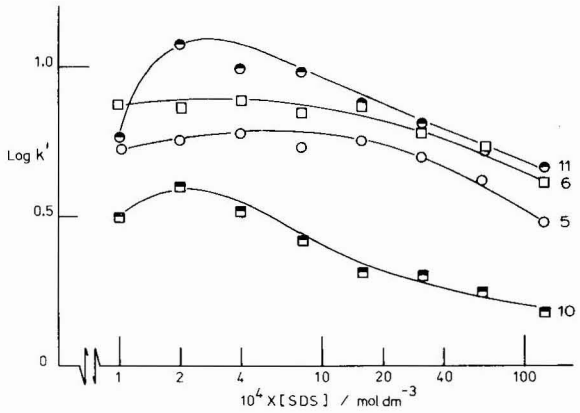


Fig. 4. As Fig. 3 except anionic surfactant was sodium lauryl sulphate (SDS).

governed primarily by the nature of the *non-ionic* surfactant. This latter assertion is supported by further comparisons of Figs. 1, 3, 4 and 5 from this report with Fig. 1 of ref. 3, Fig. 3 of ref. 4 and Figs. 2 and 4 of ref. 5, since maximum retention of charged analytes in the single cationic and anionic systems occurs at much higher concentrations of the appropriate surfactant, whereas maximum retentions of uncharged (and charged, *cf.* Fig. 5) analytes were observed at approximately the same concen-

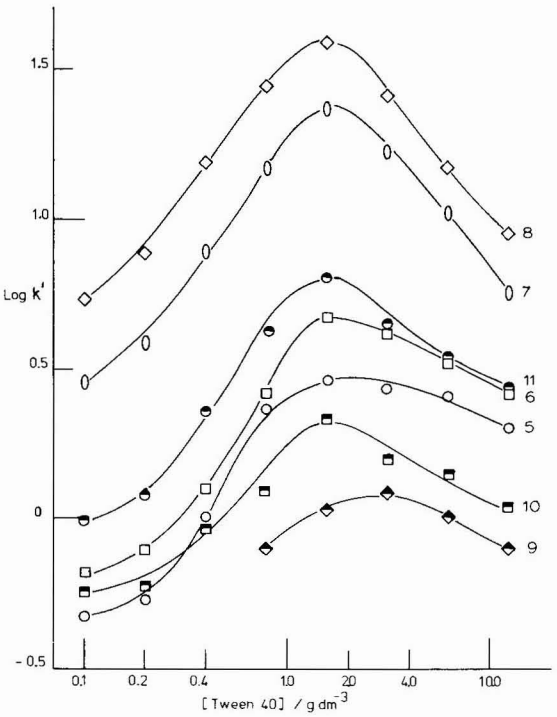


Fig. 5. Effect of variation of concentration of non-ionic (Tween 40) soap on analyte retention. Water–methanol (1:1, v/v) solution of SDS (10^{-2} mol dm^{-3}), pH 4.0 (with H_3PO_4). Analytes as in Figs. 2 and 3.

tration (*ca.* 2 g dm^{-3}) of the non-ionic surface active eluent component in *both* single and duplex soap systems.

The other feature of duplex surfactant liquid chromatographic systems which is shown very clearly in Figs. 1 and 2 and less obviously in Figs. 3 and 4 is the very different response of charged and uncharged analytes to variations in the ionic soap constituent of the eluent. The change in retaining surface charge density with increasing ionic soap content gives a steady rise in retentiveness for ionised analytes (of charge opposite to that of the soap) through a maximum shared by analytes of the same charge. The effect of the increasing surface charge density on *uncharged* analytes is simply to reduce their retention from the maximum which is observed in the absence of the ionic soap eluent component. So in many cases the order of elution (*i.e.*, selectivity) of a given set of charged and uncharged eluites may well alter drastically with changes in ionic surfactant concentration (at constant non-ionic concentration).

The more complete data collections of the present work confirm the tendencies to selective retention observed in Part II[7] of this extended study, and suggest that gradient elution systems in which either counter ion or ionic surfactant concentrations were varied might prove to be useful separation procedures.

Dynamic coating with aqueous surfactant solutions

A possible deterrent to wider use of the oxide gel–surfactant liquid chromatographic systems described so far in this study is the relatively low lipophilicity of these dynamically coated surfaces compared to current alkylbonded silica surfaces. Retention of aromatic ketones and phenols on duplex or simplex (non-ionic) soap coated silicas is apparently of the same order as found on the least hydrophobic bonded silicas. However, Fig. 8 of Part III[4] (and unpublished results associated with ref. 7) of this series shows that alteration of the organic components of eluents containing non-ionic surfactants leads to the expected large changes in retention of neutral analytes.

Taking these latter observations to their logical conclusion, especially in view of the "micellar chromatography" studies of Armstrong and Terrill[6] on separations on alumina thin layers with aqueous SDS eluents, it seemed appropriate to determine whether aqueous non-ionic surfactant solutions could also be used to generate a controlled retentive surface on silica gel. In the event, such interactions did take place and gave the very large increase in retention expected by analogy with the effects of similar eluent changes in alkyl-bonded silica chromatography.

As can be seen in Fig. 6, particularly by comparison with Fig. 8 of ref. 4, k' for acetophenone (and several other analytes not shown) in aqueous eluents is about ten times that found with water–methanol (1:1) eluents. Further inspection of Fig. 6 reveals two other differences between the aqueous and organic-modified aqueous chromatographic systems. First, and most important, the fairly sharp maximum in the retention *vs.* non-ionic detergent concentration curve so characteristic of aqueous methanol eluents (*cf.* Fig. 5) does not appear in purely aqueous systems. Instead, retention rises slowly to a limiting value defined by interaction of pure water eluent and pre-loaded (with 0.5 g dm^{-3} [Tween 20]) silica gel. The k' measurements on the [Tween 20] = 0 axis were obtained after passage of approx. 0.8 l of water through the pre-loaded column. Secondly, the order of elution of the second and third eluites is reversed from that previously observed on columns of alkyl-bonded silica or dynamically coated silica eluted with aqueous organic eluents (*cf.* Fig. 11 of ref. 7), which

suggests considerable involvement of surface silanols in the retention mechanism[12] of these fairly polar eluites. Note also that there does not appear to be any pronounced change in the chromatographic characteristics of the single non-ionic surfactant eluent–gel system as might be expected near the CMC[11] of this detergent.

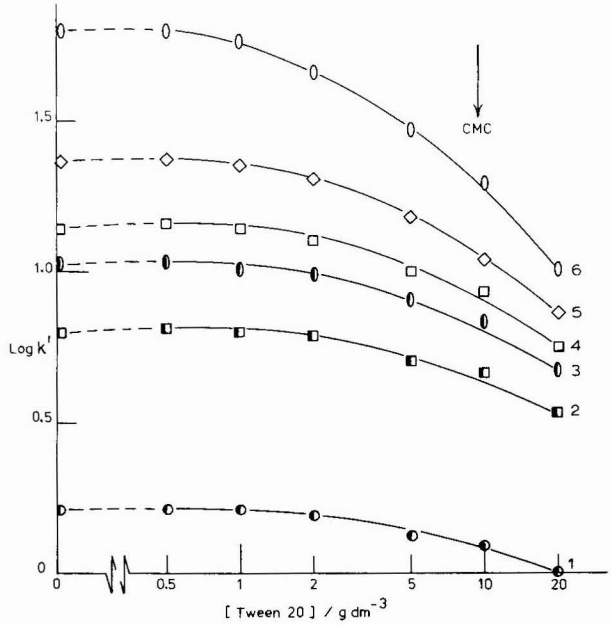


Fig. 6. Effect of variation of concentration of Tween 20 on aqueous single soap chromatographic retention of uncharged samples. Analytes: 1 = 4-methylpentan-2-one; 2 = acetophenone; 3 = 4-methoxyacetophenone; 4 = salicylamide; 5 = 4-methylphenol; 6 = 2,3-xylenol.

Fig. 7 is a simple demonstration that column efficiency with this aqueous non-ionic soap eluent is comparable to that obtained in conventional "reversed-phase" alkyl-bonded silica separation systems. Note the unusual order of elution of acetophenone, 4-hydroxyacetophenone and 4-methoxyacetophenone.

In line with the general theme of the present work, aqueous duplex soap eluents were examined with the same pre-loaded (after collection of the water elution data) silica column used for the studies described by Fig. 6. The retention *vs.* [SDS] data shown in Fig. 8 were measured in a series of eluents of *decreasing* anionic soap concentration, following the same methodology used with the aqueous single soap eluents. A very large disturbance in baseline detector signal resulted from the application of the most concentrated (*ca.* 2 × CMC of SDS, ref. 11) duplex soap eluent tested. Moreover, measurement of the column void volume by injection of pure water or aqueous potassium nitrate solution demonstrated a considerable increase over the values recorded in the absence of the anionic detergent. Apparently the high solubilising power of aqueous SDS at concentrations at or above the CMC removes most of the dynamically deposited non-ionic surfactant and hence leads to minimal retentive power for all analytes examined.

Note, however, that as the concentration of the ionic soap falls below the

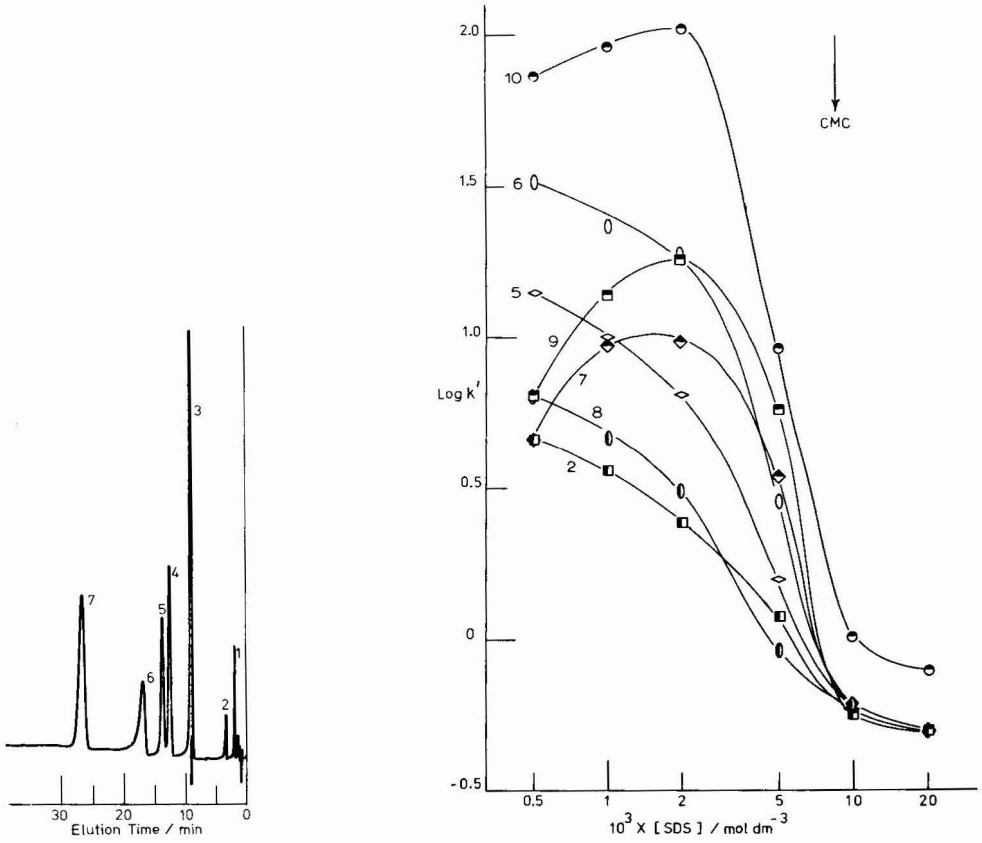


Fig. 7. Sample separation of some uncharged analytes by aqueous single non-ionic soap chromatography. Column of Hypersil, 135 × 4.6 mm I.D., eluted by aqueous solution of Tween 20 (2 g dm^{-3}) at 1 cm^3 min^{-1}. Detection by ultraviolet absorption at 254 nm. Analytes: 1 = butan-2-one; 2 = 4-methylpentan-2-one; 3 = acetophenone; 4 = 4-hydroxyacetophenone; 5 = 4-methoxyacetophenone; 6 = salicylamide; 7 = 4-methylphenol.

Fig. 8. Effect of variation of concentration of sodium lauryl sulphate on aqueous duplex soap chromatographic retention of cationic and neutral samples. [Tween 20] = 1 g dm^{-3}, pH adjusted to 3.0 with phosphoric acid after addition of Na_2HPO_4 to give $[Na^+]$ = 0.020 mol dm^{-3}. Analytes 2, 5 and 6 as in Fig. 6, and: 7 = glycyltyrosine; 8 = 4-hydroxyacetophenone; 9 = tyrosine methyl ester; 10 = 1-amino-1-(1-naphthyl)ethane.

CMC, there is a rapid generation of retentive character for both cationic and neutral analytes. The behaviour shown in Fig. 8 is distinctly different from that observed with the aqueous single soap eluent exemplified in Fig. 6 above. Concomitant with the restoration of retention at sub-CMC SDS concentrations there was the expected baseline detector perturbation and a measureable decrease in the void volume as if non-ionic Tween was again deposited on the silica surface.

Note that the relationship of retention of uncharged analytes to surfactant concentration in the duplex soap system resembles that in the single soap system, although Fig. 8 makes clear that (at moderate ionic strength) neutral eluites are not bound quite so firmly to the SDS–Tween surface as to the Tween surface. However,

the retaining power of the duplex surface for cationic analytes passes through a well defined maximum as anionic soap concentration is reduced. That this difference in selectivity for charged as opposed to uncharged eluites is a general property of all the duplex soap systems examined to date may be confirmed by examination of Figs. 1–4 and 8 and Figs. 1 and 4 of ref. 7.

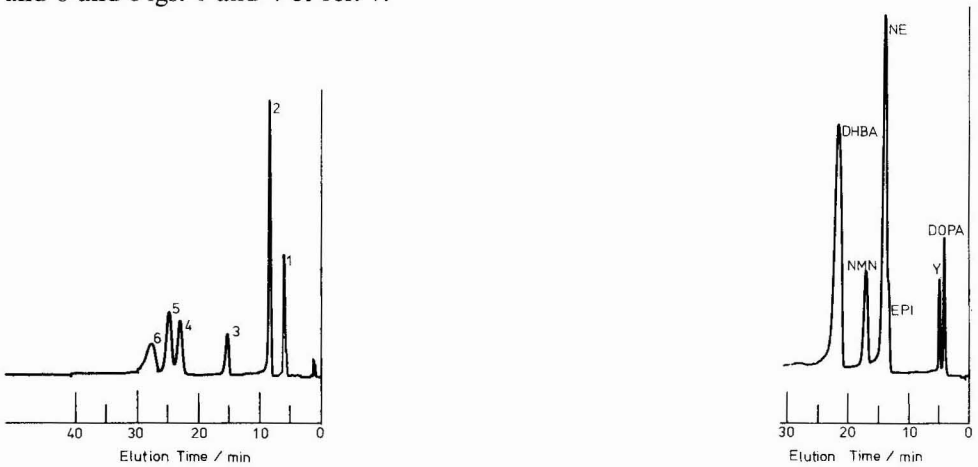


Fig. 9. Sample separation of some analytes of Fig. 8. Same column, detector setting and eluent flow-rate as Fig. 7. Eluent: aqueous solution of Tween 20 (1 g dm^{-3}) and SDS ($2 \cdot 10^{-3}$ mol dm^{-3}) containing Na_2HPO_4 ($9 \cdot 10^{-3}$ mol dm^{-3}), pH adjusted to 3.01 by addition of H_3PO_4. Analytes: 1 = 4-hydroxyacetophenone; 2 = 4-methoxyacetophenone; 3 = glycyltyrosine; 4 = 3,5-xylenol; 5 = 2,3-xylenol; 6 = tyrosine methyl ester.

Fig. 10. Sample separation of tyrosine and some of its metabolites. Column, eluent, and operating conditions as in Fig. 9 except detection at 272 nm. Analytes: DOPA = 3,4-dihydroxyphenylalanine; Y = tyrosine; EPI = epinephrine; NE = norepinephrine; NMN = normetanephrine; DHBA = 3,5-dihydroxybenzylamine (internal standard for catecholamine analyses).

A sample separation of some of the test substances used in the experiments with aqueous single and duplex soap eluents is shown in Fig. 9. Although there is evidence of increased peak asymmetry resultant from extended usage of the column, the perceived separating power has remained acceptable. Since it was clear that relatively polar analytes could be well retained with aqueous soap eluents, it seemed appropriate to examine possible separations of the biologically interesting catecholamines and their precursor amino acids. Fig. 10 shows such a trial separation. Although this model mixture does not include all the biologically significant tyrosine metabolites, the analytical possibilities inherent in this non-optimised separation appear to warrant further study, particularly since the elution order of these amines is distinctly different from that observed in aqueous or aqueous–organic buffer elution from anionic surfactant-modified alkyl-bonded silica columns[13–15].

CONCLUSIONS

Equilibration of porous silica gel with an aqueous methanolic solution of a ("duplex soap") mixture of a cationic and a non-ionic surfactant may be used to produce a liquid chromatographic column packing material which will separate neu-

tral analytes by solvophobic interactions and anionic analytes by a combination of anion-exchange and solvophobic distribution processes. Retention of charged and uncharged analytes on such dynamically coated silica is a function of the concentrations of *both* surface-active agents in the eluting solvent system, although greatest selectivity in control of relative retention is achieved by variation of the concentration of the cationic rather than the non-ionic soap.

Duplex cationic soap chromatographic systems qualitatively resemble single cationic soap–acidic oxide gel separation systems. However, maximum analyte retentions occur at much lower ionic soap concentrations on duplex soap modified oxide gel column packings than on single soap modified materials.

Replacement of the cationic soap in the above duplex soap chromatography procedure by an anionic surfactant produces a separation system which discriminates between analytes by a combination of cation-exchange and solvophobic interactions. These discriminative properties are preserved with both aqueous and aqueous–organic eluting solvents.

Although analyte retention in all single and duplex soap chromatography systems examined to date has been found to be a non-linear function of surfactant concentration in the mobile phase, very rapid changes in the retentive properties of the dynamically modified stationary phase associated with micellisation have been observed only with an aqueous duplex soap chromatography procedure as a consequence of variation of the ionic surfactant concentration in the eluent.

Each of the single and duplex "soap chromatography" procedures studied in this and earlier reports[1–7] has been found to give efficient separations of a wide variety of organic compounds. Many questions remain to be answered about these separation systems, but what has been found so far clarifies the potential value of (dynamic) single *and* duplex soap chromatography methods.

REFERENCES

1 J. H. Knox and G. R. Laird, *J. Chromatogr.*, 122 (1976) 17.
2 M. T. Gilbert and R. A. Wall, *J. Chromatogr.*, 149 (1978) 341.
3 Y. Ghaemi and R. A. Wall, *J. Chromatogr.*, 174 (1979) 51.
4 Y. Ghaemi and R. A. Wall, *J. Chromatogr.*, 198 (1980) 397.
5 Y. Ghaemi, J. H. Knox and R. A. Wall, *J. Chromatogr.*, 209 (1981) 191.
6 D. W. Armstrong and R. Q. Terrill, *Anal. Chem.*, 51 (1979) 2160.
7 R. A. Wall, *J. Chromatogr.*, 194 (1980) 353.
8 P. A. Bristow, P. N. Brittain, C. M. Riley and B. F. Williamson, *J. Chromatogr.*, 131 (1977) 57.
9 J. A. Graham and L. B. Rogers, *J. Chromatogr. Sci.*, 18 (1980) 614.
10 E. Tomlinson, C. M. Riley and T. M. Jefferies, *J. Chromatogr.*, 173 (1979) 89.
11 P. Mukerjee and K. J. Mysels, *Critical Micelle Concentrations of Aqueous Surfactant Systems*, U.S. Government Printing Office, Washington, DC, 1971.
12 A. Nahum and Cs. Horváth, *J. Chromatogr.*, 203 (1981) 53.
13 R. M. Riggin and P. T. Kissinger, *Anal. Chem.*, 49 (1977) 2110.
14 J. Wagner, M. Palfreyman and M. Zraika, *J. Chromatogr.*, 164 (1979) 41.
15 G. A. Scratchley, A. N. Masoud, S. J. Stohs and D. W. Wingard, *J. Chromatogr.*, 169 (1979) 313.

Journal of Chromatography, 212 (1981) 283–293
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

CHROM. 13,833

REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHIC SEPARATIONS OF SOME HETEROBORANE ANIONS

ZBYNĚK PLZÁK, JAROMÍR PLEŠEK and BOHUMIL ŠTÍBR
Institute of Inorganic Chemistry, Czechoslovak Academy of Sciences, 250 68 Řež near Prague (Czechoslovakia)
(Received March 30th, 1981)

SUMMARY

Separation of 23 heteroborane anions by reversed-phase ion-pair liquid chromatography on C_1-bonded-phase columns using C_{12} or C_6 *n*-alkylamine pairing ion is reported. The elution was monitored with a UV detector at 235, 254 or 280 nm well below microgram sensitivity level. The effects of the concentrations of the pairing ion and the inert salt, the pH, the pairing ion length, the stationary phase material and the methanol–water ratio on retention and selectivity were investigated.

INTRODUCTION

In the past two decades, a great variety of borane anionic species have been synthesized, ranging from polyhedral borane anions to sandwich cage heteroborane complexes. Some of these compounds were successfully applied in neutron capture therapy[1], as extraction reagents in processing spent nuclear fuel[2,3] and in homogenous catalysis[4]. Despite the fact that many closely related compounds were prepared, which differ structurally in a very subtle way, a speedy and efficient analytical method for purity assay, reaction monitoring and isolation of reaction products is still lacking. The only report dealing with separation of these compounds is the study of Wellum *et al.*[5], who separated some polyhedral borane anions using ion-exchange and partition thin-layer chromatography (TLC) systems.

Ion-pair reversed-phase high-performance liquid chromatography is now a well established technique for the analysis of many organic and biochemical systems[6,7], but reports of its application to analysis of inorganic and organometallic compounds are relatively scarce[8–10].

The present article is aimed at demonstrating the convenience of the reversed-phase ion-pair technique for direct analysis of some heteroborane anions, and at investigating the effect of factors governing the separation process.

EXPERIMENTAL

The home-made chromatograph described previously[11] comprised a VCM 300 membrane pump, a UV detector operating at 254 nm, a UVM-4 variable-wavelength

0021-9673/81/0000–0000/$02.50

(235–380 nm) detector (Development Works of Czechoslovak Academy of Sciences, Prague, Czechoslovakia), a U6K injector (Waters Assoc., Milford, MA, U.S.A.) and a differential refractometer (Model 2025/50; Knauer, Oberursel, G.F.R.).

All samples were obtained from laboratory stocks; the methods of preparation are listed in Table I. Laurylamine (Lachema, Brno, Czechoslovakia) and hexylamine (Koch-Light Labs., Colnbrook, Great Britain) were converted into hydrochloride salts and purified by crystallization from dry diethyl ether–absolute ethanol mixture. All other chemicals were p.a. grade. Methanol was passed through a column of activated silica and distilled in glass before use. Water was deionized and double distilled from all-glass still.

Packing materials were C_1-bonded phase (C_1-BP) prepared from Kieselgel for TLC (Merck, Darmstadt, G.F.R.); a fraction with average particle size 14.3 μm

TABLE I

CAPACITY RATIOS OF HETEROBORANE ANIONS

System I: column, 150 × 3.8 mm; sorbent, C_1-BP on silica (14.3 μm); eluent, $2.70 \cdot 10^{-3}$ *M n*-$C_{12}H_{25}NH_2 \cdot HCl$ in methanol–water (6:4); flow-rate, 0.92 ml/min; pressure drop, 4.8 MPa; UV detection. System II: eluent, $2.70 \cdot 10^{-3}$ *M n*-$C_{12}H_{25}NH_2 \cdot HCl$ in methanol–water (7:3); flow-rate, 1.19 ml/min; pressure drop, 5.8 MPa; other parameters as in system I. System III: eluent, $2.70 \cdot 10^{-3}$ *M n*-$C_{12}H_{25}NH_2 \cdot HCl$ in methanol–water (8:2); flow-rate, 0.93 ml/min; pressure drop, 3.7 MPa; other parameters as in system I.

Anion	*Cation*	*k′ value*			*Reference to method of preparation*
		System I	*System II*	*System III*	
7,8-$C_2B_9H_{12}^-$	K^+	6.83	0.89	–	12
7,8-$C_2B_9H_{12}^-$	$N(CH_3)_4^+$	6.39	0.84	–	12
7,8-$C_2B_9H_{12}^-$	Na^+	–	0.84	–	12
5-HS-7,8-$C_2B_9H_{11}^-$	$N(CH_3)_4^+$	–	1.05	–	13
5-iso-C_3H_7-7,8-$C_2B_9H_{11}^-$	Cs^+	16.8	1.49	–	13
5-I-7,8-$C_2B_9H_{11}^-$	$N(CH_3)_4^+$	10.4	1.16	–	13
5-Cl-7,8-$C_2B_9H_{11}^-$	K^+	–	1.08	–	13
9-Cl-7,8-$C_2B_9H_{11}^-$	$N(CH_3)_4^+$	–	1.03	–	13
9-I-7,8-$C_2B_9H_{11}^-$	$N(CH_3)_4^+$	13.1	1.22	–	13
9-OH-7,8-$C_2B_9H_{11}^-$	$N(CH_3)_4^+$	12.1	1.32	–	13
5,6-I_2-7,8-$C_2B_9H_{10}^-$	$N(CH_3)_4^+$	16.1	1.54	–	13
5,6-Cl_2-7,8-$C_2B_9H_{10}^-$	Na^+	–	1.11	–	13
9,11-I_2-7,8-$C_2B_9H_{10}^-$	$N(CH_3)_4^+$	–	2.03	–	13
9,11-Cl_2-7,8-$C_2B_9H_{10}^-$	$N(CH_3)_4^+$	–	1.22	–	13
7,9-$C_2B_9H_{12}^-$	Cs^+	7.00	0.86	–	14
10-OH-7,9-$C_2B_9H_{11}^-$	$N(CH_3)_4^+$	2.87	0.48	–	13
10-CH_3O-7,9-$C_2B_9H_{11}^-$	$N(CH_3)_4^+$	6.35	0.89	–	13
$CB_{10}H_{13}^-$	Cs^+	6.65	0.84	–	15
$CB_9H_{12}^-$	$N(CH_3)_4^+$	6.91	0.87	–	16
(1,2-$C_2B_9H_{11})_2Co^-$	Na^+	–	2.95	–	17
(1,2-$C_2B_9H_{11})_2Co^-$	Cs^+	–	2.89	0.21	
8,8′-S-(1,2-$C_2B_9H_{10})_2Co^-$	$N(CH_3)_4^+$	–	2.03	0.28	18
8,8′-C_6H_4-(1,2-$C_2B_9H_{10})_2Co^-$	Cs^+	–	2.62	0.29	18
(1,2-$C_2B_9H_{11})_2Fe^-$	Cs^+	–	4.08	–	17
(1,2-$C_2B_9H_{11})_2Fe^-$	$N(C_4H_9)_4^+$	–	4.05	0.50	17
(1,2-$C_2B_9H_{10}I)_2Co^-$	Cs^+	–	22.8	1.83	13
(1,2-$C_2B_9H_{11})_2Ni^-$	Cs^+	–	4.68	–	17

(obtained by sorting with Alpine MZR air classifier) was used. The sorbent was treated in toluene with trimethylchlorosilane–hexamethyldisilazane mixture in the usual manner[19]. The prepared material contained 5.84% C and exhibited a capacity ratio (k') of 0.23 for nitrobenzene in heptane and negative methyl red test[20]. Separon SI C_1 and Separon SI C_{18} (Laboratorni Přistroje, Prague, Czechoslovakia) are sorbents based on spherical silica with average particle size 10 μm, and with carbon content 6 and 20% C, and specific surface area 200 and 300 m^2/g, respectively. Both exhibit k' values less then 0.6 for nitrobenzene in heptane and negative methyl red test.

The columns were prepared by the slurry packing technique using an apparatus described by Coq *et al.*[21]. Equilibration of the eluent with the column packing was relatively rapid; 30 column volumes were sufficient to achieve constant k' values. The system was washed with a minimum of 50 column volumes of pure methanol prior to leaving out of operation. The capacity factor values k' were calculated in a usual way from triplicate measurements of retention times, and the hold-up time was determined by the method of Bristow and Knox[22] from baseline disturbance of the UV trace. Fresh 0.1–0.5% solutions of borane salts in methanol–water mixture corresponding to the given eluent composition were used; for sparingly soluble samples the pure methanolic solution was preferred.

RESULTS AND DISCUSSION

The chromatographic conditions used and k' data for heteroborane anions are summarized in Table I. Figs. 1–4 show some typical separations of borane anion mixtures and demonstrate the separation power of the method. Heteroborane anions are relatively strongly retained on hydrocarbonaceous bonded phase, and a methylated silica gel C_1-bonded phase was preferred to the more conventional C_{18}-bonded phase to lower the required methanol content and improve peak shapes for strongly

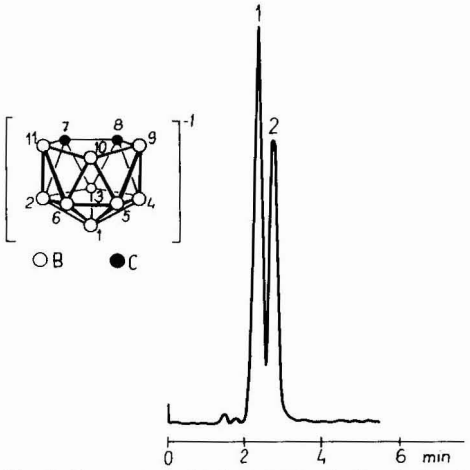


Fig. 1. Separation of 7,8-$C_2B_9H_{12}^-$ iodine derivatives: 1, 5-I-7,8-$C_2B_9H_{11}^-$; 2, 5,6-I_2-7,8-$C_2B_9H_{10}^-$. Hydrogen atoms are omitted from the structural formula. Operating conditions: sorbent, C_1-BP (14 μm); column, 150 × 3.8 mm; eluent, $2.7 \cdot 10^{-3}$ *M* *n*-$C_{12}H_{25}NH_2 \cdot HCl$ in methanol–water (7:3); flow-rate, 1.09 ml/min; pressure drop, 5.3 MPa; detector, UV 254 nm.

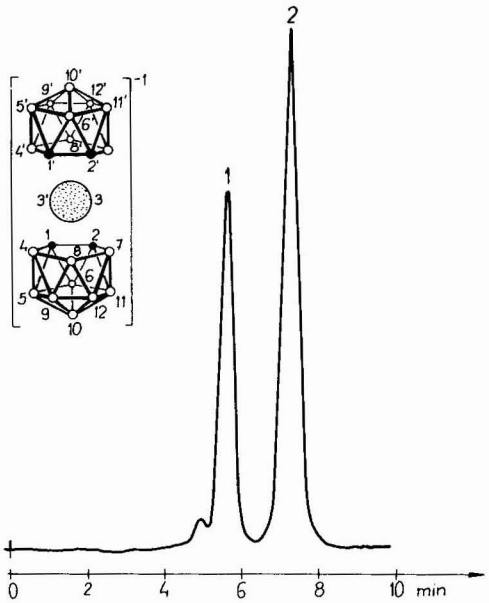


Fig. 2. Separation of sandwich complexes $(1,2\text{-}C_2B_9H_{11})_2M^-$, where M = Co (1) or Fe (2). Operating conditions: flow-rate, 0.84 ml/min; pressure drop, 4.0 MPa; other conditions as in Fig. 1.

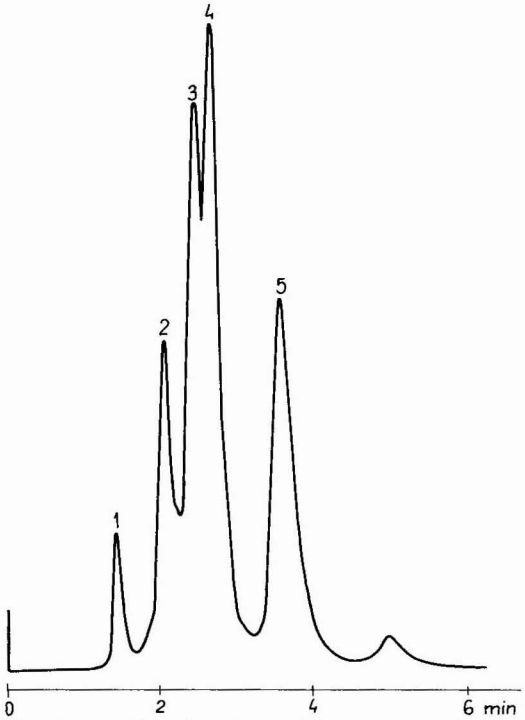


Fig. 3. Separation of a mixture of borane anions. 1 = Unknown; 2 = $7,8\text{-}C_2B_9H_{12}^-$; 3 = $5\text{-I-}7,8\text{-}C_2B_9H_{11}^-$; 4 = $9\text{-I-}7,8\text{-}C_2B_9H_{11}^-$; 5 = $9,11\text{-}I_2\text{-}7,8\text{-}C_2B_9H_{10}^-$. Operating conditions: sorbent Separon SI C_1 (10 μm); column, 200 × 3.8 mm; eluent, $2.7 \cdot 10^{-3}$ *M* $n\text{-}C_{12}H_{25}NH_2 \cdot HCl$, 0.02 *M* $NaClO_4$ in methanol–water (6:4); flow-rate, 1.15 ml/min; pressure drop, 8.5 MPa; detector, UV 254 nm.

retained solutes. Column efficiencies of 6000 theoretical plates per metre were achieved for ionic compounds, as compared with 11,500 theoretical plates per metre for the non-ionic test substance anisole on the C_1-BP sorbent. The spherical commercial sorbent Separon SI C_1 exhibited the same selectivity, the retention ratios being *ca.* 50% of the value obtained with irregular C_1-BP material.

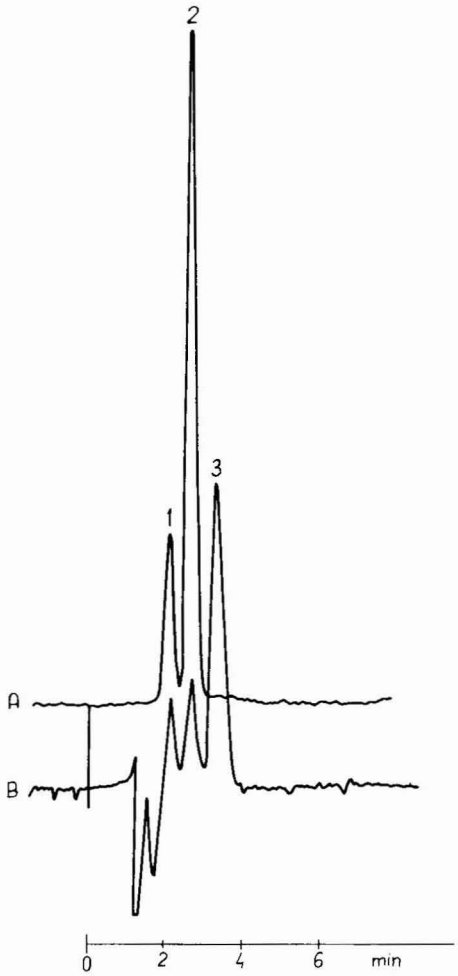


Fig. 4. Separation of non-ionic *o*-carborane from borane anion reaction products. 1 = 7,8-$C_2B_9H_{12}^-$; 2 = 9-OH-7,8-$C_2B_9H_{11}^-$; 3 = 1,2-$C_2B_{10}H_{12}$ (*o*-carborane). Operating conditions: detection A, UV 254 nm; B, refractive index; other conditions as in Fig. 2.

All borane anions studied exhibit absorption in the low UV region. For the $C_2B_9H_{12}^-$ anion and its derivatives, the absorption maximum lies below 240 nm and, when working with a variable-wavelength UV detector, the lowest available value (235 nm) was used. Sandwich heteroborane complexes exhibit absorption maxima near 280 nm. For comparison, Table II presents the minimum detectable amount of solute that causes a detector signal of double the noise intensity. However, the gain in signal intensity of the single beam variable-wavelength detector is partially de-

TABLE II

MINIMUM DETECTABLE AMOUNTS (IN μg) OF SOME BORANE ANIONS

For chromatographic conditions see Table I, system II.

Species	*UV detection wavelength*		
	*254 nm**	*280 nm***	*235 nm***
7,8-$C_2B_9H_{12}K$	0.250	–	0.105
5-iso-C_3H_7-7,8-$C_2B_9H_{11}Cs$	0.580	–	0.360
5-I-7,8-$C_2B_9H_{11}N(CH_3)_4$	0.120	–	0.167
9-I-7,8-$C_2B_9H_{11}N(CH_3)_4$	0.093	–	0.065
10-OH-7,9-$C_2B_9H_{11}N(CH_3)_4$	0.370	–	0.096
(1,2-$C_2B_9H_{11})_2CoCs$	0.030 0.139**	0.036	–
(1,2-$C_2B_9H_{11})_2FeN(C_4H_9)_4$	0.062	0.130	–
8,8′-S(1,2-$C_2B_9H_{10})_2CoN(CH_3)_4$	0.100	0.095	–
8,8′-C_6H_4(1,2-$C_2B_9H_{10})_2CoCs$	0.068	0.083	–

* Fixed-wavelength detector.
** UVM detector.

preciated by its relatively high noise level. But even with this instrumentation, including very short column, sufficient sensitivity can be routinely achieved.

For some borane anions under certain but different conditions peak splitting was observed. Similar behaviour was described in the reversed-phase ion-pair separation of benzenesulphonic acids[23] and the phenanthroline complex of ruthenium[10]. The phenomenon, which is not due to bad column performance, can be easily identified by changing the amount of sample injected, as seen in Fig. 5. Not all the principles governing the observed peak splitting are clear, but from the experiments it follows that the main factors determining the extent of peak splitting are the ratio of the concentrations of the solute and the counter ion, the composition of the mobile phase and the chemical nature of the solute.

To gain insight in chromatographic factors in ion-pair chromatography that can be used to optimize the separation process, we studied the effect of the concentra-

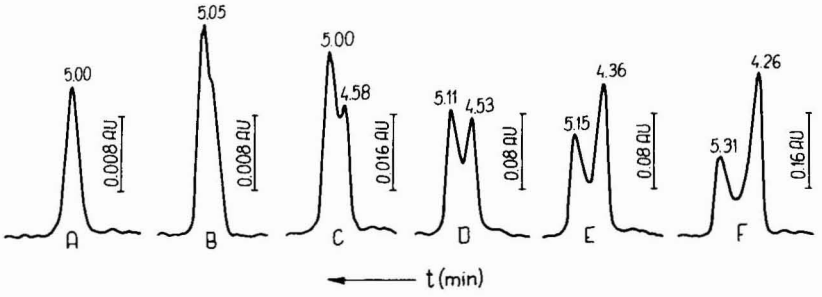


Fig. 5. Effect of a sample amount on splitting of the 9,11-I_2-7,8-$C_2B_9H_{10}^-$ peak. Sample size: A, 11.4 μg; B, 28.5 μg; C, 57 μg; D, 136.8 μg; E, 285 μg; F, 570 μg. Operating conditions: sorbent C_1-BP (14 μm); column, 300 × 3.8 mm; eluent, $2.7 \cdot 10^{-3}$ *M* n-$C_{12}H_{25} \cdot HCl$, 0.02 *M* $NaClO_4$ in methanol–water (65:35); flow-rate, 1.26 ml/min, pressure drop, 7.8 MPa; detector, UV 254 nm. The numbers above the peaks denote corresponding k' values.

tion of the pairing ion (Fig. 6) and methanol content in mobile phase (Fig. 7) on retention behaviour of borane solutes. In addition, the effect of the inert salt concentration, the pH of mobile phase (Table III) and the effect of the lengths of the pairing-ion chain and the bonded-phase hydrocarbon chain were examined.

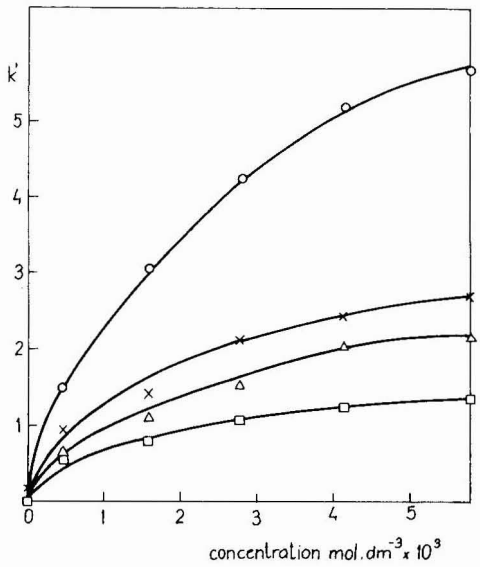


Fig. 6. Effect of the concentration of the pairing agent (laurylamine hydrochloride) on borane anion capacity ratios: △, 5-I-7,8-$C_2B_9H_{11}^-$; ×, 5-iso-C_3H_7-7,8-$C_2B_9H_{11}^-$; □, 7,8-$C_2B_9H_{12}^-$; ○, (1,2-$C_2B_9H_{11}$)$_2Co^-$. Operating conditions: sorbent, C_1-BP (14 μm); column, 150 × 3.8 mm; eluent, methanol–water (7:3); flow-rate, 1.35–1.40 ml/min; pressure drop, 6.5–7.0 MPa; detector, UV 254 nm.

Fig. 6 shows that the addition of pairing ion sharply increases the retention of borane anions which are practically not retained in methanol–water solvent system. Further increase in pairing-ion concentration increases retention without any change in elution order. The retention of borane cage anions is very sensitive to the amount of methanol in the mobile phase, as seen from Fig. 7. The observed phenomena, which are consistent with observations on other ion-pair reversed-phase systems[24], can be rationalized in terms of solvophobic theory[25,26]. Despite the fact that the exact mechanism for ion-pair chromatography has not been clearly established to date, a model involving the formation of ion pairs in the mobile phase, followed by distribution of the ion peaks to the hydrophobic stationary phase, is the easiest framework for rationalizing the observed phenomena. Thus, the decrease in methanol concentration increases the surface tension of methanol–water eluent and increases the repulsive interaction of hydrophobic ion pairs with the eluent, resulting in increased k' values. As seen from Table III, the addition of neutral salt decreases the k' values of borane anions. Added salt can compete in forming ion pairs with the pairing ion, thus decreasing the thermodynamic activity of the pairing ion. The observed decrease in retention is dependent also on the chemical nature of the anion added. The perchlorate anion is known to be a good ion-pairing agent, and its effect is thus stronger than that of the sulphate anion.

The conjugated acids of the borane anions under study are generally strong

TABLE III

EFFECT OF ACIDITY OF ELUENT AND INERT SALT CONCENTRATION ON CAPACITY RATIOS OF SOME BORANE ANIONS

Chromatographic conditions: sorbent, Separon SI C_1 (10 μm); column, 200 × 3.8 mm; eluent, $2.7 \cdot 10^{-3}$ *M* n-$C_{12}H_{25}NH_2 \cdot HCl$ in methanol–water (6:4); flow-rate, 1.25 ml/min; detector, UV 254 nm.

Species	*k′ value*				
		pH 6.8	*pH 3.6*	*pH 5.6*	
	$c_{H_2SO_4}$ *(M)*				
	0.000	*0.000*	*0.002*	*0.000*	*0.000*
	$c_{inert\ salt}$ *(M)*				
	0.000	*0.020 (Na_2SO_4)*	*0.020 (Na_2SO_4)*	*0.020 ($NaClO_4$)*	*0.040 ($NaClO_4$)*
7,8-$C_2B_9H_{12}K$	2.29	0.95	1.03	0.33	0.36
5-iso-C_3H_7-7,8-$C_2B_9H_{11}Cs$	4.83	2.02	2.24	0.77	0.85
5-I-7,8-$C_2B_9H_{11}N(CH_3)_4$	3.82	1.62	1.75	0.59	0.61
9-I-7,8-$C_2B_9H_{11}N(CH_3)_4$	4.32	1.93	2.03	0.71	0.73
5-Cl-7,8-$C_2B_9H_{11}K$	2.64	1.16	1.22	0.40	0.44
9-Cl-7,8-$C_2B_9H_{11}N(CH_3)_4$	2.96	1.29	1.37	0.47	0.53
$(1,2\text{-}C_2B_9H_{11})_2CoCs$	12.00	6.24	6.63	2.10	2.22

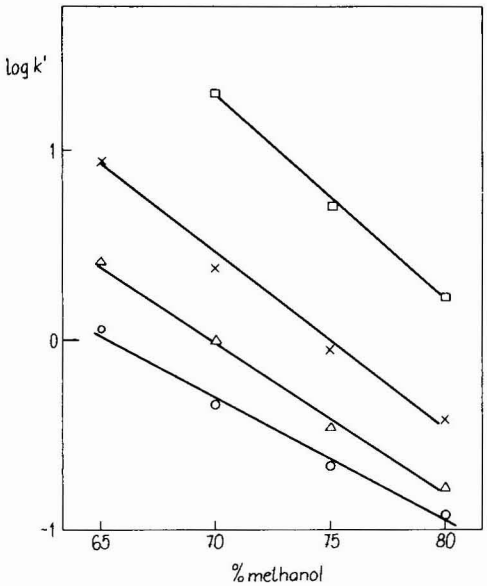


Fig. 7. Relationship between log k' and the amount of methanol in the mobile phase for some borane anions: ○, 7,8-$C_2B_9H_{12}^-$; ×, $(1,2\text{-}C_2B_9H_{11})_2Co^-$; □, $(1,2\text{-}C_2B_9H_{10}I)_2Co^-$; △, 9-I-7,8-$C_2B_9H_{11}^-$. Operating conditions: sorbent, C_1-BP (14 μm); column, 300 × 3.8 mm; eluents $2.7 \cdot 10^{-3}$ *M* n-$C_{12}H_{25}NH_2 \cdot HCl$ and 0.02 *M* $NaClO_4$ in methanol–water; flow-rate, 1.25–1.30 ml/min; pressure drop, 6.3–7.8 MPa; detector, UV 254 nm.

acids in a Brönsted sense, *e.g.* 7,8-$C_2B_9H_{13}$ can be titrated in methanol–water (1:2) solution[12] with apparent pK_a of 2.95. As might be expected, the influence of the acidity of the eluent on k' values was minimal, as demonstrated in Table III. The reproducible k' values of borane anions and symmetrical peaks can be obtained without using complicated buffer system.

Table IV shows the influence of changing the lengths of the pairing-ion chain and bonded-phase hydrocarbon chain on the retention and selectivity of some borane anions. The group contribution term, τ, is defined[25] as $\tau = \log k'_j/k'_i$, where k' are capacity ratios of solutes j and i which differ by a functional group. In this present study, the reference solute i was taken to be the unsubstituted 7,8-$C_2B_9H_{12}^-$ anion, and the sandwich complexes are formally considered as derivatives of this anion. From the results it follows that a decrease in the length of alkyl chain of the counter ion produces a corresponding decrease in the retention of a given solute, but the elution order of solutes remains unchanged. The order of the retention power of the stationary phases, $C_{18} > C_1$, can be related to the carbon loading of the support. However, the higher carbon content of the C_{18} sorbent does not bring about a corresponding increase in selectivity. To obtain a reasonable retention, mobile phases of different polarity were used. The linear relationship between τ and the surface

TABLE IV

FUNCTIONAL BEHAVIOUR OF SOME 7,8-$C_2B_9H_{12}^-$ SUBSTITUTION DERIVATIVES DETERMINED USING VARIOUS ALKYLSILICA STATIONARY PHASES AND VARIOUS PAIRING IONS

Column 150 × 3.8 mm; methanol–water eluent; pairing ion concentration, $2.70 \cdot 10^{-3}$ *M*; flow-rate, 1.20 ml/min. 7,8-$C_2B_9H_{12}^-$ structure drawing: Fig. 1.

	Sorbent					
	C_1-BP		*C_1-BP*		*Separon SI C_{18}*	
	Pairing agent					
	n-$C_{12}H_{25}NH_2$		*n-$C_6H_{13}NH_2$*		*n-$C_{12}H_{25}NH_2$*	
	Methanol concentration (%) (v/v)					
	70		*60*		*75*	
Substituent	k'	τ	k'	τ	k'	τ
H	0.89	0.00	0.72	0.00	0.89	0.00
5-Cl	1.08	0.08	0.92	0.11	1.14	0.11
5-I	1.16	0.11	1.08	0.18	1.28	0.16
5-iso-C_3H_7	1.49	0.22	1.62	0.35	1.56	0.24
5-HS	1.05	0.07	0.89	0.09	–	–
9-I	1.22	0.14	1.33	0.27	1.44	0.21
9-Cl	1.03	0.06	0.97	0.13	1.06	0.08
9-OH	1.32	0.17	1.47	0.31	1.44	0.21
9,11-I_2	2.03	0.36	2.94	0.61	2.46	0.44
9,11-Cl_2	1.22	0.14	1.25	0.24	1.28	0.16
3-$C_2B_9H_{11}Fe^-$	4.05	0.66	8.86	1.09	5.14	0.76
3-$C_2B_9H_{11}Co^-$	2.89	0.51	5.53	0.89	3.73	0.62

tension of the eluent was demonstrated by Riley *et al.*[26], and for the C_1 bonded phase–hexylamine counter-ion system the effect of the more polar eluent with higher surface tension dominates. Thus, for a given class of compounds, the C_1-bonded phase seems to be more useful than the C_{18}-bonded phase in terms of selectivity.

When we try to discuss the relationship between the chemical nature of the compounds under study and their retention behaviour, we are handicapped by the fact that, for these compounds, only a few physical data are available. Despite this fact, two general trends are obvious. As the surface area of the molecule increases so the retention increases, which is demonstrated by high positive τ values of sandwich complexes and $C_2B_9H_{12}^-$ iodine derivatives (see Table IV). Substitution of hydrogen in 7,9-$C_2B_9H_{12}^-$ by a more polar group leads, for 10-OH and 10-CH_3O groups, to a decrease of retention and, for 5-HS group in the 7,8-$C_2B_9H_{12}^-$ skeleton, to only a small increase in retention in comparison with the retention of the parent anion. The k' values of 9-OH-7,8-$C_2B_9H_{12}^-$ are higher than one might expect on the basis of the ideas given above.

CONCLUSION

Ion-pair liquid chromatography with *n*-alkylamine pairing ions, C_1-bonded phase on silica and a methanol–water eluent system presents a powerful and relatively simple analytical method for separating heteroborane anions. The methanol–water ratio, the *n*-alkylamine chain length, and the concentrations of the pairing ion and the inert salt can be varied, to achieve the required separation for the broad range of borane anions. The separation of non-ionic heteroboranes and heteroborane anions can be performed in a single analysis. The great separation efficiency allows the routine separation of structurally closely related species, including geometrical isomers, on a sub-microgram scale.

ACKNOWLEDGEMENTS

The authors are grateful to Drs. K. Baše and Z. Janoušek for providing some of the compounds used in this study, Mrs. H. Časenská for technical help and Dr. M. Tobola (Institute of Mineral Raw Materials, Kutná Hora, Czechoslovakia) for sorting the silica gel.

REFERENCES

1 H. Hatanaka and K. Sano, *Z. Neurol.*, 204 (1973) 309.
2 M. Kyrš, S. Heřmánek, J. Rais and J. Plešek, *Czech. Pat.*, 182,913 (1980); *C.A.*, 94 (1981) 128481.
3 J. Rais, P. Selucký and M. Kyrš, *J. Inorg. Nucl. Chem.*, 38 (1976) 1376.
4 M. F. Hawthorne and T. E. Paxon, *J. Amer. Chem. Soc.*, 96 (1974) 4674.
5 G. R. Wellum, E. I. Tolpin, L. P. Andersen and R. Sneath, *J. Chromatogr.*, 103 (1975) 153.
6 E. Tomlinson, T. M. Jefferies and C. M. Riley, *J. Chromatogr.*, 159 (1978) 315.
7 R. Gloor and E. L. Johnson, *J. Chromatogr. Sci.*, 15 (1977) 413.
8 G. Schwedt, *Chromatographia*, 12 (1979) 613.
9 R. N. Reeve, *J. Chromatogr.*, 77 (1979) 393.
10 J. W. O'Laughlin and R. S. Hanson, *Anal. Chem.*, 52 (1980) 2263.
11 Z. Plzák and B. Štíbr, *J. Chromatogr.*, 151 (1978) 363.
12 R. A. Wiesboeck and M. F. Hawthorne, *J. Amer. Chem. Soc.*, 86 (1964) 1642.

13 J. Plešek, Z. Janoušek and B. Štíbr, in preparation.
14 M. F. Hawthorne, D. C. Young, P. M. Garrett, D. A. Owen, S. G. Schwerin, F. N. Tebbe and P. A. Wegner, *J. Amer. Chem. Soc.*, 90 (1968) 862.
15 D. E. Hyatt, F. R. Scholer, L. J. Todd and J. L. Warmer, *Inorg. Chem.*, 6 (1967) 2229.
16 B. Štíbr, K. Baše, S. Heřmánek and J. Plešek, *J. Chem. Soc. Chem. Commun.*, (1976) 150.
17 M. F. Hawthorne, D. C. Young, T. D. Andrews, D. V. Hove, R. L. Pilling, A. D. Pitts, M. Reintjes, L. F. Warren and P. A. Wegner, *J. Amer. Chem. Soc.*, 90 (1968) 879.
18 J. Plešek and S. Heřmánek, *Collect. Czech. Chem. Commun.*, 43 (1978) 1325.
19 J. J. Kirkland and J. J. de Stefano, *J. Chromatogr. Sci.*, 8 (1970) 309.
20 K. Karch, I. Sebestian and I. Halász, *J. Chromatogr.*, 122 (1976) 3.
21 B. Coq, C. Gonnet and J.-L. Rocca, *J. Chromatogr.*, 106 (1975) 249.
22 P. A. Bristow and J. H. Knox, *Chromatographia*, 10 (1977) 279.
23 H. U. Ehmcke, H. Kelker, K. H. König and H. Ullner, *Z. Anal. Chem.*, 294 (1979) 251.
24 B. A. Bidlingmeyer, *J. Chromatogr. Sci.*, 18 (1980) 525.
25 Cs. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129.
26 C. M. Riley, E. Tomlinson and T. M. Jefferies, *J. Chromatogr.*, 185 (1979) 197.

Journal of Chromatography, 212 (1981) 295–304
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,836

SEPARATION OF CYTOCHROMES *c* BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

SHIGERU TERABE*, HIROYUKI NISHI and TEIICHI ANDO
Department of Industrial Chemistry, Faculty of Engineering, Kyoto University, Sakyo-ku, Kyoto 606 (Japan)
(Received April 1st, 1981)

SUMMARY

Six kinds of cytochrome *c* of different origin, *i.e.*, bovine, chicken, dog, horse, rabbit and tuna, were subjected to separation by reversed-phase high-performance liquid chromatography on three commercial packing materials; octadecyl-, octyl- and cyanoalkyl-silicas. The effects of reversed-phase material, mobile phase and temperature on the separation of cytochromes *c* were examined. The parameters of the mobile phase were the organic modifier, the pH, the salt concentration and additives. Under optimal conditions, five of the six cytochromes *c* were resolved in 10 min. The relative retention values cannot be explained in terms of the relative lipophilicities of the side-chains of the amino acid residues.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) is now a well-established method for the separation of native and synthetic peptides[1,2]. Several papers[3–12] have described the success of RP-HPLC in separating closely related peptides. Some detailed investigations[4,6,13–16] have been reported on the experimental conditions and parameters affecting the separation of such peptides. On the other hand, recent advances in aqueous gel permeation chromatography have permitted rapid analysis of a mixture of proteins of a wide range of molecular weights by size exclusion[2,17–19]. However, proteins of comparable sizes cannot be separated by this method.

We have already shown[8–10] that RP-HPLC is extremely useful for the separation of peptides having closely related structures, such as those that differ only in (i) the number of constituent amino acid residues by one or more, (ii) the kind of amino acid residues, including optical isomers, (iii) the sequence of amino acid residues, or (iv) the structure of the acyl groups attached to the N-terminal groups. The molecular weights of the peptides in our previous work[8] are below 6000.

In the present study, cytochromes *c* were chosen as a group of closely related proteins in order to explore the possibility of the fine separation of larger peptides by RP-HPLC. Cytochrome *c* is a hemoprotein found in the cells of all aerobic or-

0021-9673/81/0000–0000/$02.50

TABLE I

DIFFERENCES IN AMINO ACID SEQUENCES IN SIX CYTOCHROMES c[20]

The differences between the sequence of amino acid residues are indicated by italics.

	Position																				
Source	*4*	*9*	*15*	*22*	*28*	*33*	*44*	*46*	*47*	*54*	*58*	*60*	*61*	*62*	*88*	*89*	*92*	*95*	*100*	*103*	*104*
Bovine	Glu	Ile	Ala	Lys	Thr	His	Pro	Phe	Ser	Asn	Thr	Gly	Glu	Glu	Lys	Gly	Glu	Ile	Lys	Asn	Glu
Horse	Glu	Ile	Ala	Lys	Thr	His	Pro	Phe	*Thr*	Asn	Thr	*Lys*	Glu	Glu	Lys	*Thr*	Glu	Ile	Lys	Asn	Glu
Dog	Glu	Ile	Ala	Lys	Thr	His	Pro	Phe	Ser	Asn	Thr	Gly	Glu	Glu	*Thr*	Gly	*Ala*	Ile	Lys	Asn	Glu
Rabbit	Glu	Ile	Ala	Lys	Thr	His	*Val*	Phe	Ser	Asn	Thr	Gly	Glu	*Asn*	Lys	*Asp*	*Ala*	Ile	Lys	Asn	Glu
Chicken	Glu	Ile	*Ser*	Lys	Thr	His	*Glu*	Phe	Ser	Asn	Thr	Gly	Glu	*Asp*	Lys	*Ser*	*Val*	Ile	*Asp*	*Ser*	*Lys*
Tuna	*Ala*	*Thr*	Ala	*Asn*	*Val*	*Trp*	*Glu*	*Tyr*	Ser	*Ser*	*Val*	*Asn*	*Asn*	*Asp*	Lys	Gly	Gln	*Val*	*Ser*	*Ser*	–

ganisms and consists of a single polypeptide chain of *ca.* 104 amino acid residues with the heme group attached through cysteine residues at positions 14 and 17. Studies were made on the separation of five mammalian cytochromes *c* whose sequences differ by between two and eight out of 104 amino acid residues, and one from tuna which is appreciably different from the mammalian proteins, for the purpose of examining the effects of experimental parameters and conditions.

EXPERIMENTAL

Reagents and materials

All six cytochromes *c* investigated were purchased from Sigma (St. Louis, MO, U.S.A.) and used without further purification. The differences between them in the sequences of amino acid residues are listed in Table I[20]. Acetonitrile (Wako, Osaka, Japan) and methanol (Nakarai, Kyoto, Japan) were of HPLC quality, and the other chemicals used were of reagent grade. Antioxidant- and peroxide-free tetrahydrofuran (THF) was obtained by passing commercial THF through an active aluminium oxide column. Water was purified by means of an ion-exchange column followed by single distillation in glassware.

Apparatus

A Perkin-Elmer Series 2/2 liquid chromatograph equipped with a Rheodyne 7105 sample injector was used in conjunction with a Perkin-Elmer LC-65T UV detector/oven. As packing materials, Nucleosil $7C_{18}$, Nucleosil $7C_8$ or Nucleosil 5CN (Macherey, Nagel & Co., Düren, G.F.R.) were used. The columns (10 cm × 4.6 mm I.D.) were packed by the modified viscosity method recommended by the manufacturer, using a Chemco slurry-packing apparatus Model 124 (Chemco, Osaka, Japan) at *ca.* 500 kg/cm^2.

Procedure

Cytochrome *c* was dissolved in distilled water to give a concentration of 1 mg/ml. The sample solutions were kept at −20°C when not in use. Amounts of sample injected were usually of the order of micrograms.

Experiments were carried out at room temperature unless otherwise stated. The flow-rate was 1.0 or 2.0 ml/min. The detector was operated at 210, 220 or 400 nm. The mobile phase was filtered through a 0.7-μm membrane filter and degassed prior to use. The recovery of cytochrome *c* eluted from the column was measured by the method of Lowry *et al.*[21].

RESULTS AND DISCUSSION

Reversed-phase materials

Three kinds of commercial packing material, *i.e.* silica gel with chemically bonded octadecyl (Nucleosil $7C_{18}$), octyl (Nucleosil $7C_8$) and nitrile (Nucleosil 5CN) groups, were used. The order of elution of the cytochromes *c* investigated on the C_{18} column under the conditions indicated in Fig. 1 was as follows: horse > rabbit > bovine = chicken = tuna > dog. Bovine, chicken and tuna cytochromes *c* could not be resolved on the C_{18} column although various conditions were examined.

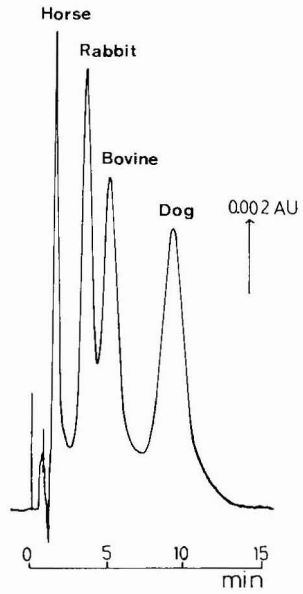


Fig. 1. Separation of cytochromes *c* on a C_{18} column. Conditions: mobile phase, mixture of 69% of 0.005 *M* phosphate buffer (pH 3.0) containing 0.1 *M* sodium sulphate, and 31% of acetonitrile; flow-rate, 2.0 ml/min; detection, absorption at 210 nm; sample size, total 6 μg in the ratio of 3:3:6:8 in the order of elution.

Five kinds of cytochromes *c* were successfully separated on the C_8 column as shown in Fig. 2, but bovine and chicken were again not separable. Cytochrome *c* from tuna was eluted between bovine and dog, the order of elution of the other cytochromes *c* being the same as for the C_{18} column. The content of acetonitrile in the

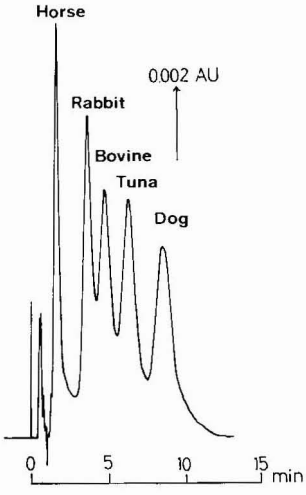


Fig. 2. Separation of cytochromes *c* on a C_8 column. Contitions: mobile phase, mixture of 72.5% of 0.005 *M* phosphate buffer (pH 3.0) containing 0.1 *M* sodium sulphate, and 27.5% of acetonitrile; flow-rate, 2.0 ml/min; detection, absorption at 220 nm; sample size, total 11 μg (2:2:2:2:3).

mobile phase was reduced to 27.5% on the C_8 column to obtain suitable retention times.

The order of elution on the CN column (Fig. 3A) was as follows: horse > rabbit = bovine = chicken > dog > tuna. It is noticeable that the order of elution of dog and tuna on the CN column was the reverse of that observed on the C_{18} and C_8 columns. The mobile phase containing a higher concentration of phosphate buffer (0.1 *M*) gave better resolution on the CN column than that employed for the C_{18} and C_8 columns.

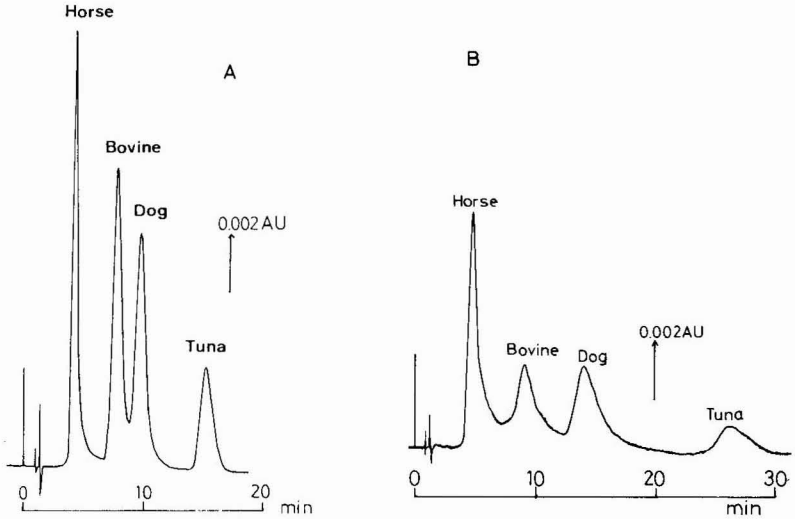


Fig. 3. Separation of cytochromes *c* on a CN column at different pH values. Conditions: mobile phase, 77.5% of 0.1 *M* phosphate buffer (A, pH 2.0; B, pH 3.0) containing 0.05 *M* sodium sulphate and 22.5% of acetonitrile; flow-rate, 1.0 ml/min; detection, absorption at 400 nm; sample size, total 10 μg (1:1:1:1).

A few initial injections of cytochrome *c* gave no appreciable peaks until the total injections amounted to *ca.* 10 μg, when the newly packed columns were used. Moreover, initially recorded peaks were unusually broad and unsymmetrical, and several additional injections were required in order to obtain satisfactory peak shapes. This observation suggests that the packing materials used have some active sites where cytochrome *c* is irreversibly adsorbed. The recovery of cytochrome *c* from the column was more than 80%, as determined by the method of Lowry *et al.*[21], after the column was conditioned by several injections of cytochrome *c*.

The capacity factors of the six cytochromes *c* as a function of the packing the naphthalene peak eluted with the methanol–water solvent system, although the efficiency of the columns for cytochromes *c* was rather low. No significant differences in the resolution were noticed between elution on the column mentioned above and elution on more efficient columns having more than 5000 theoretical plates for naphthalene.

The capacity factors of the six cytochromes *c* as a function of the packing material are shown in Fig. 4. Bovine and chicken cytochromes *c* could not be resolved on any of the three columns, as shown in Fig. 4. Their separation can be effected on an NH_2 column (Nucleosil $5NH_2$, 15 cm × 4.6 mm I.D.) with 0.005 *M* phosphate

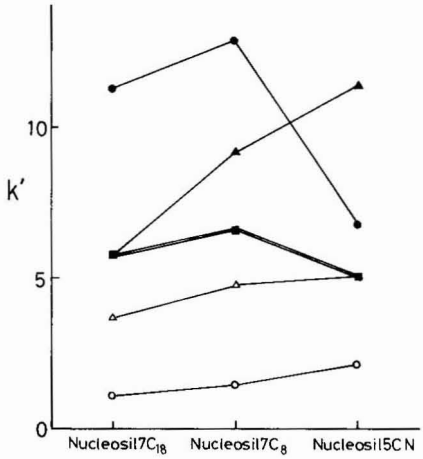


Fig. 4. Plot of capacity factor (k') of cytochrome *c* *vs.* packing material. (○) Horse, (△) rabbit, (■) bovine and chicken, (▲) tuna and (●) dog. Conditions are indicated in Figs. 1–3.

buffer (pH 6.0) containing 0.05 *M* sodium sulphate as the mobile phase, although these conditions may not be regarded as those for RP-HPLC. The order of elution was as follows: tuna > horse = chicken > rabbit = bovine = dog.

Mobile phase

Effect of organic modifier. When 30% of THF was employed instead of 31% of acetonitrile under the conditions indicated in Fig. 1, tuna cytochrome *c* was eluted last from the C_{18} column and the other cytochromes *c* were not resolved, except for horse cytochrome *c* which was eluted first. In contrast, the same order of elution was observed on the nitrile column for the following two solvent systems: one consisting of 22.5% of acetonitrile and 77.5% of 0.1 *M* phosphate buffer (pH 3.0) containing 0.005 *M* sodium sulphate, and one consisting of 40% of methanol and 60% of the buffer. A much higher content of methanol than acetonitrile was required to obtain comparable retention times. The solvent system containing methanol gave broader peaks than the acetonitrile system.

The dependence of the capacity factor on the composition of the mobile phase was investigated for four peptides of different molecular weights on the C_{18} column. The mobile phases with slightly different compositions were prepared by means of the solvent programmer of the liquid chromatograph in order to obtain reproducible compositions. The plot of the logarithm of the capacity factor against the content of acetonitrile gave straight lines with different slopes, as shown in Fig. 5. The larger the peptide molecule, the steeper the slope of the line in Fig. 5. This result is consistent with observations[6,8] that the retention times of peptides are highly dependent on the amount of the organic modifier present. In addition, it is interesting that the plot of molecular weights of peptides against the slopes of the plots in Fig. 5 gives an almost straight line.

Effect of pH. As the packing materials employed are based on silica gel, the pH of the mobile phase is limited to the range 2–8. The acidic mobile phases (pH 2–3) were preferable to the neutral in reducing peak tailings for cytochrome *c*. No signifi-

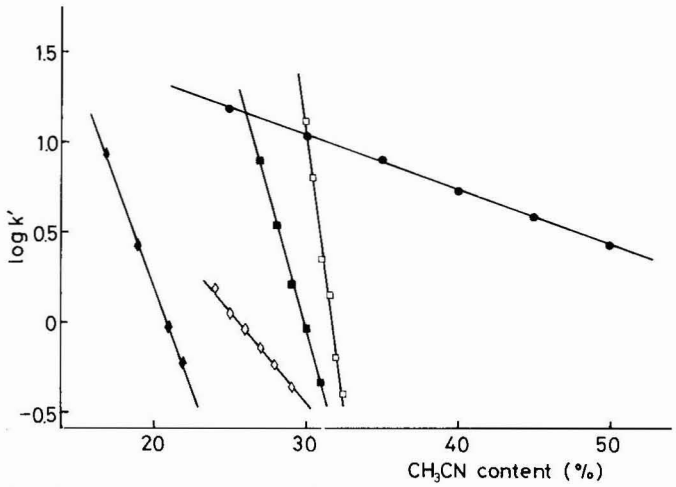


Fig. 5. Dependence of k' of cytochrome *c* on the content of acetonitrile in the mobile phase. (●) Benzene, (◇) [Gly^4,Phe^5]-LH-RH^8, (◆) ACTH-(1-26)-NH$_2$^{8}, (■) porcine insulin^8 and (□) bovine cytochrome *c*. Conditions: column, Nucleosil 7C$_{18}$ 10 cm × 4.6 mm I.D.; mobile phase, mixture of acetonitrile and 0.005 *M* phosphate buffer (pH 3.0) containing 0.1 *M* sodium sulphate; flow-rate, 1.0 ml/min.

cant differences in the chromatograms were recognized between pH 2.0 and 3.0 on the C_{18} and C_8 columns. However, alterations in retention behaviour and peak widths were noticed on the CN column between pH 2.0 and 3.0, as shown in Fig. 3.

Effect of salt concentration. The effect of the concentration of sodium sulphate was shown in Fig. 6. The capacity factor decreased with an increase in salt concentration. Although the separation was not appreciably influenced in the tested range of

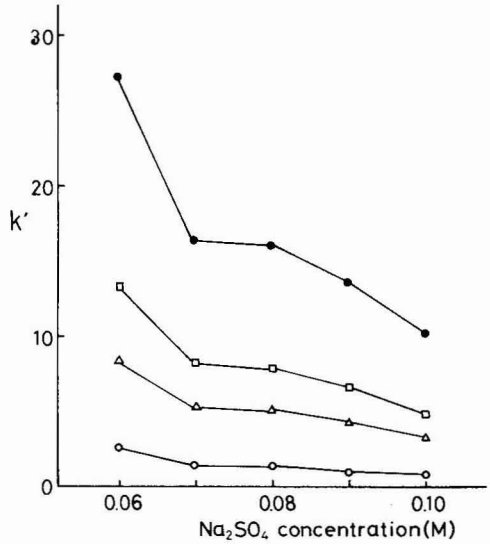


Fig. 6. Dependence of k' of cytochrome *c* on the salt concentration. (○) Horse, (△) rabbit, (□) bovine and (●) dog. Conditions are the same as shown in Fig. 1 except for the concentration of sodium sulphate.

salt concentration, the addition of some salt was found desirable in order to obtain symmetrical peaks.

Effect of additives. Capacity factors of cytochromes *c* on the C_{18} column increased with an increasing amount of EDTA disodium salt added to the mobile phase in the range 0.001–0.003 *M*, but otherwise no appreciable effect was observed. No significant change in capacity factors or peak symmetry was noticed when an ion-pairing agent, 0.005 *M* sodium 1-butanesulphonate or 0.0001–0.0004 *M* L-arginine, was added to the mobile phase.

The peak symmetry and resolution of cytochromes *c* on the C_{18} column were much improved by the addition of horse cytochrome *c* in the concentration of 3.3 mg per litre of the buffer ($2.6 \cdot 10^{-7}$ *M*), as shown in Fig. 7. The addition of cytochrome *c* in higher concentration resulted in an extra peak at the retention time of horse cytochrome *c*.

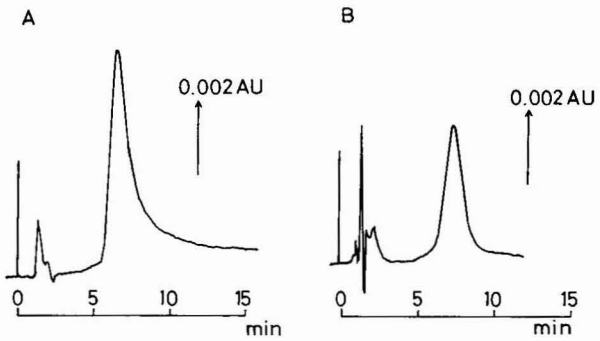


Fig. 7. Effect of cytochrome *c* added to the mobile phase. (A) No cytochrome *c* added, (B) $2.6 \cdot 10^{-7}$ *M* horse cytochrome *c* added to the buffer. Conditions: column, Nucleosil $7C_{18}$; mobile phase, mixture of 68.5% of 0.005 *M* phosphate buffer (pH 3.0) containing 0.1 *M* sodium sulphate and 31.5% of acetonitrile; flow-rate, 1.0 ml/min; detection, absorption at 220 nm; sample, bovine cytochrome *c* (A) 4 μg, (B) 2 μg.

Effect of temperature

The temperature dependence of retention in RP-HPLC is receiving increasing attention (*e.g.*, ref. 22). Plots of the logarithm of the capacity factor against the reciprocal of the temperature usually show a linear relationship, and this was in fact observed for cytochromes *c* on the CN column although the slopes of the plots were different for different cytochromes *c*. On the C_{18} column, however, no such dependence was obtained, as shown in Fig. 8. The reason for this observation is not clear. Increasing temperature was less effective for improving the separation of cytochrome *c* both on the CN and the C_{18} columns.

Relative order of elution of cytochrome c

The separation of closely related peptides by RP-HPLC has generally been explained in terms of the difference in hydrophobicity of the side-chains of the constituent amino acid residues[8,14,23]. This interpretation is invalid in some instances, however, and conformational and hydrophobic properties of the whole molecule and its size should be taken into account[8,14,16,23].

All the amino acid residues different among five cytochromes *c* shown in Table

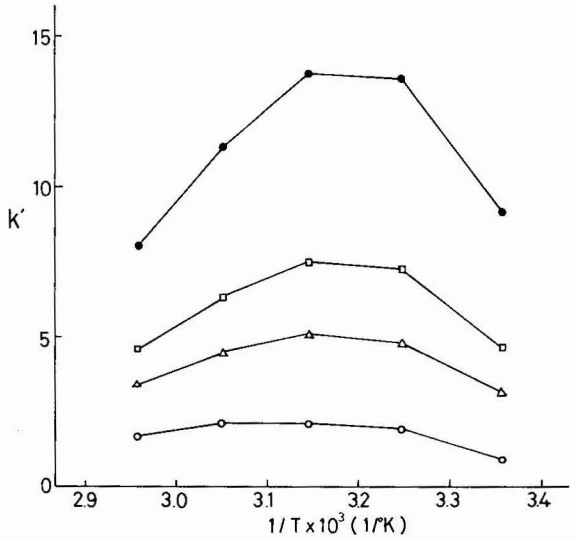


Fig. 8. Dependence of k' of cytochrome *c* on the temperature. (○) Horse, (△) rabbit, (□) bovine and (●) dog. Conditions: column, Nucleosil $7C_{18}$; mobile phase, mixture of 69 % of 0.005 *M* phosphate buffer (pH 3.0) containing 0.1 *M* sodium sulphate and 31 % of acetonitrile.

I (except for tuna) are situated on the outside of the molecule[24]. This means that the side-chains of these amino acid residues can interact with the stationary phase and, therefore, the alteration of the amino acid residue at these positions can affect the hydrophobic interaction of cytochrome *c* with the hydrocarbonaceous stationary phase.

The relative retention values of the six cytochromes *c* do not correspond with the relative hydrophobicities calculated from the relative lipophilicities[25] of the side-chains of amino acid residues at the structurally variable positions. This suggests that the minor conformational change of the molecule supposedly produced by the alteration of the amino acid residue is more dominant in determining the retention of cytochrome *c* than the change in hydrophobicity of the side-chain at the relevant position.

CONCLUSION

Five kinds of cytochrome *c*, *i.e.*, bovine, dog, horse, rabbit and tuna, have been separated by employing appropriate reversed-phase, chromatographic conditions. However, the efficiency of the columns was much lower than for small molecules. This low efficiency can be attributed partly to the low diffusitivity of cytochrome *c* in the mobile phase and partly to the irreversible adsorption of cytochrome *c* on the stationary phase. It is desirable to develop new packing materials for the separation of proteins by RP-HPLC.

REFERENCES

1 K. Krummen, *J. Liquid Chromatogr.*, 3 (1980) 1243.
2 F. E. Regnier and K. M. Gooding, *Anal. Biochem.*, 103 (1980) 1.
3 K. Tsuji and J. H. Robertson, *J. Chromatogr.*, 112 (1975) 663.
4 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 27.
5 K. S. Axelsen and S. H. Vogelsang, *J. Chromatogr.*, 140 (1977) 174.
6 J. E. Rivier, *J. Liquid Chromatogr.*, 1 (1978) 343.
7 B. Larsen, V. Viswanatha, S. Y. Chang and V. J. Hruby, *J. Chromatogr. Sci.*, 16 (1978) 207.
8 S. Terabe, R. Konaka and K. Inouye, *J. Chromatogr.*, 172 (1979) 163.
9 S. Terabe, R. Konaka and J. Shoji, *J. Chromatogr.*, 173 (1979) 313.
10 J. Shoji, J. Kato, S. Terabe and R. Konaka, *J. Antibiot.*, 32 (1979) 313.
11 A. Dinner and L. Lorenz, *Anal. Chem.*, 51 (1979) 1872.
12 M. E. F. Biemond, W. A. Sipman and J. Olivié, *J. Liquid Chromatogr.*, 2 (1979) 1407.
13 B. T. Bush, J. H. Frenz, W. R. Melander, Cs. Horváth, A. R. Cashmore, R. N. Dryer, J. O. Knipe, J. K. Coward and J. R. Bertino, *J. Chromatogr.*, 168 (1979) 343.
14 D. D. Blevins, M. F. Burke and V. J. Hruby, *Anal. Chem.*, 52 (1980) 420.
15 J. F. M. Kinkel, G. Heuver and J. C. Kraak, *Chromatographia*, 13 (1980) 145.
16 M. J. O'Hare and E. C. Nice, *J. Chromatogr.*, 171 (1979) 209.
17 A. R. Cooper and D. S. Van Derveer, *J. Liquid Chromatogr.*, 1 (1978) 693.
18 S. Rokushika, T. Ohkawa and H. Hatano, *J. Chromatogr.*, 176 (1979) 456.
19 Y. Kato, K. Komiya, H. Sasaki and T. Hashimoto, *J. Chromatogr.*, 190 (1980) 297.
20 M. O. Dayhoff, L. H. Hunt, P. J. McLaughlin and W. C. Barker, *Atlas of Protein Sequence and Structure*, Vol. 5, National Biomedical Research Foundation, Washington, DC, 1972, p. D7.
21 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
22 L. R. Snyder, *J. Chromatogr.*, 179 (1979) 167.
23 I. Molnár and Cs. Horváth, *J. Chromatogr.*, 142 (1977) 623.
24 E. E. Conn and P. K. Stumpf, *Outlines of Biochemistry*, Wiley, New York, 1976, 4th ed., p. 105.
25 R. F. Rekker, *The Hydrophobic Fragmental Constant*, Elsevier, Amsterdam, 1977, p. 301.

Journal of Chromatography, 212 (1981) 305–311
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,850

MICROEXTRACTION AND GAS CHROMATOGRAPHIC ANALYSIS OF SELECTED PETROLEUM HYDROCARBONS IN WATER AND FISH TISSUE

D. A. J. MURRAY* and W. L. LOCKHART
Department of Fisheries and Oceans, Freshwater Institute, 501 University Crescent, Winnipeg, Manitoba R3T 2N6 (Canada)
(Received February 16th, 1981)

SUMMARY

The most effective water to solvent ratio is determined for the analyses of aromatic hydrocarbons in water using hexane. The recoveries of these hydrocarbons formed in the water soluble fraction of crude oils and petroleum products are measured using a microextraction procedure. Recoveries were in the 30–40% range but are consistent for each compound. Fish muscle samples are fortified with the standards and the recoveries measured with a modified extraction procedure using dichloromethane as the primary extracting solvent. This is dispersed in water using acetone and finally extracted with hexane. Recoveries range from 90–113% with a mean value of 98%.

INTRODUCTION

Microextraction procedures for the analysis of organic contaminants in water are widely known[1–4,6–8], and are becoming more popular because of their economy of solvents, ease of extraction and speed of analysis. Since there is no need for a concentration step the problem of impurities in the solvents is reduced. Although extraction efficiencies are in the 40–60% range, consistent and reliable results are obtained.

Crude oils and petroleum products such as diesel oils and gasolines are not readily miscible with water, but when an aqueous extract is made of an oil, appreciable amounts of sparingly soluble aromatic compounds appear in the aqueous phase. The compounds selected for standardization and recovery studies are representative of those found in the water soluble fraction of crude oils and petroleum products[9–11].

A microextraction flask[3] was used to investigate the most effective water to solvent ratio at various concentration levels. A modified procedure was developed to extract these organics and measure recoveries from fish muscle samples using dichloromethane as the primary extracting solvent. The dichloromethane extract was dispersed in water with acetone. This was then extracted with 1 ml of hexane using the microextraction procedure.

0021-9673/81/0000–0000/$02.50

EXPERIMENTAL

A standard solution of ethyl benzene, 1,3,5-trimethylbenzene, 1-isopropyl 4-methyl benzene, naphthalene, 2-methyl naphthalene, 1-methyl naphthalene and 2,3-dimethyl naphthalene was made up in acetone at a concentration of 1 μg/μl of each. An internal standard of *n*-decyl benzene was made up at the same concentration in hexane.

Duplicate extractions were done with a 1-l microextraction flask in which 950 ml of water fortified with standards were extracted with 250–1000 μl of hexane. The design of the flask permitted small volumes of solvents to be conveniently recovered for direct injection into a gas chromatograph. The flask was shaken manually for 2 min to equilibrate the standards in the two phases and water was added to bring the solvent into the capillary neck of the flask for analysis.

Samples of fish muscle (5 g) were fortified at three levels of the standards (20, 10, 5 ppm) and extracted with 10 ml of dichloromethane. The slurry was passed through a coarse stainless-steel sieve and 5 ml of the liquid cleaned up on a column of dry sodium sulphate and Florisil. The compounds were eluted with dichloromethane to give 5 ml of eluate. This was transferred to the microextraction flask, 100 ml of acetone and 850 ml of water were added to produce one aqueous phase. This was then extracted with 1 ml of hexane, the internal standard was added and the solvent layer analysed by gas chromatography.

Water soluble fractions of the oils and petroleum products were prepared by shaking 50 ml of oil with 1 l of water in a separatory funnel for 5 min and allowing the layers to separate overnight. The aqueous phase was passed through two glass wool plugs in series to remove any droplets of insoluble oil. Organic materials were recovered from the aqueous phase by the microextraction procedure.

Gas chromatographic conditions

A Perkin-Elmer 900 gas chromatograph was used with an Infotronics C.R.S. 208 integrator for quantitative analysis. A 2 m × 4 mm O.D. glass column was packed with 10% Dexsil 400 coated on Chromosorb W AW, 80–100 mesh. Conditions: temperatures: flame ionization detector, 275°C; injector, 225°C; column programmed 100–250°C at 10°C/min; flow-rates: nitrogen, 25 ml/min; hydrogen, 25 ml/min; air, 200 ml/min.

A 15 m × 0.1 mm I.D. capillary column was used for the analysis of the crude oils and the water soluble fractions. It was coated with SE-30 and programmed from 50–275°C at 8°C/min after a 4-min initial hold at 50°C. A splitless injection technique was used.

RESULTS

Evaporative losses typical of conventional concentration steps were measured by concentrating 5 ml of hexane to 0.5 ml on a rotary evaporator. The hexane was fortified with 100 μl of the standard solution, 100 μl of the internal standard added and the ratios of standards to *n*-decyl benzene calculated from the chromatogram. This was repeated after the concentration step and the ratios again calculated. The figures in Table I show 27–66% losses occurred during the concentration step.

TABLE I

LOSSES OF SELECTED COMPOUNDS BY CONCENTRATION WITH A ROTARY EVAPORATOR

Compound	*b.p. (°C)*	*Ratio before concentration*	*Ratio after concentration*	*Loss (%)*
Ethyl benzene	136	1.14	0.39	66
Trimethyl benzene	165	1.12	0.49	56
Isopropyl methyl benzene	177	1.08	0.50	54
Naphthalene	218	1.26	0.75	40
2-Methyl naphthalene	241	1.06	0.70	34
1-Methyl naphthalene	245	1.01	0.70	30
2,3-Dimethyl naphthalene	268	1.05	0.77	27

Using the microextraction flask, 950 ml of clean water were spiked with 50 μl of the standard solution and extracted with 250 μl of hexane. The internal standard was added and the ratios calculated from the chromatogram and expressed as a percentage recovery. This was repeated with 500, 750 and 1000 μl of hexane. The results in Table II show that the volume of hexane to extract 1000 ml of water was approaching an optimum value of 1000 μl. Larger volumes of hexane would have a diluting effect with no appreciable increase in recoveries. This optimum water to solvent ratio of 1000:1 was used to measure recoveries at four concentration levels and the results in Table III show recoveries which are consistent for each compound.

TABLE II

RECOVERIES (%) OF SELECTED COMPOUNDS AT 50 μg/l BY MICROEXTRACTION

Compound	*Volume of hexane (µl)*			
	250	*500*	*750*	*1000*
Ethyl benzene	5.5	17.2	21.4	25.4
Trimethyl benzene	9.8	30.8	33.9	35.6
Isopropyl methyl benzene	15.2	37.1	38.2	38.5
Naphthalene	5.7	19.0	24.3	27.6
2-Methyl naphthalene	10.4	33.3	35.8	37.0
1-Methyl naphthalene	9.7	32.5	37.4	39.4
2,3-Dimethyl naphthalene	16.3	39.9	40.2	40.9

Four concentration levels of the standards were added to 5 ml of dichloromethane and 100 ml of acetone used to disperse the dichloromethane into the aqueous phase in the microextraction flask. This was then extracted with 1000 μl of hexane and the recoveries calculated as before. These recoveries in Table IV were 20–30% higher using dichloromethane but were consistent for each compound within the concentration range of 10–100 ppb (10^9).

Fish muscle was spiked with three concentration levels of the standards and extracted with dichloromethane. An aliquot of the extract was run through a clean-up

TABLE III

RECOVERIES (%) OF SELECTED COMPOUNDS IN WATER WITH 1000 µl HEXANE BY MICROEXTRACTION

Compound	*Concentration (µg/l)*				*R.S.D. (%)*
	100	*50*	*25*	*10*	
Ethyl benzene	27.1	25.4	23.6	18.5	±14.6
Trimethyl benzene	36.7	35.6	34.8	31.8	± 5.8
Isopropyl methyl benzene	38.4	38.5	38.6	35.1	± 4.3
Naphthalene	28.7	27.6	27.8	24.9	± 5.8
2-Methyl naphthalene	37.7	37.0	37.4	35.2	± 2.9
1-Methyl naphthalene	40.0	39.4	35.8	32.2	± 9.4
2,3-Dimethyl naphthalene	40.5	40.9	41.8	40.7	± 2.1

TABLE IV

RECOVERIES (%) OF SELECTED COMPOUNDS FROM DICHLOROMETHANE WITH 1000 µl HEXANE BY MICROEXTRACTION

Compound	*Concentration (µg/l)*				*R.S.D. (%)*
	100	*50*	*25*	*10*	
Ethyl benzene	33.6	32.3	32.5	26.0	±10.5
Trimethyl benzene	46.2	46.2	47.2	41.6	± 5.2
Isopropyl methyl benzene	53.5	56.0	56.2	50.6	± 4.5
Naphthalene	33.9	32.4	32.1	30.0	± 4.7
2-Methyl naphthalene	48.0	48.3	48.9	44.5	± 3.9
1-Methyl naphthalene	50.7	46.4	44.8	39.9	± 9.1
2,3-Dimethyl naphthalene	56.3	59.6	60.5	57.1	± 3.2

TABLE V

RECOVERIES (%) OF SELECTED COMPOUNDS FROM FORTIFIED FISH MUSCLE BY MICROEXTRACTION

Compound	*Concentration (ppm)*			*R.S.D. (%)*
	20	*10*	*5*	
Ethyl benzene	98.7	98.1	102.0	±5.8
Trimethyl benzene	97.7	95.7	94.2	±7.1
Isopropyl methyl benzene	97.4	93.8	89.9	±5.3
Naphthalene	98.3	100.6	113.2	±3.8
2-Methyl naphthalene	96.1	91.7	104.4	±4.0
1-Methyl naphthalene	102.6	94.3	99.0	±4.0
2,3-Dimethyl naphthalene	98.0	91.9	99.6	±4.4

column, transferred to the aqueous phase in the microextraction flask with 100 ml of acetone and re-extracted with 1 ml of hexane. The ratios were again calculated from the chromatograms and using the mean recovery figure for each compound from Table IV, the results were expressed as a percentage recovery. These recoveries from fish muscle in Table V range from 90–113% with a mean value of 98%. The relative standard deviation (R.S.D.) of results in Tables IV–V was less than 9% with the exception of ethyl benzene at the lowest concentration, possibly because it was most volatile.

DISCUSSION

It has been demonstrated that many types of organic compounds show losses during concentration steps such as rotary evaporation, use of micro Snyder column or blowing nitrogen over a solution[3,5]. The microextraction procedure overcame this disadvantage in that it extracted and concentrated in one step with minimum of loss due to handling and transfers.

Recoveries of selected compounds approached a maximum when 1 ml of hexane was used to extract 1 l of water containing 10–100 μg of contaminants. Recoveries from dichloromethane using the modified procedure were improved by 20–30% and were consistent over the concentration range investigated. When fish muscle was fortified with the standards in the 5–20 ppm range the corrected recoveries were close to 100%.

The advantage of using dichloromethane as a primary extracting solvent for fish samples was in the concentrating effect; the compounds extracted in 5 ml of dichloromethane were finally extracted into 1 ml of hexane with no evaporative step in the process. There were no detectable hydrocarbon impurities in the dichloromethane and the clean-up step using sodium sulphate and Florisil produced a clear eluate suitable for microextraction. Other solvent systems no doubt exist which would permit the transfer of organic compounds through a solvent–water–solvent phase system to achieve concentration in the final solvent.

The effect of adding inorganic salts to the aqueous layer before extraction was not investigated since consistent recoveries were achieved without this step.

Recoveries for ethyl benzene and naphthalene have been reported in the 90% range[1,2] using water to solvent ratios of 100:1 and 20:1. These high recoveries are due to the choice of pentane (b.p. 36°C) as a solvent. The use of lower water to solvent ratios partially negated the concentrating effect in that less organics were available for extraction. This was offset to some extent by higher extraction efficiencies, but the overall recovery was improved, four to five fold, when a large volume of water was extracted with a small volume of solvent. In our experience, hexane (b.p. 69°C) was found to be more suitable than pentane because it was less volatile and less soluble in water. Extracts could be stored more easily without evaporative losses and more hexane was recovered from the microextraction.

The importance of using clean water and solvents must be emphasized. Distilled water was obtained directly from a commercial still without passage through plastic pipes since these contaminated water with phthalate esters[3]. Each batch of solvents was checked for interfering contaminants before use but four small peaks persisted in the water blank chromatogram and have been attributed to impurities in the water.

The chromatograms in Fig. 1 illustrate the complex nature of the organic compounds found in crude oils and their water soluble fractions. Tentative identification have been made of some of the major components from retention time data of known compounds. The peaks numbered in the chromatogram of Alberta Crude are the *n*-alkanes present in all crude oils in varying proportions. Pristane and phytane are seen next to C_{17} and C_{18} respectively and serve as helpful points of identification. In the water soluble fraction chromatograms, the peaks numbered 1–7 are those selected compounds which appear in all water soluble fractions, again in varying proportions. Although nominally insoluble, these compounds are soluble in the low ppb range of these extractions.

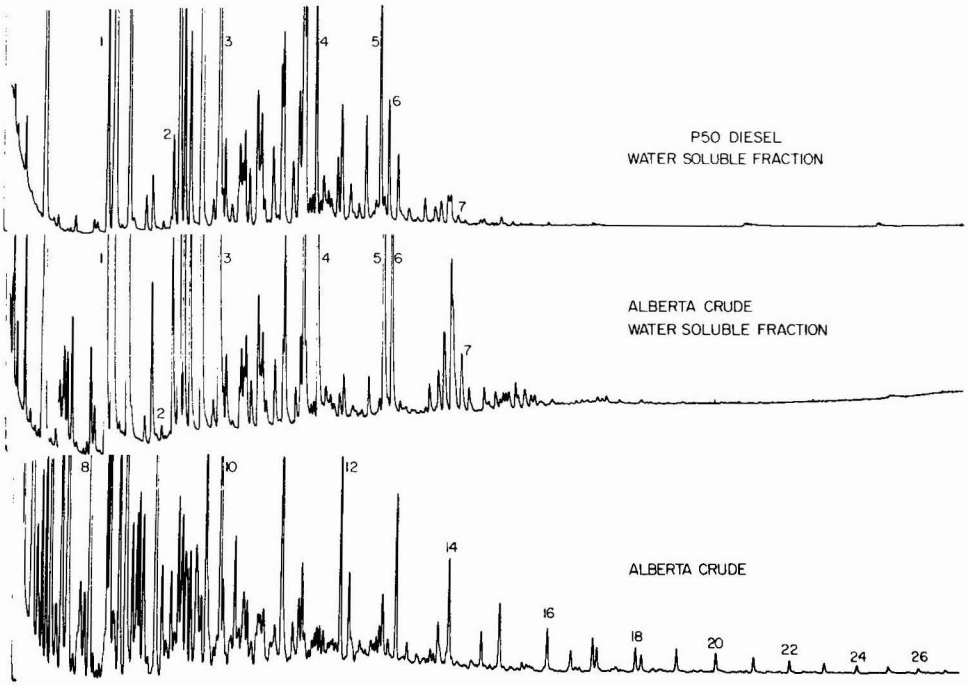


Fig. 1. Gas chromatograms of microextractions of the water soluble fractions of diesel and crude oil compared to the crude oil using a 15-m wall-coated open tubular SE-30 column and a splitless injection.

It is of interest that crude oils and petroleum products all produced similar water soluble fractions, showing the same pattern of major peaks. Further work on the sub-lethal toxic effects of these compounds on fish, invertebrates and other organisms is presently underway using the described techniques.

ACKNOWLEDGEMENTS

We thank B. G. E. de March for statistical advice and Dr. D. C. G. Muir for assistance in preparing this manuscript.

REFERENCES

1 K. E. Thrun, K. E. Simmons and J. E. Oberholtzer, *J. Environ. Sci. Health*, A15(5) (1980) 485.
2 J. W. Rhoades and C. P. Nulton, *J. Environ. Sci. Health*, A15 (1980) 467.
3 D. A. J. Murray, *J. Chromatogr.*, 177 (1979) 135.
4 K. Grob, K. Grob Jr. and G. Grob, *J. Chromatogr.*, 106 (1975) 299.
5 G. A. Junk, J. J. Richard, M. D. Grieser, D. Witiak, J. L. Witiak, M. D. Arguello, R. Vick, H. J. Svec, J. S. Fritz and G. V. Calder, *J. Chromatogr.*, 99 (1974) 745.
6 P.-F. Blanchet, *J. Chromatogr.*, 179 (1979) 123.
7 J. E. Henderson, G. R. Peyton and W. H. Glaze, in L. H. Keith (Editor), *Identification and Analysis of Organic Pollutants in Water*, Ann Arbor Sci. Publ., Ann Arbor, MI, 1976, p. 105.
8 C. Feyerabend and M. A. H. Russell, *J. Pharm. Pharmacol.*, 31 (1979) 73.
9 J. W. Anderson, J. M. Neff, B. A. Cox, H. E. Tatem and G. M. Hightower, *Mar. Biol.*, 27(1) (1974) 75.
10 S. D. Rice, A. Moles, T. L. Taylor and J. F. Karinen, in *1979 Oil Spill Conference*, American Petroleum Institute, Washington, DC, 1979, p. 549.
11 J. A. Whipple, T. G. Yocom, D. R. Smart and M. H. Cohen, in *Proceedings of the Conference on Assessment of Ecological Impacts of Oil Spills*, American Institute of Biological Sciences, Keystone, CO, 1978, p. 757.

Journal of Chromatography, 212 (1981) 313–322
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,834

NEW CHROMATOGRAPHIC SYSTEM FOR THE RAPID ANALYSIS AND PREPARATION OF COLOSTRUM SIALYLOLIGOSACCHARIDES*

RÜDIGER W. VEH
Institut für Anatomie, Ruhr-Universität, D-4630 Bochum (G.F.R.)
JEAN-CLAUDE MICHALSKI
Laboratoire de Chimie Biologique, Université des Sciences et Techniques de Lille I, F-59650 Villeneuve d'Ascq (France)
ANTHONY P. CORFIELD
Biochemisches Institut, Christian-Albrechts-Universität, D-2300 Kiel (G.F.R.)
MICHAEL SANDER-WEWER and DAGMAR GIES
Institut für Anatomie, Ruhr-Universität, D-4630 Bochum (G.F.R.)
and
ROLAND SCHAUER*
Biochemisches Institut, Christian-Albrechts-Universität, Olshausenstrasse 40–60, D-2300 Kiel (G.F.R.)
(Received March 30th, 1981)

SUMMARY

A new thin-layer chromatographic system on silica gel for the separation of sialyloligosaccharides is described. Calibration of the system with standard milk and colostrum sialyloligosaccharides is presented. The use of the system in monitoring different oligosaccharides is demonstrated for the purification of bovine colostrum sialyllactose isomers and a commercial sialyllactose product, and is discussed with respect to other biological fluids.

The large-scale preparation of pure sialyllactose isomers from bovine colostrum is achieved using an improved ion-exchange separation on Dowex 1-X2 (< 400 mesh) employing isomolar elution at 20 m*M* for monosialyloligosaccharides and 200 m*M* for disialyllactose. The purification of four major monosialyltrisaccharides, the 2–3 and 2–6 isomers of N-acetylneuraminyllactose, N-glycolylneuraminyl2–3lactose and N-acetylneuraminyl2–6-N-acetyllactosamine, and the disialyltetrasaccharide di-N-acetylneuraminyllactose is reported. The detection and partial purification of three new minor monosialyloligosaccharides is described.

INTRODUCTION

Sialyloligosaccharides are present in large amounts in the milk and colostrum of mammals[1] and also in urine[2,3], where the excretion of particular oligosaccharides

* Glycolipids and oligosaccharides are abbreviated according to the recommendations of the IUPAC–IUB Lipid Nomenclature Document, *Lipids*, 12 (1977) 455–468.

0021-9673/81/0000–0000/$02.50

has been correlated with genetic disorders in man[4]. The isolation of the smaller oligosaccharides, in particular sialyllactose, from colostrum or milk has provided widely used substrates for the assay of sialidase activity[5].

The occurrence of the α2–3 and α2–6 derivatives of N-acetylneuraminyllactose (IINeu5AcLac) in colostrum has long been established[6], and has usually been prepared and detected using paper chromatography. However, the existence of other isomers, sialyl-N-acetyllactosamine (IINeu5AcGalβl–4GlcNAc) and N-glycolylneuraminyllactose (IINeu5GlLac) present in bovine colostrum[7], is not usually considered although commercially available sialyllactose is obtained principally from this source.

We present a new thin-layer and column chromatographic system, where rapid identification and large-scale preparation of the different sialyllactose isomers and other sialyloligosaccharides can be achieved. Alternative systems have been reported in the literature by Maury[2] and more recently by Holmes and O'Brien[8] and Momoi and Wiegandt[9]. However, good resolution of sialyllactose isomers has not been reported previously. The preparation of sialyllactose isomers by ion-exchange chromatography has previously proved to be more difficult, and the results reported by Schneir and Rafelson[10] and Smith *et al.*[11] with Dowex and DEAE-cellulose ion exchangers give only partial separation of all isomers.

The new system will be of value in the screening for these oligosaccharides in biological fluids, especially in the preparation of highly purified oligosaccharides, and in analysis of sialyllactose cleavage in sialidase assays.

MATERIALS AND METHODS

Materials

Bovine colostrum was obtained from the Bundesanstalt für Milchforschung (Kiel, G.F.R.) and was collected within 1 h of milking and frozen at −15°C until use.

Sialyllactose was purchased from Boehringer (Mannheim, G.F.R.). Silica gel 60 and cellulose thin-layer plates (0.1 mm layer), solvents and chemicals of analytical grade were products of E. Merck (Darmstadt, G.F.R.). Dowex 1-X2, minus 400 mesh, was purchased from Bio-Rad (München, G.F.R.).

The following standard compounds were prepared from the sources indicated: II^3Neu5AcLac, II^6Neu5AcLac, $II^3(Neu5Ac)_2$Lac and II^6Neu5AcGalβ1–4GlcNAc from bovine colostrum; IV^3Neu5AcLcOse$_4$, IV^6Neu5AcLcnOse$_4$ and IV^3Neu5Ac-III^6Neu5AcLcOse$_4$ from human milk[12]; II^3Neu5GlLac (after ozonolysis of II^3Neu-5GlLacCer) from equine erythrocytes, by the method of Wiegandt and Bücking[13].

Analytical methods

Sialic acids were determined by the periodic acid–resorcinol method[14], by the periodic acid–thiobarbituric acid and orcinol–Fe^{3+} micromethods[15] and by gas–liquid chromatography (GLC)[15]. The methanolysis products of the oligosaccharides were analysed by GLC as described previously[16]. Methylation analysis was performed according to Hakomori[17].

Thin-layer chromatography (TLC) of sialic acids was performed on cellulose, developing with *n*-butanol–*n*-propanol–0.1 *M* HCl (1:2:1, v/v/v) and visualizing with the orcinol–Fe^{3+} spray reagent[15]. Oligosaccharides were separated on silica gel 60

thin-layer plates using ethanol–*n*-butanol–pyridine–water–acetic acid (100:10:10:30:3, v/v) and visualizing with orcinol–Fe^{3+} spray reagent or orcinol–H_2SO_4 (200 mg of orcinol in 100 ml of aqueous 20% H_2SO_4) sprays. To identify the nature of sialic acids in individual oligosaccharides, two-dimensional TLC was employed. The oligosaccharide sample was applied to the corner of a 20 × 20 cm cellulose thin-layer plate and run in the *n*-butanol–*n*-propanol–0.1 *M* HCl system described above. After drying, the plate was sprayed with 0.1 *M* HCl and incubated, with an overlying glass plate to prevent drying out, for 30 min at 80°C. The plate was then removed and dried, and the hydrolysis was repeated. On completion of the second hydrolysis the plate was developed in the same solvent and the components visualized with the orcinol–Fe^{3+} spray reagent. Standard Neu5Ac and Neu5Gl, as well as oligosaccharides, were run in the appropriate dimensions.

Paper chromatography was carried out on Whatman No. 3 paper in pyridine–ethyl acetate–acetic acid–water (5:5:1:3, v/v). Fowl plague virus (donated by Professor R. Rott, University of Giessen, Giessen, G.F.R.) was used as a source of sialidase; incubations were carried out as described elsewhere[5,19].

Preparative methods

Bovine colostrum (1 l) was mixed with chloroform–methanol (2:1, v/v; 5 l) and stirred vigorously for 20 min at 4°C. The suspension was centrifuged for 20 min at 4000 *g* and the upper light-yellow aqueous layer separated from the intermediate cake of precipitated protein and the lower deep-yellow organic phase. Aqueous phases were pooled (*ca.* 2 l for 1 l of colostrum) and concentrated by rotary evaporation under reduced pressure, and 300–400 ml were applied to a column (100 × 10 cm I.D.) of Sephadex G-25 fine. The column was eluted with distilled water using a peristaltic pump at a flow-rate of 3 ml/min, and the effluent was monitored for sialic acid content by the orcinol–Fe^{3+} assay and at 280 nm for glycopeptides. All procedures were conducted at 4°C. The fractions (10 ml) were combined to give three pools, as indicated in Fig. 1. This is a slight modification of the method of Ohman and Hyg-

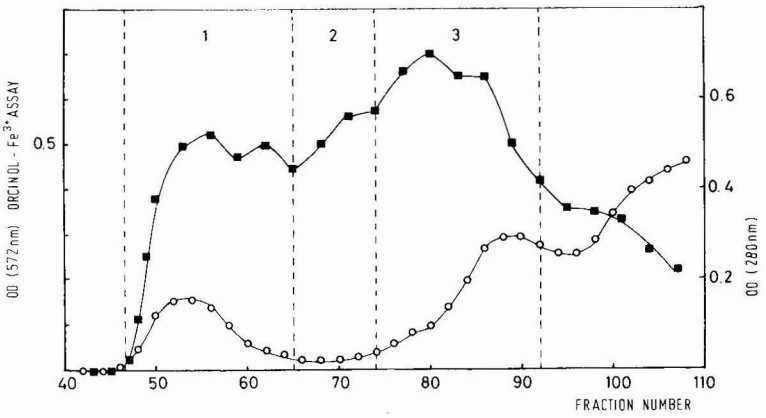


Fig. 1. Elution profile of 300 ml of concentrated aqueous phase after extraction of bovine colostrum with chloroform–methanol (2:1) on Sephadex G-25 fine eluted with water. The effluent was monitored for the absorbance at 280 nm (○) and with the orcinol–Fe^{3+} assay for sialic acids (■). Fractions were pooled as indicated to give pools 1–3.

stedt[18]. The pools were freed of lactose and neutral oligosaccharides by chromatography on Dowex 1-X8 acetate, 200–400 mesh. Sialyllactose isomers were eluted with 50 m*M* pyridinium acetate (pH 5.0), and disialyllactose with 200 m*M* pyridinium acetate (pH 5.0) to give a crude mixture of monosialyllactose isomers and disialyllactose.

Separation of sialyllactose isomers was carried out on a column (20 × 1 cm I.D.) of Dowex AG 1-X2 (minus 400 mesh, acetate form at 4°C). A sample (10 mg) of crude sialyllactose from Sephadex G-25 chromatography was dissolved in 10 ml of 2 m*M* pyridinium acetate buffer (pH 5.0) and applied to the column. The column was washed with 50 ml of the same buffer and eluted with a linear gradient (2 × 250 ml) from 10 m*M* to 50 m*M* pyridinium acetate (pH 5.0). The buffer concentration at the beginning of the sialyllactose elution was found to be 20 m*M*, and this concentration could be used for isomolar elution of the column with good separation of the different isomers. The separation of larger amounts (800 mg) of the crude mixture of sialyllactose isomers was carried out using the same technique. The sialyllactose was dissolved in 80 ml of 2 m*M* pyridinium acetate buffer (pH 5.0) and loaded on to a Dowex AG 1-X2 column (95 × 3 cm I.D.). Elution was carried out at a flow-rate of 50 ml/h using 20 m*M* buffer, and fractions of 16 ml were collected. After 8 l of buffer had been passed through the column the disialyllactose was eluted by raising the buffer concentration to 200 m*M*.

RESULTS AND DISCUSSION

The separation of sialyloligosaccharide standards by a new TLC system on silica gel is shown in Fig. 2a, which shows that a good resolution of the major sialyloligosaccharides occurring in the milk of mammals is achieved with this system. Separation of larger sialyloligosaccharides from glycoproteins[20] and gangliosides is also possible (data not shown). The new system is superior to other reported systems (*e.g.* refs. 2, 3, 8 and 9) in its resolution and application in the identification of sialyllactose isomers, and is more rapid and sensitive than paper chromatography. Thus a rapid analysis of sialyloligosaccharides is possible.

The TLC system has been used to monitor the sialyloligosaccharide components during their preparation from bovine colostrum (Fig. 2b). Fractionation of the aqueous phase of colostrum extracted with chloroform–methanol on Sephadex G-25 yielded three pools (Fig. 1). The first pool contained glycopeptides, glycolipids and small amounts of disialyllactose, and pools 2 and 3 contained the sialyllactose isomers. Disialyllactose was found chiefly in pool 2 and monosialyllactose isomers in pool 3, which was also contaminated with lactose, eluting later but still overlapping with sialyllactose owing to the high concentration in colostrum.

Removal of residual lactose by ion-exchange chromatography without resolution of individual components yielded between 0.7 and 1.2 g of sialyloligosaccharide mixture per litre of colostrum (fifteen experiments).

Resolution of the individual components from the combined pools 2 and 3 was carried out on an anion-exchange column (Fig. 3). After the concentration of buffer necessary to commence elution of sialyllactose isomers had been determined, an isomolar elution was found to give a good separation of these isomers; this approach had not previously been utilized for oligosaccharide separations. The chromatogra-

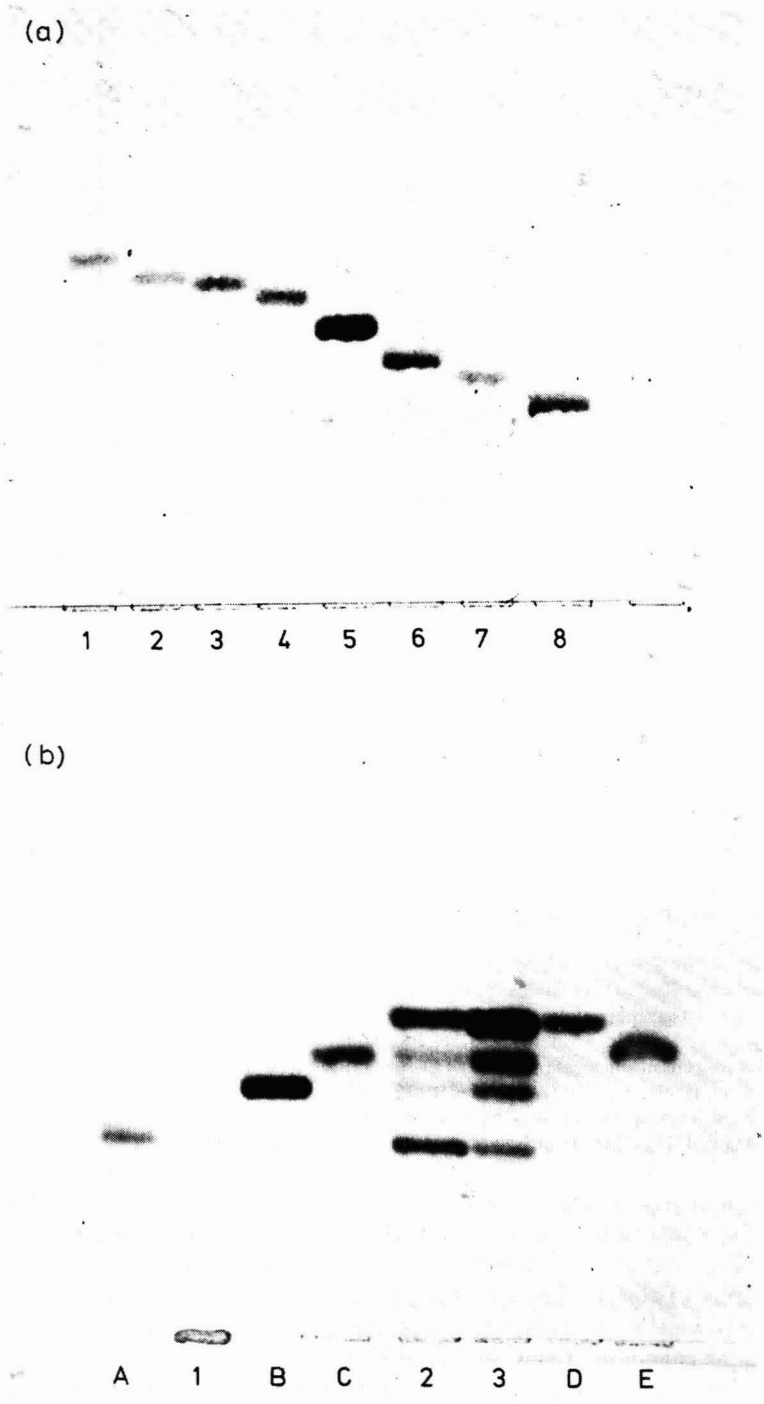


Fig. 2.

(Continued on p. 318)

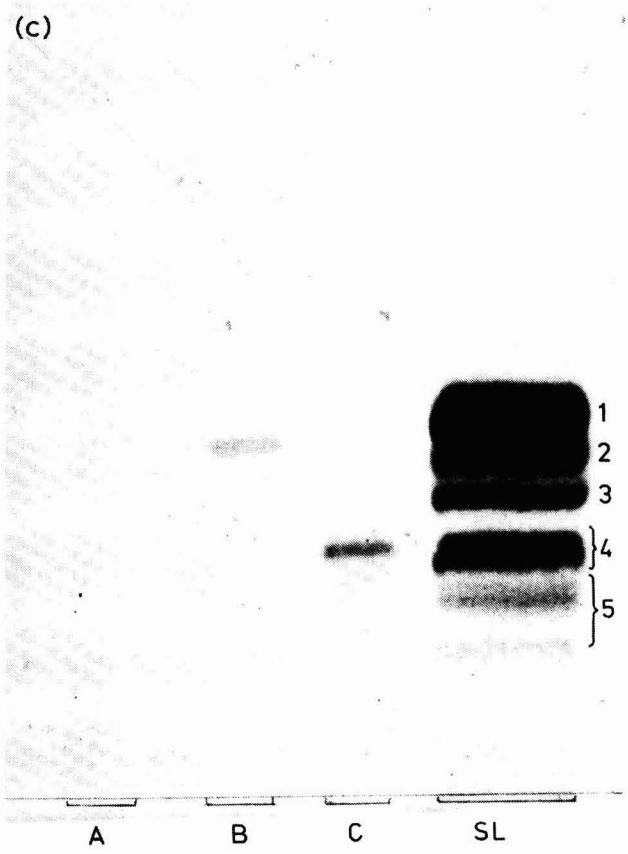


Fig. 2. (a) Chromatography of standard sialyloligosaccharides on silica gel thin-layer plates in ethanol–*n*-butanol–pyridine–water–acetic acid (100:10:10:30:3, v/v). 1 = II^3Neu5AcLac (1.0); 2 = IV^3Neu5AcLcOse$_4$ (0.94); 3 = II^3Neu5GlLac (0.93); 4 = II^6Neu5AcGalβ1–4GlcNAc (0.89); 5 = II^6Neu5AcLac (0.80); 6 = IV^6Neu5AcLcnOse$_4$ (0.70); 7 = II^3(Neu5Ac)$_2$Lac (0.65); 8 = IV^3Neu5AcIII^6Neu5AcLcOse$_4$ (0.57). The figures in brackets are the $R_{II^3Neu5AcLac}$ values for each component. (b) TLC as in (a) of the pools 1–3 from Sephadex G-25 chromatography of bovine colostrum sialyloligosaccharides. The pools 1–3 and standards II^3(Neu5Ac)$_2$Lac (A), II^6Neu5AcLac (B), II^6Neu5AcGalβ1–4GlcNAc (C), II^3Neu5AcLac (D) and II^3Neu5GlLac (E) were run on silica gel plates. (c) TLC as in (a) of a commercial sample of sialyllactose (SL) and the standards II^3Neu5AcLac (A), II^3Neu5GlLac (B) and II^3(Neu5Ac)$_2$Lac (C). The commercial sample shows bands corresponding to II^3Neu5AcLac and II^3Neu5GlLac (1), II^6Neu5AcGalβ1–4GlcNAc (2), II^6Neu5AcLac (3), II^3(Neu5Ac)$_2$Lac (4) and higher molecular weight oligosaccharides (5).

phy revealed four main trisaccharide components, identified as II^3Neu5AcLac, II^6Neu5AcLac, II^6Neu5AcGalβ1–4GlcNAc and II^3Neu5GlLac. These compounds were analysed by GLC and compared with reference oligosaccharides on TLC and paper chromatography (Table I). The structure of II^6Neu5AcGalβ1–4GlcNAc was also confirmed by methylation analysis. Cross-contamination between these fractions was less than 0.5%, as calculated from GLC and TLC analyses.

Disialyllactose was also separated by ion-exchange chromatography, and its structure was confirmed by GLC after methanolysis. The isolated product gave only one band on TLC (Fig. 2a and b), although several bands eluted with 200 m*M*

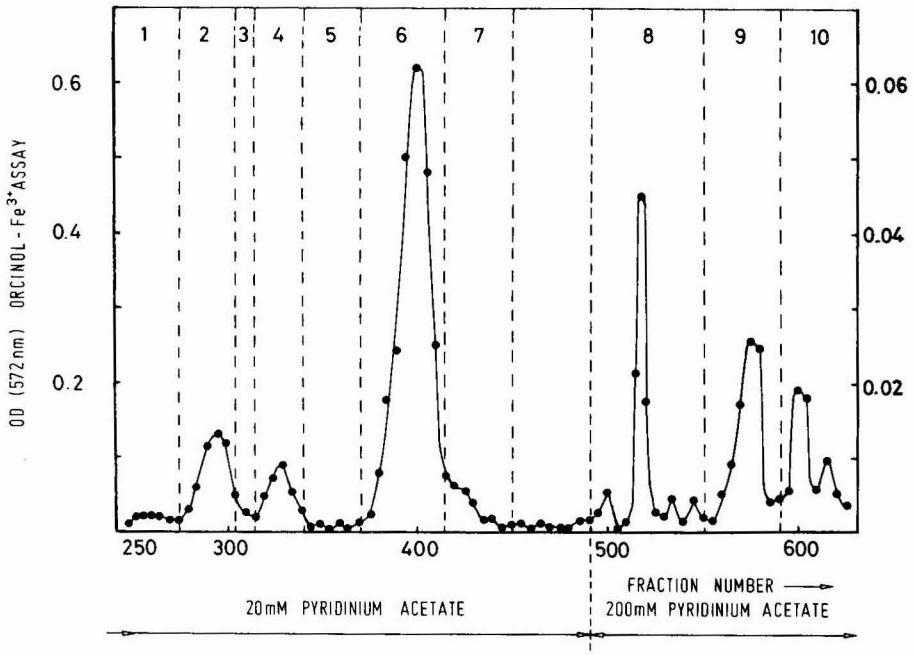


Fig. 3. The elution profile of crude sialyllactose from Sephadex G-25 chromatography (pools 2 and 3), on Dowex AG 1-X2 eluted with 20 m*M* and 200 m*M* pyridinium acetate (pH 5.0) as indicated. Samples were measured using the orcinol–Fe^{3+} assay. From fraction 490 onwards the scale is reduced by a factor of 10 as indicated on the right-hand axis. The fractions were pooled to give samples 1–10 as indicated; fractions 450–490 were discarded. Further details are given in the text.

pyridinium acetate (Fig. 3). The reason for this behaviour of disialyllactose is unclear at present. The structure of the disialyllactose was further analysed by incubation with fowl plague virus sialidase, and separation of the products on the TLC system. The formation of lactose was detected at different times of incubation and with different enzyme concentrations. No formation of II^{6}Neu5AcLac was detected. The virus sialidase cleaves 2–3 and 2–8 glycosidic linkages to Neu5Ac but 2–6 linkages are very slowly cleaved[5,19]. Thus incubation with the sialidase should result in an enrichment of II^{6}Neu5AcLac if this linkage exists in disialyllactose. The results showed only lactose and very small amounts of II^{3}Neu5AcLac, the other expected product, but no II^{6}Neu5AcLac. This result was further supported by 360 MHz nuclear magnetic resonance spectroscopy of the disialyllactose isolated, because only evidence for a 2–3 linkage was found, and so the presence of a 2–6 linkage could be ruled out[21].

In addition to these major oligosaccharide components, three minor oligosaccharides were detected and analysed (Table I) for the molar ratios of the component monosaccharides by GLC and for the type of sialic acid by a new method combining two-dimensional TLC with intermediate mild acid hydrolysis (Fig. 4). On the basis of these results and their elution positions on anion-exchange chromatography (Fig. 3), it is tentatively concluded that the components 3 and 5 in Table I are II^{6}Neu5GlGalβ-1–4GlcNAc and II^{6}Neu5GlLac, respectively. Component 1 contained 2 mol of galactose per mole of glucose and Neu5Ac. The structure of this component has not yet

TABLE I

ANALYSIS OF SIALYLOLIGOSACCHARIDES SEPARATED BY DOWEX CHROMATOGRAPHY

Crude sialyllactose (800 mg) was fractionated on Dowex 1-X2 as detailed in the text, and fractions 1–10 were collected as indicated in Fig. 3. Lactose was washed through the column and constituted the weight not recovered as sialyloligosaccharides 1–10.

Pool	*Fraction No.*	*Amount (mg)*	*Sialic acid*	*GLC (a) or TLC (b)*	*Monosaccharides molar ratio–GLC*				*Silica gel* $R_{II^3Neu5AcLac}$	*Comment*
					Glc	*Gal*	*GlcNAc*	*Neu5Ac*		
1	251–275	7	Neu5Ac	b	1	2	–	1	0.97	Unknown
2	276–305	70	Neu5Ac	b, a	–	1	1	1	0.89	Neu5Ac2–6N-acetyllactosamine
3	306–315	6	Neu5Gl	b	–	1	1	1	0.83	Neu5Gl2–6N-acetyllactosamine
4	316–340	37	Neu5Ac	b, a	1	1	–	1	0.79	Neu5Ac2–6lactose
5	341–370	6	Neu5Gl	b	1	1	–	1	0.80	Neu5Gl2–6lactose
6	371–415	332	Neu5Ac	b, a	1	1	–	1	1.00	Neu5Ac2–3lactose
7	416–450	19	Neu5Gl	b, a	1	1	–	1	0.93	Neu5Gl2–3lactose
8	490–550	–	Neu5Ac	b, a	–	–	–	1	0.66	free Neu5Ac
9	551–590	8	Neu5Ac	b, a	1	1	–	2	0.65	disialyllactose
10	591–630	7	Neu5Ac	b, a	1	1	–	2	0.65	disialyllactose

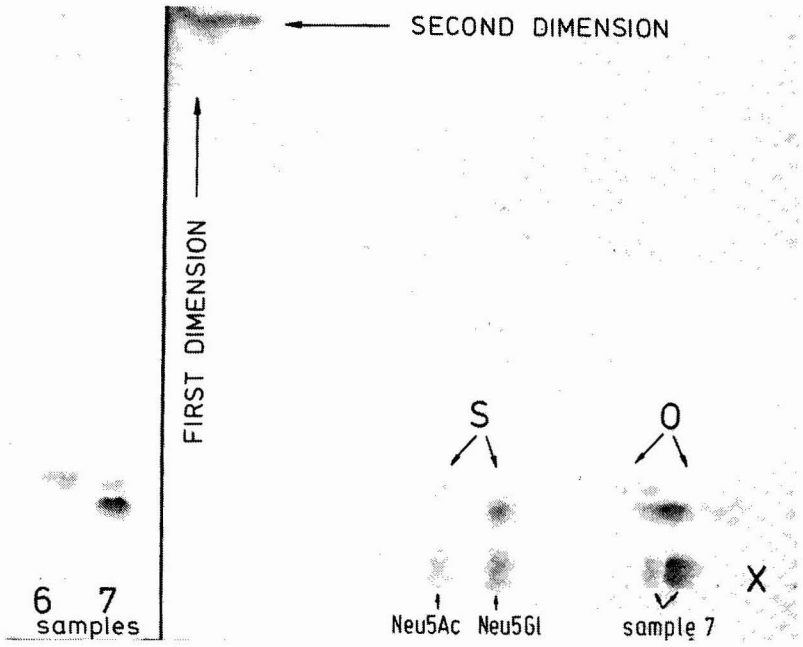


Fig. 4. Two-dimensional chromatography of sample 7 (see Table I) from ion-exchange chromatography (Fig. 3) on cellulose thin-layer plates. Development in both directions was with *n*-butanol–*n*-propanol–0.1 *M* HCl (1:2:1, v/v/v). After application of the sample at × and development of the plate in the first dimension, the plate was dried, sprayed with 0.1 *M* HCl and incubated at 80°C for 60 min as described in the text. After drying, a mixture of sample 7 with Neu5Ac and Neu5Gl standards was applied to position × and development in the second dimension carried out. In addition, samples 6 and 7 (see Table I) were run only in the first dimension for comparison (left-hand side of the chromatogram). The products of two-dimensional chromatography, visualized with the orcinol–Fe^{3+} spray reagent, are indicated as sialic acid (S) or oligosaccharide (O) products.

been determined. These minor components were difficult to analyse as they still contained oligosaccharides from neighbouring fractions (Figs. 3 and 4).

Using this method it is possible to process gram amounts of crude sialyllactose isomers and to prepare homogenous products in amounts suitable for routine analysis of sialidases, for example. The yields of the major sialyloligosaccharides in a typical experiment are noted in Table I for a crude mixture containing smaller amounts of disialyllactose. Typical yields for 1 l of colostrum were: II^{3}Neu5AcLac, 530 mg; II^{6}Neu5AcLac, 65 mg; II^{3}Neu5GlLac, 32 mg; II^{6}Neu5AcGalβ1–4GlcNAc, 110 mg; II^3(Neu5Ac)$_2$Lac, 285 mg.

Thus, in contrast to previous methods, the isomolar elution technique employed here combines good resolution of individual components with the scaling-up of the applied sample size and represents a significant improvement[10,11,18].

The availability of relatively large amounts of sialyllactose from colostrum and its suitability as a substrate for sialidases has led to its widespread commercial availability. Although the occurrence of the four major trisaccharides has been described[7], subsequent workers have reported only the 2–3 and 2–6 isomers of sialyllactose, or noted other minor and unidentified oligosaccharides[10,18]. Disialyllactose occurs as a

major component in bovine colostrum, as has been reported earlier[1,7,18], and is also present in the commercial sample of sialyllactose in amounts similar to those found in the present work (Fig. 2c). The commercial sample shows the presence of two bands in the region of disialyllactose and other slower migrating sialyloligosaccharides not found in the present work. A major band migrates as II^{6}Neu5AcGalβ1–4GlcNAc, and II^{3}Neu5GlLac is probably included in the large II^{3}Neu5AcLac band (Fig. 2c). Use of these samples for sialidase or other assays must take into account the different behaviour of each isomer in such experiments[5,19].

Using the TLC system presented here, a rapid analysis is possible of sialyloligosaccharides from colostrum, milk, urine and other biological fluids, as well as commercial samples, and the ion-exchange method provides a new system for the large-scale preparation of pure sialyllactose isomers.

ACKNOWLEDGEMENTS

Jean-Claude Michalski was recipient of an EMBO fellowship during this work. The technical assistance of Marzog El Madani (Kiel) is gratefully acknowledged. The authors thank Dr. W. Kaufmann, Bundesanstalt für Milchforschung, Kiel, for donating bovine colostrum. This work was supported by the Deutsche Forschungsgemeinschaft (Grant Scha 202/9) and the Fonds der Chemischen Industrie.

REFERENCES

1 A. Kobata, in M. I. Horowitz and W. Pigman (Editors), *The Glycoconjugates*, Academic Press, New York, Vol. 1, 1977, pp. 423–440.
2 P. Maury, *Biochim. Biophys. Acta*, 252 (1971) 48–57.
3 P. Maury, *Biochim. Biophys. Acta*, 252 (1971) 472–480.
4 G. Strecker and J. Montreuil, *Biochimie*, 61 (1979) 1199–1246.
5 A. P. Corfield, J.-C. Michalski and R. Schauer, in G. Tettamanti (Editor), *Proc. Int. Symp. on Sialidases and Sialidoses, Genova*, Edi Ermes Publ., Milan, 1981, in press.
6 R. Kuhn, *Naturwissenschaften*, 46 (1959) 43–50.
7 R. Kuhn and A. Gauhe, *Chem. Ber.*, 98 (1965) 395–413.
8 E. W. Holmes and J. S. O'Brien, *Anal. Biochem.*, 93 (1979) 167–170.
9 T. Momoi and H. Wiegandt, *Hoppe-Seyler's Z. Physiol. Chem.*, 361 (1980) 1201–1210.
10 M. L. Schneir and M. E. Rafelson, Jr., *Biochim. Biophys. Acta*, 130 (1966) 1–11.
11 D. F. Smith, D. A. Zopf and V. Ginsburg, *Anal. Biochem.*, 83 (1976) 602–608.
12 L. Grimmonprez, S. Bouquelet, B. Bayard, G. Spik, M. Monsigny and J. Montreuil, *Eur. J. Biochem.*, 13 (1970) 484–492.
13 H. Wiegandt and H. W. Bücking, *Eur. J. Biochem.*, 15 (1970) 287–292.
14 G. W. Jourdian, L. Dean and S. Roseman, *J. Biol. Chem.*, 246 (1971) 430–435.
15 R. Schauer, *Methods Enzymol.*, 50C (1978) 64–89.
16 J. P. Kamerling, G. J. Gerwig, J. F. G. Vliegenthart and J. R. Clamp, *Biochem. J.*, 151 (1975) 491–495.
17 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
18 R. Ohman and O. Hygstedt, *Anal. Biochem.*, 23 (1968) 391–402.
19 R. Drzeniek, *Curr. Top. Microbiol. Immunol.*, 59 (1972) 35–74.
20 B. Bayard, E. P. Kerckaert, D. Roux and G. Strecker, *Protides Biol. Fluids, Proc. Colloq.*, 27 (1979) 153–156.
21 J. F. G. Vliegenthart, Bio-Organic Chemistry Department, University of Utrecht, Utrecht, The Netherlands, personal communication.

Journal of Chromatography, 212 (1981) 323–330
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,826

DETERMINATION OF ADRIAMYCIN (DOXORUBICIN) AND RELATED FLUORESCENT COMPOUNDS IN RAT LYMPH AND GALL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

SHINYA SHINOZAWA*
Department of Hospital Pharmacy, Okayama University Medical School, 2-5-1, Shikata-cho, Okayama 700 (Japan)
and
TAKUZO ODA
Department of Biochemistry Cancer Institute, Okayama University Medical School, 2-5-1, Shikata-cho, Okayama 700 (Japan)
(First received January 27th, 1981; revised manuscript received March 27th, 1981)

SUMMARY

The concentrations of adriamycin (ADM) and related fluorescent compounds in lymph and gall were determined by high-performance liquid chromatography (HPLC) after a single intravenous injection into AH 109A tumour-bearing rats. HPLC was carried out by using Zorbax Sil as the stationary phase and chloroform–isopropanol–acetic acid–water–sodium acetate buffer (pH 4.5) (100:100:14:14:1) as the mobile phase, with a fluorescence spectrophotometric detector at an excitation wavelength of 470 nm and an emission wavelength of 585 nm. The detection limit for ADM was down to 1.0 ng/ml. In the thoracic duct lymph, the concentration of total ADM equivalent values (total ADM values) was maximal 30 min after injection and, after a subsequent decrease, increased gradually from 60 to 180 min. The ratio of total ADM in lymph to that in plasma at 180 min was 1.5 times that at 30 min. In gall, the total ADM showed a maximal level of 20.0 μg/ml at 30 min.

INTRODUCTION

Adriamycin (ADM) is an anthracycline antibiotic used extensively for the treatment of leukaemia and various malignant tumours[1]. Its initial half-life in plasma after intravenous injection is very short[2–5] and it has a strong affinity to tissue proteins[3,6–8] and deoxyribonucleic acid[9]. Its biliary excretion is the major excretory pathway in several animal species[10], but its passing into lymph has not yet been examined in detail.

In this study we devised a high-performance liquid chromatographic (HPLC) method for the determination of ADM and applied it to the transport of ADM into rat thoracic lymph and gall.

0021-9673/81/0000–0000/$02.50

EXPERIMENTAL

Reagents

Adriamycin hydrochloride, adriamycinone and adriamycinol were kindly donated by Farmitalia (Milan, Italy) and daunomycin was obtained from Meiji Seika (Tokyo, Japan). Chloroform and isopropanol were of HPLC grade. Acetic acid and sodium acetate were of analytical reagent grade.

Animal experiments

AH 109A ascites tumour (2×10^6 cells per animal) was inoculated on the back of Donryu male rats (body weight, *ca.* 160–170 g). The animals had free access to food and water. Seven days later, the rats were anaesthetized with diethyl ether, injected intravenously with 1.0 mg/kg of ADM or daunomycin (DUM) solution (1 mg/ml) with a syringe with a Harvard Apparatus infusion pump to the right inferior vena cava (0.53 ml/min), and the blood was collected with a heparinized syringe from the left inferior vena cava. The plasma was isolated. For studying the passing of ADM and DUM into lymph, the rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and the thoracic duct was cannulated according to the method of Bollman *et al.*[11]: a heparinized vinyl catheter (I.D. 0.5 mm; Imamura Rubber, Tokyo, Japan) was inserted into the duct and fixed with aid of a drop of Aron Alpha A tissue cement (Sankyo, Tokyo, Japan). The thoracic duct lymph was collected. For studying the passing of ADM into gall, the rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and the common bile duct was cannulated with a heparinized vinyl catheter. The gall was collected. Before or after these cannulations the rats were injected intravenously with 1.0 mg/kg of ADM or DUM solution (1 mg/ml) with a syringe with a Harvard Apparatus infusion pump to right inferior vena cava (0.53 ml/min).

Determination of ADM and related fluorescent compounds

ADM, related fluorescent compounds and DUM in the plasma, lymph and gall of AH 109A tumour-bearing rats were determined by modifying the previously reported HPLC method[12]. In brief, a Hitachi Model 635A high-performance liquid chromatograph was connected to a Hitachi Model 650-10S high-sensitivity fluorescence spectrophotometer, the results were recorded on a Hitachi Model 056 recorder and calculations were performed using a Hitachi Model 834-30 Chromato-Processor as an integrator, based on the ratio of the peak area to that of standard ADM or DUM (external standard method). The stationary phase was Zorbax Sil (5 μm) packed in a stainless-steel tube (150 × 4.6 mm I.D.). The mobile phase was chloroform–isopropanol–acetic acid–water–sodium acetate buffer (pH 4.5) (100:100:14:14:1) at a flow-rate of 1.0 ml/min, with a fluorescence spectrophotometric detector at an excitation wavelength of 470 nm and an emission wavelength of 585 nm. ADM was extracted from the biological samples by the method described previously, using chloroform–methanol (4:1)[6]. All operations with ADM and related fluorescent compounds were carried out in near darkness.

RESULTS

The use of Zorbax Sil as the stationary phase and chloroform–isopropanol–acetic acid–water–sodium acetate buffer (pH 4.5) (100:100:14:14:1) as the mobile phase resulted in a good separation of ADM, adriamycinol, adriamycinone and DUM. A typical example of the HPLC analysis of rat gall is shown in Fig. 1. Peaks 1–7 (P1 metabolite–P7 metabolite) representing ADM-related compounds were detected. P2 was identified as adriamycinone and P6 as adriamycinol by HPLC and thin-layer chromatography[6]. The contaminating biological blank was detected at the same site as P5 but in trace amounts. The detection limit for ADM was down to 1.0 ng/ml. Tables I and II show the concentrations of ADM and related fluorescent compounds in AH 109A tumour-bearing rat plasma and thoracic duct lymph after a single intravenous injection (1 mg/kg), as determined by HPLC. AD–NE indicates the total concentration of P2 plus P3 metabolites. The values are expressed as means ± standard errors (ng adriamycin equivalent value/ml).

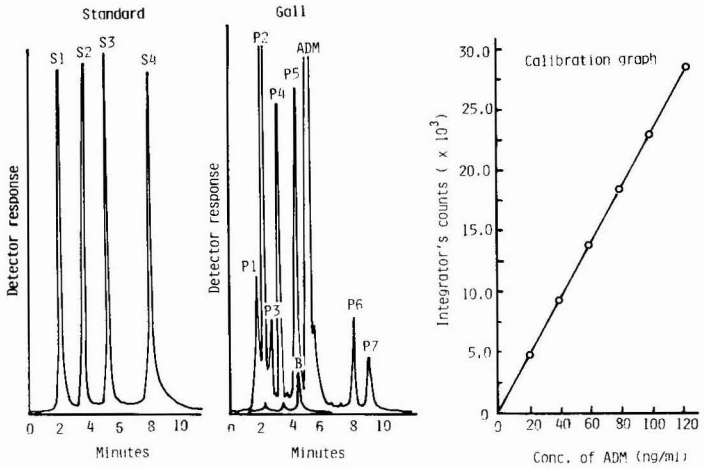


Fig. 1. High-performance liquid chromatogram of a standard sample mixture (standard), ADM-administered rat gall and calibration graph of adriamycin obtained with a Hitachi Model 635A high-performance liquid chromatograph with a Zorbax Sil column (150 × 4.6 mm I.D.) at room temperature. Mobile phase, chloroform–isopropanol–acetic acid–water–sodium acetate buffer (pH 4.5) (100:100:14:14:1); flow-rate, 1.0 ml/min; detector, Hitachi Model 650-10S fluorescence spectrophotometer (excitation, 470 nm; emission, 585 nm); S1 = adriamycinone; S2 = daunomycin; S3 = adriamycin; S4 = adriamycinol; P2 = adriamycinone; P6 = adriamycinol; ADM = adriamycin; B = gall blank.

ADM and related fluorescent compounds were eliminated rapidly from plasma, but in the thoracic duct lymph the concentration of AD–NE and the total ADM equivalent fluorescent values (total ADM values) showed maxima 30 min after injection and, after a subsequent decrease, increased gradually from 60 to 180 min. The total ADM values in plasma and thoracic duct lymph are shown in Fig. 2 in comparison with the values of total DUM equivalent fluorescent values (total DUM values). Total DUM values in the plasma showed were maximal at 5 min and decrease rapidly from 5 to 30 min, then they are almost constant up to 360 min. Total

TABLE I

CONCENTRATIONS OF ADRIAMYCIN AND RELATED FLUORESCENT COMPOUNDS IN AH 109A TUMOUR-BEARING RAT PLASMA AFTER A SINGLE INTRAVENOUS INJECTION

The values are means ± S.E. for at least eight animals.

Time after injection (min)	*Substance (ng adriamycin equivalent value/ml)*					
	*AD–NE**	*P4 metabolite*	*P5 metabolite*	*Adriamycin*	*Adriamycinol*	*Total*
5	211.29 ± 39.42	9.29 ± 2.73	35.31 ± 12.93	159.82 ± 80.32	5.07 ± 1.59	418.11 ± 113.69
30	100.12 ± 14.72	13.49 ± 1.31	21.56 ± 9.70	24.40 ± 9.31	2.13 ± 0.23	168.19 ± 25.20
180	65.39 ± 9.84	6.80 ± 1.38	25.47 ± 10.97	16.37 ± 3.36	1.53 ± 0.44	113.00 ± 16.72
360	48.01 ± 13.26	6.99 ± 2.95	23.45 ± 8.07	8.36 ± 2.97	1.41 ± 0.62	84.56 ± 14.76

* AD–NE = P2 metabolite plus P3 metabolite.

TABLE II

CONCENTRATIONS OF ADRIAMYCIN AND RELATED FLUORESCENT COMPOUNDS IN AH 109A TUMOUR-BEARING RAT THORACIC LYMPH AFTER A SINGLE INTRAVENOUS INJECTION

The values are means ± S.E. for at least five animals.

Time after injection (min)	*Substance (ng adriamycin equivalent value/ml)*					
	*AD–NE**	*P4 metabolite*	*P5 metabolite*	*Adriamycin*	*Adriamycinol*	*Total*
30	532.95 ± 247.71	5.55 ± 4.22	58.24 ± 53.74	104.06 ± 35.59	3.51 ± 1.78	704.29 ± 329.19
60	271.49 ± 84.98	4.88 ± 2.03	31.88 ± 25.16	92.25 ± 33.65	3.77 ± 1.35	404.37 ± 123.53
90	394.77 ± 179.44	5.08 ± 2.54	9.05 ± 4.17	57.63 ± 24.62	3.76 ± 1.18	470.28 ± 183.96
120	390.46 ± 152.46	2.84 ± 2.37	10.57 ± 5.20	112.18 ± 80.03	3.31 ± 1.13	519.36 ± 216.64
150	447.30 ± 102.79	1.55 ± 1.34	3.66 ± 1.91	67.51 ± 27.52	3.86 ± 1.95	539.88 ± 106.97
180	647.84 ± 300.64	2.54 ± 1.71	11.05 ± 5.32	114.14 ± 65.62	2.53 ± 1.42	779.71 ± 322.15
0–180	468.33 ± 105.78	4.35 ± 1.54	19.24 ± 13.60	94.54 ± 38.21	3.28 ± 0.49	589.74 ± 130.10

* AD–NE = P2 metabolite plus P3 metabolite.

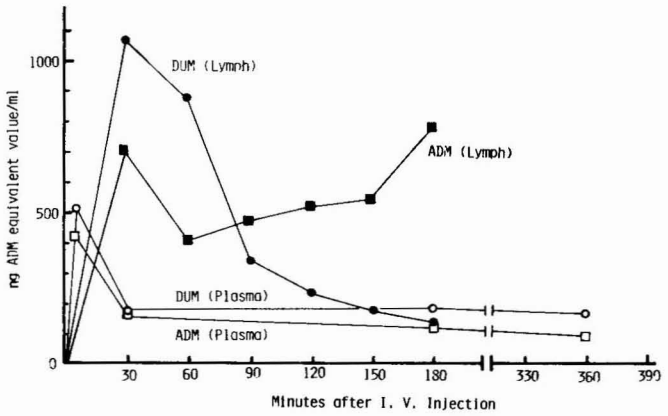


Fig. 2. Concentrations of total adriamycin (ADM) and daunomycin (DUM) (equivalent values) in AH 109A tumour-bearing rat plasma [ADM (plasma) and DUM (plasma)] and thoracic duct lymph [ADM (lymph) and DUM (lymph)] after a single intravenous injection, examined by HPLC. The values are means for at least five animals.

DUM values in the rat thoracic duct lymph differed from the ADM value, showing a maximum at 30 min, then decreasing gradually to near to the its plasma value at 180 min.

Table III shows the ratios of ADM and related compounds in lymph to those in plasma 30 and 180 min after injection: the AD–NE, ADM and total ADM values at 180 min were up to 1.5 times the corresponding values at 30 min.

The passing of ADM into rat gall is illustrated in Fig. 3. The total ADM level was maximal at 30 min (20.0 μg/ml), being rich in ADM (unchanged form), and thereafter decreased gradually up to 120 min. At 450 min, the level was still (5.0 μg/ml).

DISCUSSION

We have previously reported a method for the determination of ADM in serum and tissues with Zorbax Sil as the stationary phase and 3.8% sodium acetate in isopropanol as the mobile phase[6,12]. However, there were problems with broad chromatographic peaks and the long analytical time (22 min). In this study, we devised a method based on these points that gave sharp chromatographic peaks, with a rapid (10 min) and highly sensitive determination of ADM.

For the identification of metabolites of ADM, opinions vary about contamination of aglycones and polar metabolites (containing adriamycinol)[3,5,6,13–15], but in some work no metabolites were detected[2]. In our experiments, the main metabolites in rat plasma, lymph and gall were adriamycinone, P4 metabolite, P5 metabolite and adriamycinol, and other minor metabolites were also detected.

ADM was shown to be passed into lymph or gall in high concentrations. Because lecithin and bile acid, the main constituents of gall, have been related to the passing of drugs into the lymph[16], a high concentration of ADM in lymph was considered to be correlated with its high excretion into gall.

Arena *et al.*[7] first reported on the pharmacokinetics of ADM in mice, stating that the concentrations in most tissues were at least one or two orders of magnitude

TABLE III

RATIOS OF ADRIAMYCIN AND RELATED FLUORESCENT COMPOUNDS IN LYMPH TO THOSE IN PLASMA FROM AH 109A TUMOUR-BEARING RATS AFTER A SINGLE INTRAVENOUS INJECTION

The values are means of at least five determinations.

Time after injection (min)	*Ratio of lymph to plasma levels*					
	*AD–NE**	*P4 metabolite*	*P5 metabolite*	*Adriamycin*	*Adriamycinol*	*Total*
30	5.32	0.41	2.70	4.26	1.65	4.19
180	9.91	0.37	0.43	6.97	1.65	6.90

* AD–NE = P2 metabolite plus P3 metabolite.

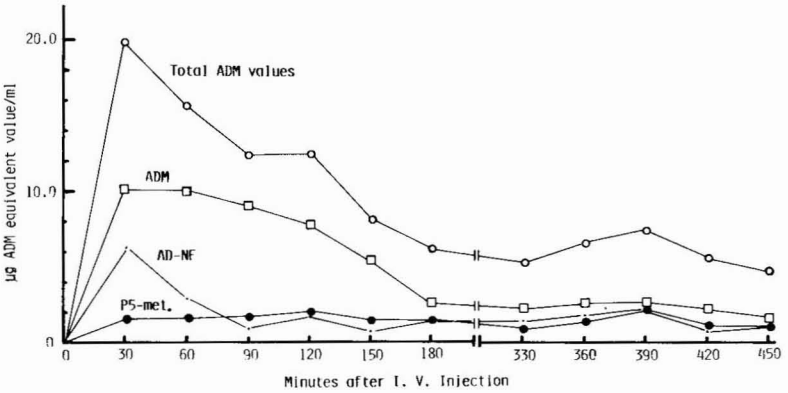


Fig. 3. Concentrations of adriamycin and related fluorescent compounds in AH 109A tumour-bearing rat gall after a single intravenous injection. Total ADM values = total adriamycin equivalent values; ADM = adriamycin; AD-NE = P2 metabolite plus P3-metabolite; P5-met. = P5 metabolite. The values are means for at least three animals.

greater than blood levels, and demonstrated that biliary excretion was the primary route of elimination. The high retention of ADM in tissues in various animals has been reported[3,6–8]. For this reason, it is considered that ADM is structurally similar to quinone compounds present in animal tissues, and can enter coenzyme Q and related enzyme systems[17] or has an especially strong affinity to deoxyribonucleic acid[9] and some negatively charged phospholipids[18].

ACKNOWLEDGEMENTS

This work was supported in part by a grant in aid for Japanese Cancer Research from the Ministry of Education, Science and Culture. We thank Dr. H. Satoh, Sasaki Institute, for provinding rat AH 109A ascites tumour. Thanks are due to Dr. Y. Araki for providing facilities for this work.

REFERENCES

1 G. Bonadonna, S. Monfardini, M. de Lena, F. Fossati-Bellani and G. Beretta, *Proc. 1st Int. Symp. Adriamycin, Milan, Italy, September 9–10, 1971*, Springer, Berlin, Heidelberg, New York, 1972, p. 139.
2 R. Rosso, C. Ravazzoni, M. Esposito, R. Sala and L. Santi, *Eur. J. Cancer*, 8 (1972) 455.
3 D. W. Yesair, E. Schwarzbach, D. Shuck, E. P. Denine and M. A. Asbell, *Cancer Res.*, 32 (1972) 1177.
4 R. S. Benjamin, C. E. Riggs and N. R. Bachur, *Clin. Pharmacol. Ther.*, 14 (1973) 592.
5 R. Baurain, D. Deprez-De Campeneere and A. Trouet, *Anal. Biochem.*, 94 (1979) 112.
6 S. Shinozawa, Y. Mimaki, Y. Araki and T. Oda, *J. Chromatogr.*, 196 (1980) 463.
7 E. Arena, N. D'Alessandro, L. Dusochet, N. Gebbia, F. Gerbasi, M. Palazzoadriano, A. Raineri, L. Rausa and E. Tubaro, *Arzneim.-Forsch.*, 21 (1971) 1258.
8 P. A. Harris and J. F. Gross, *Cancer Chemother. Rep.*, 59 (1975) 819.
9 A. Di Marco and F. Arcamone, in M. Staquet (Editor), *Adriamycin Rev., 2nd EORTC Int. Symp.*, European Press, Ghent, 1975, p. 11.
10 P. M. Wilkinson, M. Israel, W. J. Pegg and E. Frei, *Cancer Chemother. Pharmacol.*, 2 (1979) 121.
11 J. L. Bollman, J. C. Cain and J. H. Grindlay, *J. Lab. Clin. Med.*, 33 (1948) 1349.
12 S. Shinozawa, Y. Mimaki, H. Tomano, Y. Araki and T. Oda, *J. Chromatogr.*, 190 (1980) 489.
13 J. J. Langone and H. V. Vunakis, *Biochem. Med.*, 12 (1975) 283.
14 K. K. Chan and C. D. Wong, *J. Chromatogr.*, 172 (1979) 343.

15 R. S. Benjamin, C. E. Riggs, Jr. and N. R. Bachur, *Cancer Res.*, 37 (1977) 1416.
16 T. Noguchi, K. Taniguchi, T. Yoshifuji, S. Muranishi and H. Sezaki, *Chem. Pham. Bull.*, 25 (1977) 2231.
17 T. Kishi and K. Folkers, *Cancer Treat. Rep.*, 60 (1976) 223.
18 E. Goormaghtigh, P. Chatelain, J. Caspers and J. M. Ruysschaert, *Biochim. Biophys. Acta*, 597 (1980) 1.

Journal of Chromatography, 212 (1981) 331–338
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,895

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF PILOCARPINE HYDROCHLORIDE, ISOPILOCARPINE, PILOCARPIC ACID AND ISOPILOCARPIC ACID IN EYE-DROP PREPARATIONS

JOHN M. KENNEDY* and PAMELA E. McNAMARA
National Biological Standards Laboratory, P.O. Box 462, Canberra City, A.C.T. 2601 (Australia)
(Received April 16th, 1981)

SUMMARY

A high-performance liquid chromatographic method is described which allows the simultaneous estimation of pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid. The method offers advantages over existing chromatographic procedures in that pilocarpine and the degradation products are eluted within 15 min making the procedure suitable for routine quality control.

Pilocarpine eye-drop preparations available on the Australian market were determined to contain between 0.4 and 1.9% isopilocarpine and between 2.2 and 6.3% total pilocarpic acids*. These findings are comparable to those reported by Weber in a survey of pilocarpine containing products available in the U.S.A.

INTRODUCTION

The alkaloid pilocarpine is used topically as a miotic in the treatment of glaucoma. In aqueous solution degradation can occur either through epimerisation to isopilocarpine or hydrolysis to pilocarpic and isopilocarpic acids[1,2]. Both mechanisms result in a loss of pharmacological activity.

Analytical procedures for pilocarpine based on classical titrimetric or spectrophotometric procedures are described in a number of texts[3–8]. None of these methods is capable of distinguishing the epimers of pilocarpine.

The monographs for pilocarpine salts in the British Pharmacopoeia[6] and the United States Pharmacopoeia (USP)[8] include requirements for specific rotation which may be interpreted as indirectly assigning limits to the total impurity levels. Weber[9] calculated that the range of specific rotation permitted in USP XIX monograph[8] for pilocarpine hydrochloride allows a maximum of either 6% isopilocarpine, or 3% pilocarpic acid. The monographs for pilocarpine preparations, however do not include requirements for specific rotation presumably because excipients may interfere with optical rotation measurements.

* Subsequent reference to pilocarpic acid content shall be understood to mean the total content of pilocarpic and isopilocarpic acids.

0021-9673/81/0000–0000/$02.50

A survey of the literature revealed that nuclear magnetic resonance[2,10] and chromatographic procedures[9,11–14] had been devised for the quantitation of pilocarpine and degradation products.

Urbanyi *et al.*[11] reported the separation of the pilocarpine epimers by ion exchange on Aminex A-7. Weber[9] modified this procedure and estimated the content of pilocarpic acids by cyclizing them to pilocarpine and measuring the resultant increase in alkaloid content of the sample. Weber conceded the inconvenience of the two stage analysis and the possibility that cumulative errors could lead to inaccurate results.

Wahba Khalil[12] joined a μBondapak C_{18} column and a μBondapak CN column in series and used as mobile phase borate buffer (adjusted to pH 9.2)–tetrahydrofuran (7:3). Our investigations to establish the usefulness of this procedure were abandoned after it was determined that the mobile phase had a tendency to dissolve the silica base of the column packing material.

Mitra *et al.*[13] enhanced the detectability of the pilocarpine epimers by forming *p*-nitrobenzyl bromide derivatives which were subsequently separated by ion-pair chromatography on a μBondapak C_{18} column. A sample chromatogram included in the paper showed that the isopilocarpine derivative eluted as an unresolved rider on the tail of the pilocarpine peak which may lead to potential quantitation difficulties. No mention of detection or estimation of the pilocarpic acids was made.

Noordam *et al.*[14] separated pilocarpine and its degradation products on a C_{18} column using a mobile phase of water–methanol (97:3) containing 5% of potassium dihydrogen orthophosphate adjusted to pH 2.5 with orthophosphoric acid. Our attempts to repeat the method gave poor resolution and excessive tailing. The sample chromatogram included in the paper by Noordam *et al.* indicates that these workers experienced similar problems.

In this paper we present a simple method involving minimal sample preparation which would be suitable for routine quality control of pilocarpine eye-drop formulations. Emphasis has been placed on reducing analysis times while maintaining resolution consistent with the needs of quantitation. The procedure described has been used to survey a wide range of pilocarpine eye-drop preparations available in Australia.

EXPERIMENTAL

Materials

Pilocarpine nitrate was obtained from BDH (Poole, Great Britain) and isopilocarpine nitrate from Koch-Light Labs. (Colnbrook, Great Britain). Both alkaloids were used as received. All other reagents and chemicals were either analytical grade or high purity. Water was freshly distilled.

Equipment

The high-performance liquid chromatographic (HPLC) system comprised a Varian 8500 constant-flow pump; an Altex Model 905-42 syringe-loading sample injector fitted with a 20-μl loop; a Waters Assoc. (Milford, MA, U.S.A.) μBondapak phenyl column 30 cm × 3.9 mm; a Varian Aerograph temperature-controlled waterbath; a Perkin-Elmer LC 55 spectrophotometer fitted with a Coleman 55-204 UV accessory and a Hewlett-Packard Model 3380A integrator.

The pH measurements were made with a Radiometer (Copenhagen, Denmark) PHM64 research pH meter which was standardised with Radiometer buffers 51316 (pH 4.01) and s1001 (pH 6.5).

The mobile phase was filtered immediately prior to use with a Millipore filtration assembly XX10047 30 using 0.5-μm filters.

Chromatographic conditions

The column was maintained at 40 $\pm$ 1°C. The mobile phase was a 5% (w/v) aqueous solution of potassium dihydrogen orthophosphate adjusted to pH 2.5 with orthophosphoric acid. Flow-rate was 1 ml/min. The UV detector was set at 215 nm. Integrator settings were attenuation 4, slope sensitivity 0.3 and chart speed 0.5 cm/min.

Samples and standards were chromatographed in the following order, standard 1 followed by 4 samples, consisting of alternated duplicates from 2 batches, then an injection of standard 2 and a group of 4 samples from another 2 batches, followed by a standard 1 and so on.

Before commencing the day's analysis mobile phase was pumped through the column for 1.5 h at a rate of 2 ml/min to establish stable baseline conditions. At the conclusion of each day the column was flushed with 30 ml of water and 30 ml of methanol–water (60:40, v/v).

Preparation of samples and standards

Standards and samples were prepared in 0.2 *M* Na_2SO_4 solution, in order to minimise disturbances of ionic equilibria in the column. Weber[9] had earlier demonstrated the stability of pilocarpine in this medium.

Eye-drop preparations assayed contained pilocarpine as the hydrochloride but the more readily available nitrate salt was chosen as the reference standard. In the preparation of standards 1 and 2 below it is necessary to make allowance for the isopilocarpine content of the pilocarpine nitrate standard.

Each standard contained nominally 1.108 mg/ml of pilocarpine nitrate; in addition standard 1 contained 4.43 μg/ml and standard 2 0.554 μg/ml of isopilocarpine nitrate. These standard solutions (1 and 2) correspond to a nominal content of 1 mg/ml of pilocarpine hydrochloride containing respectively 4% and 0.5% (w/w) of added isopilocarpine hydrochloride. It was anticipated that these levels of isopilocarpine contamination would represent the extremes which may occur in the samples to be surveyed.

Eye-drop preparations were diluted to a nominal concentration of 1 mg/ml of pilocarpine hydrochloride with 0.2 *M* Na_2SO_4. The dilutions were carried out on the day of analysis.

We were unable to locate a source of either pilocarpic or isopilocarpic acid. It was therefore necessary to determine an equivalence factor which related the peak areas of the pilocarpic acids to their concentration. This determination involved the quantitative hydrolysis of a known amount of pilocarpine, following the procedure described below.

About 80 mg of pilocarpine nitrate was weighed accurately and diluted to 200 ml with water. A 25-ml aliquot was refluxed for 1 h with 5 ml of 1 *M* sodium hydroxide. The resultant solution was adjusted to about pH 7 with 0.5 *M* ortho-

phosphoric acid (universal indicator paper), transferred quantitatively to a 50-ml volumetric flask and diluted to volume with water. The hydrolysis procedure was performed in triplicate.

A 25-ml aliquot of the original pilocarpine nitrate solution was diluted to 50 ml with water. Each solution was chromatographed and the mean equivalence factor calculated dividing the pilocarpine peak area by the mean of the pilocarpic acid peaks.

Linearity of standard curves

The relationship between pilocarpine peak areas and concentration was investigated in the concentration range corresponding to a sample assay range of between 70 and 110% of labelled content. A linear regression fit gave a correlation coefficient (r) of 0.99 ($n = 9$).

Solutions of pilocarpine nitrate were spiked with isopilocarpine to investigate the linearity of the relationship between peak height and concentration of isopilocarpine in the concentration range equivalent to 1–5% isopilocarpine contamination. A linear regression fit gave $r = 0.99$ ($n = 15$).

RESULTS AND DISCUSSION

Our investigation of the methods in the literature indicated that the method of Noordam *et al.*[14] appeared to be the most promising for routine quality control of pilocarpine eye-drops. In practice, however, poor resolution due to excessive tailing of the peaks made quantitation difficult. The method was modified as follows.

The ODS column was replaced by a μBondapak phenyl column, which reduced analysis times while improving peak shapes and resolution. Increasing column temperatures led to reduced capacity factors and sharper peaks and a plot of temperature against resolution showed a definite maximum at 40°C. In agreement with the observations of Noordam *et al.*[14] we found that decreasing pH and increasing ionic strength resulted in less peak tailing. However, a compromise was necessary because use of a mobile phase with too low a pH resulted in overlap of the pilocarpic acids, and pilocarpine peaks. A pH of 2.5 was optimum. We adopted the suggestion of Noordam *et al.* that use of a mobile phase consisting of 5% potassium dihydrogen orthophosphate provided a reasonable compromise between peak shape and the life of the column. A discussion of the influence of pH and temperature on chromatographic separations has been given by Horváth *et al.*[15] and Gant *et al.*[16] respectively.

Initially difficulties were experienced with the quantitation of isopilocarpine. It was noted that the response factor for isopilocarpine (peak height per unit concentration) changed slowly but uniformly during the course of a day's analysis. This behaviour appeared to be linked to the age of the mobile phase since results from later experiments conducted with freshly prepared mobile phase showed no drift. Before this link was established the rate of drift was determined by making a series of injections of standard at particular times recorded throughout the day's analysis. The appropriate response factor for each sample could then be interpolated and used to calculate the isopilocarpine content. The analysis results obtained with this drift compensation compared very closely to those obtained when the same samples were re-analysed with freshly prepared mobile phase. Smuckler[17] and Rabel[18] reported

chromatographic problems arising from the gradual accumulation of un-identified UV absorbing impurities in solutions of KH_2PO_4. In order to check the relevance of their findings to our observed drift in response factor, mobile phases were prepared using both highly purified and artificially contaminated KH_2PO_4, prepared according to Smuckler's[17] procedure. No discernable difference was noted in the resultant chromatograms. To date no explanation has been found for the observed drift but the age of the mobile phase appears to be a factor.

The results of the survey are given in Table I. The samples consisted of 29 batches from 4 different manufacturers which are identified by the letters A to D. Weber[9] surveyed 12 batches from 10 manufacturers available on the U.S.A. market. Table II compares the results of our survey to those obtained in Weber's survey and shows the mean pH and mean contents of pilocarpine, isopilocarpine and pilocarpic acid. The range and standard deviation of each set of values are also included.

TABLE I

EXPERIMENTAL RESULTS OF SURVEY

Manufacturer	*Pilocarpine content*		*Isopilocarpine percent of total alkaloids**	*Pilocarpic acid percent of total alkaloids**	*pH*
	Manufacturer label claim (%)	*Percent label claim determined*			
A	0.5	94.4	0.63	2.74	4.46
A	0.5	91.7	0.65	2.38	4.53
B	0.5	96.5	1.23	4.91	4.49
C	0.5	97.6	1.21	5.06	4.49
D	0.5	103.3	1.90	6.26	5.02
A	1.0	96.8	0.51	3.91	4.18
A	1.0	94.5	0.74	3.36	4.24
B	1.0	94.3	0.95	3.79	4.24
C	1.0	93.1	0.96	3.83	4.24
D	1.0	101.1	1.75	5.35	4.81
A	2.0	94.1	0.74	4.01	4.08
A	2.0	95.9	0.67	3.89	4.09
B	2.0	94.0	0.84	3.27	4.19
C	2.0	95.0	0.84	3.13	4.20
D	2.0	103.1	1.15	4.70	4.50
A	3.0	93.0	0.43	4.75	3.98
A	3.0	92.2	0.43	4.61	3.99
B	3.0	96.2	1.37	4.79	3.92
C	3.0	98.5	1.17	3.98	4.36
D	3.0	105.4	1.06	4.98	4.23
A	4.0	93.9	0.63	2.20	3.97
A	4.0	97.4	0.66	4.24	3.96
B	4.0	96.2	1.06	4.16	4.14
C	4.0	95.0	1.04	4.22	4.14
D	4.0	106.2	0.84	3.72	4.25
A	6.0	93.9	0.58	4.42	3.95
A	6.0	95.6	0.76	4.27	4.19
B	6.0	96.3	1.45	4.25	4.40
D	6.0	103.6	1.35	4.23	4.19

* Total content of pilocarpine and isopilocarpine.

TABLE II

MEAN RESULTS OF U.S.A. SURVEY COMPARED TO AUSTRALIAN SURVEY

The first result in each block is the mean and standard deviation, the second is the range of values determined.

	Results	
	U.S.A. survey	*Australian survey*
pH	4.36 ± 0.60 3.55–5.20	4.26 ± 0.26 3.92–5.02
Pilocarpine as % of labelled strength	98.7 ± 3.8 93.7–104	96.9 ± 4.0 91.7–106.2
Isopilocarpine as % of total alkaloids	1.5 ± 0.9 0.4–3.4	1.0 ± 0.4 0.4–1.9
Pilocarpine acids as % of total alkaloids	3.9 ± 2 0.6–6.8	4.1 ± 0.9 2.2–6.3

An investigation of the relationship between isopilocarpine content (as a percentage of total alkaloids) and pH for the data obtained in our survey is shown in Fig. 1. The results of the linear regression analysis, were slope 0.47 (0.54), intercept pH 3.8 (3.5) and r 0.68 (0.84). The results are similar to the results reported by Weber which are given in parentheses. The discrepancy in r values is largely due to the outlying data point circled in Fig. 1. Rejection of this point changes the slope to 0.53 and r to 0.78. A linear regression analysis of content of pilocarpic acids against pH of the samples gave poor correlation ($r = 0.28$) which is in agreement with the data reported by Weber.

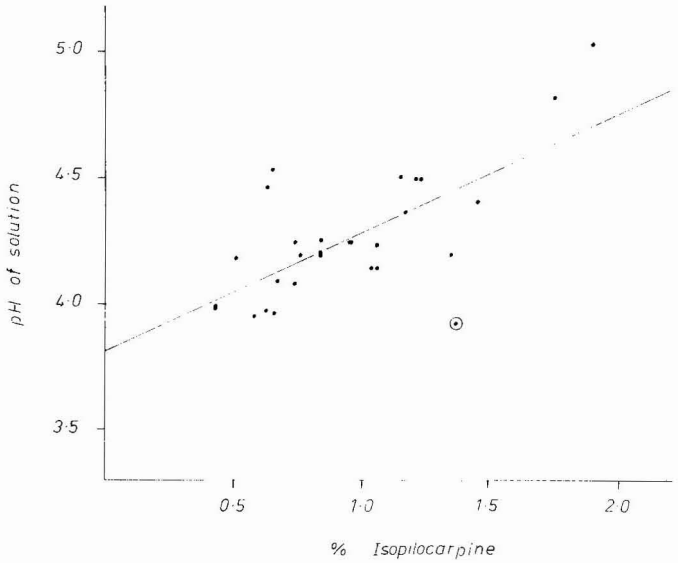


Fig. 1. Correlation of isopilocarpine content and pH of eye-drops solution.

The main difference between the two surveys was the isopilocarpine content of the preparations. Weber found a higher mean content (1.5) and a greater range (0.4–3.4) of individual contents than was found in the Australian formulations, mean (1.0) range (0.4–1.9). This difference is consistent with the following considerations. As demonstrated by Weber and corroborated by our data, isopilocarpine content is often proportional to pH (see Fig. 1). The majority of samples examined by Weber had pH values of either above 5.0 or below 4.0. This contrasts with our narrow pH range of between 4.0 and 4.5 with one sample at pH 5.0. Thus the wider range of isopilocarpine contents found by Weber would appear to be consistent with the wider pH range of the samples examined.

The samples taken for this survey had been manufactured between 4 and 25 months prior to analysis. There does not appear to be a linear relationship between age and content of either degradation product ($r = 0.31$), gradient (m) = 0.02 for isopilocarpine and $r = 0.04$, $m = 0.23$ for content of pilocarpic acids. This lack of correlation between age and content of degradation products may suggest good long-term stability for pilocarpine eye-drop preparations. The differences in degradation product concentrations between samples may be accounted for by differences in history of sample preparation and source of pilocarpine. It is intended to establish the stability of the samples analysed in this survey by re-analysing them at a later date.

Of the 41 batches analysed in the two surveys, only 3 samples were found to have isopilocarpine contents in excess of 2% of total alkaloid content. These 3 samples were part of Weber's survey and were found to contain 2.5, 2.6 and 3.4% respectively of isopilocarpine. These same 3 samples also showed atypically high pH

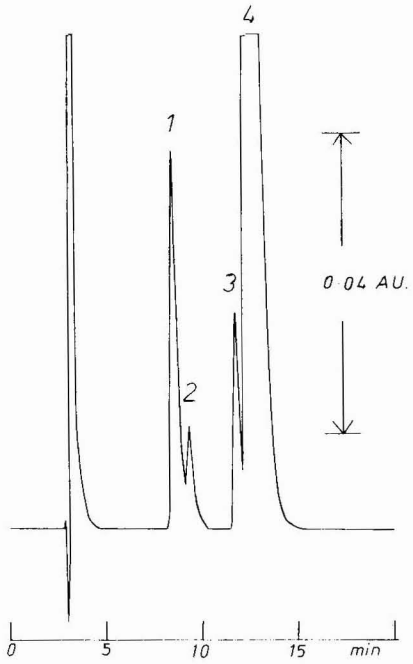


Fig. 2. Separation of standard pilocarpine (4), isopilocarpine (3), isopilocarpic acid (2), and pilocarpic acid (1).

values of 5.20, 5.00 and 5.05 respectively. The normally low isopilocarpine levels found suggest therefore that classical techniques which determine total alkaloid content may give a reasonable indication of pharmacological activity. It should be noted however than Urbanyi *et al.*[11] reported that one preparation of pilocarpine nitrate contained 25.2% isopilocarpine.

CONCLUSION

The HPLC method described allows the simultaneous quantitation of pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid in eye-drop preparations. The analysis time, sample preparation and low cost mobile phase make the method suitable for routine quality control and stability trials.

Experience indicates that the mobile phase should be freshly prepared, as ageing results in a drifting isopilocarpine peak height response factor. The cause of this drift is unknown.

As baseline resolution of the pilocarpine epimers was not achieved (Fig. 2) it is recommended that epimer resolution be optimised for each system by manipulation of mobile phase, pH and temperature.

ACKNOWLEDGEMENT

We thank Mr. J. Boots for assistance with the data processing.

REFERENCES

1 P.-H. Chung, T.-F. Chin and J. Lach, *J. Pharm. Sci.*, 59 (1970) 1300.
2 M. A. Nunes and E. Brochmann-Hanssen, *J. Pharm. Sci.*, 63 (1974) 716.
3 T. Higuchi and E. Brochmann-Hanssen, *Pharmaceutical Analysis*, Interscience, New York, 1961, Ch. VIII.
4 E. Brochmann-Hanssen, P. Schmid and J. D. Benmaman, *J. Pharm. Sci.*, 54 (1965) 783.
5 Y. A. Mohamed and M. A. El-sayed, *Pharmazie*, 30 (1975) 60.
6 *British Pharmacopoeia 1968*, Pharmaceutical Press, London, 1968, p. 775.
7 *British Pharmaceutical Codex 1973*, Pharmaceutical Press, London, 1973, pp. 382, 694.
8 *The United States Pharmacopoeia*, Mack Publishing Co., Eastern, PA, 19th rev., 1975, p. 384.
9 J. D. Weber, *J. Ass. Offic. Anal. Chem.*, 59 (1976) 1409.
10 G. A. Neville, F. B. Hasan and I. C. P. Smith, *J. Pharm. Sci.*, 66 (1977) 638.
11 T. Urbanyi, A. Piedmont, E. Willis and G. Manning, *J. Pharm. Sci.*, 65 (1976) 257.
12 S. K. Wahba Khalil, *J. Pharm. Sci.*, 66 (1977) 1625.
13 A. K. Mitra, C. L. Baustian and T. J. Mikkelson, *J. Pharm. Sci.*, 69 (1980) 257.
14 A. Noordam, K. Waliszewski, C. Olieman, L. Maat and H. C. Beyerman, *J. Chromatogr.*, 153 (1978) 271.
15 Cs. Horváth, W. Melander and I. Molnar, *Anal. Chem.*, 49 (1977) 142.
16 J. R. Gant, J. W. Dolan and L. R. Snyder, *J. Chromatogr.*, 185 (1979) 153.
17 H. W. Smukler, *J. Chromatogr. Sci.*, 8 (1970) 581.
18 F. M. Rabel, *J. Chromatogr. Sci.*, 18 (1980) 46.

Journal of Chromatography, 212 (1981) 339–346
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,846

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF SODIUM NITROPRUSSIDE

DAVID MICHAEL BAASKE*

Analytical Development, Product Development Section, Research and Development, American Critical Care, McGaw Park, IL 60085 (U.S.A.)

MARILYN DIX SMITH

Lederle Laboratories, Analytical Development & Product Stability, Quality Assurance Section, Pearl River, NY 10965 (U.S.A.)

and

NANCY KARNATZ and JAMES E. CARTER

Analytical Development, Product Development Section, Research and Development, American Critical Care, McGaw Park, IL 60085 (U.S.A.)

(Received March 23rd, 1981)

SUMMARY

A rapid high-performance liquid chromatographic method for the determination of nitroprusside in commercial lyophilized products or in intravenous admixture solutions is described. The method is stability-indicating. Reversed-phase liquid chromatography was performed using a microparticulate (10 μm) phenyl column with a mobile phase acetonitrile–phosphate/tetrabutylammonium hydroxide buffer (pH 7.1) (30:70) and detection at 210 nm. A coefficient of variation of less than 3.1 % was achieved over the concentration range studied (10–50 μg/ml). Total analysis time was 9 min.

This method was used to show that there is a small loss of nitroprusside due to photodegradation during intravenous infusion, even when the admixture container is wrapped in foil as recommended and used expeditiously.

INTRODUCTION

Sodium nitroprusside is hydrated sodium nitrosyl pentacyanoferrate $Na_2Fe(CN)_5NO \cdot 2H_2O$. It is a potent, rapid-acting hypotensive agent when administered intravenously. Sterile sodium nitroprusside (U.S.A. Pharmacopoeia, USP) is lyophilized sodium nitroprusside. The USP assay for this product is a polarographic determination of the ferric ion at a dropping mercury electrode. Polarographic methods for nitroprusside[1–3] require specialized instrumentation not available in many laboratories and are not suitable for automation for routine analysis. Colorimetric[4,5] and spectrophotometric[1,6] methods have been described for the determination of sodium nitroprusside and its photodegradation products. However, they are either

0021-9673/81/0000-0000/$02.50

not stability-indicating or they rely on unproven assumptions about the underlying chemistry of the photodegradation. Coulometric, gravimetric and titrimetric methods have also been described[7] but they also are time-consuming or require unusual instrumentation.

High-performance liquid chromography (HPLC) has become the method of choice for the determination of stability of organic pharmaceuticals, but has had little application in the quantitation of inorganic or quasi-inorganic compounds. A method for the determination of nitrate and nitrite by HPLC with ultraviolet (UV) detection was recently described[8]. This paper reports a method using ion-pair reversed-phase HPLC with UV detection for the rapid direct measurement of nitroprusside in vials or diluted intravenous solutions, and which provides the potential for measuring breakdown products. In the presence of light, sodium nitroprusside rapidly undergoes a wide variety of reactions to yield numerous products depending on the characteristics of the impinging light[1]. Therefore its stability in an infusion set was questionable.

EXPERIMENTAL

Materials

Sodium nitroprusside (Fisher Scientific, Fairlawn, NJ, U.S.A.) was used as the standard. Sterile sodium nitroprusside, USP (Roche Labs., Nutley, NJ, U.S.A.) and dextrose (5%) injection, USP (American McGaw, Irvine, CA, U.S.A.) were used to prepare admixtures. Distilled-in-glass acetonitrile (Burdick & Jackson, Muskegon, MI, U.S.A.), tetrabutylammonium hydroxide (Eastman, Rochester, NY, U.S.A.) sodium nitrate, sodium nitrite, potassium ferricyanide, potassium ferrocyanide and potassium phosphate monobasic (Mallinckrodt, St. Louis, MO, U.S.A.) were used without further purification. Purified water was further purified (Milli-Q water purification system, Millipore, Bedford, MA, U.S.A.) prior to use.

Apparatus

The liquid chromatographic system consisted of a solvent pumping system, an automatic sampler equipped with a 50-μl sample loop, a variable-wavelength detector (detection wavelength 210 nm) and a 10-mV recorder (Perkin-Elmer, Norwalk, CT). A 30 cm $\times$ 3.9 mm I.D. column packed with phenyl bonded to 10-μm silica gel (Water Assoc., Milford, MA, U.S.A.) was employed.

Mobile phase

The mobile phase was prepared fresh daily by thoroughly mixing 300 ml of acetonitrile and 700 ml of buffer. The buffer was 0.01 *M* potassium phosphate monobasic and 0.005 *M* tetrabutylammonium hydroxide adjusted to a final pH of 7.1 with phosphoric acid. The mobile phase was filtered through a 0.5-μm filter (Millipore) prior to use and pumped at a constant flow-rate of 2 ml/min (<2000 p.s.i.).

Standards

Sodium nitroprusside stock standard solutions were prepared at 1 mg/ml in 5% dextrose in water USP (D_5W). The flask was wrapped in foil to protect it from light.

The stock standard nitroprusside solution was diluted with D_5W to yield standards of 50, 40, 25 and 10 μg/ml, which were also wrapped in foil for protection from light.

A 1.5-ml volume of each standard and each sample was placed in autosampler vials (which had been made opaque with interior latex paint), capped and injected in duplicate by the autosampler. Peak heights were used to construct calibration curves and for sample analysis. Peak areas may also be used.

Analysis of unit dosage

The lyophilized sodium nitroprusside vials were reconstituted with exactly 5.0 ml of D_5W. The vial was sealed and shaken to dissolve the sodium nitroprusside. Exactly 1.0 ml of this solution was quantitatively transferred to a 200-ml volumetric flask and brought to volume with D_5W. The flask was wrapped with foil to protect it from light. Quadruplicate samples (50 μg/ml) were prepared from each vial.

Infusion set study

Admixture solutions were prepared to contain sodium nitroprusside 100 μg/ml as per package insert instructions. Solutions were immediately wrapped in foil. Prior to the attachment of the infusion set a control sample was taken from the admixture. Nitroprusside degradation while traversing the infusion set was defined in relation to this control, which was taken as 100% of theoretical. The infusion set spike was inserted into the IV bottle which was then inverted and hung. The tubing was filled by gravity flow upon release of the roller clamp. Flow-rate regulation was accomplished by measuring delivered volume over time at 10 drops/min. Flow-rates were monitored throughout the test period. An initial 3-ml sample was taken from each set. Additional samples were taken at 30, 60, 90, 120, 180, 240 and 300 min. The samples were stored in foil-wrapped glass vials with rubber stoppers at 10°C until assayed. These samples were diluted 1:1 with D_5W prior to analysis.

Light studies

Admixtures were prepared as described above in label free bottles and stored in a high intensity light cabinet (1400–2000 foot-candles), in sunlight and under 24 h/day flourescent lights. A control wrapped in foil was stored in the dark. These samples were diluted 1:1 with D_5W prior to analysis.

RESULTS AND DISCUSSION

Chromatography

Using ion-pair chromatography with tetrabutylammonium hydroxide (TBA, 0.005 *M*) as the ion-pairing agent, the effect of the eluent composition on the retention time of sodium nitroprusside and its major degradation products as well as potassium ferricyanide and ferrocyanide was investigated. Potassium ferricyanide and ferrocyanide were used to determine whether this HPLC system could differentiate ferrocyanide, ferricyanide and nitroprusside.

As the pH of the aqueous phase was increased, the retention time for nitroprusside, its major unidentified photodegradation product, ferrocyanide and ferricyanide increased as shown in Fig. 1. At the pH values studied, nitroprusside deg-

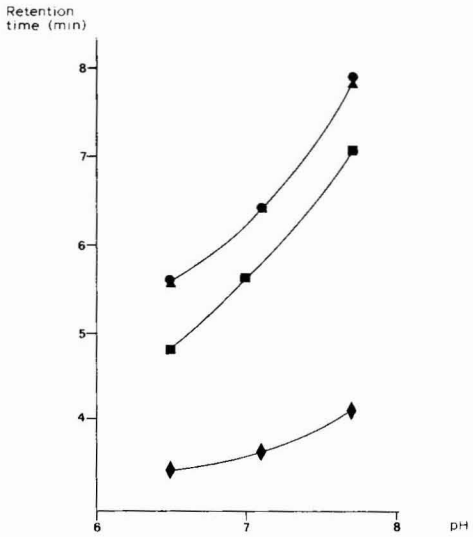


Fig. 1. Effect of the pH of the mobile phase on the retention times of nitroprusside, its major photodegradation product, ferricyanide and ferrocyanide. ●, Nitroprusside; ■, ferrocyanide; ▲, ferricyanide; ◆, degradation product.

radation products eluted first, then ferrocyanide, ferricyanide and finally nitroprusside. Ferricyanide and nitroprusside eluted at about the same retention times at all pH values.

A pH of 7.1 was chosen for the chromatographic procedure because at this pH the greatest separation of degradation products and nitroprusside occurs and the integrity of the column can be maintained.

As the percentage of acetonitrile in the eluent decreases, the retention times for nitroprusside, its major photodegradation products, ferrocyanide and ferricyanide increased as shown in Fig. 2. A mobile phase composition of acetonitrile–buffer (30:70) was chosen for the cnromatographic procedure. Under these conditions nitroprusside is resolved from its photodegradation products and sample time is minimized for routine analysis.

The eluent used in the HPLC procedure for the determination of sodium nitroprusside in the commercial lyophilized product, admixture solutions, infusion set studies and photodegradation studies was acetonitrile–0.005 *M* tetrabutylammonium hydroxide, 0.01 *M* potassium phosphate (pH = 7.1) (30:70). Chromatograms of a sample of nitroprusside admixture solution, reference standard, infusion set sample and photodegraded sample under the above chromatographic conditions are given in Fig. 3. Several extra peaks near the solvent front, presumably degradation products, are present in the photodegraded sample. The retention times of these degradation products are similar to those of nitrite and nitrate.

Precision

Four replicate nitroprusside samples (50, 40, 25 and 10 μg/ml) were automatically injected, and their peak heights were measured (Table I). A correlation coef-

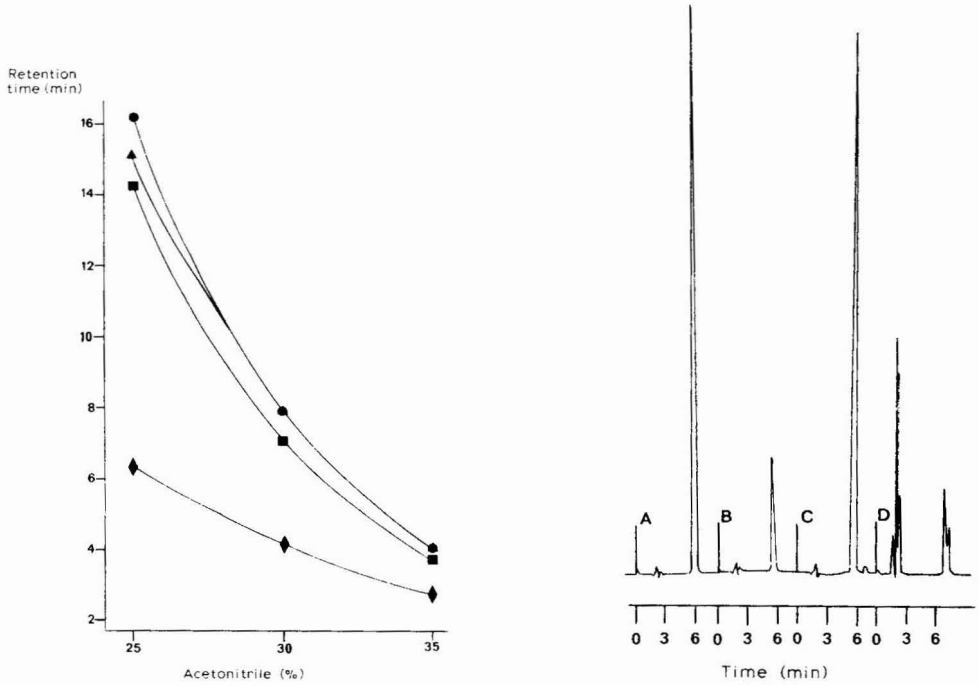


Fig. 2. Effect of acetonitrile content of the mobile phase on the retention times of nitroprusside. Its major photodegradation product, ferricyanide and ferrocyanide. ●, Nitroprusside; ■, ferrocyanide; ▲, ferricyanide; ◆, degradation product.

Fig. 3. Actual chromatograms of nitroprusside admixtures. (A) Sodium nitroprusside admixture in dextrose (5%); nominal concentration is 50 μg ml. (B) Sodium nitroprusside standard at a nominal concentration of 10 μg/ml. (C) Sodium nitroprusside sample at a nominal concentration of 50 μg/ml after passage through an infusion set exposed to normal room light. (D) Sodium nitroprusside admixture after 3 days in a 1400–2000 foot-candle light cabinet. Nominal initial concentration of 50 μg/ml.

ficient of greater than 0.999, a coefficient of variation (C.V.) of less than 3.1% and an accuracy of greater than 98.2% were consistently obtained. Although similar results were obtained using peak areas, for consistency peak heights were employed for all analyses.

TABLE I

PRECISION DATA FOR SODIUM NITROPRUSSIDE EMPLOYING PEAK HEIGHTS

Nitroprusside concentration (μg/ml)	*Mean (n = 4) peak height (mm)*	*Adjusted group mean*	*S.D.*
50	182.25	96.6	2.4
40	144.25	100.7	2.1
25	91.00	99.7	1.2
10	36.38	99.7	1.1
Overall	—	99.9	1.8

Potency study

Four vials from two commercial lots of lyophilized sodium nitroprusside were analyzed (Table II). A C.V. between different vials of the same lot of less than 1 % was found. The pooled C.V. was less than 1.4 %.

TABLE II

POTENCY OF SODIUM NITROPRUSSIDE VIALS IN MILLIGRAMS

Label claim is 50 mg.

	Lot A	*Lot B*		
		Vial A	*Vial B*	*Vial C*
Mean (n = 4)	48.16	48.04	48.92	50.02
Standard deviation	0.652	0.477	0.376	0.286

Admixture infusion set study

An admixture of sodium nitroprusside was connected to an infusion set and kept in the dark, which protected the nitroprusside from the effects of light but permitted gas exchange to occur. Duplicate experiments gave no evidence of nitroprusside degradation or adsorption.

Admixture solutions of sodium nitroprusside protected from light with aluminium foil were connected to administration sets and exposed to normal room light. Duplicate experiments were performed. Every delivered potency was equal to, or less than, the bottle control. This is the opposite of what is seen in the light-protected sets. A comparison of the light-exposed and -protected sets (Table III) shows that the light-exposed sets have a lower delivered potency over the entire time span of the experiment. This supports the theory that the loss of nitroprusside potency seen during i.v. infusion is due to light instability and not adsorption to plastic. Although the maximum difference is only 3.5 %, an examination of the chromatograms of samples exposed to light shows very small peaks at approximately the retention times of the light-degradation products. While the peaks are too small to be definitive, they also indicate photodegradation.

TABLE III

POTENCIES DELIVERED FROM i.v. INFUSION SETS

Nominal concentration 100 μg/ml (10 drops/min)

Time (min)	*Sets protected from light (% bottle control)*	*Sets exposed to light (% bottle control)*
0	102.5	100.0
30	101.5	98.8
60	101.3	98.7
90	101.3	98.0
120	101.1	98.0
180	101.4	97.9
240	101.4	98.5
300	101.4	98.4

Because photodegradation would be a function of exposure time, and the exposure time is constant at a uniform drip-rate, each data point can be thought of as a replicate sample. Performing a T-test on the two data sets shows that while the nitroprusside loss due to light degradation is small it is statistically significant.

Photodegradation study

Sodium nitroprusside admixtures were exposed to various types and intensities of light to help assess their stability (Table IV). When properly protected from light the admixtures show good stability. Even when exposed to normal fluorescent light generally acceptable stability was seen for the first few hours. After 6 h on a laboratory bench with the room lights on the sample still exhibited 93% of its initial potency. However, since potentially toxic degradation products may form it would always be wise to follow package insert directions to wrap the infusion bottle with foil and use the admixture quickly after preparation. It is particularly interesting to note that direct sunlight was the most potent source of photodegradation tested, causing a 32% potency loss in only one hour. This ubiquitous potential source of degradation must be considered during all phases of admixture preparation, transportation and use. Finally, a high-intensity light cabinet was found to provide an effective, convenient model system for studying the photodegradation of sodium nitroprusside admixtures as all light sources gave similar chromatographically detected breakdown patterns.

TABLE IV

SODIUM NITROPRUSSIDE POTENCY (μg/ml) IN DEXTROSE (5%) ADMIXTURES STORED UNDER LIGHT CONDITIONS

Time (h)	*Control foil-wrapped dark cabinet*	*Room light, fluorescent light, 24 h/day*	*Window light eastern window*	*High-intensity light cabinet, 1400–2000 foot-candles*
Initial	50.54	52.12	50.54	50.76
1	50.46	51.97	34.44	45.26
2	52.39	52.52	27.56	38.38
4	51.04	50.55	22.51*	28.78
6	49.81	48.46	14.89	23.74*
7	48.83	46.74	11.94	21.53
24	49.39	43.30	11.87	9.68
31	49.22	41.45	8.49	7.65
48	51.47	38.57	6.76	4.09
60	47.62	31.66	3.78	1.18
144	49.07	23.63*	1.73	0.69

* From this time the solution was blue.

CONCLUSION

An analytical method has been developed which allows quantitation of sodium nitroprusside in admixture solutions, in commercially available lyophilized vials and during photodegradation studies. This HPLC method was used to show that while

there is no adsorption to the infusion set there is a small loss of nitroprusside due to photodegradation even when the infusion bottle is wrapped in foil.

ACKNOWLEDGEMENT

We acknowledge the assistance of Mr. Jatin Shah in the preparation of the infusions.

REFERENCES

1 M. J. Frank, J. B. Johnson and S. H. Rubin, *J. Pharm. Sci.*, 65 (1976) 44.
2 J. Masek, *Inorg. Chim. Acta Rev.*, 3 (1969) 101.
3 I. M. Kolthoff and P. E. Toren, *J. Amer. Chem. Soc.*, 75 (1953) 1197.
4 N. L. Costa, J. Danon and R. M. Xavier, *J. Phys. Chem. Solids*, 23 (1962) 1783.
5 B. Jaselskis and J. C. Edwards, *Anal. Chem.*, 32 (1960) 381.
6 T. Martin and J. A. Patel, *Amer. J. Hosp. Pharm.*, 26 (1969) 51.
7 R. Rucki, *Anal. Profiles Drug Subst.*, 6 (1977) 487.
8 R. G. Gerritse, *J. Chromatogr.*, 171 (1979) 527.

Journal of Chromatography, 212 (1981) 347–355
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,876

ANALYSIS OF THE ANTI-COCCIDIAL DRUG, HALOFUGINONE, IN CHICKEN TISSUE AND CHICKEN FEED USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

ALAN ANDERSON*, EDWARD GOODALL, GRAHAM W. BLISS and ROBERT N. WOODHOUSE

Department of Analytical Chemistry, Huntingdon Research Centre, Huntingdon PE18 6ES (Great Britain)

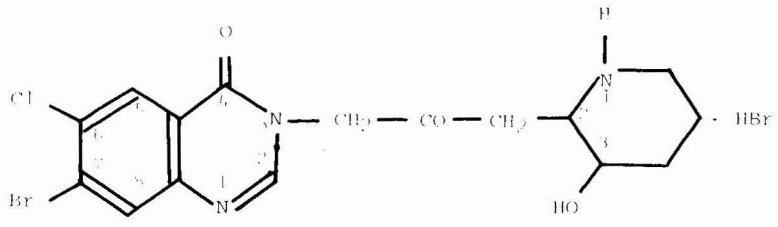
(Received April 10th, 1981)

SUMMARY

Methods are described for the analysis of the anti-coccidial drug, halofuginone, in chicken tissue at concentrations as low as 1 ppb (0.001 ppm) and in chicken feed at a concentration of 3 ppm, using high-performance liquid chromatography. The tissue analysis involves: enzymatic release of the halofuginone followed by ethyl acetate extraction under basic conditions, partition into ammonium acetate buffer, concentration using Sep-pak™ C_{18} cartridge. The feed analysis involves: ethyl acetate extraction under basic conditions, partition into hydrochloric acid, concentration using XAD-2 column chromatography. Both methods use high-performance liquid chromatography with ultraviolet detection for the final analysis. Precision and accuracy data for both methods are given.

INTRODUCTION

Halofuginone (DL-*trans*-7-bromo-6-chloro-3-{3-(3-hydroxy-2-piperidyl) acetonyl}-4(3H)-quinazolinone hydrobromide) (I) is an anti-coccidial drug proposed for incorporation in poultry feed to permit continuous administration to broiler chickens. For the purposes of registration, sensitive and precise methods were required for the analysis of halofuginone in chicken tissues, particularly liver and kidney, and in chicken feed at the inclusion concentration of 3 ppm. A previous paper[1] from this laboratory has described a high-performance liquid chromatography (HPLC) method for analysing feed at 3 ppm. To eliminate the interference



(I)

0021-9673/81/0000 0000/$02.50

caused by matrix co-extractives from some feeds, modification of the HPLC conditions of this method was necessary. This paper describes the modification, with precision and accuracy data generated over 2 years, and the extension of the method to the analysis of chicken tissues at concentrations as low at 1 ppb (0.001 ppm).

EXPERIMENTAL

Apparatus

HPLC. A Waters Assoc. (Hartford, Great Britain) M-6000A pump fitted with a μBondapak C_{18} (30 cm × 3.9 mm I.D.) pre-packed column (Waters Assoc.) was used. Injection was achieved by means of a WISP 710A automatic sample injector (Waters Assoc.). The eluate from the column was monitored using a LC–UV variable-wavelength UV detector (Pye-Unicam, Cambridge, Great Britain). Output from the detector was measured on either a Spectra-Physics SP4100 computing integrator (St. Albans, Great Britain) or W + W series 1100 chart recorder (Basle, Switzerland).

General. Sample maceration was achieved using a Polytron model PCU-2 macerator (Kinematica, Switzerland). Glass columns (30 × 1 cm I.D.) with sintered filters were used for macroreticular resin separations. A Beckman Model 3500 digital meter with combination electrodes was used for pH measurements. Centrifugation was accomplished using a MSE "GF-8" centrifuge (Crawley, Great Britain) capable of accepting 200-ml glass centrifuge bottles. Büchi "Rotavapor R" rotary film evaporators (RFE; Orme Scientific, Middleton, Great Britain) were used for reduced pressure evaporations.

Materials

Analytical-grade halofuginone (micronised) was obtained from Roussel Uclaf (Paris, France). The macroreticular resin, Amberlite XAD-2 standard grade (BDH, Poole, Great Britain), was freed from chloride ions by water washing and purified by overnight Soxhlet extraction with methanol. The purified resin was stored, under methanol, in stoppered glass bottles. Immediately prior to use, a slurry of the purified resin (10 g), in methanol, was transferred to a glass chromatography column and, after draining of the methanol, washed thoroughly with glass distilled water. Sep-pak™ C_{18} cartridges (Waters Assoc.) were pre-washed sequentially with methanol (2 ml) and glass-distilled water (5 ml). All chemicals were analytical-reagent grade and all solvents HPLC grade. The enzyme preparation, Trypsin, was laboratory-reagent grade (Fisons Scientific, Loughborough, Great Britain). Unmedicated (control) poultry diet was prepared at Huntingdon Research Centre to a fixed commercial recipe and control and treated chicken tissues were obtained from the Department of Animal Science at Huntingdon Research Centre.

Acetate buffer (0.25 *M*, pH 4.3) was prepared by dissolving ammonium acetate (19.4 g) and glacial acetic acid (30 ml) in glass-distilled water and diluting to 1 l, with glass-distilled water. Acetate buffer (0.125 *M*, pH 4.3) was prepared by diluting 500 ml of the 0.25 *M*, pH 4.3 acetate buffer to 1 l, with glass-distilled water.

Chromatography

The HPLC mobile phase consisted of acetonitrile acetate buffer (0.25 *M*)–water (5:3:12, v/v/v), re-adjusted to pH 4.3 with glacial acetic acid after mixing.

Sample injection volume was 40 μl for feed analysis and 100 μl for tissue analysis. Chromatography was carried out at ambient temperature (23 $\pm$ 3°C) using a mobile phase flow-rate of 2 ml/min. The eluate from the HPLC column was monitored at a wavelength of 243 nm. Chart recordings were made at a chart speed of 0.5 cm/min using a detector sensitivity of 0–0.04 a.u.f.s. (W + W) or 0–1 volt unattenuated (SP4100). Under these conditions the retention volume of halofuginone was 14 ml.

Procedures

Finished feeds (approximately 3 ppm halofuginone). A representative sample of feed (10 g) was macerated with sodium carbonate solution (10 ml; 10% w/v) and ethyl acetate (100 ml). The phases were separated by centrifugation and the ethyl acetate phase decanted. The residue was re-extracted with ethyl acetate (100 ml) and the combined ethyl acetate extracts washed with salt-saturated sodium carbonate solution (50 ml; 5%, w/v). The ethyl acetate phase was separated, extracted with hydrochloric acid (2 × 50 ml, 0.1 *M*) and the combined hydrochloric acid extracts washed, by gentle agitation, with ethyl acetate (10 ml). Residual ethyl acetate was removed from the acid solution (RFE at 40°C, 5 min). The acid solution was passed through an Amberlite XAD-2 macroreticular resin column prepared as under *Materials* and the column washed with hydrochloric acid (20 ml, 0.1 *M*). Any residual acid solution was removed from the column using low-pressure compressed air. The column was equilibrated with methanol for 10 min and eluted with methanol (100 ml). The solvent was evaporated (RFE at 40°C) and the residue dissolved in HPLC mobile phase (10 ml), filtered through a Whatman GF/F paper and analysed by HPLC.

Tissues. Trypsin (0.5 g) and water (10 ml) were added to a representative sample (20 g) of homogenised tissue, mixed, and the pH adjusted to 8 $\pm$ 1 by the dropwise addition of sodium carbonate solution (10%, w/v). The mixture was incubated for 3 h (water bath, 40°C), allowed to cool to ambient temperature and macerated for 3 min with sodium carbonate solution (10 ml; 10%, w/v) and ethyl acetate (100 ml). After centrifuging, the organic phase was decanted, the residue re-extracted with ethyl acetate (100 ml) and the combined extracts washed with salt-saturated sodium carbonate solution (50 ml; 5%, w/v).

The organic layer was extracted with ammonium acetate buffer (2 × 50 ml, 0.125 *M*) and the combined aqueous extracts washed, by gentle agitation, with ethyl acetate (10 ml). Residual ethyl acetate was removed from the aqueous solution (RFE at 40°C, 5 min). The solution was transferred quantitatively to a volumetric flask (100 ml) and diluted to volume with acetate buffer (0.125 *M*). An aliquot (10 ml) of the solution, after filtration through a Whatman GF/F paper, was passed through a pre-washed Sep-pak C_{18} cartridge. This was repeated with a second aliquot (10 ml) and the cartridge washed with glass-distilled water (3 ml). Halofuginone was eluted from the cartridge with methanol (5 ml), the eluate collected in a tapered test tube (10 ml) and evaporated to dryness using a stream of dry nitrogen. The residue was dissolved in HPLC mobile phase (200 μl) and analysed by HPLC.

Standard solutions

Halofuginone (50 mg) was dissolved in acetate buffer (250 ml, 0.25 *M*) to produce a stock standard solution. Portions of the stock standard solution were diluted with HPLC mobile phase to provide calibration solutions in the range 0–5 μg halofuginone/ml.

TABLE I

PRECISION AND ACCURACY (FINISHED FEEDS)

The following procedural recovery data has been generated by three analysts over a 2-year period.

Concentration (nominal)	3 ppm
Number of determinations	85
Mean recovery	92.7%
Standard deviation	±4.2%

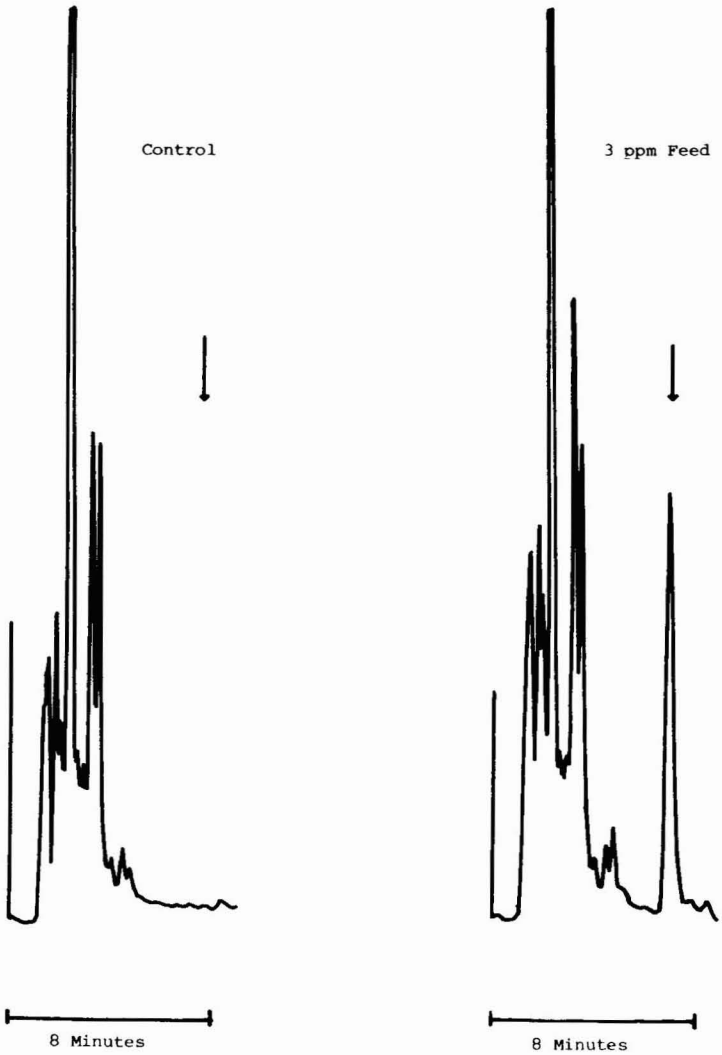


Fig. 1. Chromatograms obtained for extracts from control feed and feed containing halofuginone at 3 ppm.

Procedural recoveries

Finished feed. The stock standard halofuginone solution was diluted to a concentration of approximately 10 μg halofuginone/ml with glass-distilled water and portions (3 ml) of this solution used to fortify control feed (10 g) to produce halofuginone concentrations of 3 ppm. The mixture was analysed immediately as described under *Procedures.*

Tissues. The stock standard halofuginone solution was diluted with glass-distilled water to a concentration such that the addition of 100 μl of the solution to control homogenised tissue (20 g) gave halofuginone concentrations in the range 0.015–1.0 ppm in the tissue. The mixture was analysed as described under *Procedures.*

Calibration and calculation

Calibration curves of chromatographic peak height or peak area *versus* concentration of halofuginone (μg/ml) were constructed using the data obtained by injections of aliquots of the standard solutions prepared as described under *Standard solutions.*

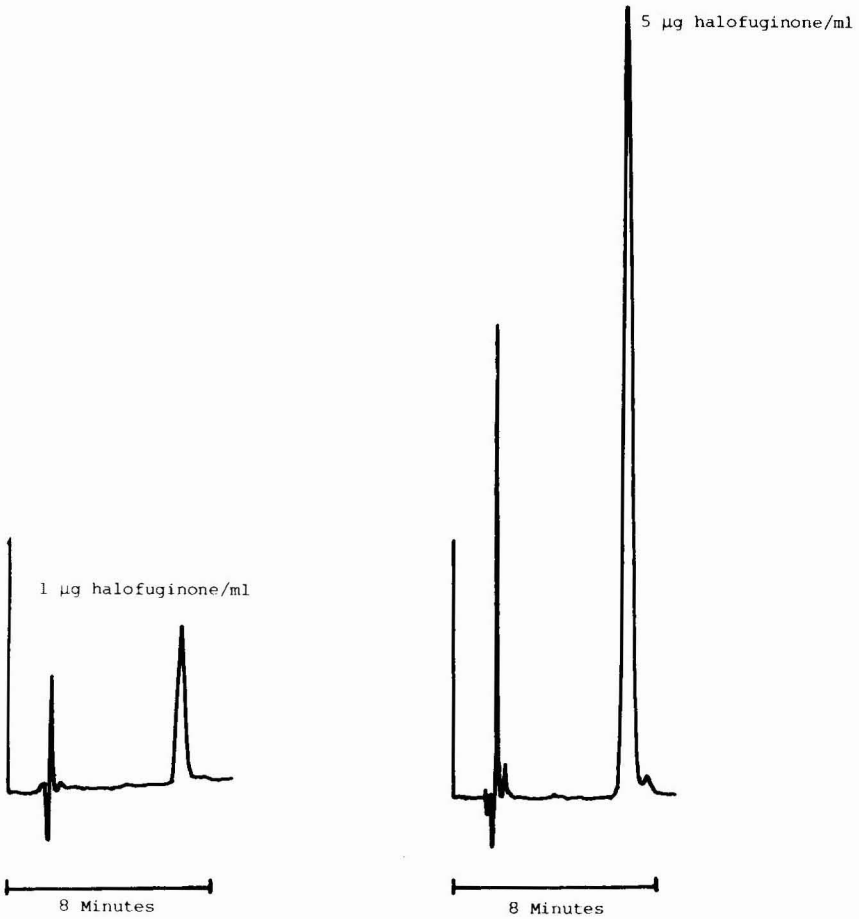


Fig. 2. Chromatograms obtained for calibration standards of halofuginone.

Concentrations of halofuginone in samples were determined by measurement of the chromatographic peak height or peak area at the characteristic retention volume for halofuginone and, after interpolation to the appropriate calibration curve, application of the necessary dilution factors.

RESULTS AND DISCUSSION

HPLC was found to be superior to gas–liquid chromatography for the analysis of halofuginone at 3 ppm in finished feed[1]. Using the HPLC method detailed in this paper we have analysed approximately 500 commercial finished feeds from many different feed compounders without interference from feed co-extractives. During this

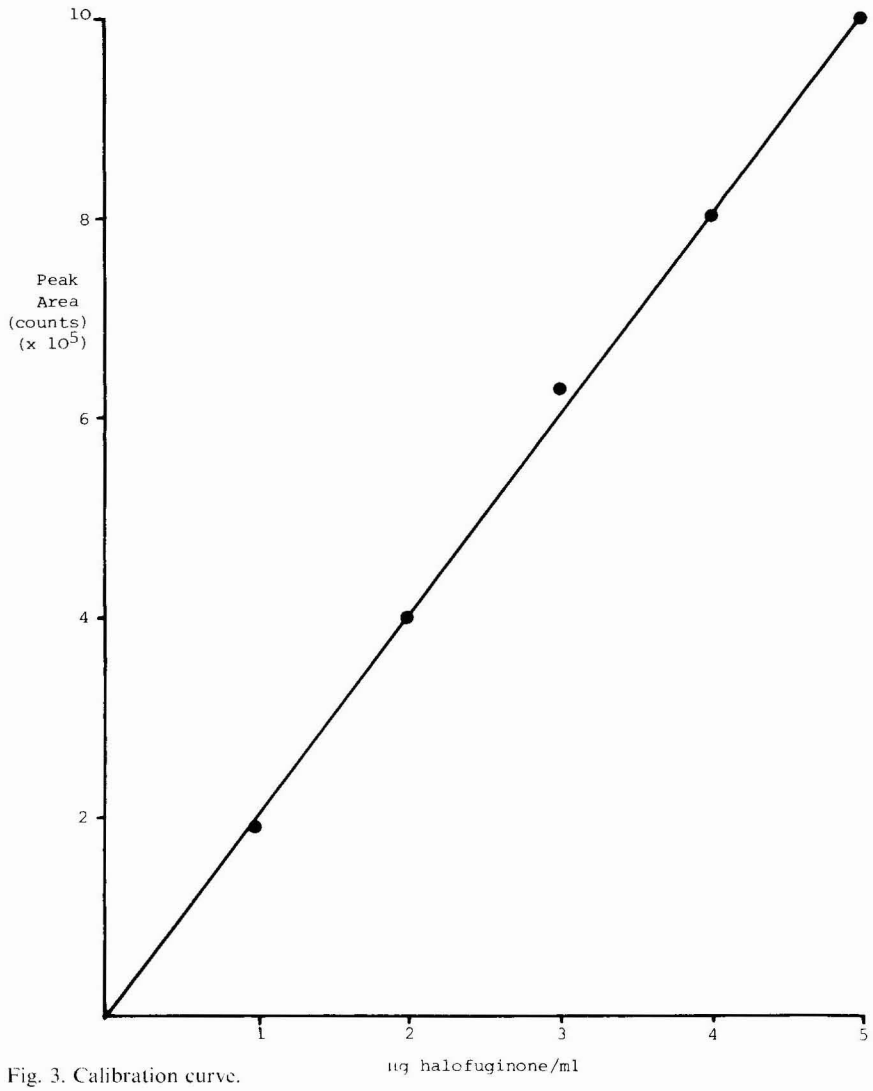


Fig. 3. Calibration curve.

TABLE II

PRECISION AND ACCURACY (TISSUES)

The following procedural recovery data illustrates the precision and accuracy of the method used for analysing tissues.

Tissue	*Fortification range (ppm)*	*No. of determinations*	*Mean recovery (%)*	*Recovery range (%)*
Liver	0.015–1.03	18	81.0	68–93*
Kidney	0.015–1.03	17	84.3	69–100
Muscle	0.03	7	98.7	74–117
Skin and fat	0.03	10	87.3	72–104

* One recovery was obtained at 124% (0.03 ppm).

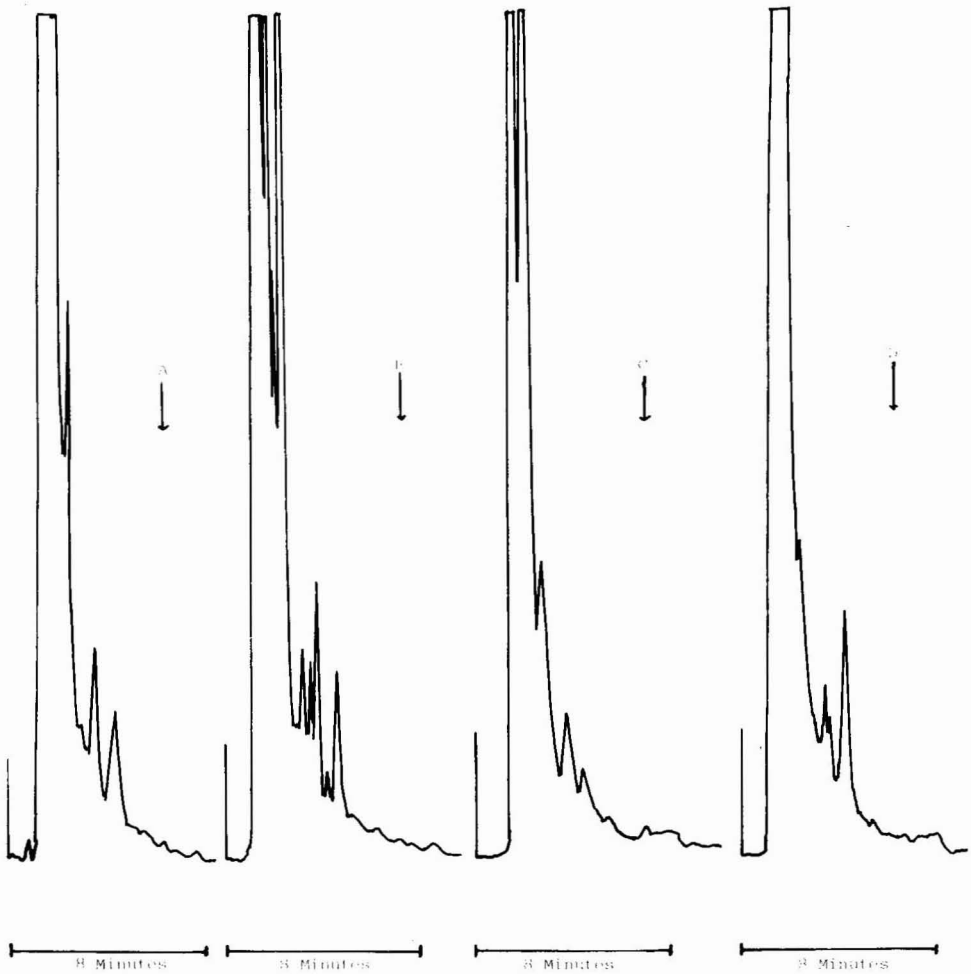


Fig. 4. Chromatograms obtained for extracts from control chicken tissues. Control tissue extracts: liver (A), kidney (B), muscle (C), and skin and fat (D).

time a total of 85 procedural recoveries have been undertaken, by three different analysts. The precision and accuracy data are shown in Table I, indicating the method to be both precise and accurate. Typical chromatography for the feed analysis is shown in Fig. 1, with typical calibration chromatograms and calibration curve shown in Figs. 2 and 3, respectively. The method has proved to be both very selective, so eliminating interference from other compounded animal products, and stability indicating. Analysis of small-scale (1–7 kg) mixes of halofuginone with poultry feed and radioactive experiments have shown that the procedural recovery data, although generated by aqueous addition of halofuginone to feed, is a good reflection of the accuracy of the method.

The HPLC method for analysing halofuginone in feed was modified to develop a procedure capable of detecting halofuginone in tissues at concentrations as low as 0.001 ppm (1 ppb). To reduce solvent volumes, the decision was taken to replace the XAD-2 resin column with a Sep-pak C_{18} cartridge. Previous work had suggested that

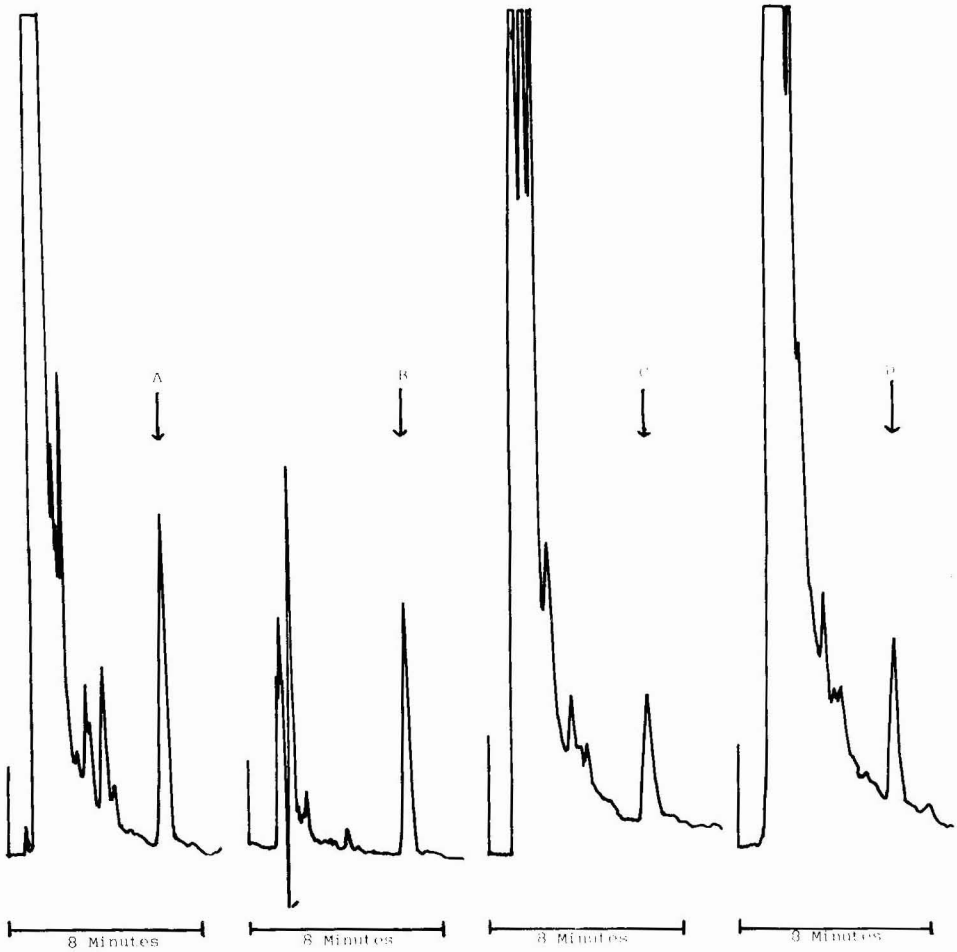


Fig. 5. Chromatograms obtained for extracts from control chicken tissues fortified with halofuginone. A, Liver (0.1 ppm halofuginone); B, kidney (1 ppm); C, muscle (0.03 ppm); D, skin and fat (0.03 ppm).

the use of 0.1 *M* hydrochloric acid resulted in breakdown of the Sep-pak C_{18} support. Therefore, the hydrochloric acid, used to extract halofuginone from the ethyl acetate, was replaced with ammonium acetate buffer. An aliquot of the buffer solution was passed through a Sep-pak C_{18} cartridge, enabling collection of the eluate in a small tapered glass tube and the ability to concentrate to a final mobile phase volume of 200 μl to achieve the required sensitivity. The use of Sep-pak C_{18} for feed analysis is also possible, and precision and accuracy data are presently being generated using this modification. It was also necessary to use the enzyme, trypsin, to release halofuginone from the tissues.

The precision and accuracy data of the method used for analysing tissues are given in Table II with typical chromatography, obtained from control tissues and tissues fortified with halofuginone, shown in Figs. 4 and 5 respectively. The accuracy of the method was not significantly different over the concentration range of the procedural recoveries or for different tissues, although recoveries were generally higher from muscle. The lowest concentration of halofuginone at which procedural recoveries were undertaken was 0.015 ppm, although the limit of sensitivity, defined as the concentration of halofuginone producing a peak with a height three times instrument noise, was estimated at 0.001 ppm (1 ppb).

This method has now been used successfully to analyse tissues from chickens dosed for 14 days with radioactive halofuginone, in order to determine the target tissue and the ratio of halofuginone to total radioactive residues.

ACKNOWLEDGEMENT

We thank Roussel Uclaf for sponsoring this work.

REFERENCES

1 A. Anderson, D. H. Christopher and R. N. Woodhouse, *J. Chromatogr.*, 168 (1979) 471–480.

Journal of Chromatography, 212 (1981) 356–360
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,835

Note

Preparative purification of the peptide des-enkephalin γ-endorphin

Comparison of high-performance liquid chromatography and counter-current chromatography

MARTHA KNIGHT*

Pharmacology Section, Experimental Therapeutics Branch, National Institute of Neurological and Communicative Disorders and Stroke, 9000 Rockville Pike, Building 36, Room 5A31, Bethesda, MD 20205 (U.S.A.)

YOICHIRO ITO

Laboratory of Technical Development, National Heart, Lung and Blood Institute, Bethesda, MD 20205 (U.S.A.)

and

THOMAS N. CHASE

Pharmacology Section, Experimental Therapeutics Branch, National Institute of Neurological and Communicative Disorders and Stroke, Bethesda, MD 20205 (U.S.A.)

(Received March 30th, 1981)

Considerable importance is attached to the development of procedures for the purification of synthesized peptides where good chromatographic fractionation can be achieved. Partition chromatography in volatile solvent systems has for some time been a useful procedure for large amounts of material. Amounts of peptide in the range 100–500 mg were easily separated in the counter-current distribution machine of the Craig–Post design. This large apparatus, however, is no longer commonly available. Methods utilizing more modern instruments which are relatively compact and accessible were evaluated for their applicability to purifying large amounts of synthesized peptides. These methods provide potentially easy and routine initial fractionation steps. One procedure studied in this experiment is counter-current chromatography performed in the horizontal flow-through coil planet centrifuge[1,2]. This liquid–liquid partition system allows excellent recovery of material and is versatile in that either phase is used as the mobile phase. The other procedure evaluated was high-performance liquid chromatography (HPLC) using a semi-preparative C_{18} column, 30 cm × 7.8 mm I.D. μBondapak (Waters Assoc., Milford, MA, U.S.A.). In this case the resolution was expected to be better. Both these procedures were tried in the purification of the dodecapeptide β-LPH (66–77), or des-enkephalin γ-endorphin, a β-endorphin fragment studied for its possible neuroleptic-like behavioral effects[3]. The results were compared on the basis of purity and recovery.

EXPERIMENTAL

All chemicals were analytical grade reagents. *n*-Butanol was from Burdick &

Jackson (Muskegon, MI, U.S.A.). Acetonitrile and methanol were HPLC grade from Waters Assoc. Water used in HPLC was deionized and glass distilled.

The peptide des-enkephalin γ-endorphin (Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu) was synthesized by the automated solid-phase procedure on a Beckman 990B instrument[4]. The N-α-Boc amino acids in 2.5 molar excess were coupled in the desired sequence to 0.92 g Leu substituted chloromethyl 1% divinylbenzene cross-linked polystyrene resin (0.4 mmol) using dicyclohexylcarbodi-imide as the coupling agent. A 4-molar excess of Boc-Gln was coupled as the *p*-nitrophenyl ester in dimethylformamide. The side-chain-protected N-α-Boc amino acids were benzyl Thr, benzyl Ser, ε-2Cl carbobenzoxy Lys, γ-benzyl Glu and Gln *p*-nitrophenyl-ester (purchased from BaChem or Chemalog). Each coupling step was repeated with half the amount of coupling amino acid. The peptide resin was cleaved and deprotected with 10 ml of anhydrous hydrogen fluoride and 1 ml of anisole for 45 min at 0°C.

Counter-current chromatography was carried out in the horizontal flow-through coil planet centrifuge with 2.6 mm I.D. PTFE tubing coiled in 1000 turns with a capacity of 260 ml. The column was charged with 230 mg of peptide in 5 ml of each phase of the solvent system, 0.1% trifluoroacetic acid–*n*-butanol (1:1, v/v). The sample had a K value of 0.19 in this system. The coil was filled with lower phase and the upper phase served as the mobile phase. The upper phase was pumped at 24 ml/h and the column-coil was rotated at 400 rpm. Fractions of 6 ml or 15 min were collected. The solvent front emerged at tube 28 (168 ml). The mobile phase was pumped for three times the column volume, then the column contents were pumped out emerging between fractions 130 and 170. The fractions were assayed by Folin-Lowry determination[5], and tubes containing peptide were pooled, evaporated in a rotary evaporator and lyophilized.

For preparative HPLC a 50–100 mg sample was dissolved in 0.02 M acetic acid (0.1%) and passed through a Millipore filter and injected with the 6UK manual injector in a volume of 2 ml onto the semi-preparative C_{18} μBondapak column, pumped at a flow-rate of 3 ml/min with 0.1% acetic acid. After 5 min, a linear gradient of methanol was pumped from 0 to 60% methanol in 60 min. Two 6000A Waters pumps were used with a 720 systems controller. The column was run through the chromatography conditions prior to loading the sample. Absorbance at 210 nm at the full scale of 2 was monitored. Fractions of 1 min were collected and assayed by the Folin-Lowry method, and tubes containing peptide were pooled and concentrated as described above.

Solutions of 10 mg/ml were made of the different chromatographic products and these were analyzed by TLC, analytical HPLC, and amino acid analysis of hydrolysates. Analytical HPLC was done on the 30 cm × 4 mm I.D. C_{18} μBondapak column in 0.1% phosphoric acid and 17% acetonitrile at a flow-rate of 3 ml/min. Absorbance at 210 nm at 0.04 absorbance units was recorded.

RESULTS AND DISCUSSION

The yield of synthesized product after lyophilization was 382 mg of off-white powder. On cellulose TLC in the system *n*-butanol–acetic acid–water (4:1:1, v/v/v), there were two spots (not discrete but smeared) with R_F values of 0.41 and 0.13.

The results of an HPLC experiment are shown in Fig. 1. Absorbance at 210 nm was monitored. The peaks, determined by the Folin-Lowry method, were well separated. The yield of material from a chromatography of 50 mg was 2 mg in the peak emerging at 37 min and 9 mg in the peak emerging at 48 min. In a chromatography of 100 mg, 3 and 29 mg were obtained, respectively. Amino acid analysis showed the expected integral molar ratios of the amino acids in the material of the second peak.

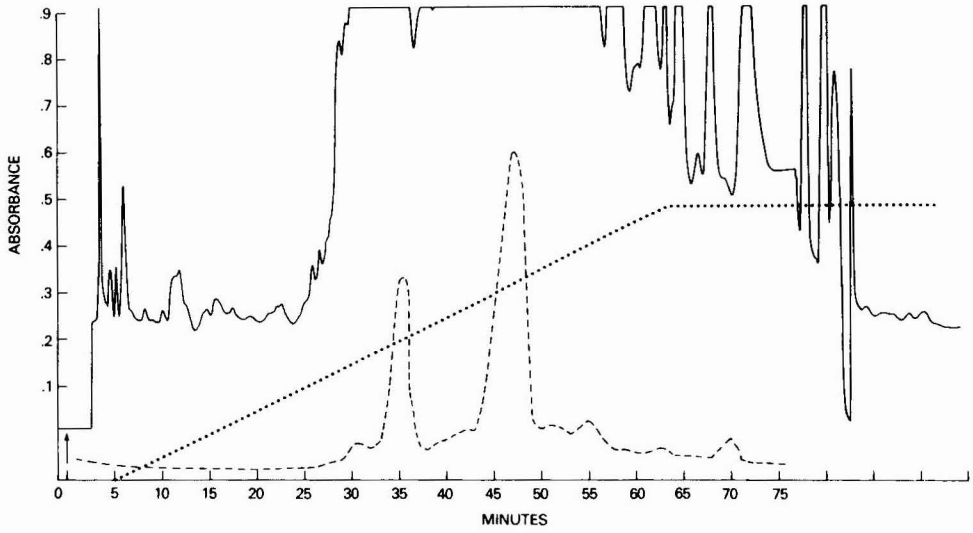


Fig. 1. Preparative HPLC of 50 mg des-enkephalin γ-endorphin. The procedure is described in the text. The absorbance recording of the Model 440 variable-wavelength detector (Waters Assoc.) at 210 nm with the full scale of 2 units is the solid line. The dashed line is absorbance at 700 nm. Samples of 20 μl were taken from 3-ml fractions and analyzed by the Folin-Lowry method. The dotted line indicates percentage of methanol. The column was pumped at 3 ml/min in 0.1% acetic acid, and 5 min after injection of sample a gradient of methanol at 1%/min was started.

Since the yield was so low the other procedure was tried. A sample of 230 mg was run in the horizontal flow-through coil planet centrifuge. As seen in Fig. 2, only a single peak was found and it was not symmetrical, indicating lack of resolution. The yield of the major fraction of the peak was 148 mg and of the following material, 33 mg. Much more material was recovered in this procedure. Fig. 3 contains the traces of the analytical HPLC of each of the fractions from both procedures. In the upper panel (A) the major peak of the reversed-phase chromatography was pure. The major peak of the counter-current chromatogram contained impurities but was highly purified (C). This partially purified product was passed through the reversed-phase column, affording complete purification but low yield (23%).

Des-enkephalin γ-endorphin, a neutral, non-aromatic, moderate-sized peptide would be expected to be resolved only with difficulty from impurities of similar structure, such as deletion or truncated peptides or diastereoisomers. The resolution of the semi-preparative reversed-phase column in purifying this peptide was excellent. The impurities were separated well from the major product by 10 min. This separation was not complete in the counter-current chromatography. Nevertheless, the coil

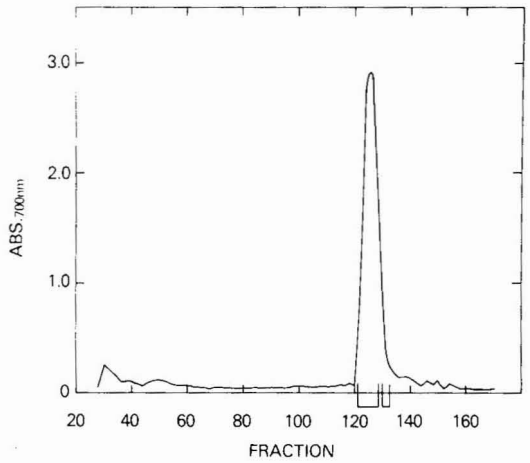


Fig. 2. Analysis of horizontal flow-through planet centrifuge fractions. The absorbance of 100-μl samples analyzed by the Folin-Lowry method is shown. Fractions 121–128 were pooled as the major product and fractions 129–132 as the minor product. The solvent front emerged at tube 28, the lower phase at tube 121 and column contents between tubes 130 and 170. The conditions of the chromatography are described in the text.

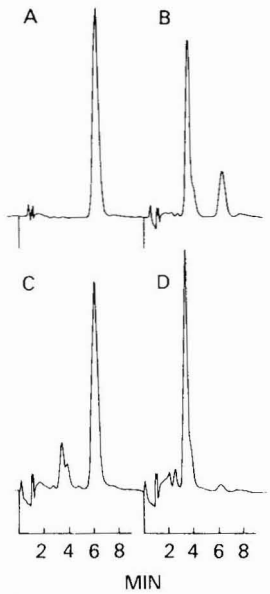


Fig. 3. Analytical HPLC of 5-μg samples of each chromatographic product. Recording at 210 nm absorbance (0.04 units full scale) is shown. The products of the HPLC preparative fractionation are shown in the upper panel. A is the large peak emerging at 47 min and B is the smaller peak at 35 min (Fig. 1). In the lower panel C is the major component of the counter-current chromatography fraction (121–128) and D is the remainder of the material (129–132) (Fig. 2).

could have been run longer or recycled to achieve a better separation. Compared with the major product, the impurities were less hydrophobic eluting earlier in the reversed-phase and later in the counter-current chromatography.

A serious problem with the reversed-phase chromatography, however, was the recovery. Pure peptide attained by this method (29 % yield) was one half that of the counter-current procedure (65 %). The counter-current fraction appeared to be highly purified (at least 85 %) in the analytical HPLC. With other peptides the horizontal flow-through coil planet centrifuge has provided chromatographically pure products[6,7]. Conditions will have to be developed to improve the recovery of peptide from the reversed-phase column. Other volatile solvents or flow-rates or gradients could be tried. The column packing material may be affecting the results as well. The flow-rate utilized was not very high and back pressure was *ca.* 3500 p.s.i. Furthermore, in one example (D-Ala^2 enkephalin Arg^6) which has a relatively early retention time, the recovery from HPLC after two chromatographic runs on a column was quite high (50 %). Thus the recovery may depend on the structure of the peptide. It is still preferable to perform a chromatography that provides quantitative recovery and then resort to HPLC for further purification. If high recoveries were consistently possible with HPLC then this method could be used exclusively.

REFERENCES

1 Y. Ito and R. L. Bowman, *J. Chromatogr.*, 147 (1978) 221.
2 Y. Ito and G. J. Putterman, *J. Chromatogr.*, 193 (1980) 37.
3 N. W. Pedigo, T. D. Reisine, T. Schallert, D. H. Overstreet, N. C. Ling, P. Ragan and H. I. Yamamura, in E. Costa and G. Trabucchi (Editors), *Advances in Biochem. Psychopharmacology*, Vol. 22, Raven Press, New York, 1980, p. 549.
4 J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*, W. H. Freeman Press, San Francisco, CA, 1969.
5 O. H. Lowry, N. S. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
6 M. Knight, *Ph.D. Dissertation*, Johns Hopkins University, Baltimore, MD, 1980.
7 M. Knight and W. A. Klee, *J. Biol. Chem.*, 254 (1979) 10,426.

Journal of Chromatography, 212 (1981) 361–365
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

CHROM. 13,927

Note

Determination of N-methyl pyridinium-2-aldoxime chloride and its hydrolytic by-products by ion-pair high-performance liquid chromatography

NESBITT D. BROWN*, M. PATRICIA STRICKLER, H. KENNETH SLEEMAN and BHUPENDRA P. DOCTOR

Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20012 (U.S.A.)

(Received April 27th, 1981)

During the past two decades, much work has been conducted by various research groups to develop effective cholinesterase reactivators for use as therapeutic agents in reversing the deleterious effects of organophosphate poisoning of the nervous system in mammals[1–5]. As a result of these efforts, several groups of compounds have been tested in animals in an attempt to understand better their modes of action. N-Methyl pyridinium-2-aldoxime chloride (2-PAM) is an oxime that falls within this area of research. Although much research has been performed on this compound, many questions remain unanswered; this is particularly true with respect to the pharmacokinetic actions of 2-PAM in humans.

In studies conducted by Ellin and co-workers[6,7], 2-PAM was shown to degrade via two routes when exposed to various hydrogen and hydroxyl ion concentrations *in vitro* (Fig. 1). The results obtained from these studies are relevant in view of the fact that 2-PAM may be subject to these conditions when administered to human subjects, either orally or intramuscularly as a therapeutic drug. It has been determined that parenteral solutions containing pyridinium oximes are most stable at pH values

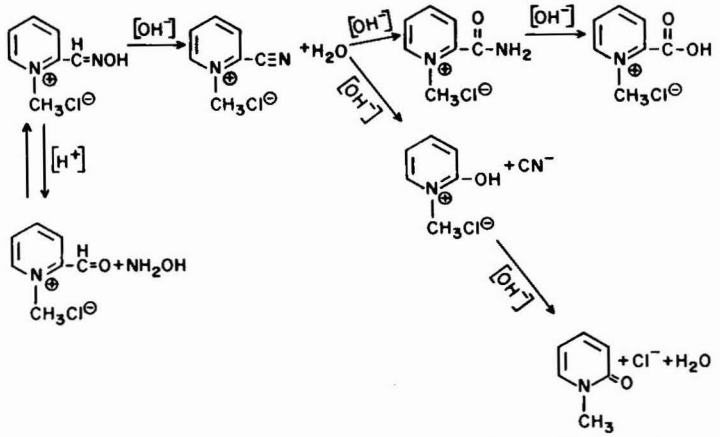


Fig. 1. Decomposition mechanism for 2-PAM in acid and alkaline solutions at 95°C.

0021-9673/81/0000–0000/$02.50

below 5. Within an *in vivo* system, where the normal pH value is approximately 7.4, the reaction kinetics for 2-PAM should follow the reaction scheme of the hydroxyl ion degradation.

Specific and sensitive methods are required to establish the decomposition mechanism for 2-PAM *in vivo*. In this paper, we describe a relatively simple procedure for separating and quantifying major degradative by-products of 2-PAM in both basic and acidic solutions. The method involves the separation of 2-PAM and its by-products by employing ion-pair reversed-phase high-performance liquid chromatography (HPLC). Amounts as low as 25 ng are detected by this method. Analysis time requires less than 6 min per sample. In addition, high accuracy and reproducibility are obtained by using this procedure. The method offers an excellent alternative to many of the previously described procedures used for analyzing the pyridinium oximes.

EXPERIMENTAL*

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) Model ALC/GPC 204 liquid chromatograph, equipped with two Model 6000A high-pressure pumps, a 660 solvent programmer, a U6K loop injector, a 280-nm UV detector and a Model 730 data module was used in this study.

Reagents

All solvents and chemicals used in the study were either of spectroquality or of analytical grade. Acetonitrile was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). PIC-B7 reagent (1-heptane sulfonic acid) was purchased from Waters Assoc. Stock standard solutions (5 $\mu g/\mu l$) of 2-PAM, 1-methyl-2-pyridone (Aldrich, Milwaukee, WI, U.S.A.), 2-formyl-N-methyl pyridinium chloride hydrate, 2-carbamoyl-1-methyl pyridinium chloride monohydrate, and 2-carboxy-N-methyl pyridinium chloride (Ash Stevens, Detroit, MI, U.S.A.) were prepared in methanol–water (1:1).

Procedure

A pre-packed 30 cm × 3.9 mm I.D. μBondapak C_{18} column (Waters Assoc.) was employed to chromatograph all compounds used in this study. The mobile phase consisted of a 0.01 *M* solution of PIC-B7 mixed with acetonitrile. PIC-B7 reagent was prepared by dissolving 20 ml of the pre-packaged reagent into 480 ml of glass-distilled water. The pH of the solution was 3.4. A PIC-B7–acetonitrile ratio of 80:20 was used in an isocratic mode to separate each compound. The flow-rate was 1.5 ml/min. Column pressures ranged between 1500 and 1700 p.s.i. All separations were performed at ambient temperature. Samples were introduced into the column through a continuous-flow loop injector. Peak areas and heights were measured and computed with an on-line integrator.

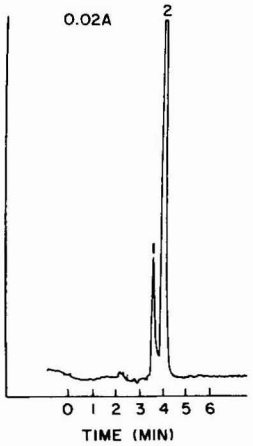
* The manufacturers' names and products are given as scientific information and do not constitute an endorsement by the U.S. Government.

RESULTS AND DISCUSSION

The therapeutic efficacy of 2-PAM to reactivate organophosphorus-inhibited cholinesterases is completely dependent upon the potency of the antidote, based on the stability of the active compound in the formulation, prior to its administration. In certain cases where the anticholinergic activity of the antidote is unknown due to the deterioration of the parent compound caused by prolonged storage conditions, specific methods are needed to characterize the formulation.

In a series of recently published reports[8–10] we studied the degradative fate of several anticholinergic compounds after exposure to various pH and temperature gradients. The data compiled from these studies were used to predict the shelf life of these compounds when stored in different climatic regions. In this paper, we studied the reaction kinetics of 2-PAM by subjecting it to elevated temperatures in acidic and basic solutions, thus simulating long-term storage.

In order to identify the hydrolytic by-products of 2-PAM, a series of standard solutions containing the major degradation products of this oxime were prepared and chromatographed (Figs. 2 and 3). Concentrations ranged from 100 to 1500 ng/μl. Linearity was established for each compound chromatographed. Experimental samples of 2-PAM were prepared in 0.1 *N* hydrochloric acid and 0.1 *N* sodium hydroxide.



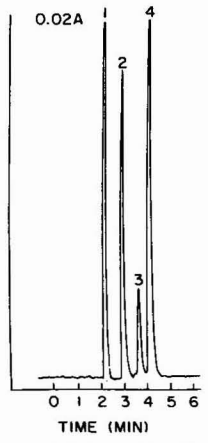
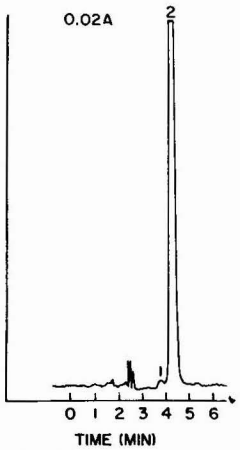


Fig. 2. Separation of a standard solution containing (1) 100 ng of 2-formyl-N-methyl pyridinium chloride and (2) 1.25 μg of 2-PAM. Column: 300 × 3.9 mm I.D. μBondapak C_{18}. Mobile phase: PIC-B7–acetonitrile (80:20). Flow-rate: 1.5 ml/min. Column temperature: ambient.

Fig. 3. Separation of a standard solution of (1) 2-carboxy-N-methyl pyridinium chloride (560 ng), (2) 1-methyl-2-pyridone (560 ng), (3) 2-carbamoyl-1-methyl pyridinium chloride (280 ng) and (4) N-methyl pyridinium-2-aldoxime chloride (560 ng).

Each group was heated at 95°C for various time periods. The chromatograms below depict the results of this experiment. Chromatograms of the acid hydrolysates showed a pattern of degradation with 2-formyl-N-methyl pyridinium chloride as the principal hydrolytic by-product. The first signs of degradation were observed after 5 min of heating. Heating was continued for 60 min. Samples were collected every minute during the first 5 min, followed by 5-min intervals during the next 30 min and in 10-

min time periods, thereafter. During the 60-min hydrolysis, only 10% of the 2-PAM was degraded to 2-formyl-N-methyl pyridinium chloride. No other by-products were observed in the chromatographic separations. Figs. 4 and 5 represent two time frames of the reaction kinetics for the hydrolytic break-down of 2-PAM in acid.



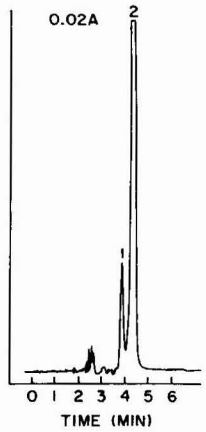
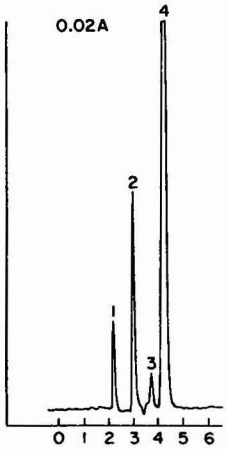


Fig. 4. Chromatogram of a 1.50-μg sample of 2-PAM heated in 0.1 *N* HCl at 95°C for 5 min. 1 = 2-Formyl-N-methyl pyridinium chloride; 2 = 2-PAM.

Fig. 5. Chromatogram showing the hydrolysis of 2-PAM in acid after 60 min. 1 = 2-Formyl-N-methyl pyridinium chloride; 2 = 2-PAM.

Chromatograms of the alkaline hydrolysates showed contrasting results for 2-PAM. Hydrolytic degradation occurred rapidly. Whereas one major by-product was formed during acid hydrolysis, a series of by-products were formed in the basic solution. The chromatograms in Figs. 6 and 7 show the rate of formation of break-down products occurring during hydrolysis.



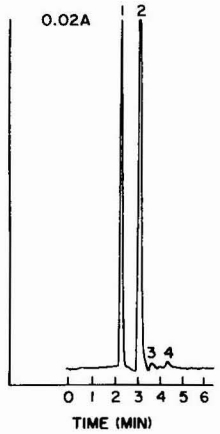


Fig. 6. Chromatogram of a 1.50 μg sample of 2-PAM hydrolyzed in 0.1 *N* NaOH at 95°C for 5 min. (1) 2-Carboxy-N-methyl pyridinium chloride, (2) 1-methyl-2-pyridine, (3) 2-carbamoyl-1-methyl pyridinium chloride and (4) 2-PAM.

Fig. 7. Chromatogram of the 60 minute alkaline hydrolysate of 2-PAM. (1) 2-Carboxy-N-methyl pyridinium chloride, (2) 1-methyl-2-pyridone, (3) 2-carbamoyl-1-methyl-pyridinium chloride, (4) and 2-PAM.

It was noted that after exposure of 2-PAM to alkaline hydrolysis for 5 min, three degradative by-products were observed in the mixture. As the reaction time increased, the rate of degradation of 2-PAM was accelerated. Upon completion of the 60-min hydrolysis, it was shown that 1-methyl-2-pyridone (61.4%) and 2-carboxy-N-methyl pyridinium chloride (37.8%) were the major by-products formed during the hydrolysis. A small amount of 2-carbamoyl-1-methyl pyridinium chloride was also formed.

Studies conducted by Kramer[11], from analysis of human urine, following oral administration of 2-PAM showed that a metabolite possessing a carboxyl function and weak acidic group was produced. The trend observed by Kramer is similar to what we saw during basic hydrolysis *in vitro*.

In light of the evidence, which indicates that the degradative fate of 2-PAM *in vivo* is similar to *in vitro* degradation, the method described in this paper may possibly be used to answer some of the questions pertaining to the metabolism of 2-PAM in human subjects.

ACKNOWLEDGEMENTS

We thank Dr. Jurgen von Bredow for his technical support in helping us to complete this study. We also express our sincere appreciation to Mr. Stewart Galloway for his excellent secretarial assistance.

REFERENCES

1 F. Hobbiger and P. W. Sadler, *Brit. J. Pharmacol.*, 14 (1959) 192.
2 W. D. Erdman, *Arzneimittel-Forsch.*, 15 (1965) 135.
3 J. H. Fleisher, L. W. Harris and E. F. Murtha, *J. Pharmac. Exp. Ther.*, 156 (1967) 345.
4 E. Meeter and O. L. Wolthuis, *Eur. J. Pharmacol.*, 2 (1968) 377.
5 L. W. Harris, J. H. Fleisher and H. I. Yamamura, *Eur. J. Pharmacol.*, 14 (1971) 38.
6 R. I. Ellin, *J. Amer. Chem. Soc.*, 80 (1958) 6588.
7 R. I. Ellin, J. S. Carlese and A. A. Kondritzer, *J. Pharm. Sci.*, 51 (1962) 141.
8 N. D. Brown and H. K. Sleeman, *J. Chromatogr.*, 138 (1977) 449.
9 N. D. Brown and H. K. Sleeman, *J. Chromatogr.*, 150 (1978) 225.
10 N. D. Brown and H. K. Sleeman, B. P. Doctor and J. P. Scovill, *J. Chromatogr.*, 195 (1980) 146.
11 B. Kramer, *Biochem. Pharmacol.*, 11 (1962) 299.

Journal of Chromatography, 212 (1981) 366–369
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,849

Note

Gas chromatographic–mass spectrometric method for the quantitative analysis of carbofuran in water

THOMAS R. NELSEN* and MICHAEL H. GRUENAUER
FMC Corporation, Agricultural Group, 100 Niagara Street, Middleport, NY 14105 (U.S.A.)
(Received February 16th, 1981)

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methyl carbamate) is a broad spectrum insecticide/nematicide[1]. A variety of analytical methods involving gas chromatography (GC)[2–5] and high-performance liquid chromatography[6–9] have been reported for the analysis of carbofuran in environmental matrices. All these methods have varying degrees of specificity. We sought a reliable quantitative method with a high degree of specificity for confirmation of low-level carbofuran residues in water samples involved in various environmental monitoring programs. This paper reports a rapid specific method which allows accurate quantitation of carbofuran in water at the 10 μg/l level, with a lower limit of detection of 0.5–1 μg/l.

EXPERIMENTAL

All solvents employed were Baker (Phillipsburgh, NJ, U.S.A.) Resi-Analyzed®. Standards were analytical grade (>99% purity) prepared in FMC laboratories.

Gas chromatography–mass spectrometry

A Hewlett-Packard HP5992B microprocessor controlled GC–mass spectrometry (MS) system was used. The system was equipped with a 122 cm × 2 mm I.D. silanized glass column packed with Tenax GC 60–80 mesh. The GC–MS system included a HP5990B quadrapole mass spectrometer coupled to a HP9825 computer and a HP9885 flexible disk. Operating conditions were: column temperature, 260°C; injector temperature, 260°C; carrier gas, helium (20 ml/min); ion source, 170°C; electron impact energy, 70 eV; electron multiplier voltage, 2.8 eV.

Methodology

Extraction. Water samples (100 g) were acidified by the addition of three drops of concentrated hydrochloric acid. The acid solution was extracted twice with 150 ml dichloromethane (DCM).

Conversion of carbofuran to 2,3-dihydro-2,2-dimethyl-7-hydroxybenzofuran (7-phenol). The DCM extract was washed twice with 150 ml 0.5 *N* sodium hydroxide and the aqueous phase discarded. The DCM was reduced in volume to *ca.* 10 ml using a Kuderna–Danish (KD) evaporative concentrator on a steam bath. The extract was

0021-9673/81/0000–0000/$02.50

taken just to dryness with a gentle stream of nitrogen. A 25-ml volume of 0.5 *N* NaOH was added to the residue and the solution stirred 0.5 hours at room temperature. The contents of the flask were washed two times with 50 ml DCM and the DCM discarded. The basic hydrolysis solution was made acidic by the addition of 5 ml concentrated hydrochloric acid and the solution extracted twice with 50 ml DCM. The DCM extract was dried by filtration through anhydrous sodium sulfate. The filter pad was rinsed with an additional 25 ml DCM. The combined extracts were placed in a KD concentrator and 50 ml methanol and five drops of diethylene glycol (as a keeper) were added. The solution was reduced in volume to *ca.* 5 ml on a stream bath and quantitatively transferred to a graduated centrifuge tube with methanol. The methanolic extract was reduced in volume to exactly 1 ml under a gentle stream of nitrogen for analysis.

Quantitation. Quantitation was accomplished by comparing the area of abundance at the 164 ion (molecular ion of 7-phenol) in a sample to that of a standard injection of a known quantity of 7-phenol (typically 1 ng).

The following formula was employed

$$\text{ppm} = \frac{\text{area of unknown} \times \text{ng standard}}{\text{area of standard} \times \text{mg water injected}} \times \text{CF}$$

where CF = 1.35; the molecular weight ratio of carbofuran (221) to 7-phenol (164).

The detection system was shown to be linear over the 0.25–2.0 ng range. A standard injection was made after each sample and the average of all standards was used to calculate μg/l values. An indication of the precision of calculation is the coefficient of error (standard deviation/mean) observed for the alternate standard injections (typically six standard injections). The coefficient of error ranged from 0.03–0.07 in the work reported.

Sensitivity. Method sensitivity (quantitatively reliable measurement of re-

TABLE I

RECOVERY OF CARBOFURAN FROM FORTIFIED WATER SAMPLES

Sample number	*Fortification level (μg/l)*	*Recovery level (μg/l)*	*Recovery (%)*
1	10	6.1	61.0
2	10	6.3	63.0
3	10	7.3	73.0
4	10	6.9	69.0
5	20	12.9	64.5
6	30	24.5	81.6
7	30	23.8	79.3
8	40	32.0	80.0
9	40	29.9	74.7
10	50	46.1	92.2
		Average	73.8
		Standard deviation	9.8

TABLE II

MASS MAJOR FRAGMENTS OF CARBOFURAN AND 7-PHENOL

Ion	*Carbofuran (relative abundance)*	*7-Phenol (relative abundance)*
221	10	–
165	12	12
164	100	100
149	63	76
147	9	11
131	18	30
123	16	26
122	17	25
121	11	20
103	9	14

sponse) was validated down to the 10 μg/l level by satisfactory recovery of carbofuran (as 7-phenol) from artificially fortified control samples. Method detectability, recognition of MS detector response was possible at 0.1 ng injected on column (0.7 μg/l).

RESULTS AND DISCUSSION

Table I lists the recoveries obtained from fortification experiments.

The conversion of carbofuran to 7-phenol was incorporated into the method for two reasons: first 7-phenol ionizes at 70 eV approximately five times as well as

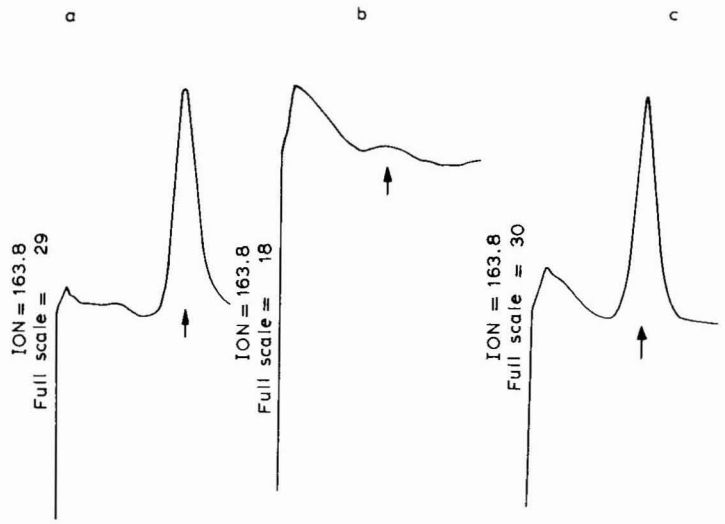


Fig. 1. Typical chromatograms from the analysis of carbofuran in water. a, 1 ng 7-phenol, area = 2341 units (scale = 29 abundance units); b, control water sample 200 mg injected (scale = 18 abundance units); c, control water sample fortified with 10 μg/l carbofuran 200 mg injected, area = 2384 units (scale = 30 abundance units). Response is equivalent to 6.9 μg/l carbofuran.

carbofuran. This is evidenced by comparison of mass to total ion abundance for each compound. Carbofuran gives essentially the same mass spectra as 7-phenol (see Table II); second the conversion allows one to take advantage of 7-phenol's acidity for sample cleanup. The initial base wash removes acidic coextractives while the DCM wash of the hydrolyzed mixture removes neutral and basic coextractives. This simple acid–base partition provides all the sample cleanup required for the analysis.

Control experiments demonstrated the quantitative nature of the hydrolysis of carbofuran to 7-phenol. It was also possible to demonstrate that the 7-phenol anion was unextractable from the basic hydrolysis mixture. Finally, control experiments showed that base wash of the initial DCM extract did not remove any carbofuran while it removed 7-phenol quantitatively.

Fig. 1 shows typical selected ion chromatograms derived through this method.

REFERENCES

1 W. T. Thomson, *Agricultural Chemicals Book I*, Thomson Publications, Fresno, CA, 1977, p. 31.
2 L. I. Butler and L. M. McDonough, *J. Ass. Offic. Anal. Chem.*, 54 (1971) 1357.
3 J. A. Coburn, B. D. Ripley and A. S. Y. Chau, *J. Ass. Offic. Anal. Chem.*, 59 (1976) 188.
4 R. F. Cook, in G. Zweig (Editor), *Analytical Methods for Pesticides, Plant Growth Regulators, and Food Additives, Vol. 7*, Academic Press, New York, 1973, p. 187.
6 L. Wong and F. M. Fisher, *J. Agr. Food Chem.*, 23 (1975) 315.
6 J. F. Lawrence, *J. Agr. Food Chem.*, 25 (1977) 211.
7 J. F. Lawrence and R. Leduc, *J. Agr. Food Chem.*, 25 (1977) 1362.
8 J. F. Lawrence and R. Leduc, *J. Chromatogr.*, 152 (1978) 507.
9 T. R. Nelsen and R. F. Cook, *J. Agr. Food Chem.*, 27 (1979) 1186.

Journal of Chromatography, 212 (1981) 370–373
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,840

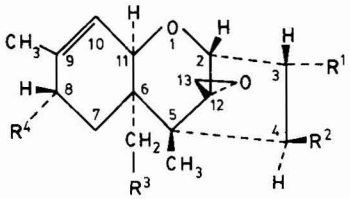
Note

High-performance liquid chromatography of trichothecenes

I. Detection of T-2 toxin and HT-2 toxin

RAINER SCHMIDT, ELENA ZIEGENHAGEN and KLAUS DOSE*
Institut für Biochemie, Joh. Gutenberg Universität Mainz, Joh. Joachim Becher Weg 30, 65 Mainz (G.F.R.)
(Received March 30th, 1981)

The fungi *Fusarium tricinctum*, *F. solani* and *F. sporotrichioides* produce a large number of metabolites including the highly toxic trichothecenes diacetoxyscirpenol (DAS), T-2 toxin and HT-2 toxin[1] (Fig. 1). The absence of conjugated unsaturation in most of the trichothecenes explains their lack of UV absorption[2]. The determination of these compounds is therefore difficult. It can be achieved by thin-layer chromatography (TLC) followed by spraying with sulphuric acid[3], or derivatization with a silylating reagent and subsequent gas chromatography[4,5].



Trichothecene	R^1	R^2	R^3	R^4
T-2 toxin	OH	CH_3COO	CH_3COO	$OCOCH_2CH(CH_3)_2$
HT-2 toxin	OH	OH	CH_3COO	$OCOCH_2CH(CH_3)_2$
DAS	OH	CH_3COO	CH_3COO	H

Fig. 1. Structures of naturally occurring trichothecenes.

So far, methods for isolation of trichothecenes have relied heavily on column chromatography on alumina and silica gel, or TLC[6,7]. We have found, however, that single runs with column chromatography or TLC yield incomplete separations. Therefore multiple chromatography techniques have to be applied. Moreover, application of TLC in trichothecene isolation can be hazardous inasmuch as the operator may have direct contact with mycotoxins, especially when scratching fractions from the TLC plate.

In view of these problems with classical separation techniques, we have reconsidered the use of high-performance liquid chromatography (HPLC) for the separation and determination of trichothecenes.

EXPERIMENTAL

Production of trichothecenes by Fusaria

Flasks containing 60 g of rice and 30 ml of water were stoppered with cotton-wool plugs. After 2 h they were autoclaved for 20 min at 121°C. Then the rice was

0021-9673/81/0000–0000/$02.50

inoculated with a suspension of spores of *F. tricinctum* sp. 897, *F. solani* sp. 900 or *F. sporotrichioides* sp. 941 (Professor Dr. Leistner, Bundesanstalt für Fleischforschung, 8650 Kulmbach, G.F.R.) in 0.2% Tween-80 and incubated at room temperature for 6 days.

Sample preparation

The extraction of moulds has been described previously[8]. Extraction with acetonitrile–water (containing 4% of potassium chloride) was followed by partitioning against *n*-hexane and dichloromethane. After evaporation of the solvent under reduced pressure, the residue was dissolved in 400 μl methanol. A 200-μl volume of the solution thus obtained was pipetted on to a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.) and eluted with 5 ml methanol–water (80:20). The prepurified extract was transferred into a 10-ml Luer-Lok syringe containing a Swinney filter holder and a 0.5-μm Millipore filter through which the sample was filtered. After evaporation of the solvent, the residue was redissolved in a small amount of methanol and injected into the HPLC system.

For the separation of non-polar trichothecenes like T-2 toxin a modified sample preparation is advantageous. If the extract placed on the Sep-Pak C_{18} cartridge is eluted with 5 ml of 20% methanol, T-2 toxin is retained while more polar compounds pass through the bed. T-2 toxin is then eluted with 5 ml of 80% methanol. This fractionation results in a less complex chromatogram.

HPLC

Liquid chromatographic separations were performed on the apparatus recently described[9]. The prepacked μBondapak C_{18} (particle size 10 μm) column (30 cm × 7.9 mm I.D.) was obtained from Waters Assoc. The detection of the metabolites was carried out by a differential refractometer (M 401, Waters Assoc.).

RESULTS

In the course of our investigations the differential refractometer has proved to be suitable for detection of as little as 1 μg T-2 toxin per injection at the highest detector sensitivity up to semipreparative quantities of about 1 mg.

In Table I are summarized the retention times for T-2 toxin, HT-2 toxin and DAS in different methanol–water mixtures. A strong decrease in retention with increasing methanol concentration (55 to 65%) is evident. Therefore the solvent has to be prepared carefully. The retention times for the solvent containing 60% methanol are highly reproducible. Several injections of extracts from mouldy rice and pure T-2

TABLE I
RETENTION TIMES (min) OF DIACETOXYSCIRPENOL, HT-2 TOXIN AND T-2 TOXIN IN DIFFERENT METHANOL–WATER SOLVENT SYSTEMS ON A μBONDAPAK C_{18} COLUMN

Solvent (flow-rate 2.0 ml/min)	*DAS*	*HT-2 toxin*	*T-2 toxin*
Methanol–water (5.5:4.5)	12.3	19.3	30.6
Methanol–water (6:4)	9.8	13.0	17.6
Methanol–water (6.5:3.5)	8.1	10.3	13.0

TABLE II

REPRODUCIBILITY OF RETENTION TIMES FOR T-2 TOXIN, HT-2 TOXIN AND DIACETOXYSCIRPENOL IN HPLC

Parameter	*T-2 toxin*	*HT-2 toxin*	*DAS*
No. of injections, *N*	28	29	12
Retention time (min)			
Range	17.1–18.5	12.6–13.4	9.5–10.2
Mean	17.6	13.0	9.8
Standard deviation (min)	0.47	0.26	0.24
Coefficient of variation (%)	2.66	2.01	2.49

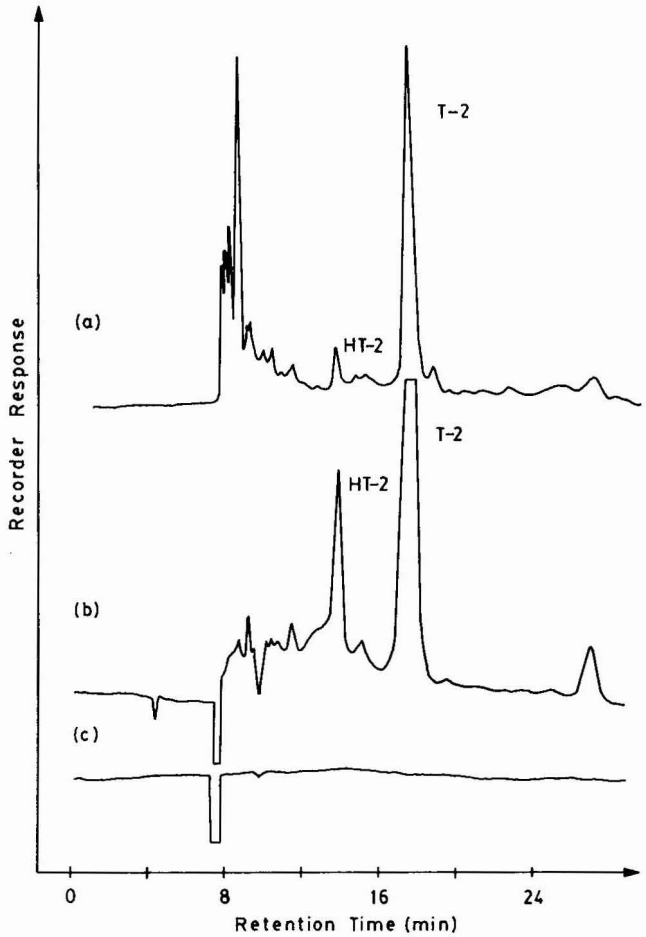


Fig. 2. Elution patterns of extracts from rice after HPLC with methanol–water (6:4) as solvent. (a) Extract from mouldy rice, using sample preparation with 80% methanol; (b) extract from mouldy rice, using modified sample preparation for removal of polar compounds with 20% methanol and elution with 80% methanol; (c) extract from non-mouldy rice.

toxin, HT-2 toxin and DAS (Makor Chemicals, Jerusalem, Israel) gave reproducible mean retention times of 17.6 min for T-2 toxin, 13.0 min for HT-2 toxin and 9.8 min for DAS (coefficients of variation 2.66, 2.01 and 2.49, respectively). The relatively high variation can be attributed to slight differences in the methanol concentration of the mobile phase (Table II).

For the separation of extracts from mouldy rice the solvent methanol–water (6:4) was suitable. The chromatograms in fig. 2 show the elution pattern for an extract from non-mouldy rice (no characteristic peaks) and for two extracts from mouldy rice which had been treated differently. The chromatograms of the extracts from cultures of *F. tricinctum* sp. 897, *F. solani* sp. 900 and *F. sporotrichioides* sp. 941 showed no remarkable differences. The accuracy of the retention times permitted a clear distinction between T-2 toxin, HT-2 toxin and DAS that was lacking in the original extracts. This result was confirmed by TLC of the fractions. At least two more metabolites (t_R 14.9 and 30.5 min), preliminarily classified as trichothecenes, have been detected on the HPLC chromatogram. Both fractions exhibit the same reaction with sulphuric acid on silica gel TLC plates (a green-yellow fluorescence) as T-2 toxin, HT-2 toxin and DAS.

ACKNOWLEDGEMENTS

This research was supported by the Bundesminister für Jugend, Familie und Gesundheit (412-6080-3/19). We thank Professor Dr. Leistner, Kulmbach, for providing us with spores of *Fusaria* and we gratefully acknowledge the microbiological work carried out by Ms. A. Bieger.

REFERENCES

1 J. Palti, *Toxigenic Fusaria, their Distribution and Significance as Causes of Disease in Animal and Man*, Verlag Paul Parey, Berlin, 1978.
2 C. J. Mirocha, S. V. Pathre and C. M. Christensen, in T. D. Wyllie and L. G. Morehouse (Editors), *Mycotoxic Fungi, Mycotoxins, Mycotoxicoses — An Encyclopedic Handbook*, Vol. 1, Marcel Dekker, New York, 1977, Ch. 2.5.
3 Z. Ďuračková, V. Betina and P. Nemec, *J. Chromatogr.*, 116 (1976) 141.
4 C. O. Ikediobi, I. C. Hsu, J. R. Bamburg and F. M. Strong, *Anal. Biochem.*, 43 (1971) 327.
5 N. Milama and H. Lelievre, *Analusis*, 7 (1979) 232.
6 J. R. Bamburg and F. M. Strong, in S. Kadis, A. Ciegler and S. J. Ajl (Editors), *Microbial Toxins*, Vol. 7, Academic Press, New York, 1971, p. 207.
7 L. Leistner, R. Amano, N. Halls, M. Manabe, N. Sato, F. Tauchmann and D. Trapper, *Mykotoxine-Report Juni 1977*, Institut für Bakteriologie und Histologie der Bundesanstalt für Fleischforschung, Kulmbach, 1977.
8 R. Schmidt, A. Bieger, E. Ziegenhagen and K. Dose, *Z. Anal. Chem.*, 308, No. 2 (1981).
9 R. Schmidt, J. Mondani, E. Ziegenhagen and K. Dose, *J. Chromatogr.*, 207 (1981) 435.

Journal of Chromatography, 212 (1981) 374–378
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,839

Note

High-performance paper electrophoresis

II. Comparison of separations obtained by high-voltage paper electrophoresis and high-performance paper electrophoresis

S. FANALI and L. OSSICINI*
Istituto di Cromatografia del C.N.R., Area della Ricerca di Roma, P.O. Box 10, 00016 Monterotondo Stazione, Rome (Italy)
(Received April 4th, 1981)

During the last few years special attention has been paid to studies on fast separation processes, and so-called "high-performance" methods have been developed in both electrophoresis and chromatography.

Lederer[1] has recently developed a rapid electrophoretic method that can yield good separations within a few minutes by an extremely simple arrangement which has been called "high-performance paper electrophoresis". So far this method has been used experimentally to separate, for example, mixtures of metal ions. We have now carried out a more extensive study and have compared the results with those previously obtained by high-voltage electrophoresis[2–7].

EXPERIMENTAL AND RESULTS

The apparatus used consisted of an LKB power supply (Model 337 IE), a plastic electrophoretic cell without a cooling system and glass plates 10 cm long and 5 cm wide and varying in thickness from 2 to 5 mm. The paper strips (Whatman No. 1 or 3MM) were usually 10 cm long and 5–10 mm wide and were placed between the glass plates. Unless indicated otherwise in the figure legends, the length of the experiments varied from 3–15 min. The electric field applied was 40–80 V/cm (in the figure legends we give the values of the voltage applied, as the distance between the electrodes was always 10 cm).

The separations described below were chosen in order to establish the limits of utilization of the method. All of the separations studied had already been achieved by high-voltage electrophoresis[2–7].

Separation of cobalt(III) complexes: separation of $Co(en)_3^{3+}$, $Co(NH_3)_6^{3+}$, $Co(o\text{-}phen)_3^{3+}$ *and* $Co(dip)_3^{3+}$

For the separations we used 0.3 and 0.5 *N* aqueous solutions of sodium trichloroacetate, sodium sulphate and sodium perchlorate. As shown in Figs. 1–4, good separations of all the compounds could be obtained in trichloroacetate, whereas in sulphate and perchlorate solutions $Co(dip)_3^{3+}$ and $Co(o\text{-phen})_3^{3+}$ were not separated.

0021-9673/81/0000–0000/$02.50

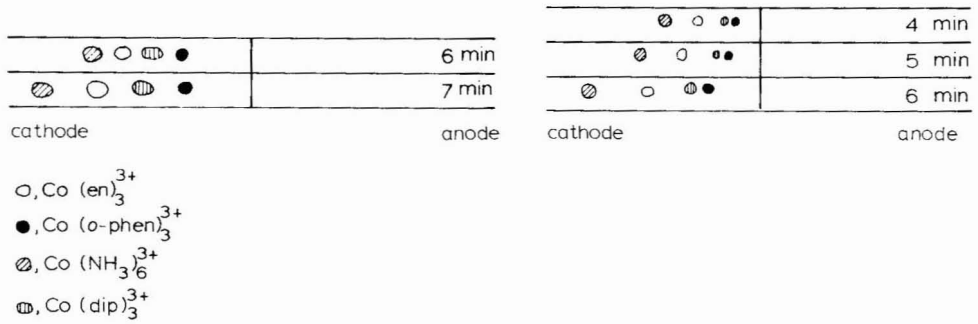


Fig. 1. Electropherograms of cobalt(III) complexes on Whatman 3MM paper at 500 V. Solution of sodium trichloroacetate (0.3 *N*).

Fig. 2. Electropherograms of cobalt(III) complexes on Whatman 3MM paper at 500 V. Solution of sodium trichloroacetate (0.5 *N*). Spots as in Fig. 1.

Fig. 3. Electropherograms of cobalt(III) complexes on Whatman 3MM paper at 500 V. Solution of sodium perchlorate (0.3 *N*). Spots as in Fig. 1.

Fig. 4. Electropherograms of cobalt(III) complexes on Whatman 3MM paper at 500 V; time, 6 min. A, Solution of Na_2SO_4 (0.3 *N*); B, solution of Na_2SO_4 (0.5 *N*).

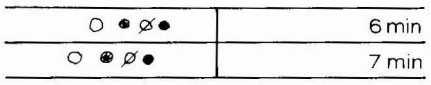
Figs. 1 and 2 show also that with more concentrated solutions (0.5 *N*) at the same voltage separations could be obtained even in 4 min with a relatively short electrophoretic run.

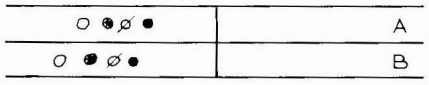
In all of the experiments we used Whatman 3MM paper because thick paper causes more heating owing to the Joule effect, which favours the separation. As reported previously[2-4], because of the difference in ion-pair formation, we also observed an inversion of the electrophoretic mobility when we used solutions of sulphate instead of perchlorate and trichloroacetate; Co(III) $(en)_3^{3+}$, for example, was the fastest in perchlorate and trichloroacetate and the slowest in sulphate.

Separation of cobalt(III) mixed complexes: (a) $Co(en)_3^{3+}$, $Co(en)_2(o\text{-}phen)^{3+}$, $Co(en)(o\text{-}phen)_2^{3+}$ and $Co(o\text{-}phen)_3^{3+}$ and (b) $Co(en)_3^{3+}$, $Co(en)_2(dip)^{3+}$, $Co(en)(dip)_2^{3+}$ and $Co(dip)_3^{3+}$

As in the previous instance, we utilized aqueous solutions of trichloroacetate, perchlorate and sulphate. Figs. 5–7 show how it is possible to separate these complexes in all of the solutions examined; the best separations were obtained with 0.5 *N* solutions, but separations could also be obtained with 0.3 *N* solutions; the optimal voltage was 500 V (50 V/cm); the length of the experiments can be reduced to 3 min.

Regarding the series of mixed complexes, $Co(en)_3^{3+}$–$Co(en)_2(dip)^{3+}$–$Co(en)(dip)_2^{3+}$–$Co(dip)_3^{3+}$, good separations could be obtained with 0.3 and 0.5 *N* solutions of sodium perchlorate (under different conditions of voltage and time) and with 0.3 and 0.5 *N* solutions of sodium perchlorate (Figs. 8 and 9). In contrast to the results obtained by high-voltage electrophoresis[4], the four complexes could not be separated in trichloroacetate solutions.

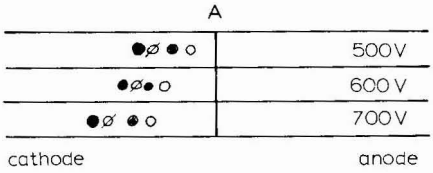




○, $Co(en)_3^{3+}$
⊘, $Co(en)_2(o\text{-phen})^{3+}$
⊘, $Co(en)(o\text{-phen})_2^{3+}$
●, $Co(o\text{-phen})_3^{3+}$

Fig. 5. Separation of mixed complexes of cobalt(III) on Whatman 3MM paper at 500 V. Solution of sodium trichloroacetate (0.5 *N*).

Fig. 6. Separation of mixed complexes of cobalt(III) on Whatman 3MM paper at 500 V. A, Sodium perchlorate (0.3 *N*), time = 6 min; B, sodium perchlorate (0.5 *N*), time = 5 min. Spots as in Fig. 5.



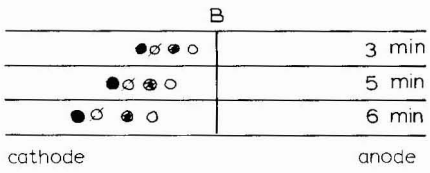
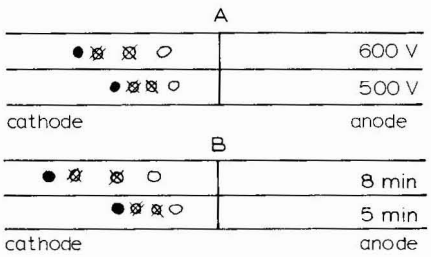
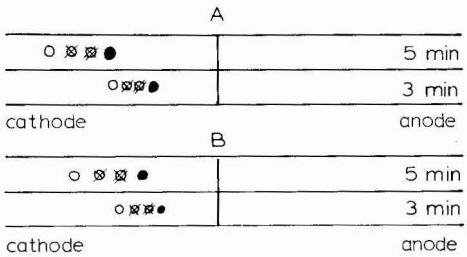


Fig. 7. Electropherograms of mixed complexes of cobalt(III) on Whatman 3MM paper; solution of Na_2SO_4 (0.5 *N*). A, Time = 3 min; B, voltage = 500 V. Spots as in Fig. 5.





○, $Co(en)_3^{3+}$
⊠, $Co(en)_2(dip)^{3+}$
⊠, $Co(en)(dip)_2^{3+}$
●, $Co(dip)_3^{3+}$

Fig. 8. Electropherograms of mixed complexes of cobalt(III) on Whatman 3MM paper; solution of Na_2SO_4 (0.3 *N*). A, Time = 5 min; B, voltage = 500 V.

Fig. 9. Electropherograms of cobalt(III) complexes on Whatman 3MM paper at 500 V. A, Solution of sodium perchlorate (0.3 *N*); B, solution of sodium perchlorate (0.5 *N*). Spots as in Fig. 8.

Separation of some inorganic anions: separation of ClO_3^-, BrO_3^- and IO_3^-

Fig. 10 shows the separation of chlorate, bromate and iodate ions in 0.1 *N* HCl solutions. At 700 V the time required could be reduced to 60 sec.

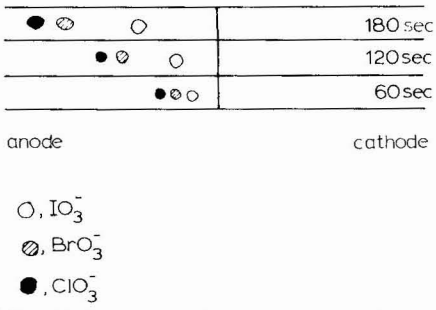
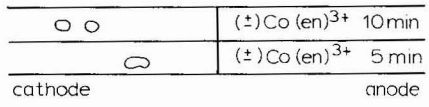


Fig. 10. Separation of some inorganic anions on Whatman No. 1 paper at 700 V. Electrolyte, 0.1 *N* HCl.

Separation of optical isomers

We separated optical antipodes of $Co(en)_3^{3+}$ with various electrophoretic systems; the results are shown in Figs. 11–14. With solutions of antimonyl tartrate a longer time is necessary (10–15 min). The time required could be considerably reduced (Figs. 13 and 14) if mixtures of aluminium chloride and D(−)- or L(+)-tartrate were used. As we were studying thermolabile substances, we used the lighter Whatman No. 1 paper (which gives a low temperature) or Whatman 3MM paper, and in these instances the glass plates between which the paper strips were placed were much thicker (>5 mm) in order to absorb the heat generated. With this technique it was impossible to separate the optical isomers of $Co(en)_3^{3+}$ with solutions of optically active tartrate, whereas this separation was achieved with high-voltage electrophoresis[6].



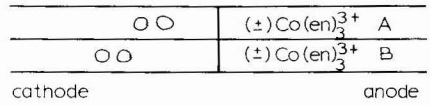
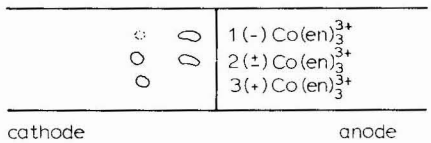


Fig. 11. Electropherograms of (±)-cobalt(III) $(en)_3^{3+}$ on Whatman 3MM paper at 800 V. Electrolyte, antimony potassium (+)-tartrate solution (0.1 *N*).

Fig. 12. Electropherograms of (±)-cobalt(III) $(en)_3^{3+}$ at 500 V for 15 min. Electrolyte, antimony potassium (+)-tartrate solution (0.1 *N*). A, Whatman No. 1 paper; B, Whatman No. 3MM paper.



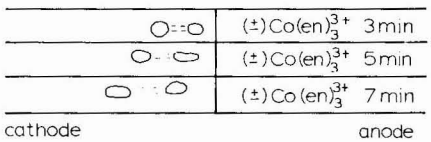


Fig. 13. Separation of two enantiomers of $Co(en)_3$ on Whatman No. 1 paper at 500 V for 7 min. Electrolyte, mixture of aluminium chloride (0.24 *M*) and disodium D(−)-tartrate (0.36 *M*). The strip paper was 2 cm wide.

Fig. 14. Separation of two enantiomers of $Co(en)_3^{3+}$ on Whatman No. 1 paper at 500 V for 7 min. Electrolyte, mixture of aluminium chloride (0.24 *M*) and disodium D(−)-tartrate (0.36 *M*).

CONCLUSION

A general comparison of the results obtained by this method with those obtained by high-voltage electrophoresis leads to the following conclusions:

(1) It is possible to obtain separations by high-performance paper electrophoresis whenever the differences in the electrophoretic mobility in high-voltage electrophoresis are 2 cm or more; in this event the time required is reduced from 1 h to about 10 min.

(2) When the mobility differences are less than 1.5–2 cm it is still possible to obtain separations utilizing the increase in the electrophoretic mobility due to the temperature increase. In this instance, the method can only be used with non-thermolabile substances.

(3) Of the different factors that influence the separations, the most important is the variation of temperature due to the Joule effect.

(4) The extreme shortness of the time necessary for the experiments (which generally does not exceed 5 min) makes high-performance paper electrophoresis particularly suitable, for example, for controlling the purity of complexes prepared in the laboratory.

ACKNOWLEDGEMENT

Thanks are due to Professor M. Lederer for his interest in this work and his helpful suggestions.

REFERENCES

1 M. Lederer, *J. Chromatogr.*, 171 (1979) 403.
2 M. Mazzei and M. Lederer, *J. Chromatogr.*, 31 (1967) 196.
3 M. Lederer and M. Mazzei, *J. Chromatogr.*, 35 (1968) 201.
4 T. Prosperi, M. Sinibaldi and M. Lederer, *Gazz. Chim. Ital.*, 103 (1973) 995.
5 M. Sinibaldi and M. Lederer, *J. Chromatogr.*, 60 (1971) 241.
6 L. Ossicini and C. Celli, *J. Chromatogr.*, 115 (1975) 655.
7 V. Cardaci, L. Ossicini and T. Prosperi, *Ann. Chim. (Rome)*, 68 (1978) 713.

Journal of Chromatography, 212 (1981) 379–381
Elsevier Scientific Publishing Company, Amsterdam Printed in The Netherlands

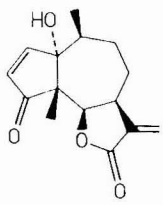
CHROM. 13,925

Note

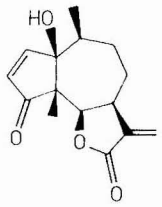
Separation and identification of stereoisomers of sesquiterpene lactones by multiple development of thin-layer chromatograms

ANNA K. PICMAN*, IRMA PANFIL* and G. H. NEIL TOWERS
Department of Botany, University of British Columbia, Vancouver, B. C. V6T 2B1 (Canada)
(First received March 17th, 1981; revised manuscript received April 24th, 1981)

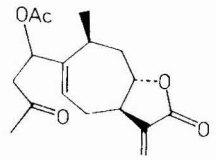
Because diastereoisomers have different physical properties, including different solubilities in a given solvent, they are often separated chromatographically[1]. However, thin-layer chromatography (TLC) techniques with various solvent systems which have been generally used for separation of sesquiterpene lactones[2] are inadequate for the separation of their stereoisomers. For example, two naturally occurring diastereoisomers, parthenin and hymenin (I, II), give identical R_F values on TLC using silica gel in various solvent systems even with heat-activated plates or plates impregnated with $AgNO_3$. In addition, they cannot be distinguished under UV light, using iodine vapours, $KMnO_4$ spray or vanillin or *p*-dimethylaminobenzaldehyde spray reagents[3].



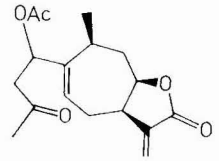
I, PARTHENIN



II, HYMENIN



III, XANTHININ



IV, XANTHUMIN

A better separation of similar compounds can be sometimes achieved through the multiple development of TLC plates in the same solvent system[4]. We report here the use of the multiple-development TLC technique for the separation of parthenin and hymenin. This method appears to be equally suitable for the separation of other isomeric sesquiterpene lactones.

EXPERIMENTAL

Methods

Silica gel plates, without gypsum and with fluorescent indicator (Polygram;

* Permanent address: Institute of Organic Chemistry, Polish Academy of Sciences, Kasprzaka 44/52, Warszawa, Poland.

0021-9673/81/0000–0000/$02.50

Brinkmann, Westbury, NY, U.S.A.) were used. Strips of silica gel plate (12 cm) with parthenin and hymenin or xanthinin(III) and xanthumin(IV) applied to the same spot (approximately 1 μg of each compound) were developed 1–10 × in a standard chamber with heptane–diethyl ether–ethyl acetate (30:65:5), the plates being air dried in between runs. The compounds were visualized with the vanillin spray reagent[3]. Parthenin and hymenin give a bluish green color, xanthinin brownish red, and xanthumin reddish brown. Under these conditions parthenin moves ahead of hymenin and xanthinin ahead of xanthumin.

To determine which of the two stereoisomers, parthenin or hymenin, is present in crude extracts of *Parthenium hysterophorus*, two 20 × 20 cm plates were used. One plate was spotted (in one corner) with parthenin, the other one with hymenin, and to these spots the crude plant extract was added. The TLC plates were developed in chloroform–acetone (6:1) to separate parthenin (or hymenin) from other extract constituents, air dried, and then developed in a second direction in the above solvent system ten times. The presence of parthenin in an extract was indicated by two spots on a plate spotted with hymenin but only one spot on a plate spotted with parthenin. On the other hand, the presence of hymenin was indicated by two spots on a plate spotted with parthenin.

Materials

Parthenin and hymenin were isolated from *P. hysterophorus* collected in Texas (U.S.A.) and Argentina, respectively. The samples of xanthinin were provided by the late T. A. Geissman (University of California, Los Angeles, CA, U.S.A.) and T. J. Mabry (University of Texas, Austin, TX, U.S.A.). Xanthumin was a gift of E. Rodriguez (University of California, Irvine, CA, U.S.A.).

RESULTS AND DISCUSSION

The multiple development of TLC plates in the same solvent system facilitates the separation of stereoisomers which after a single development of plates move as one spot, the technique taking advantage of the slightly different solubilities of otherwise very similar compounds in a given solvent system. To achieve a separation the solvent system must be one in which the compounds move very slowly on the chromatographic plates.

In case of both mixtures (parthenin and hymenin, xanthinin and xanthumin) 1–6 developments of the TLC plates did not sufficiently separate the stereoisomers (Fig. 1). To separate parthenin and hymenin or xanthinin and xanthumin clearly it is necessary to develop the TLC plates 8 × or 10 ×, respectively (Fig. 1).

The method described proved to be useful in a comparative study of the sesquiterpene lactones of *P. hysterophorus* (*Compositae*) collected from various populations throughout its range of distribution. In general, North and Central American plants as well as Indian specimens were found to contain parthenin (I), while South American samples had hymenin (II) as the major sesquiterpene lactone[5]. Parthenin is responsible for an epidemic of allergic contact dermatitis in India[6] which is stereospecific[7]. Both stereoisomers were never found together in a single plant.

This technique, however, also makes it possible to identify stereoisomers of sesquiterpene lactones in cases where they are present in the same sample. "Xan-

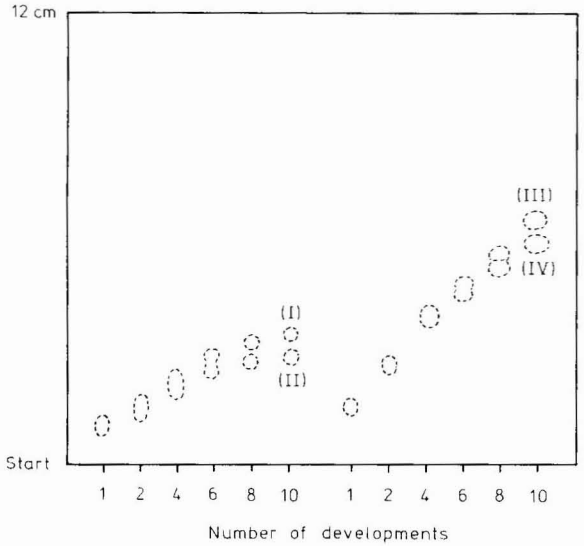


Fig. 1. Separation of parthenin (I) and hymenin (II), and xanthinin (III) and xanthumin (IV) by multiple-development technique.

thinin" which was available in our laboratory proved to be a mixture of authentic samples of stereoisomers, xanthinin(III) and xanthumin(IV). The shades of colors formed with the vanillin spray reagent are slightly different for these compounds. Both, xanthinin and xanthumin, co-occur in *Xanthium strumarium* (*Compositae*). The species is separated into the "italicum" morphological complex with the predominant sesquiterpene lactone being xanthinin and the "chinese" complex with the major compound being xanthumin. Some hybrids between these two complexes contain both stereoisomers[8]. Our TLC method could be successfully used in such comparative chemical studies. In addition, this technique is particularly useful when only small samples of plants are available for analyses and when other techniques such as nuclear magnetic resonance spectroscopy which may require the isolation and purification of a compound, cannot be successfully applied.

ACKNOWLEDGEMENTS

We thank those who kindly provided samples of sesquiterpene lactones and the Natural Sciences and Engineering Research Council of Canada for financial support.

REFERENCES

1 M. Brenner and G. Pataki, *Helv. Chim. Acta*, 44 (1961) 1420.
2 H. Yoshioka, T. J. Mabry and B. N. Timmermann, *Sesquiterpene Lactones: Chemistry, NMR and Plant Distribution*, University of Tokyo Press, Tokyo, 1973.
3 A. K. Picman, R. L. Ranieri, G. H. N. Towers and J. Lam, *J. Chromatogr.*, 189 (1980) 187.
4 E. Stahl, *Thin-Layer Chromatography*, Springer, New York, 1969.
5 A. K. Picman and G. H. N. Towers, in preparation.
6 G. H. N. Towers, J. C. Mitchell, E. Rodriguez, P. V. Subba Rao and F. D. Bennett, *J. Sci. Ind. Res.*, 36 (1977) 672.
7 P. V. Subba Rao, A. Mangala, G. H. N. Towers and E. Rodriguez, *Cont. Derm.*, 4 (1978) 199.
8 C. McMillan, P. I. Chavez and T. J. Mabry, *Biochem. System. Ecol.*, 3 (1975) 137.

Journal of Chromatography, 212 (1981) 382
Elsevier Scientific Publishing Company, Amsterdam Printed in The Netherlands

CHROM. 13,838

Book Review

Chemische Reaktionsdetektoren für die schnelle Flüssigkeits-Chromatographie — Grundlagen und Anwendungen in der Spurenanalyse, by G. Schwedt, Hüthig, Heidelberg, 1980, 213 pp., price DM 38.00, ISBN 3-7785-0687-0.

To write a book on reaction detectors in high-performance liquid chromatography (HPLC) is certainly a very pertinent endeavour these days. On the other hand, one wonders whether there is enough material published for an entire book on this subject and the result, consequently, is rather on the meagre side.

The book starts with a general introduction to the subject, which is useful to the newcomer to reaction detector technology.

The main section on applications gives a good overview of all the work done in this area, with a fair coverage of the literature up to 1980. The reader will find some practical information on the application of this technique to many important groups of compounds, particularly in the bioanalytical area. Although the author gives many details of procedures, one still has to resort to the original literature in most instances in order to be able to reproduce a technique; thus, all details may be given on a separation system, but not enough detail to tell you how to make the reagent solution (and hence the reaction medium) compatible with the mobile phase. The various methods are cited with no critical remarks about their relative merits and some of the examples given in detail in tabular form seem to be poorly chosen.

The book also contains a theoretical section on diffusion effects in various reactor designs, but this seems to contribute more to confusion than to diffusion! The equations are not numbered, which makes discussion difficult. Some equations contain errors (*e.g.*, on pp. 29, 30, 32 and 48), sigma values have been added instead of adding sigma-squared values, some unusual symbols have been used and graphical representations resulting from some of these equations are presented without any further explanation about their usefulness to the practitioner.

Nevertheless, with all its shortcomings, the book will be useful to the novice in the area of reaction detectors in HPLC, or to workers who need some ideas on how to handle specific groups in complex matrices. It fulfills a useful purpose, even if only owing to the lack of a better up-to-date alternative source.

Amsterdam (The Netherlands) R. W. FREI

Author Index

Erratum

J. Chromatogr., 210 (1981) 301–309
Page 306, Table II, the heading of the 2nd column should read: *HCl*.

Vol. 212, No. 3

journal of chromatography news section

NEW BOOKS

Polymer catalysts and affiants – Polymers in chromatography, edited by B. Sedláček, C.G. Overberger and H.F. Mark, Wiley, Chichester, New York, 1981, 260 pp., price US$ 31.50, £ 14.50, ISBN 0-471-09014-X.

Essentials of nuclear chemistry, by H. J. Arnikar, Wiley, Chichester, New York, 1981, *ca.* 250 pp., price *ca.* US$ 15.50, £ 5.95, ISBN 0-85226-033-4.

MEETING

CHROMATOGRAPHY '82, THE 17th INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY

The 17th International Symposium on Advances in Chromatography will be held April 5–8, 1982, in Las Vegas, NV, U.S.A. The scope of the meeting will include papers and informal discussion groups by outstanding researchers from throughout the world in all fields of chromatography. In particular, new developments in gas, liquid, and high-performance thin-layer chromatography will be included. There will also be a commercial exhibition of the latest instrumentation and books. Participation in the symposium will be on the basis of invited papers as well as unsolicited contributions. Authors desiring to present papers must submit 200-word abstracts by Sept. 14th, 1981. Complete manuscripts of accepted authors will be due on Oct. 14th, 1981. Special separate, intensive 2-day courses in (a) High Resolution Capillary Columns, (b) HPLC, (c) Gas Chromatography–Mass Spectrometry, and (d) HPTLC will be held on Saturday and Sunday, April 3 + 4, just prior to the meeting. All correspondence pertaining to the symposium, short courses, and exhibition space should be directed to: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A.

CALENDAR OF FORTHCOMING MEETINGS

Sept. 20–25, 1981 Philadelphia, PA, U.S.A.	**8th National Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies (FACSS)** Contact: M.A. Kaiser, E.I. du Pont de Nemours & Co., Experimental Station E228/200, CR&D Department, Wilmington, DE 19898, U.S.A.
Sept. 28–Oct. 1, 1981 Barcelona, Spain	**16th International Symposium Advances in Chromatography** Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel. (713) 749-2623. (Complete program published in Vol. 209, No. 3)
Sept. 28–Oct. 4, 1981 Moscow, U.S.S.R.	**International Symposium on Chemical Physics** Contact: Prof. N.M. Emmanuel, Institute of Chemical Physics, Academy of Sciences of U.S.S.R., Vorobyevskoye Chaussee 2-6, SU–117334 Moscow, U.S.S.R.

Sept. 29–Oct. 2, 1981 Basle, Switzerland	**ILMAC 81; 8th International Exhibition of Laboratory, Chemical Engineering, Measurement and Automation Techniques in Chemistry** Contact: D. Gammeter, Secretariat ILMAC 81, Postfach, CH-4021 Basle, Switzerland. Tel. 061 20 20 20. (Further details published in Vol. 212, No. 2)
Oct. 22–23, 1981 Montreux, Switzerland	**Workshop on Liquid Chromatography – Mass Spectrometry** Contact: Prof. Dr. R.W. Frei, Free University, Department of Analytical Chemistry, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands (Further details published in Vol. 207, No. 3).
Oct. 27–29, 1981 London, Great Britain	**Petroanalysis 81** Contact: Miss I.A. McCann, Conference Officer, Institute of Petroleum, 61 New Cavendish Street, London W1M 8AR, Great Britain. (Tel: 01–636 1004, Telex: 264380)
Oct. 28–30, 1981 Gatlinburg, TN, U.S.A.	**Resource Recovery and Environmental Issues of Industrial Solid Wastes** Contact: J.S. Watson, Oak Ridge National Laboratory, P.O. Box X, Oak Ridge, TN 37830, U.S.A.
Nov. 9–10, 1981 Berlin, G.F.R.	**Symposium on Practical Aspects of HPLC** Contact: Dr. I. Molnár, Wissenschaftliche Geratebau Dr. H. Knauer GmbH, Hegauer Weg 38, D-1000 Berlin 37, G.F.R. (Further details published in Vol. 207, No. 2).
Nov. 16–17, 1981 Washington, DC, U.S.A.	**International Symposium on HPLC of Proteins and Peptides** Contact: Ms. S.E. Schlessinger, Symposium Manager, 400 East Randolph, Chicago, IL 60601, U.S.A. (Further details published in Vol. 208, No. 2)
Nov. 23–25, 1981 Barcelona, Spain	**2nd International Congress on Analytical Techniques in Environmental Chemistry** Contact: Dr. Joan Albaigés, General Secretary, Plaza de Espana, Barcelona-4, Spain. Tel. 223 31 01.
Dec. 2–3, 1981 Paris, France	**Journées de Chromatographie en Phase Liquide** Contact: H. Colin, Laboratoire C.A.P., Ecole Polytechnique, Route de Saclay, 91128 Palaiseau Cedex, France.
Jan. 4–9, 1982 Orlando, FL, U.S.A.	**1982 Winter Conference on Plasma Spectrochemistry** Contact: 1982 Winter Conference, c/o ICP Information Newsletter, Chemistry -GRC Towers, University of Massachusetts, Amherst, MA 01003, U.S.A. Tel. (413) 545–2294.
Jan. 19–20, 1982 Amsterdam, The Netherlands	**Symposium on "Detection in High-Performance Liquid Chromatography"** Contact: Mrs. Peschier, Hewlett-Packard Nederland B.V., Analytical Department, van Heuven Goedhartlaan 121, 1181 KK Amstelveen, The Netherlands. (Tel.: 020-47 20 21). (Further details published in Vol. 212, No. 2)
March 8–12, 1982 Atlantic City, NJ, U.S.A.	**1982 Pittsburgh Conference and Exhibition on Analytical Chemistry and Applied Spectroscopy** Contact: Mrs. Linda Briggs, Program Secretary, Pittsburgh Conference, Department J-057, 437 Donald Road, Pittsburgh, PA 15235, U.S.A. (Further details published in Vol. 212, No. 2)
March 28–April 2, 1982 Las Vegas, NV, U.S.A.	**National American Chemical Society Meeting** Contact: A.T. Winstead, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
April 5–8, 1982 Las Vegas, NV, U.S.A.	**International Symposium "Advances in Chromatography"** Contact: Prof. A. Zlatkis, Chemistry Department, University of Houston, Central Campus, 4800 Calhoun, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623.

April 14–16, 1982 Amsterdam, The Netherlands	**12th Annual Symposium on the Analytical Chemistry of Pollutants** Contact: Prof. Dr. Roland W. Frei, The Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. (Further details published in Vol. 206, No. 1)
April 15–17, 1982 Tokyo, Japan	**International Symposium "Advances in Chromatography"** Contact: Prof. A. Zlatkis, Chemistry Department, University of Houston, Central Campus, 4800 Calhoun, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623.
April 19–22, 1982 Barcelona, Spain	**International Congress on Automation in Clinical Laboratory** Contact: Dr. R. Galimany, Sección de Automatización, Laboratorio de Analisis Clinicos, C.S. "Principes de Espana", Hospitalet de Llobregat, Barcelona, Spain.
April 21–23, 1982 Neuherberg near Munich, G.F.R.	**Second International Workshop on Trace Element Analytical Chemistry in Medicine and Biology** Contact: Dr. P. Schramel, Gesellschaft fuer Strahlen- und Umweltforschung, Institut fuer Angewandte Physik, Physikalisch-Technische Abteilung, Ingolstaedter Landstrasse 1, D-8042 Neuherberg, G.F.R.
April 27–30, 1982 Munich, G.F.R.	**Biochemische Analytik 82** Contact: Prof. I. Trautschold, Medizinische Hochschule Hannover, Karl-Wiechert-Allee 9, 3000 Hannover 61, G.F.R. (Further details published in Vol. 224, No. 3)
May 2–6, 1982 Interlaken, Switzerland	**2nd International Symposium on Instrumental TLC (HPTLC)** Contact: Dr. R.E. Kaiser, Institute for Chromatography, P.O. Box 1141, D-6702 Bad Dürkheim, G.F.R.
May 11–14, 1982 Ghent, Belgium	**4th International Symposium on Quantitative Mass Spectrometry in Life Sciences** Contact: Prof. A. De Leenheer, Symposium Chairman, Laboratoria voor Medische Biochemie en voor Klinische Analyse, De Pintelaan 135, B-9000 Ghent, Belgium.
June 6–12, 1982 Frankfurt, G.F.R.	**European Meeting on Chemical Engineering and ACHEMA Exhibition Congress 1982** Contact: DECHEMA P.O. Box 970146, D-6000 Frankfurt/M 97, G.F.R.
June 7–11, 1982 Philadelphia, PA, U.S.A.	**VI International Symposium on Column Liquid Chromatography** Contact: R.A. Barford, ERRC – SEA, U.S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118, U.S.A. (Further details published in Vol. 211, No. 3)
June 28–30, 1982 East Lansing, MI, U.S.A.	**35th American Chemical Society Annual Summer Symposium** Contact: A.I. Popov, Chemistry Department, Michigan State University, East Lansing, MI 48824, U.S.A.
July 11–16, 1982 Washington, DC, U.S.A.	**6th International Conference on Computers in Chemical Research and Education (ICCCRE)** Contact: Dr. Stephen R. Heller, Chairman, 6th ICCCRE, EPA, MIDSD, PM–218, 401 M Street, S.W., Washington, DC 20460, U.S.A. Tel: (202) 755–4938, Telex: 89–27–58.
July 11–16, 1982 Louvain-la-Neuve, Belgium	**6th IUPAC Conference on Physical Organic Chemistry** Contact: Prof. A, Bruylants, Université Catholique de Louvain, Laboratoire de Chimie Generale et Organique, Batiment Lavoisier 1 Place Louis Pasteur, 1348 Louvain-la-Neuve, Belgium.

July 12–16, 1982 Amherst, MA, U.S.A.	**IUPAC Macromolecular Symposium** Contact: James C.W. Chien, Department of Polymer Science & Engineering, University of Massachusetts, Amherst, MA 01003, U.S.A.
July 19–22, 1982 Prague, Czechoslovakia	**Prague Microsymposium "Selective Sorbents"** Contact: Dr. F. Svec, c/o Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Heyrovského n.2, 162 06 Prague, Czechoslovakia.
Aug. 11–13, 1982 Hameenlinna, Finland	**6th European Symposium on Polymer Spectroscopy (ESOPS 6)** Contact: Professor Johan Lindberg, Department of Wood and Polymer Chemistry, University of Helsinki, Meritullinkatu 1 A, SF 00170 Helsinki 17, Finland.
Aug. 15–21, 1982 Perth, Australia	**The 12th International Congress of Biochemistry** Contact: Brian Thorpe, Department of Biochemistry, Faculty of Science Australian National University, Canberra A.C.T. 2600, Australia.
Aug. 23–27, 1982 Budapest, Hungary	**22nd International Conference on Coordination Chemistry** Contact: Prof. M.T. Beck, Institute of Physical Chemistry, Kossuth Lajos University, Debrecen 10, H–4010, Hungary.
Aug. 29–Sept. 4, 1982 Kyoto, Japan	**5th International Congress of Pesticide Chemistry** Contact: Rikagaku Kenysho (The Institute of Physical and Chemical Research), 2–1 Hirosawa Wako-shi Saitama Pref. 351, Japan.
Aug. 30–Sep. 3, 1982 Vienna, Austria	**9th International Mass Spectrometry Conference** Contact: Interconvention, P.O. Box 105, A–1014 Vienna, Austria. (Further details published in Vol. 206, No. 1)
Aug. 31–Sept. 2, 1982 Vienna, Austria	**5th International IUPAC Symposium on Mycotoxins and Phycotoxins** Contact: Prof. P. Krogh, Department of Veterinary Microbiology, Purdue University, West Lafayette, IN 47907, U.S.A.
Sept. 12–17, 1982 Kansas City, MO, U.S.A.	**National American Chemical Society Meeting** Contact: A.T. Winstead, American Chemical Society, 1155 Sixteenth Street, NW, Washington DC 20036, U.S.A.
Sept. 13–17, 1982 London, Great Britain	**14th International Symposium on Chromatography** Contact: The Executive Secretary, Chromatography Discussion Group, Trent Polytechnic, Burton Street, Nottingham, NG1 4BU, Great Britain. (Further details published in Vol. 211, No. 3)
Sept. 19–24, 1982 Philadelphia, PA, U.S.A.	**9th National Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies (FACSS)** Contact: Division of Analytical Chemistry, American Chemical Society, Department of Chemistry, Notre Dame, IN 46556, U.S.A.
July 17–23, 1983 Edinburgh, Great Britain	**SAC 83: Sixth International Conference on Analytical Chemistry** Contact: Miss P.E. Hutchinson, Royal Chemistry Society, Analytical Division, Burlington House, London W1V 0BN, Great Britain. Tel. 01–734 9971.
Aug. 28–Sept. 2, 1983 Amsterdam, The Netherlands	**9th International Symposium on Microchemical Techniques** Contact: Symposium Secretariat, c/o Municipal Congress Bureau, Oudezijds Achterburgwal 199, 1012 DK Amsterdam, The Netherlands. Tel: (020) 552 3459.

PUBLICATION SCHEDULE FOR 1981

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

MONTH	N 1980	D 1980	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography			203 204 205/1 205/2	206/1 206/2 206/3	207/1 207/2 207/3	208/1 208/2 209/1	209/2 209/3 210/1	210/2 210/3 211/1	211/2 211/3 212/1 212/2	212/3 213/1 213/2	The publication schedule for further issues will be published later.			
Chromatographic Reviews							220/1							
Biomedical Applications	221/1	221/2	222/1	222/2	222/3	223/1	223/2	224/1	224/2	224/3				

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. A free reprint can be obtained by application to the publisher)

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

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

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.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

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. 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 author's 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. Please check a recent issue for the lay-out of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

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.

More and more primary literature? Less and less time to read it?

take TrAC

trends in analytical chemistry

A monthly publication of short, critical reviews and new on trends and developments in analytical chemistr

How much better informed you could be if only you had the time to keep up with the latest developments.

Time we cannot give you, but we can give you concise, critical information on what is going on in the analytical sciences. Every month, as it happens.

It's all in TrAC – Trends in Analytical Chemistry – new for the 1980's from Elsevier and yours now at a low introductory rate.

Introductory Offer

SIXTEEN ISSUES FOR THE PRICE OF TWELVE!

Volume 1 – 1981/82 – of **Trends in Analytical Chemistry** will have sixteen issues: March 1981 and monthly from October 1981 to December 1982. Order the **Personal Edition** before December 1981 and receive all sixteen issues for US $42.50 (USA and Canada), £20.00 (UK), 91.50 Dutch guilders (Europe), 95.50 Dutch guilders (elsewhere). Or order the **Library Edition** for US $133.25 or 260.00 Dutch guilders throughout the world.

All issues of both editions are sent by air worldwide.

**) The Dutch guilder price is definitive.*

Take just a minute to order either edition now – you will enjoy the time it saves you later.

ELSEVIER

TrAC is your opportunity to learn from researchers in related fields, to g first-hand, detailed reports on important developments in methodolo and instrumentation. **TrAC** brings you current information on trends a techniques from laboratories all over the world.

Lab managers will find in **TrAC** evaluations of new methods and tec niques which will enable them to make better-informed purchasi decisions. As a training aid **TrAC** is more up-to-date than any textbo

TrAC is written in clear, jargon-free language, avoiding highly specializ terminology and provides you with a working knowledge of relat methodology and techniques.

In every issue you will find:

- short critical reviews written for an interdisciplinary audience
- feature articles
- insights into the function, organization and operation of industria government or research laboratories
- news on topics of general interest
- teaching aids – TrAC is more up-to-date than any textbook
- articles on the history of analytical chemistry
- reports on meetings and book reviews

Trends in Analytical Chemistry comes in either the monthly **Perso Edition** or the special **Library Edition** which includes the monthly issu plus a hardbound volume containing all the review articles published o the year and indexed for easy retrieval.

Order Form

Special Introductory Offer for the Personal Edition valid until December 31, 1981

To **ELSEVIER Dept. TrAC. AP**
P.O. Box 330, 1000 AH Amsterdam, The Netherlands
52 Vanderbilt Avenue, New York, NY 10017

US residents may call (212) 867-9040 and charge their American Express, Master Charge Visa/Bankamericard account.

Yes! Please enter my subscription now – Volume 1 – 1981/82
☐ Personal Edition ☐ Library Edition
I enclose my ☐ personal cheque ☐ bankcheque

Orders from individual subscribers must be prepaid.

☐ Please send me a free sample copy first.

Name: _____ Position: _____ Date: _____

Address: _____

City: _____ State: _____ Postal Code _____

-2 PLAN