ISSN 0021-9673

BLISHED
EEKLY

VOL. 188 NO. 2 FEBRUARY 1, 1980
THIS ISSUE COMPLETES VOL. 188

JOURNAL OF CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

PUBLICATION SCHEDULE FOR 1980

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

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

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

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

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

Publication. The *Journal of Chromatography* (including *Biomedical Applications* and *Chromatographic Reviews*) has 22 volumes in 1980. The subscription price for 1980 (Vols. 181–202) is Dfl. 2838.00 plus Dfl. 352.00 (postage) (total *ca.* US$ 1556.00). The subscription price for the *Biomedical Applications* section only (Vols. 181–183) is Dfl. 399.00 plus Dfl. 48.00 (postage) (total *ca.* US$ 218.00). Journals are automatically sent by air mail to the U.S.A. and Canada at no extra costs, and to Japan, Australia and New Zealand with a small additional postal charge. Back volumes of the *Journal of Chromatography* (Vols. 1 through 180) are available at Dfl. 140.00 (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, N.Y. 10017. Tel: (212) 867-9040.

For further information, see page 3 of cover.

Printed in The Netherlands

CONTENTS

(Continued overleaf)

Contents (continued)

Book Reviews

75 Years of Chromatography

A Historical Dialogue

L. S. ETTRE *and* A. ZLATKIS (Editors).

Journal of Chromatography Library - Volume 17

On the occasion of the 75th anniversary of the invention of chromatography, this book compiles the personal stories of 59 pioneers of the various chromatographic techniques (including five Nobel Prize laureates). In their contributions to this volume, these pioneers review the events which influenced them to enter the field; explain the background of their inventions; summarize their activities and results during their professional lives; and discuss their interactions with other scientists and other disciplines.

This book is more than a nostalgic recollection of the past for those who have been in chromatography for some time. It also provides, for the younger generation of chromatographers, a unique record of how present-day knowledge was accumulated.
The final chapter is devoted to "Those who are no longer with us".

Contributors:

E. R. Adlard
H. Boer
E. Cremer
D. H. Desty
G. Dijkstra
L. S. Ettre
P. Flodin
C. W. Gehrke
J. C. Giddings
E. Glueckauf
M. J. E. Golay
D. W. Grant
E. Heftmann
G. Hesse
G. H. Higgins
E. C. Horning
M. G. Horning
C. Horváth
J. F. K. Huber
A. T. James
J. Janák
R. E. Kaiser
A. Karmen
J. G. Kirchner
J. J. Kirkland
A. V. Kiselev
E. sz. Kováts
E. Lederer
M. Lederer
A. Liberti
S. R. Lipsky
J. E. Lovelock
* A. J. P. Martin ④
* S. Moore ③
H. W. Patton
C. S. G. Phillips
J. Porath
V. Pretorius
G. R. Primavesi
N. H. Ray
L. Rohrschneider
K. I. Sakodynskii
G. Schomburg
G.-M. Schwab
R. D. Schwartz
C. D. Scott
R. P. W. Scott
* G. T. Seaborg ②
M. S. Shraiber
L. R. Snyder
E. Stahl
* W. H. Stein ⑤
H. H. Strain
F. H. Stross
* R. L. M. Synge ①
R. Teranishi
J. J. van Deemter
A. A. Zhukhovitskii
A. Zlatkis

(*Nobel Prize laureates)

Feb. 1979 *530 pages* *US $49.75/Dfl. 112.00* *ISBN 0-444-41754-0*

ELSEVIER

P.O. Box 211,
1000 AE Amsterdam
The Netherlands

52 Vanderbilt Ave
New York, N.Y. 10017

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

Journal of Chromatography, 188 (1980) 297–303

CHROM. 12,405

CALCULATION OF GAS CHROMATOGRAPHIC RETENTION INDICES OF ISOALKANES BASED ON A TOPOLOGICAL APPROACH

D. PAPAZOVA
Economic Combine "Neftochim", Institute of Petroleum, Burgas 8104 (Bulgaria)
N. DIMOV
Analytical Department, Chemical Pharmaceutical Research Institute, Sofia 1156 (Bulgaria)
and
D. BONCHEV
Higher School of Chemical Technology, Burgas 8010 (Bulgaria)
(First received June 6th, 1979; revised manuscript received September 10th, 1979)

SUMMARY

The combination of topological and structural molecular characteristics seems to be a promising tool for the prediction of retention indices. The Wiener number and the total and mean information on both distance equality and distance magnitude were combined with seven structural elements, and a linear equation with a regression coefficient 0.9986 was deduced. The mean deviation of retention indices calculated for 118 isoalkanes on squalane from their experimental values was less than 8 retention index units.

INTRODUCTION

The precise calculation of the gas chromatographic retention indices of hydrocarbons on a rigorous thermodynamic basis[1,2] is not yet possible because of the lack of a detailed non-aqueous solution theory. Additivity approaches and other mathematical methods[3–6] are insufficiently accurate. The handling of the retention index as a result of two contributions, the physico-chemical index (*PCI*) and a structural number (*StN*)[7–9], allows the calculation of a theoretical retention index (I^{theor}) for hydrocarbons on different stationary phases with very good accuracy. The calculation of *PCI*, however, requires the vapour pressure of the hydrocarbon at the analysis temperature. Such data, especially for higher hydrocarbons, are not always available Therefore, a calculation based on the chemical structure and conformation of compounds seems to be more useful.

A remarkably good correlation has been obtained between a number of physico-chemical properties of an organic compound and its topological invariants[10]. The Wiener number[11,12], the Randić molecular connectivity[13–15], etc., made use of molecular topology for retention index prediction with a maximum error of 15 retention

index units (i.u.). The Wiener method of factor analysis is a promising approach, providing an insight into solute–solvent interactions[16,17]. Related to this, this paper describes a study of the importance of different topological invariants in retention index calculations. We assumed that the equation[18]

$$I_t^{\text{theor}} = PCI_t + StN \tag{1}$$

could be transformed into

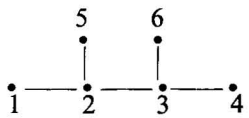
$$I_t^{\text{theor}} = a + bx + \sum_{i=1}^{n} c_t y_i \tag{2}$$

where a, b and c_i are constants, x is a suitable topological increment, y_i are suitable structural increments and t is the temperature of analysis.

TOPOLOGICAL INVARIANTS CALCULATION

The following topological invariants were chosen for examination in this study: Wiener number (W), total (I_D^E) and mean ($\bar{I}_D^E$) information on distance equality or diversity, and total (I_D^W) and mean ($\bar{I}_D^W$) information on distance magnitude. A brief outline of these quantities is sufficient here; more details can be found, for example, in refs. 11, 12 and 19–24.

A molecule of any hydrocarbon could be represented, according to the graph theory, by its molecular graph, in which carbon atoms are depicted by points and chemical bonds by edges. For example, the molecular graph of 2,3-dimethylbutane is



The corresponding topological distance matrix, which uniquely describes the molecule, is symmetrical:

$$D = \begin{array}{c|cccccc} i \backslash j & 1 & 2 & 3 & 4 & 5 & 6 \\ \hline 1 & 0 & 1 & 2 & 3 & 2 & 3 \\ 2 & 1 & 0 & 1 & 2 & 1 & 2 \\ 3 & 2 & 1 & 0 & 1 & 2 & 1 \\ 4 & 3 & 2 & 1 & 0 & 3 & 2 \\ 5 & 2 & 1 & 2 & 3 & 0 & 3 \\ 6 & 3 & 2 & 1 & 2 & 3 & 0 \end{array}$$

Every entry $d_{i,j}$ is defined by the number of the edges between i and j atoms. The total number, N, of $d_{i,j}$ is obviously

$$N = \frac{n(n-1)}{2} \tag{3}$$

where n is the number of carbon atoms in the molecule. The sum, W, of all the elements of the triangular submatrix,

$$W = \frac{1}{2} \sum_{i,j=1}^{n} d_{i,j} \tag{4}$$

is the so-called path number or Wiener number[11], which is a measure of the compactness of the molecule.

The matrix elements could be classed in k subsets according to their magnitude. In the above example there are three such subsets, those of distances equal to 1, 2 and 3, respectively.

The distance $d = 1$ appears 5 times in the triangular submatrix, while distances $d = 2$ and $d = 3$ appear 6 and 4 times, respectively. Denoting in general these appearance numbers by n_i, one can express in a concise form the essence of the distance matrix, writing the sequence $1^{n}{}_{1}2^{n}{}_{2}3^{n}{}_{3} \ldots$ (or, in our example, $1^{5}2^{6}3^{4}$), which coincides with the Randić molecular code. Now, the Wiener number is easily calculated by the equation

$$W = (5 \times 1) + (6 \times 2) + (4 \times 3) = 29$$

Additional indices characterizing the distance matrix on the basis of information theory[24] are given in refs. 22 and 23.

The distance matrix elements are regarded as the elements of every structure, distributed according to a certain criterion into a number of classes. A certain probability is ascribed to each of the elements belonging to a certain class. Thus, a probability distribution is constructed to which an information measure (information content) is specified. Two criteria are used for classing the distance matrix elements: their equality and their magnitude. Correspondingly, two different information measures are defined on the topological distance matrix. The total information, $I_{\mathrm{D}}^{\mathrm{E}}$, and mean information, $\bar{I}_{\mathrm{D}}^{\mathrm{E}}$, on distance equality or diversity are given in bits, according to the equations

$$I_{\mathrm{D}}^{\mathrm{E}} = \frac{n(n-1)}{2} \cdot \log_2\left[\frac{n(n-1)}{2}\right] - \sum_{i=1}^{k} n_i \log_2 n_i \tag{5}$$

and

$$\bar{I}_{\mathrm{D}}^{\mathrm{E}} = 2I_{\mathrm{D}}^{\mathrm{E}}/n(n-1) \tag{6}$$

The total and mean information on distance magnitude, $I_{\mathrm{D}}^{\mathrm{W}}$ and $\bar{I}_{\mathrm{D}}^{\mathrm{W}}$, respectively, are given in bits according to the equations

$$I_{\mathrm{D}}^{\mathrm{W}} = W \log_2 W - \sum_{i=1}^{k} n_i d_i \log_2 d_i \tag{7}$$

and

$$\bar{I}_{\mathrm{D}}^{\mathrm{W}} = I_{\mathrm{D}}^{\mathrm{W}}/W \tag{8}$$

where d_i represents the distinct topological distances in a molecular graph and n_i their appearance number. In our example, $n_1 = 5$, $n_2 = 6$, $n_3 = 4$ and $d_1 = 1$, $d_2 = 2$, $d_3 = 3$.

RESULTS AND DISCUSSION

Experimental retention indices of 56 isoalkanes separated on squalane were taken from ref. 25. The validity of the linear equation

$$I^{calc} = a + bI_{inv} \tag{9}$$

where I_{inv} is any kind of topological invariant in use and a and b are constants, was examined. Independently of the type of I_{inv}, the differences between the calculated (I^{calc}) and the experimental retention indices (I^{exp}) vary from 1 to 59 i.u. Such discrepancies are not acceptable and I^{calc} cannot be a useful aid in gas chromatography. A combination of one of the topological indices and three of the most important structural elements[26] (n_R, n_B and n_0) was then studied in order to find which of the topological invariants could best replace *PCI*, where n_R is the number of substituents in the isoalkane, n_B is the number of butane chains according to Altenburg[27], for example for *n*-butane $n_B = 1$, for isobutane $n_B = 0$ and for 2,2-dimethylbutane $n_B = 3$, and n_0 is the total number of carbon atoms in the isoalkane.

The resulting regression equations and the corresponding correlation coefficients, R, are as follows:

$$I_1^{calc} = 254.96 + 0.96W - 17.43n_R + 13.18n_B + 46.07n_0 \tag{10}$$

with $R = 0.9862$;

$$I_2^{calc} = 241.48 + 1.10I_D^E - 16.95n_R + 13.28n_B + 47.78n_0 \tag{11}$$

with $R = 0.9862$;

$$I_3^{calc} = 196.79 + 0.09I_D^W - 20.19n_R + 11.92n_B + 59.68n_0 \tag{12}$$

with $R = 0.9856$;

$$I_4^{calc} = 67.99 + 58.70\bar{I}_D^E - 15.03n_R + 13.98n_B + 61.59n_0 \tag{13}$$

with $R = 0.9868$;

$$I_5^{calc} = 87.13 + 5.35\bar{I}_D^W - 23.00n_R + 10.73n_B + 76.22n_0 \tag{14}$$

with $R = 0.9852$.

Again, none of the topological indices under study dominates the others. It seems, however, that $\bar{I}_D^E$ is the most suitable index to be inserted in eqn. 1 instead of *PCI*. This further allows us to keep constant $\bar{I}_D^E$ as x in eqn. 2 and to choose

suitable structural elements. The simplest equation[28] found for the calculation of retention indices of isoalkanes is

$$I_t^{\text{theor}} = PCI_t + c_0 + c_1x_1 + c_2x_2 \tag{15}$$

with

$$x_1 = n_R + n_i - n_d \tag{16}$$

and

$$x_2 = n_{CH_3}n_B/n_L(n_0 - n_q) \tag{17}$$

where c_0, c_1 and c_2 are constants; n_i is the number of carbon atoms in the substituents, counting from, and including the third atom from either end of the straight chain of the isoalkane; for example, for 3-methylpentane, 2,3-dimethylpentane and 4-methylheptane $n_i = 1$, and for 3-ethylhexane and 2-methyl-3-ethylhexane $n_i = 2$; n_d is the number of carbon atoms between substituents R in the alkane, when $R \geqslant 2$; for example, for 2,3-dimethylpentane $n_d = 0$ and for 2,4-dimethylpentane $n_d = 1$; n_{CH_3} is the number of methyl groups in the isoalkane; n_L is the number of carbon atoms in the straight chain of the isoalkane; and n_q is the number of quaternary carbon atoms in the isoalkane.

Replacing *PCI* in eqn. 15 by $\bar{I}_D^E$, the new values of c_i are calculated from a 56-membered matrix. A considerably better coincidence was found between the experimental and re-calculated retention indices. It is, however, still unsatisfactory. We therefore examined a polynominal equation that includes all significant structural elements by a step regression analysis. The starting model was

$$I = F(\bar{I}_D^E, n_0, n_R, n_L, n_d, n_{CH_3}, n_i, n_q) \tag{18}$$

The best linear model was found to be

$$I = 128.8 - 12.9\bar{I}_D^E - 21.6n_R + 21.4n_B + 57.8n_0 - 12.5n_i + 16.8n_L \tag{19}$$

with $R = 0.9986$.

The mean deviation of I^{calc} from I^{exp} for all C_6–C_8 isoalkanes is now less then 4 i.u. (Table I). The accuracy achieved is still not completely satisfactory, but the replacement of *PCI* by $\bar{I}_D^E$ is evidently possible. To support this concept, a calculation of the retention indices for 118 C_6–C_{10} isoalkanes was made. The results obtained corroborate both the possibility of using $\bar{I}_D^E$ instead of *PCI* and the significance of the structural elements chosen. The mean difference between experimental and calculated values of the retention index is now 8 i.u., but the result is encouraging. We assume also that a further simplification of the model, for example by the use of suitable combinations of structural elements, is possible. A simple equation for retention index calculations, based solely on topological and structural characteristics of solutes, would be very useful for laboratory purposes. At this stage in our investigation, some helpful conclusions can be made:

TABLE I

DIFFERENCES (Δ) BETWEEN CALCULATED (ACCORDING TO EQN. 19) AND EXPERIMENTAL RETENTION INDICES OF C_6–C_8 ISOALKANES[29]

Hydrocarbon	I_{50}^{SQ}	I^{calc}	Δ
2-Methylpentane	569.7	574.6	+4.9
3-Methylpentane	584.2	587.2	+3.0
2,3-Dimethylbutane	567.2	564.8	−2.4
2,2-Dimethylbutane	536.8	540.1	+3.3
2-Methylhexane	666.6	659.8	−6.8
3-Methylhexane	676.2	675.2	−1.0
2,4-Dimethylpentane	629.8	629.9	+0.1
3-Ethylpentane	686.0	683.1	−2.9
2,2-Dimethylpentane	625.6	628.0	+2.4
2,3-Dimethylpentane	671.7	665.1	−6.6
3,3-Dimethylpentane	658.9	652.6	−6.3
2-Methylheptane	764.9	760.3	−4.6
3-Methylheptane	772.3	771.7	−0.6
4-Methylheptane	767.2	770.1	+2.9
2,5-Dimethylhexane	728.4	726.5	−1.9
2,2-Dimethylhexane	719.4	724.2	+4.8
2,4-Dimethylhexane	731.9	730.6	−1.3
2,3-Dimethylhexane	760.1	760.8	+0.7
3,4-Dimethylhexane	770.6	770.8	+0.8
3,3-Dimethylhexane	743.6	747.9	+4.3
2-Methyl-3-ethylpentane	761.4	764.9	+3.5
2,2,4-Trimethylpentane	689.9	687.2	−2.7
2,3,4-Trimethylpentane	762.4	747.8	−4.6
3-Methyl-3-ethylpentane	774.0	775.8	+1.8
2,2,3-Trimethylpentane	737.1	745.3	+8.2
2,3,3-Trimethylpentane	759.4	756.9	−2.5

(1) the calculation of the retention indices of isoalkanes without vapour pressure data is possible;

(2) the topological index $\bar{I}_D^E$ expresses to an acceptable degree those physicochemical properties of isoalkanes that are included in the term *PCI* (eqn. 1);

(3) the chosen structural elements proved their significance when the initial set of 56 isoalkanes was extended to 118;

(4) though $\bar{I}_D^E$ is calculated on the basis of the molecular graph, some structural characteristics of the molecules are evidently not involved with the necessary influence. Otherwise, the topological indices of molecules are likely to be insufficient for the satisfactory description of solute properties. Therefore, combination with the structural elements mentioned above is necessary. In this respect, the extended set of topological and structural characteristics might be regarded as a new approach aimed at predicting molecular solute properties which will be of interest to theoretical chemists.

ACKNOWLEDGEMENTS

This work was supported by Economic Combine „Neftochim", Burgas. Assistance with the calculations by Ms. S. Dekalo of the Petrochemical Computer Centre is gratefully acknowledged.

REFERENCES

1 C. E. Figgins, T. H. Risby and P. C. Jurs, *J. Chromatogr. Sci.*, 14 (1976) 453.
2 A. N. Korol, *J. Chromatogr.*, 129 (1976) 125.
3 B. G. Berezkin, *Neftekhimiya*, 1 (1961) 169.
4 J. Takács, C. Szita and G. Tarján, *J. Chromatogr.*, 56 (1971) 1.
5 M. Randić, *J. Chromatogr.*, 161 (1978) 1.
6 G. Spivakovskii, A. I. Tishchenko, I. I. Zaslavskii and N. S. Wulfson, *J. Chromatogr.*, 144 (1976) 1.
7 N. Dimov and D. Papazova, *J. Chromatogr.*, 137 (1977) 265.
8 D. Papazova and N. Dimov, *J. Chromatogr.*, 137 (1977) 259.
9 N. Dimov and D. Papazova, *J. Chromatogr.*, 148 (1978) 11.
10 J. Chrétien and J. E. Dubois, *J. Chromatogr.*, 158 (1978) 43.
11 H. Wiener, *J. Amer. Chem. Soc.*, 69 (1947) 17; 69 (1947) 2536; *J. Phys. Chem.*, 62 (1948) 425.
12 J. R. Platt, *J. Chem. Phys.*, 15 (1947) 419; 56 (1962) 328.
13 M. Randić, *J. Amer. Chem. Soc.*, 97 (1975) 6609.
14 L. B. Kier and L. H. Hall, *Molecular Connectivity in Chemistry and Drug Research*, Academic Press, New York, 1976.
15 J. Chretien and J. Dubois, *Anal. Chem.*, 49 (1977) 747.
16 P. H. Wiener and D. Howery, *Can. J. Chem.*, 50 (1972) 448.
17 R. B. Selzer and D. G. Howery, *J. Chromatogr.*, 115 (1975) 139.
18 N. Dimov, *J. Chromatogr.*, 119 (1976) 109.
19 F. Harary ,*Graph Theory*, Addison-Wesley, Reading, Mass., 2nd ed., 1971.
20 A. T. (Editor), *Chemical Applications of Graph Theory*, Academic Press, New York, 1976.
21 D. H. Rouvray, *Inf. Commun. Match Math. Chem.*, 1 (1975) 125.
22 D. Bonchev and N. Trinajstić, *J. Chem. Phys.*, 67 (1977) 4517.
23 D. Bonchev and N. Trinajstić, *Int. J. Quantum Chem., Symp.*, 12 (1978) 293.
24 L. Brillouin, *Science and Information Theory*, Academic Press, New York, 1956.
25 A. Matukuma, in C. L. A. Harbourn and R. Stock (Editors), *Gas Chromatography, 1968*, Institute of Petroleum, London, 1969, p. 55.
26 N. Dimov and D. Papazova, *Chromatographia*, 12 (1979) 720.
27 K. Altenburg, in H. G. Struppe (Editor), *Gas Chromatographie 1968*, Akademie Verlag, Berlin, 1968, p. 1.
28 N. Dimov and D. Papazova, *Chromatographia*, 12 (1979) 443.
29 J. Rijks and C. Cramers, *Chromatographia*, 7 (1974) 99.

Journal of Chromatography, 188 (1980) 305–313

CHROM. 12,426

USE OF CHLORPYRIFOS AS AN EVALUATION STANDARD FOR GAS CHROMATOGRAPHIC DETECTORS

R. GREENHALGH

Chemistry and Biology Research Institute, Agriculture Canada, Ottawa, Ontario K1A OC6 (Canada)

and

W. P. COCHRANE

Laboratory Services Division, Food Production and Marketing Branch, Agriculture Canada, Ottawa, Ontario K1A OC5 (Canada)

(First received June 21st, 1979; revised manuscript received October 2nd, 1979)

SUMMARY

The performance of a range of element-selective detectors was studied using chlorpyrifos (O,O-diethyl-O-3,5,6-trichloro-2-pyridyl phosphorothioate) as a standard. It involved determination of the minimum detectable amount and linearity of the response of the flame photometric, alkali flame ionization, Hall electrolytic conductivity and electron capture detectors.

The minimum detectable amount varied with both the model and type of detector, with the electron capture detector (frequency-modulated mode) being the most sensitive and the flame photometric detector (S-mode) the least. A study of various models of electron capture detector demonstrated the superior performance of the frequency-modulated type to the d.c. or d.c. pulse mode in both sensitivity and linearity. The responses of various types of alkali flame ionization detector were similar and *ca.* 10 times more sensitive than the flame photometric detector (P-mode). The N/P response of the alkali flame ionization detector was dependent on the hydrogen flow-rate. The multi-element response of the detector is suppressed by using a flame energized source. The response of the flame photometric detector to chlorpyrifos analogues showed a variation in the minimum detectable amount of 2.1–4.5 for the P=O/P=S ratio, which indicated it to be a detector effect.

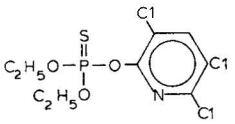
The results obtained warrant the consideration of chlorpyrifos as a common evaluation standard for the sensitivity and linearity data of gas chromatographic detectors used in pesticide residue analysis.

INTRODUCTION

Information regarding the performance of gas chromatographic (GC) detectors is of interest to the analyst concerned with the choice of equipment, the suitability of an analytical procedure or the amount of sample clean-up necessary. Detector performance is usually described in terms of sensitivity, selectivity, limit of detection

and linearity of its response. Although the literature abounds with such data[1–6], it is often difficult to correlate for comparative purposes because of the multiplicity of reference standards used. This problem applies especially to the evaluation of element selective detectors, such as the flame photometric (FPD), alkali flame ionization (AFID) and Coulson (CECD) or Hall electrolytic conductivity (HECD).

The desirability of reporting detector performance in some standard manner was emphasized as early as 1957[7]. Although various proposals have been advanced in the intervening years[1,8–10], no standard protocol has yet been adopted. As more interlaboratory comparative studies with detectors are carried out, so the need for a common performance standard increases. The use of chlorpyrifos (O,O-diethyl-O-3,5,6-trichloro-2-pyridyl phosphorothioate) has been previously suggested as a standard reference compound for the comparison of sensitivity and linearity data[11]. It has the advantage that it contains the heteroatoms Cl, N, P and S which enable it to be used with all the various element-selective detectors used in residue analysis today.



In addition, it is a stable compound, has good GC characteristics and can be detected by the detected by the electron capture detector (ECD). This paper reports further on its application for evaluating the sensitivity and linearity of various types and models of GC detectors. Comments are made on the response of chlorpyrifos and its analogues with a multi-element selective AFID detector.

EXPERIMENTAL

The work was carried out using various Pye model 104 gas chromatographs fitted with Pye 3-electrode AFID (RbCl), FID, d.c. pulse mode and frequency-modulated ECD. A Bendix SPED (FPD), and a Perkin-Elmer NPD (AFID) were also adapted for use with this gas chromatograph. In addition a Hewlett-Packard Model 5713 equipped with frequency-modulated ECD and NPD (AFID), and a Microtek MT220 fitted with a HECD were used in the study.

All the glass columns used had similar dimensions, 2.3 m × 4 mm I.D., packed with 100–120 mesh Gas-Chrom Q coated with 3% OV-17. The columns were extensively conditioned by heat[12] and repeated injection of the compounds under study, before the response factors were determined. The latter was especially important for the oxons. All responses reported are the average of five replicate injections.

Analytical standards of chlorpyrifos and its oxon were obtained from Dow Chemical, Midland, Mich., U.S.A. The analogues, O,O-diethyl-O-3,5,6-trichlorophenyl phosphorothioate, O,O-diethyl-O-2-pyridyl phosphorothioate; O,O-diethyl-O-phenyl phosphorothioate and the oxons were prepared by standard methods involving reaction of the sodium salt of the corresponding phenol with O,O-diethyl phosphoro- and phosphorothio-chloridate. The products were characterized by NMR and GC following purification by column chromatography and vacuum distillation. All the compounds had a purity of 98%.

RESULTS AND DISCUSSION

The sensitivity of a GC detector is directly related to the efficiency with which a sample is converted into the species detected. For ionization detectors it is measured as the response in coulombs/gram. Alternatively, detector performance may be expressed as the minimum detectable amount (MDA). The latter has been arbitrarily accepted as the amount of material injected which gives a response equivalent to twice the noise level and is usually expressed in units of g/sec or g atom/sec[1].

In practice, the MDA is preferred to sensitivity since it is based on peak height and takes into account the noise level of the detector. The difference between the two can be illustrated by comparison of the response of the Pye AFID and the Perkin-Elmer NPD (P-mode), where the sensitivities were found to be 105 and 10 C/gP and the noise levels were 1 and 8×10^{-2} pA, respectively. This results in both detectors having a similar MDA for chlorpyrifos although the sensitivities differ by a factor of 10.

The MDA values for chlorpyrifos determined with various types of selective element detectors are given in Table I. The values differ according to the type of detector with the ECD being the most sensitive and the FPD (S-mode) the least. However, the data indicates that chlorpyrifos is sufficiently sensitive to all types of detectors to warrant its use as a common standard. Comparing the three FPDs, these MDAs are very similar in the P-mode (526 nm) but vary in the S-mode (394 nm). The poor sensitivity of the Bendix SPED (S-mode) in this case was attributed to the fibre optics, which showed reduced light transmission with extended use at high temperature (230°). The AFIDs examined included the Pye AFID, which has a flame-heated RbCl source, and the Hewlett-Packard and Perkin-Elmer NPDs, which operate with an electrically heated source. The responses of the various types of AFIDs to chlorpyrifos are similar and approximately 10 times more sensitive than for the FPD (P-mode). It is of interest to note that the HECD in the halogen mode is as sensitive to chloryrifos (3 Cl) as the FPD in the P-mode.

Chlorpyrifos was employed as the reference standard for an interlaboratory study to determine the sensitivity of ECDs used in routine residue analysis[13]. Each

TABLE I

THE MINIMUM DETECTABLE AMOUNT OF CHLORPYRIFOS DETERMINED ON VARIOUS TYPES OF GC DETECTOR*

Detector	*MDA (pg/sec)*	*Detector*	*MDA (pg/sec)*
AFID		FPD	
Pye	0.12	Trator (P)	2.4
Perkin-Elmer (NP)	0.13	(S)	4.0
(P)	0.15	Bendix (P)	2.1
Hewlett-Packard (NP)	0.12	(S)	107
		Pye (P)	1.0
		(S)	32
HECD		ECD	
(halogen)	2.32	Pye (frequency-modulated)	0.01

* Mode of operation given in parenthesis: NP, nitrogen/phosphorus; P, phosphorus; halogen, chlorine; S, sulfur.

laboratory was asked to determine the MDA of chlorpyrifos (standard supplied) under their own conditions of operation. The results from the various types of ECD design (Table II) show surprisingly good agreement. In general, the frequency-modulated ECDs (constant current) were more sensitive than the d.c. or d.c. pulsed mode ECDs (constant frequency), especially when nitrogen was employed as the carrier gas. The only direct comparison of the two carrier gases, argon–methane and nitrogen, was made with the Analog Technology Corp. (ATC) detector, which gave MDA of 0.012 and 0.007 pg/sec, respectively. A larger pulse width is required for nitrogen (0.6 μsec) than for argon–methane owing to the slow diffusion rate of the ions. The responses of the two d.c. detectors, one with a ^{63}Ni and the other a ^{3}H source were similar, but both were less than that obtained with the frequency-modulated detectors.

TABLE II

INTERLABORATORY STUDY OF THE MDA OF CHLORPYRIFOS BY d.c.-PULSED AND FREQUENCY-MODULATED ECDs*

Frequency-modulated detectors			*d.c. or d.c.-pulsed detectors*		
Detector	*Carrier gas*	*MDA (pg/sec)*	*Detector*	*Carrier gas*	*MDA (pg/sec)*
HP 5739	argon–methane	0.015	MT 220	nitrogen	0.07
5713	argon–methane	0.05	MT 220	nitrogen	0.03
5710	argon–methane	0.07	MT 220	nitrogen	0.11
5710	argon–methane	0.02	MT 220	nitrogen	0.08
5710	argon–methane	0.04	MT 220	nitrogen	0.14
ATC 2**	argon-methane	0.012	MT 220	nitrogen	0.04
		Av. 0.034			Av. 0.078
ATC 2**	nitrogen	0.007	Varian***	nitrogen	0.01
MT 290	nitrogen	0.009	Varian	nitrogen	0.09
Fisher	nitrogen	0.003	Varian	nitrogen	0.20
MT 550	nitrogen	0.04	Varian	nitrogen	0.01
GCD	nitrogen	0.04	Varian	nitrogen	0.03
		Av. 0.019			Av. 0.068

* Except where indicated ^{63} Ni ECDs were employed.
** Scandium ^{3}H.
*** ^{3}H.

The main advantage of the frequency-modulated ECD lies in the extended linear range, which facilitates the use of this detector with automated analysis. The difference in linearity when operating d.c.-pulse and frequency-modulated modes is illustrated in Fig. 1 for the Pye 104 ECD. With nitrogen as the carrier gas, the detector is linear for a 10^4 range of concentration in the frequency-modulated mode, as opposed to 10^2–10^3 in the d.c.-pulse mode. A direct comparison of the sensitivities in the two modes is difficult, being dependent on the detector current setting in the frequency-modulated mode and pulse spacing in the d.c.-pulse mode. The increased sensitivity in the d.c.-pulse mode observed with increasing pulse space (Fig. 1) is compound dependent and is the result of greater reaction time allowed before collecting the ions[14].

The AFIDs respond to both nitrogen- and phosphorus-containing insecticides and herbicides. They are, however, more sensitive to phosphorus than nitrogen. The

response of the Perkin-Elmer AFID to chlorpyrifos and atrazine is shown in Fig. 2 when operated in both the P and NP modes. In the P-mode, the alkali source is energized by a high energy flame, as opposed to the source being electrically heated in the NP-mode. The response to phosphorus is effectively the same in the two modes, whereas the nitrogen response is suppressed *ca.* 50-fold in the P-mode, relative to the NP-mode.

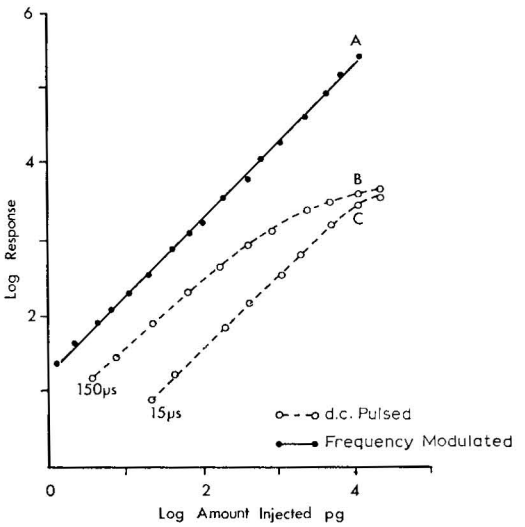


Fig. 1. Linearity of a Pye 104 ECD operating (a) in the frequency-modulated mode (b) d.c.-pulse mode, 150-μsec pulse space and (c) d.c.-pulse mode, 15-μsec pulse space, for chlorpyrifos.

The AFID response to nitrogen compounds has been reported to be proportional to the alkali ion concentration in the plasma and inversely proportional to the hydrogen flow-rate[15]. In the case of chlorpyrifos, the response increased with both hydrogen flow-rate and standing current. This effect is shown in Fig. 3 for atrazine (N) and chlorpyrifos (N,P) and diethylphenyl phosphorothioate (P) with the Perkin-Elmer AFID in the NP-mode. The result shows that the optimum flow-rate for the nitrogen compound is 2–3 ml/min, in agreement with the work of Kolb *et al.*[16] who suggested an optimum of 1–3 ml/min hydrogen flow-rate. Chlorpyrifos, although it contains both nitrogen and phosphorus, responds as a phosphorus compound, which has an optimum hydrogen flow-rate of 4–5 ml/min. These results indicate that the P/N selectivity is not constant, being dependent on the hydrogen flow-rate. Calculations based on the sensitivity of chlorpyrifos and atrazine indicate that it is three times greater at 5 ml/min than 1 ml/min hydrogen flow-rate. This detector in the NP-mode has a linear range of 10^5 for both chlorpyrifos and atrazine, whereas in the P-mode for chlorpyrifos it is reduced to 10^4 (Fig. 4). The linear range was similar for the Pye and Hewlett-Packard AFIDs, using chlorpyrifos as the standard.

Although the HECD is principally used as a selective nitrogen detector in residue analysis, it can also function as a halogen selective detector. This is achieved by operating in the reducing mode using hydrogen reaction or carrier gas and no catalyst. The linear range for chlorpyrifos (3 Cl) is compared with that of lindane

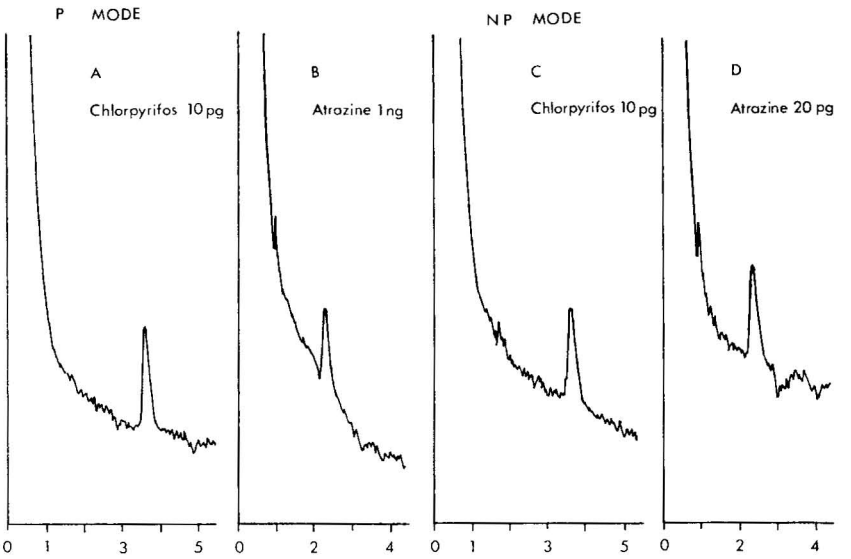


Fig. 2. Chromatograms of chlorpyrifos and atrazine obtained with a Perkin-Elmer NPD (AFID) operating in the P-mode (a) chlorpyrifos 10 pg, (b) atrazine 1 ng and in the NP mode, (c) chlorpyrifos 10 pg and (d) atrazine 20 pg. Conditions: P-mode, carrier 30 ml/min nitrogen; detector 34 ml/min hydrogen, 25 ml/min air; NP-mode, carrier 30 ml/min helium; detector, 3 ml/min hydrogen, 100 ml/min air, standing current 3 pA.

(6 Cl) in Fig. 5. As expected, the response to lindane is greater than for chlorpyrifos but the linear range of 10^5 is the same and similar to that reported by Hall[17]. Recently, a screening procedure for carbamates and urea herbicides using the heptafluoropropyl derivatives has been reported using this mode[18]. In the reductive mode with hydrogen reaction gas and nickel catalyst, the detector can be made selective to nitrogen

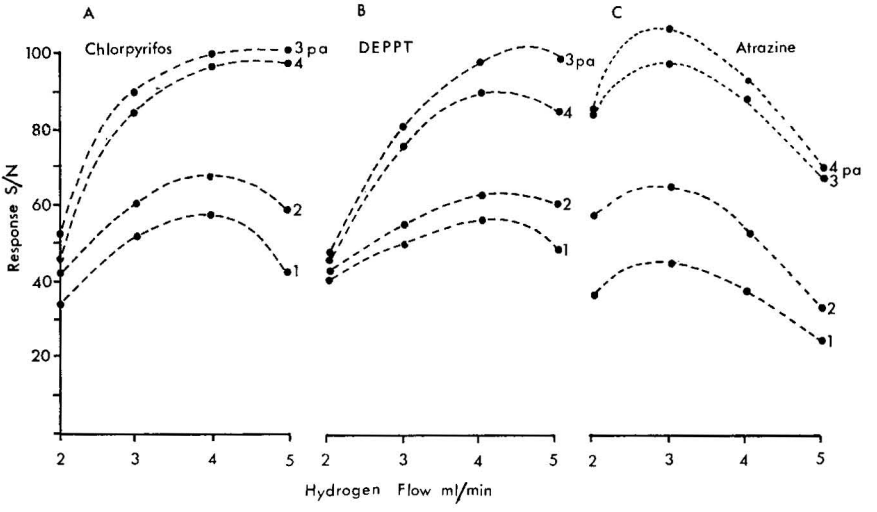


Fig. 3. Variation of the response (signal/noise) with hydrogen flow-rate and standing current with a Perkin-Elmer NPD (AFID) in the NP mode for (a) chlorpyrifos, (b) diethyl phenyl phosphorothioate (DEPPT) and (c) atrazine.

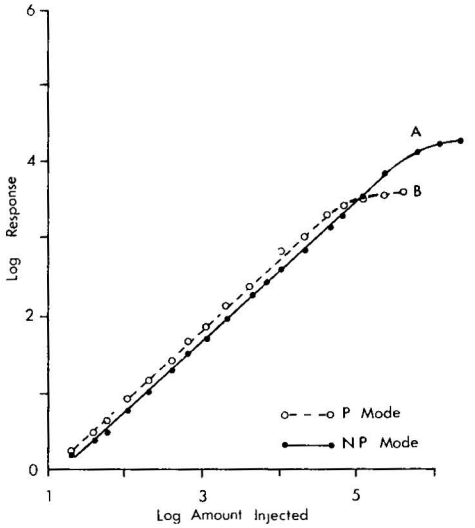


Fig. 4. Linearity of a Perkin-Elmer NPD (AFID) operating (a) in the NP-mode and (b) in the P-mode for chlorpyrifos. Conditions as in Fig. 2.

reaction gas anc nickel catalyst, the detector can be made selective to nitrogen by removal of the HCl and H_2S formed using a strontium hydroxide scrubber. A linear range of only 10^2–10^3 was obtained in the nitrogen mode for chlorpyrifos. Difficulties were experienced in obtaining reproducible results in the nitrogen mode, particularly at low levels of chlorpyrifos (<20 ng). The results were obtained on one

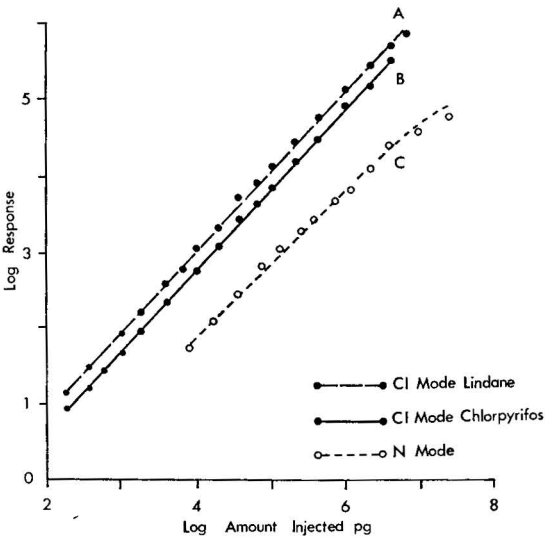


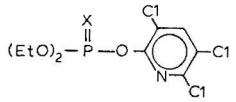
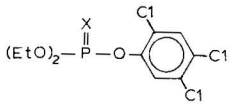
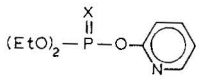
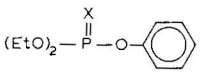
Fig. 5. Linearity of the Hall ECD operating in the halogen mode for (a) lindane and (b) chlorpyrifos, and (c) in the N-mode for chlorpyrifos. Conditions: N-mode, carrier 70 ml/min helium, reactor gas 30 ml/min hydrogen; furnace temp. 900°; nickel catalyst with $Sr(OH)_2$ scrubber; solvent, 50% 2-propanol–water, 0.7 ml/min. Halogen mode, carrier 40 ml/min helium, reactor gas 20 ml/min hydrogen; furnace temp. 850°; solvent 50% 2-propanol–water, 0.2 ml/min.

of the first commercial models produced in 1974 [19] and since then two commercial modifications of this detector have been produced.

Response of a GC detector depends on the efficiency of formation of the activated species measured, such as HPO in the case of the FPD (P-mode). The atoms attached to phosphorus will affect this process depending on the decomposition reaction and the bond energies involved. With the AFID, the sensitivity to phosphorothioates (P=S) is greater than to the oxon analogues (P=O), also substitution of the oxygen atoms attached to phosphorus by nitrogen results in a progressive reduction in sensitivity[20]. Using an OV-17 column, the effect on the response of the Bendix SPED (P-mode) of various chlorpyrifos analogues, involving changes of substitution at phosphorus and in the phenyl moiety, was examined. The MDA for the analogs are shown in Table III. Substitution in the phenyl moiety of the phosphorothioates has only a small effect on the sensitivity, but an appreciable effect in the case of the phosphates. The P=O/P=S ratio varies from 4.5 for chlorpyrifos to 2.1 for diethylphenyl phosphorothioate. The response was also determined on the more polar DEGS phase, which gave similar values. These results indicate that the variation in response is a detector rather than column effect. Replacement of the sulfur by an oxygen atom in the P=X bond appreciably reduced the formation of the measurable species. Substitution of the phenyl moiety produces only a minor effect on the phosphorothioates but a major one on the phosphates.

TABLE III

RESPONSE (LDA) OF BENDIX SPED (P-MODE) TO CHLORPYRIFOS ANALOGUES

Structure	*Minimum detectable amount (pg P/sec)*		*P = O/P = S ratio*
	Thion (X = S)	*Oxon (X = O)*	
	0.177	9.796	4.5
	0.164	0.517	3.15
	0.248	0.806	3.25
	0.15	0.32	2.1

In summary, the response of both element- and group-selective detectors to chlorpyrifos varies according to the type of detector. Similar linear ranges are obtained when standards containing one specific heteroatom are compared on different element-selective detectors. Because of the latter fact and its sensitivity to these

detectors, chlorpyrifos is recommended as a common standard for the evaluation of GC detectors.

ACKNOWLEDGEMENTS

The authors are indebted to Mrs. M. Wilson for her capable technical assistance and P. T. Holland for helpful discussions.

REFERENCES

1 C. H. Hartmann, *Anal. Chem.*, 43 (1971) 113A.
2 J. Ševčík, *Detectors in Gas Chromatography*, Elsevier, Amsterdam, 1976.
3 A. R. L. Moss, *Pye Scan.*, 4 (1964) 5.
4 D. F. S. Natush and T. Thorpe, *Anal. Chem.*, 45 (1973) 1185A.
5 D. J. David, *Gas Chromatographic Detectors*, Wiley-Interscience, New York, 1974.
6 L. S. Ettre, *J. Chromatogr. Sci.*, 16 (1978) 396.
7 D. H. Desty, *Nature (London)*, 179 (1957) 241; 180 (1957) 22.
8 H. W. Johnston, Jr. and F. H. Stross, *Anal. Chem.*, 31 (1959) 1206.
9 G. L. Sutherland, *Residue Rev.*, 10 (1965) 85.
10 R. K. Skogerbee and C. L. Grant, *Spectrosc. Lett.*, 3 (1970) 215.
11 W. P. Cochrane and R. Greenhalgh, *Chromatographia*, 9 (1976) 225.
12 W. P. Cochrane, B. P. Wilson and R. Greenhalgh, *J. Chromatogr.*, 75 (1973) 207.
13 W. P. Cochrane, R. B. Maybury and R. Greenhalgh, *J. Environ. Sci. Health*, B14 (1979) 197.
14 F. W. Karasek, O. S. Tatone and D. M. Kane, *Anal. Chem.*, 45 (1973) 1210.
15 B. Kolb and J. Bischoff, *J. Chromatogr. Sci.*, 12 (1976) 621
16 B. Kolb, M. Aver and P. Pospisil, *J. Chromatogr. Sci.*, 15 (1977) 53.
17 R. C. Hall, *J. Chromatogr. Sci.*, 12 (1976) 152.
18 J. E. Lawrence and J. J. Ryan, *J. Chromatogr.*, 130 (1977) 97.
19 B. P. Wilson and W. P. Cochrane, *J. Chromatogr.*, 106 (1975) 174.
20 R. Greenhalgh and W. P. Cochrane, *J. Chromatogr.*, 70 (1972) 37.

Journal of Chromatography, 188 (1980) 315–322

CHROM. 12,406

ÉTUDE DU MÉLANGE BINAIRE SQUALANE–STÉARONITRILE COMME PHASE STATIONNAIRE

I. COMPARAISON DES RÉSULTATS PROVENANT D'UNE ÉTUDE PAR CHROMATOGRAPHIE EN PHASE GAZEUSE AVEC CEUX RÉSULTANT DES MESURES DE TENSION SUPERFICIELLE

M. LAFOSSE et M. DREUX

Laboratoire de Chimie Organique Physique et Chromatographie, U.E.R. Sciences, 45045-Orléans-Cedex (France)

(Reçu le 18 juin 1979, manuscrit modifié reçu le 11 septembre 1979)

SUMMARY

Study of mixed solvents: squalane–stearonitrile. I. Comparison of results by gas chromatography and surface tensions

We have compared surface tension measurements of binary mixtures with chromatographic results obtained on these mixed stationary phases. At any concentration of stearonitrile the structure of the bulk mixture change. When measuring surface tension, it takes a long time before equilibrium is reached ,but equilibrium is reached quickly by chromatography. Therefore the change of structure of the binary mixture is easily observed by chromatographic measurements.

INTRODUCTION

Littlewood et Wilmott[1] ont étudié les mélanges binaires squalane–dodécanol-1 et squalane–lauronitrile comme phases stationnaires en chromatographie en phase gazeuze. En employant comme variable la proportion en poids du groupe polaire –OH ou –CN dans le mélange de solvants, les auteurs ont observé que le volume de rétention spécifique (V_g) des solutés polaires varie linéairement en fonction de ce paramètre. Par contre V_g s'écarte de la linéarité pour les solutés peu polaires.

D'après ces résultats chromatographiques, les auteurs[1] ont entrepris de comparer l'organisation du lauronitrile et du dodécanol en solution dans le squalane. Écartant l'idée d'une dimérisation ils pensent que l'on a une "polymérisation en chaîne" pour le lauronitrile comme pour le dodécanol. Saum[2] au contraire, a proposé pour les mononitriles une structure fortement dimérisée où les groupes –CN ont tendance à s'associer par paire antiparallèle par interaction dipolaire. Cette structure symétrique en quadrupole ne peut correspondre aux moments dipolaires et aux con-

stantes diélectriques élevées des mononitriles. C'est pourquoi Murray et Schneider[3] ont suggéré une structure en T:

Cette structure évite la juxtaposition d'une forte densité d'électrons π que l'on trouve dans celle proposée par Saum. Cependant Dannhauser et Flueckinger[4] pensent que cette structure est difficilement stable lorsque R est grand, ce qui est le cas du stéaronitrile.

Récemment Guérin[5] a interprété le comportement diélectrique des nitriles en adoptant un modèle supposant la dimérisation partielle du soluté.

Nous avons repris ces travaux pour essayer de mieux définir l'organisation des molécules d'alkyl-nitriles en solution dans le squalane. Dans le présent article nous comparons des résultats chromatographiques concernant la variation de V_g de quelques solutés sur des phases mélangées squalane–stéaronitrile et les mesures de tension superficielle de ces mêmes phases. Nous avons écarté le lauronitrile employé par Littlewood et Willmot[1] car ce liquide par ailleurs toxique a une tension de vapeur trop élevée à 60° pour que les colonnes de chromatographie soient stables dans le temps.

PARTIE EXPÉRIMENTALE

Les mesures chromatographiques ont été réalisées à 60° de la même manière que celles décrites dans un précédent article[6]. Les colonnes sont constituées d'un tube de longueur 1 m en acier inoxydable rempli de Gas-Chrom Q (80–100 mesh) garni de mélange squalane–stéaronitrile en taux d'imprégnation de 20%. Le gaz vecteur est l'azote U. Les mesures de constantes diélectriques ont été effectuées avec un dipolmètre type DM 01 (Wissenschaftliche Technische Werkstatten, R.F.A.).

La tension superficielle des différentes solutions squalane–stéaronitrile a été mesurée dans une microcuve thermostatée à 60° par une circulation d'eau. Les mesures ont été effectuées par la méthode à l'arrachement d'une lame de platine à l'aide de l'appareil Dognon–Abribat (Prolabo, Paris, France). Au cours du temps, entre chaque mesure, la lame n'est pas complètement immergée pour éviter de perturber la solution et elle ne pénètre dans la solution qu'à la profondeur nécessaire pour obtenir l'angle de raccordement nul. Au dessus de la solution règne une atmosphère d'azote pour éviter la présence de vapeur d'eau, d'oxygène et de poussières.

Le squalane (Merck, Darmstadt, R.F.A.) est vérifié exempt de squalène. Le stéaronitrile (Aldrich-Europe, Beerse, Belgique) est distillé de façon à obtenir un solide blanc dont le point de fusion est 40°. La pureté des deux produits est contrôlée par chromatographie en phase gazeuse.

RÉSULTATS

Résultats chromatographiques

Les Figs. 1–3 illustrent la variation de V_g à 60° de trois solutés (benzène, iodure d'allyle, chlorobenzène) (Tableau I) en fonction de la fraction molaire X_A de stéaronitrile dans la phase mixte squalane–stéaronitrile. Dans un prochain article[7] nous présenterons une étude plus complète sur une série de solutés.

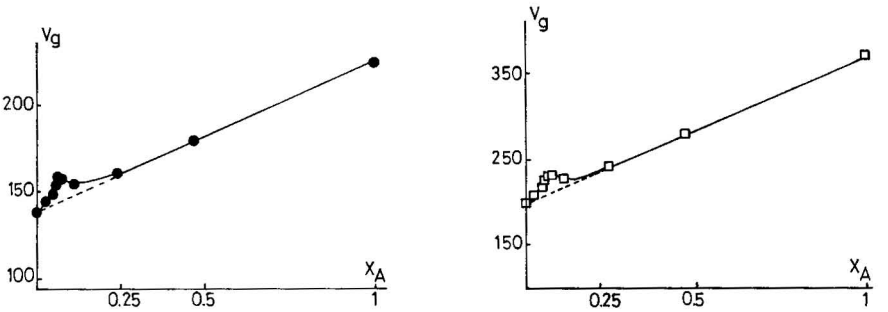


Fig. 1. Variation à 60° du volume de rétention spécifique V_g (cm³) du benzène en fonction de la fraction molaire X_A de stéaronitrile dans le mélange squalane–stéaronitrile.

Fig. 2. Voir Fig. 1. Soluté: iodure d'allyle.

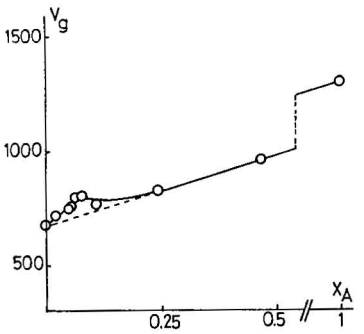


Fig. 3. Voir Fig. 1. Soluté: chlorobenzène.

La pente de la variation est différente selon que X_A est inférieur ou supérieur à 0.25. Il y a donc une différence de comportement du mélange binaire vis à vis des solutés pour cette concentration en stéaronitrile. Cette anomalie dans la courbe des V_g pourrait être due à une variation du volume molaire des mélanges. En fait il n'en est rien puisqu'à 60° les mesures des densités des mélanges ($\varrho_{A,S}$) suivent la relation classique:

$$\frac{1}{\varrho_{A,S}} = \frac{1}{\varrho_S} + \left(\frac{1}{\varrho_S} - \frac{1}{\varrho_A}\right) w_A$$

où ϱ_S et ϱ_A sont respectivement les densités du squalane (S) et du stéaronitrile (A) et W_A est la fraction massique en nitrile; (A,S) étant le mélange des deux liquides.

TABLEAU I

VOLUME DE RÉTENTION SPÉCIFIQUE V_g EN cm³ DE SOLUTÉS EN FONCTION DU TAUX DE STÉARONITRILE (X_A) DANS LE MÉLANGE SQUALANE-STÉARONITRILE À 60°

Taux d'imprégnation	X_A	*Solutés*		
		Iodure d'allyle	*Chlorobenzène*	*Benzène*
21.02	0	198.6	674.5	137.6
18.08	0.023	208.3	719.9	144.0
20.02	0.052	218.1	747.6	148.1
20.63	0.055	226.1	760.9	152.7
20.21	0.064	231.3	794.7	158.5
20.18	0.078	231.3	800.1	157.5
20.13	0.110	226.1	768.0	153.7
20.18	0.243	240.8	825.4	159.7
20.50	0.469	279.7	957.4	179.1
20.48	1	370.0	1288.3	222.9

Résultats concernant les tensions superficielles

La Fig. 4 représente la variation de la tension superficielle à 60° en fonction du temps pour les différents mélanges de X_A. On constate que pour les faibles valeurs de X_A, la tension superficielle σ ne varie presque pas avec le temps. Lorsque X_A atteint la valeur d'environ 0.25 (soit 0.62 mole·l^{-1}), σ décroît au cours du temps jusqu'à atteindre une valeur limite σ^∞. Nous avons porté sur la Fig. 5 les valeurs σ_0 prises au temps zéro et σ^∞ lorsque l'équilibre est atteint (au bout de 6 h à 60°). Les valeurs de σ_0 sont les moins précises, car lorsque σ décroît avec le temps (t), la valeur absolue $\left|\frac{d\sigma}{dt}\right|$ de la variation de la tension superficielle avec le temps est grande au temps zéro. La grandeur σ^∞ est la plus intéressante comme étant caractéristique du système à l'équilibre.

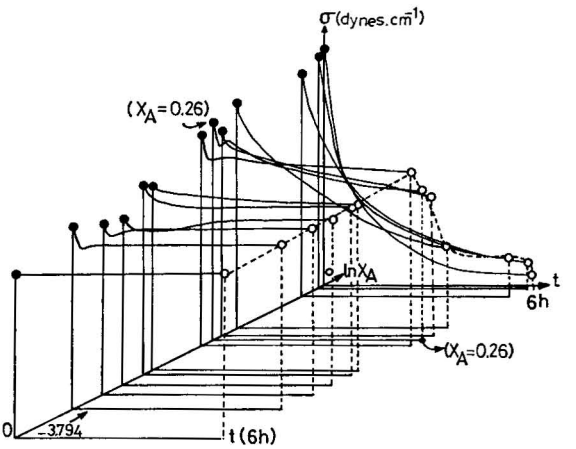


Fig. 4. Variation à 60° de la tension superficielle σ (dynes/cm) du mélange squalane–stéaronitrile en fonction de la fraction molaire X_A, (logarithme népérien: ln X_A) et du tempts t. ●, Temps zéro; ○, équilibre.

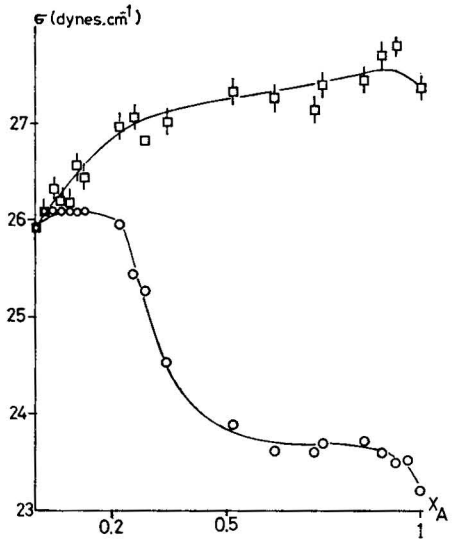


Fig. 5. Variation à 60° de σ_0 (□) au temps initial et de σ^∞ (0) à l'équilibre en fonction de X_A.

La variation de la tension superficielle σ en fonction du temps a déjà été signalée par différents auteurs dans le cas de mélange aqueux[8–11]. Dans la présente étude, la tension superficielle du stéaronitrile pur décroît au cours du temps alors que celle de l'acide stéarique dans les mêmes conditions ne varie pas (acide stéarique Merck avec l'acide oléique comme impureté). Le Tableau II donne à 50° et à 60° les valeurs de σ en fonction du temps. Cette tension superficielle dynamique implique que la structure au sein du liquide pur s'organise parallèlement à ce qui s'observe en surface. Au temps zéro, σ_0 a une valeur voisine de celle calculée par le Parachor (28 dynes·cm^{-1} à 60°) ce qui signifie qu'avant équilibre le stéaronitrile a une structure désordonnée comme tout liquide. On retrouve cette structure initiale désordonnée (soit $\sigma = \sigma_0$) en agitant la solution ce qui a pour effet de perturber l'équilibre ainsi créé.

TABLEAU II

TENSION SUPERFICIELLE σ DU STÉARONITRILE EN FONCTION DU TEMPS

50°		*60°*	
t (min)	*σ (dynes/cm)*	*t (min)*	*σ (dynes/cm)*
0	29.55	0	27.36
6	28.38	26	26.07
17	27.79	43	25.20
37	27.61	85	24.58
70	27.11	120	24.20
99	26.59	160	23.86
264	26.07	228	23.63
413	25.37	288	23.36
478	25.15	323	23.21
558	25.12	360	23.21
591	24.87	480	23.21
1440	24.33		

DISCUSSION

Alors que les mesures de tension superficielle montrent que l'équilibre est atteint au bout de plusieurs heures, il est à noter que l'on n'observe pas de variation de la rétention au cours du temps. En effet, le benzène, le cyanure d'allyle et le méthanol ont été chromatographiés sur une colonne remplie de Gas-Chrom Q recouvert de stéaronitrile (taux d'imprégnation 20%). Le temps de rétention ne varie pas sur plusieurs heures depuis l'instant où la colonne se stabilise à 60°. On peut expliquer cette stabilité en considérant que le temps nécessaire pour la mise en température est suffisant pour que la phase stationnaire déposée en film très fin soit à l'équilibre lors des chromatographies. Ainsi les volumes de rétention mesurés par chromatographie permettent d'évaluer les interactions des solutés avec le système squalane–stéaronitrile à l'équilibre[7].

La question est donc d'étudier le type d'interactions qui existent dans le système squalane–stéaronitrile. La mesure de la constante diélectrique du stéaronitrile à 60° montre une valeur élevée ($\varepsilon = 6.10$) ainsi que celle de la polarisation moléculaire à dilution infinie $(M_A P_A)_{W_A \to 0} = 321.3$, ce qui est conforme à la littérature[5].

L'étude de la variation de la polarisation moléculaire $M_{A,S}P_{A,S}$ du mélange à 60° en fonction de la fraction massique W_A en stéaronitrile (Fig. 6) montre un abaissement de la courbe, ce qui s'explique par une auto-association des molécules de nitrile dans ce domaine ($M_{A,S} = X_A M_A + X_S M_S$ où M_A et M_S sont les masses moléculaires du stéaronitrile et du squalane).

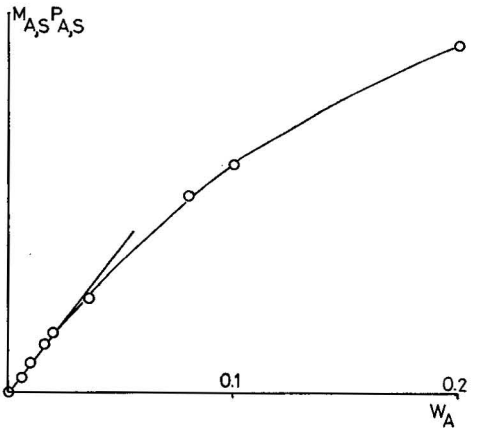


Fig. 6. Polarisation moléculaire $M_{A,S}$ $P_{A,S}$ du mélange binaire à 60° en fonction de la fraction massique W_A de stéaronitrile. (W_A = masse stéaronitrile/masse stéaronitrile + masse squalane).

Compte-tenu du fait que pour les acides notamment l'acide stéarique, ε est faible[12], les molécules sont dimérisées et qu'il n'existe pas de micelles inverses avec le dodécane[13], on peut penser qu'avec les alkylnitriles, la formation de micelles inverses est probable. Lorsque la concentration de nitrile dans le squalane devient suffisante ($X_A > 0.25$) les molécules de stéaronitrile s'organisent pour donner à l'équilibre un interface air–solution dont la tension superficielle est comprise entre celle du pentadécane et celle du tétradécane.

Ce phénomène est à rapprocher de celui des molécules d'éthanol dans l'eau. L'éthanol abaisse la tension superficielle de l'eau. Ceci s'explique par une concentration des molécules d'éthanol en surface avec les groupes hydroxyles orientés dans l'eau, où ils peuvent créer des liaisons hydrogènes[14]. Les chaînes alkyles sont tournées vers la surface. Cette explication ne vaut pas pour le mélange squalane–stéaronitrile car si la tension de surface s'abaisse environ comme celle du pentadécane, les groupes –CN à dipoles très élevés ne peuvent s'orienter vers l'intérieur du liquide apolaire. La seule explication réside pour X_A élevé dans la formation de micelles inverses avec tous les groupes polaires orientés vers un centre et les chaînes alkyles lipophiles vers le squalane. Comme le signalait déjà Littlewood et Willmott[1] les alkyl-nitriles dispersés dans le squalane doivent se "polymériser" et ne pas s'arrêter au stade dimère. Si l'on admet que la formation de micelles a lieu à 60° pour $X_A = 0.25$ environ, cela correspond à une "concentration micellaire critique" de l'ordre du dixième de gramme par décilitre de solvant, concentration très élevée que l'on note dans le cas de micelles en milieu non aqueux (benzène, tétrachlorure de carbone)[15].

CONCLUSION

La confirmation de l'existence de micelles inverses, la taille et la structure de telles micelles ainsi que l'orientation des molécules pour $X_A < 0.25$ sera déterminée par diffusion Rayleigh dépolarisée. La chromatographie permet de suivre en parallèle de tels mélanges comme elle a déjà été employée pour étudier des changements de phase[16,17]. Elle nous permettra dans un prochain article de donner les coefficients d'interaction de divers solutés avec les groupes nitriles non associés.

RÉSUMÉ

Nous avons comparé des mesures de tension superficielle relatives à des mélanges binaires aux résultats chromatographiques obtenus en employant ces mélanges comme phase stationnaire. Le mélange squalane–stéaronitrile étudié présente une organisation de structure qui change pour une certaine concentration et stéaronitrile. Par mesure de tension superficielle l'état d'équilibre est long à obtenir, alors qu'en chromatographie il est atteint rapidement d'ou une étude facilitée de l'organisation des solutions.

BIBLIOGRAPHIE

1 A. B. Littlewood et F. W. Willmott, *Anal. Chem.*, 38 (1966) 1031.
2 A. M. Saum, *J. Polym. Sci.*, 42 (1960) 57.
3 F. E. Murray et W. G. Schneider, *Can. J. Chem.*, 33 (1955) 797.
4 W. Dannhauser et A. F. Flueckinger, *J. Phys. Chem.*, 68 (1964) 1814.
5 M. Guérin, *Thèse Docteur Es-Sciences*, Université de Poitiers, 1975.
6 M. Lafosse et M. Durand, *J. Chromatogr.*, 171 (1979) 183.
7 M. Lafosse, *J. Chromatogr.*, soumis pour publication.
8 B. I. Tamamushi, in K. Shinoda (Rédacteur), *Colloidal Surfactants*, Academic Press, New York, 1963, p. 185.
9 J. J. Kipling, *Adsorption from solutions of non electrolytes*, Academic Press, New York, 1965, p. 236.
10 A. W. Neumann et W. Tanner, *Tenside*, 4 (1967) 220.

11 J. Kloubek et A. W. Neumann, *Tenside*, 6 (1969) 4.
12 *Table of Dielectric Constants of Pure Liquids*, NBS Circular, 514 (1951).
13 F. W. Fowkes, in K. Shinoda (Rédacteur), *Solvent properties of Surfactants Solutions*, Marcel Dekker, New York, 1967.
14 G. N. Lewis et M. Randall, *Thermodynamics*, 2ème édition, Mc Graw-Hill, New York, 1961, p. 476.
15 P. Becher, in M. J. Shick, (Rédacteur), *Non Ionic Surfactants*, Vol. 2, Marcel Dekker, New York, 1966, p. 513.
16 J. Serpinet, *Thèse Docteur es-sciences*, Université Claude Bernard, Lyon, 1974.
17 H. Purnell, *New Developments in Gas Chromatography*, Vol. 11, Interscience, 1973, pp. 87, 187.

Journal of Chromatography, 188 (1980) 323–331

CHROM. 12,428

COORDINATION POLYMERS AS ADSORBENTS AND STATIONARY PHASES IN GAS CHROMATOGRAPHY

VI. SEPARATION PROPERTIES OF ALKALINE-EARTH METAL DI-*n*-HEXYLPHOSPHINATES

JACEK NAWROCKI, WALENTY SZCZEPANIAK and WIESŁAW WASIAK
Institute of Chemistry, A. Mickiewicz University, 60-780 Poznań (Poland)
(Received July 26th, 1979)

SUMMARY

The separation properties of alkaline-earth metal di-*n*-hexylphosphinates in relation to hydrocarbons have been described. The possibilities of specific interactions of unsaturated hydrocarbons with alkaline-earth metals are noted. A number of chromatographic separations on magnesium di-*n*-hexylphosphinate are reported.

INTRODUCTION

Alkaline-earth metal compounds have rarely been used as stationary phases or adsorbents in gas chromatography (GC). $BaCl_2$ (refs. 1, 2) and $BaSO_4$ (ref. 3) have been studied more extensivety; the application of halides of Sr and Ba as adsorbents has also undergone careful investigations[4]. $CaCl_2$ and other metal chlorides have been used for silica gel modification[5], while a mixture of CaF_2 and CsF was employed for separation of fluorine compounds[6]. Zeolites of Mg, Ca, Sr and Ba[7] has also been.

It seemed more interesting to find out the chromatographic properties of alkaline-earth metal di-*n*-hexylphosphinates (DHP) because DHP–Mg has already been shown to exhibit good separation properties[8,9]. Thus, DHP–Ca, DHP–Sr and DHP–Ba have been synthesized, applied as adsorbents in GC and their separation properties compared for 22 C_6 hydrocarbons.

EXPERIMENTAL

Synthesis of phosphinates

DHP–Mg was synthesized as described previously[8]; DHP–Ca was obtained analogously.

DHP–Sr. A 5-g amount of di-*n*-hexylphosphinic acid[10] was dissolved in 50 ml of methanol and neutralized with a stoichiometric amount of 1 *N* KOH. Water was added to give a total volume of 100 ml, and then 100 ml of chloroform. To this mixture, an aqueous solution of $SrCl_2$ (2.8448 g $SrCl_2 \cdot 6H_2O$ in 50 ml of water) was added

dropwise and the solution mixed continuously with a magnetic stirrer. The final solution was stirred for 24 h. The chloroform layer was separated, and the water layer was washed twice with 25 ml of chloroform. The combined chloroform layers were evaporated and a white precipitate of DHP–Sr · 1/2 H_2O was obtained. The compound was dehydrated by drying for 48 h in a vacuum above molecular sieves 4A and 5A.

DHP–Ba. A 5 g amount of di-*n*-hexylphosphinic acid was dissolved in 50 ml of methanol and neutralized with the stoichiometric amount of 1 *N* KOH. A gelatinous precipitate of DHP–Ba was obtained when $BaCl_2$ (2.609 g in 50 ml of water) was added to the solution. A 50-ml volume of water was added and the solution then evaporated to *ca.* 50 ml in volume. This procedure was repeated twice. Then, the precipitate was filtered off, rinsed well and dried, first at 100° and then above molecular sieves 4A and 5A in a vacuum for 48 h.

The phosphinates were identified by means of IR spectra and elemental analysis. The analyses results and some properties of the phosphinates are presented in Table I.

Porasil C DMCS was coated with phosphinates so that each surface concentration was 10^{-4} mole of phosphinate per gram of adsorbent. Therefore 5% DHP–Mg, 5.16% DHP–Ca, 5.64% DHP–Sr and 6.15% DHP–Ba were precipitated on the support. These packings were conditioned at 150° in a stream of Ar for 15 h. Stainless-steel columns (1 m × 4 mm) were used. Some separations were carried out with the help of a steel micropacked column (1.5 m × 0.8 mm). The particle size of each packings was 0.10–0.12 mm.

TABLE I

ELEMENTAL ANALYSIS AND SOME PROPERTIES OF ALKALINE-EARTH METAL DI-*n*-HEXYLPHOSPHINATES

Phosphinate	*Analysis (%)**			*M.p. (°C)*	*Decomposition temp. (°C)*	*Colour*
	C	*H*	*M*			
DHP–Mg	58.72	10.68	4.96	100	300	white
	58.70	10.64	4.62			
DHR–Ca	56.89	10.35	7.89	107–108	330	white
	56.68	10.29	7.62			
DHR–Sr	52.01	9.45	15.80	108–109	330	white
	52.00	9.33	15.53			
DHP–Ba	47.74	8.51	23.07	109–110	330	white
	47.52	8.24	23.14			

* Calculated followed by found values.

Decomposition temperatures of phosphinates were obtained with the aid of a Paulik, Paulik, Erdey Derivatograph (MOM, Budapest, Hungary). A Böetius microscopic table was used to determine the melting points.

RESULTS AND DISCUSSION

Retention times and volumes were measured for 22 C_6 hydrocarbons on all of the packings at four temperatures ranging from 70 to 90°. The k' values for the columns of alkaline-earth metal di-*n*-hexelphosphinates are collected in Table II.

TABLE II

CAPACITY RATIO k' FOR COLUMNS CONTAINING ALKALINE-EARTH METAL DI-*n*-HEXYLPHOSPHINATES AT 70°

Compound	k'			
	DHP–Mg	*DHP–Ca*	*DHP–Sr*	*DHP–Ba*
Pentane	1.48	1.32	1.34	1.35
Heptane	6.03	5.56	5.82	5.49
Octane	12.19	11.41	12.25	11.09
Hexane	2.94	2.72	2.79	2.73
1-Hexene	2.93	2.58	2.73	2.65
1-Hexyne	4.25	3.44	3.98	3.71
1,5-Hexadiene	2.84	2.45	2.64	2.55
2,4-Hexadiene	5.89	4.43	5.08	4.83
cis-2-Hexene	3.50	2.95	3.22	3.08
trans-2-Hexene	3.31	2.82	3.01	2.91
cis-3-Hexene	3.23	2.80	3.02	2.91
trans-3-Hexene	3.20	2.78	2.97	2.87
Cyclohexane	4.53	3.51	4.02	3.92
Cyclohexene	5.66	4.02	4.84	4.63
1,3-Cyclohexadiene	6.00	4.03	4.92	4.68
1,4-Cyclohexadiene	7.95	5.10	6.37	6.01
Benzene	6.64	4.21	5.65	4.97
3-Methylpentane	2.70	2.46	2.55	2.48
3-Methylpentene-1	2.37	2.15	2.26	2.19
cis-3-Methylpentene-2	3.39	2.89	3.18	3.04
trans-3-Methylpentene-2	3.61	3.07	3.35	3.23
Methylcyclopentane	3.56	2.91	3.20	3.14
2-Methylpentene-1	2.89	2.57	2.77	2.67
4-Methylpentene-1	2.34	2.17	2.26	2.20
trans-4-Methylpentene-2	2.58	2.33	2.45	2.38

The highest k' values and the biggest differences between individual compounds have been observed for the column containing DHP–Mg. This column, then, can be expected to show the best separation properties. It is interesting to compare phosphinate separation properties for *cis* and *trans* isomers (Table III).

TABLE III

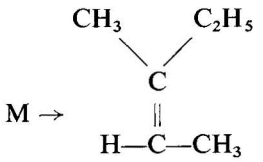
COMPARISON OF THE SEPARATION PROPERTIES OF PHOSPHINATES AND SOME LIQUID STATIONARY PHASES IN RELATION TO *cis* AND *trans* ISOMERS

Values are of α = *cis*/*trans*. ODPN = β,β'-oxydipropionitrile (2.5-m column); DMS = dimethylsulpholane (2.5-m column); TCP = tricresylphosphate (2.5-m column); SQ = squalane (600-ft. column).

Compound	*DHP–Mg* 70°	*DHP–Ca* 70°	*DHP–Sr* 70°	*DHP–Ba* 70°	*ODPN** 27°	*DMS** 27°	*TCP** 27°	*SQ** 49°
2-Hexene	1.060	1.047	1.070	1.061	1.22	1.13	1.11	1.07
3-Hexene	1.009	1.008	1.017	1.014	1.16	1.05	0.98	1.00
3-Methylpentene-2	0.939	0.943	0.950	0.942	0.94	0.90	0.89	0.90

Of the columns studied here. DHP–Sr gave the best separation of *Cis* and *trans* isomers of *n*-hydrocarbons, but branched hydrocarbon, were best separated on DHP–Mg. It is worthy of note that *trans*-3-methylpentene-2 is eluted later than the corresponding *cis*-isomer regardless of the lower boiling point of the *trans*-form. The retention index of the *cis* isomer on squalane is also lower than that of *trans*-isomer[11,12]. A similar case has been previously described[13], and *trans*-4-methylpentene-2 shows a higher stability constant with $PdCL_2$ than the corresponding *cis*-isomer. Phosphinate phases show little of the selectivity of liquid phases; it must be remembered, however, that the phosphinate data were obtained at a higher temperature.

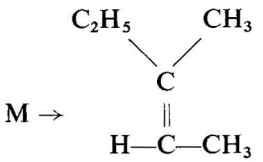
Hively[14] suggests that the terms *cis* and *trans* in relation to such compounds as 3-methylpentene-2, 3-methylhexe-3 and 3-methylhexene-2 should be reversed, so that the *trans*-isomer becomes the compound of higher boiling temperature, as verified by Cornforth *et al.*[15]. From the point of view of possible specific interactions between a metal and a multiple bond, it seems that a form with a lower boiling point



$t_B = 67.63°$ (ref. 16)

$t_B = 68–69°$ (ref. 15)

might cause stronger interactions than a form having a higher boiling point



$t_B = 70.45°$ (ref. 16)

$t_B = 70–71°$ (ref. 15)

due to obvious, smaller steric hindrance ($CH_3 < C_2H_5$). Such an interpretation also explains the reversed order of elution of *cis*- and *trans*-3-methylpentene-2 on phosphinates.

Table IV presents specific ${}^{pT}V_g$ and relative (r) retention volumes of hydrocarbons corresponding to 10^{-4} mole of phosphinate, *e.g.* to *ca.* 1 g of adsorbent. The ${}^{pT}V_g$ values are similar for all of the phosphinates. The relative retention volumes have been considered for three groups of sorbztes:

n-Hydrocarbons. The highest values may be observed on DHP–Sr and DHP–Mg. They are very approximate. Lower values were found on DHP–Ba and the lowest on DHP–Ca.

Cyclic hydrocarbons. The highest values and the best separation properties were observed on DHP–Mg, then DHP–Sr and DHP–Ba respectively and the lowest on DHP–Ca.

3-Methylpentane derivatives. The results were similar to those for cyclic hydrocarbons.

It may be concluded that the best separation properties (in relation to *n*-hydrocarbons) are expected on the column containing DHP–Mg. Relative retention volumes of unsaturated hydrocarbons show that usually these compounds are more strongly

TABLE IV

SPECIFIC AND RELATIVE RETENTION VOLUMES OF C_6 HYDROCARBONS

Compound	${}^{p}{}^{T}V_g$ *(ml/g)*				r			
	DHP–Mg	*DHF–Ca*	*DHP–Sr*	*DHP–Ba*	*DHP–Mg*	*DHP–Ca*	*DHP–Sr*	*DHP–Ba*
Pentane	2.403	2.648	2.289	2.623				
Heptane	9.813	11.130	9.952	10.669				
Octane	19.847	22.840	20.955	21.538				
Hexane	4.87	5.437	4.765	5.303	1.000	1.000	1.000	1.000
1-Hexene	4.765	5.163	4.674	5.152	0.978	0.950	0.981	0.972
1-Hexyne	6.927	6.882	6.815	7.208	1.422	1.266	1.430	1.359
1,5-Hexadiene	4.627	4.911	4.514	4.955	0.950	0.903	0.947	0.934
2,4-Hexadien	9.588	8.873	8.694	9.376	1.968	1.632	1.825	1.768
cis-2-Hexene	5.704	5.902	5.506	5.957	1.171	1.086	1.156	1.130
trans-2-Hexene	5.383	5.637	5.145	5.647	1.105	1.037	1.080	1.065
cis-3-Hexene	5.253	5.599	5.171	5.655	1.078	1.030	1.085	1.066
trans-3-Hexene	5.206	5.556	5.084	5.576	1.069	1.022	1.067	1.051
Cyclohexene	7.374	7.028	6.876	7.613	1.000	1.000	1.000	1.000
Cyclohexene	9.218	8.040	8.277	8.998	1.250	1.144	1.204	1.182
1,3-Cyclo-hexadiene	9.771	8.063	8.412	9.085	1.325	1.147	1.223	1.193
1,4-Cyclo-hexadiene	12.950	10.213	10.904	11.676	1.756	1.453	1.586	1.534
Benzene	10.814	8.428	9.656	9.665	1.466	1.199	1.404	1.269
3-Methylpentane	4.389	4.916	4.361	4.822	1.000	1.000	1.000	1.000
3-Methyl-pentene-1	3.860	4.309	3.859	4.257	0.879	0.876	0.885	0.883
cis-3-Methyl-pentene-2	5.524	5.793	5.446	5.907	1.258	1.178	1.249	1.225
trans-3-Methyl-pentene-2	5.884	6.141	5.732	6.272	1.341	1.249	1.314	1.301
Methylcyclo-pentane	5.792	5.828	5.480	6.101				
2-Methyl-pentene-1	4.708	5.148	4.743	5.187				
4-Methyl-pentene-1	3.806	4.335	3.864	4.277				
trans-4-Methyl-pentene-2	4.203	4.668	4.194	4.625				

retained than the respective alkanes. Hydrocarbons possessing higher unsaturation, *e.g.*, 1-hexyne and dienes, usually have higher relative retention volumes than their respective alkenes. At the same time, 1-hexene and 1,5-hexadiene have relative (with respect to hexane) retention volumes lower than 1.

This does not mean that these compounds do not have specific interaction with phosphinates. The specific interactions of alkaline-earth metal ions with hydrocarbons will be discussed elsewhere.

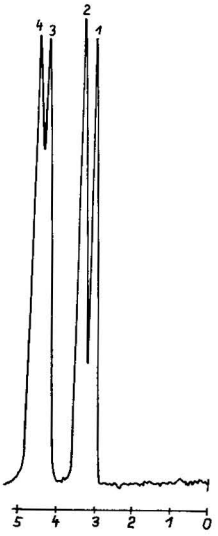
Application of DHP–Mg to chromatographic separations

Since the retention data show that DHP–Mg pactings exhibit the best separation properties, have applied this phase to the separation of model mixtures. Two

types of columns were used for chromatographic separations: classic and micropacked.

In gas chromatography DHP–Mg can be used in two ways: as an adsorbent, below the melting point; or as a liquid stationary phase, above the melting point[9]. Fig. 1 shows the separation on DHP–Mg, below its melting point, of several hydrocabons of similar structure and properties, including three isomers. A similar separation of these compounds on squalane requires *ca.* 14 min on a micropacked column[17]. Fig. 2 shows a separation of several cyclic hydrocarbons.

DHP–Mg applications are not limited to hydrocarbons. Particularly interesting separation properties are shown by DHP–Mg above its melting point. Figs. 3–6 show separations of homologous series of compounds.



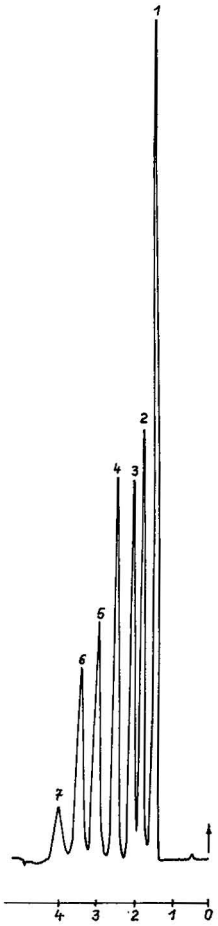


Fig. 1. Separation of some C_6 hydrocarbons. Peaks: 1 = 3-methylpentene-1; 2 = 3-methylpentane; 3 = *cis*-3-methylpentene-2; 4 = *trans*-3-methylpentene-2. Column: 1 m × 4 mm I.D., packed with 5% DHP–Mg on Porasil C DMCS; T_k = 59°; flow-rate of argon, F_0 = 17.9 ml/min.

Fig. 2. Analysis of some cyclic hydrocarbons. Peaks: 1 = cyclopentene; 2 = hexane; 3 = methylcyclopentane; 4 = cyclohexane; 5 = cyclohexene; 6 = benzene; 7 = 1,4-cyclohexadiene. Column as in Fig. 1; T_k = 72°; F_0 = 27.2 ml/min.

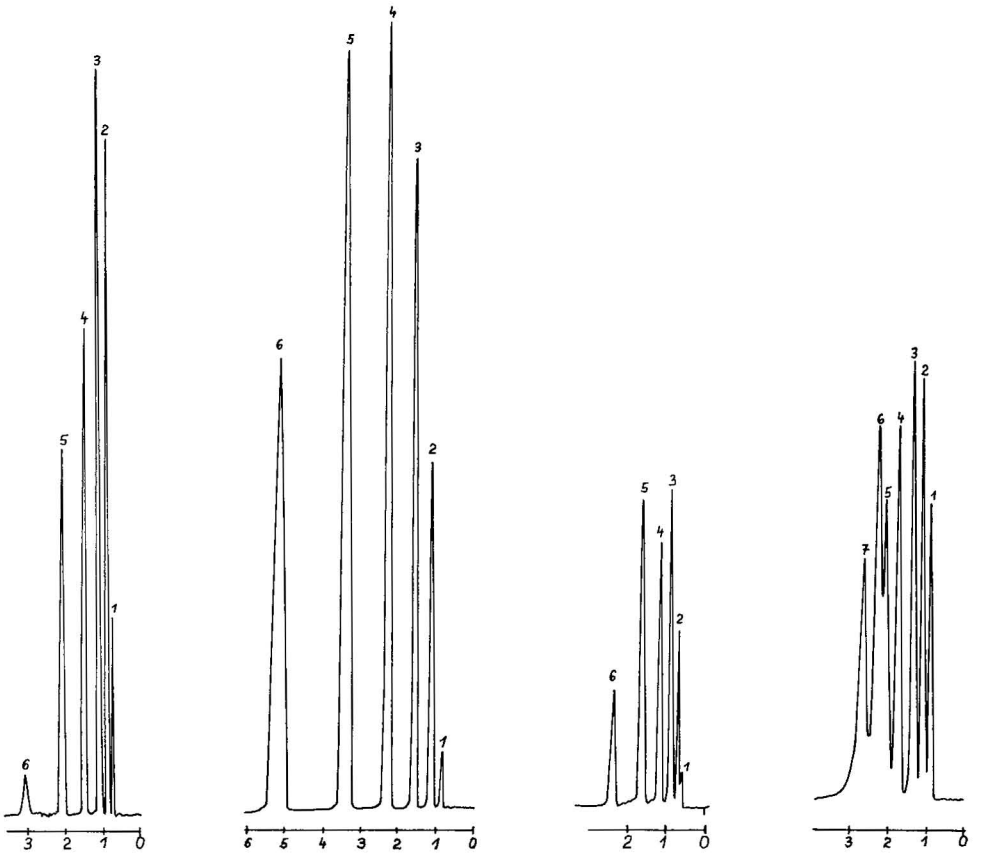


Fig. 3. Separation of C_5–C_{10}. Peaks: 1 = pentane; 2 = hexane; 3 = heptane; 4 = octane; 5 = nonane; 6 = decane. Column as in Fig. 1; T_k = 129°; F_0 = 26.6 ml/min.

Fig. 4. Separation of 1-chloroalkanes. Peaks: 1 = 1-chloropentane; 2 = 1-chlorohexane; 3 = 1-chloroheptane; 4 = 1-chlorooctane; 5 = 1-chlorononane; 6 = 1-chlorodecane. Column: 1.5 m × 0.8 mm I.D., packed with 5% DHP–Mg on Porasil C DMCS; T_k = 137°; F_0 = 4.5 ml/min.

Fig. 5. Separation of aldehydes. Peaks: 1 = propanal; 2 = butanal; 3 = pentanal; 4 = hexanal; 5 = heptanal; 6 = octanal. Column as in Fig. 4; T_k = 131.2°; F_0 = 4.3 ml/min.

Fig. 6. Separation of esters of acrylic and methacrylic acids. Peaks: 1 = methyl acrylate; 2 = methyl methacrylate; 3 = ethyl methacrylate; 4 = isobutyl acrylate; 5 = *sec.*-butyl methacrylate; 6 = isobutyl methacrylate; 7 = *n*-butyl methacrylate. Column as in Fig. 4; T_k = 124.2°; F_0 = 5.5 ml/min.

Other interesting examples of the application of DAP–Mg are the separations of halogen derivatives of ethane and analysis of cichlorobenzene isomers, which requires only *ca.* 12 min, while in the case of a 18-m micropacked column containing liquid crystals such an analysis takes *ca.* 16 min[18], with other phases much longer[19]. Only a mixture of silicone oil SP-1200 and Bentone 34 enables a faster separation of these isomers[20].

Finally, Figs. 9 and 10 show the separation of aromatic hydrocarbons.

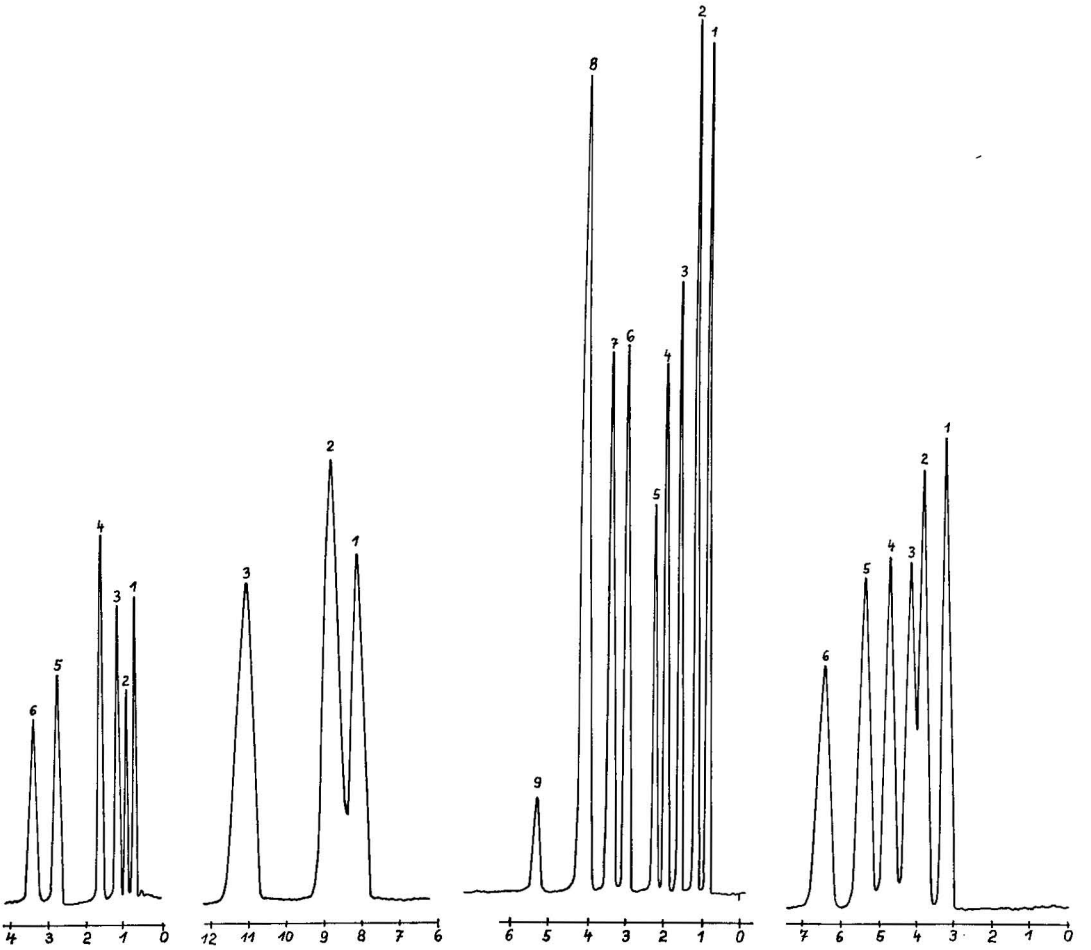


Fig. 7. Analysis of chloroethane derivatives. Peaks: 1 = chloroethane; 2 = 1,1-dichloroethane; 3 = 1,2-dichloroethane; 4 = 1,1,2-trichloroethane; 5 = 1,1,2,2-tetrachloroethane; 6 = pentachloroethane. Column as in Fig. 1; T_k = 130.6°; F_0 = 20.3 ml/min.

Fig. 8. Separation of dichlorobenzenes. Peaks: 1 = *para*-; 2 = *meta*-; 3 = *ortho*-. Column as in Fig. 4; T_k = 107°, F_0 = 4.4 ml/min.

Fig. 9. Separation of aromatic hydrocarbons. Peaks: 1 = benzene; 2 = toluene; 3 = ethylbenzene; 4 = styrene; 5 = *n*-propylbenzene; 6 = *p*-cymene; 7 = *n*-butylbenzene; 8 = *sec*.-pentylbenzene; 9 = *p*-diisopropylbenzene. Column as in Fig. 4; T_k = 129.4°; F_0 = 6.02 ml/min.

Fig. 10. Separation of aromatic C_9 isomers. Peaks: 1 = isopropylbenzene; 2 = *n*-propylbenzene; 3 = 3-ethyltoluene; 4 = 1,3,5-; 5 = 1,2,4-; 6 = 1,2,3-trimethylbenzene. Column as in Fig. 4; T_k = 101.6°, F_0 = 5.6 ml/min.

CONCLUSIONS

We have confirmed the good separation properties of phosphonates that have been described before[21]. Retention data indicate the existence of specific interactions between alkaline-earth metal atoms and unsaturated hydrocarbons. Our next paper will be conserned with a quantitative evaluation of these interactions.

REFERENCES

1 L. D. Belyakova, A. M. Kalpakian and A. V. Kiselev, *Chromatographia*, 7 (1974) 14.
2 R. L. Gaob, L. D. Belyakova and A. M. Kalpakian, *J. Chromatogr.*, 91 (1974) 699.
3 L. D. Belyakova, A. V. Kiselev and G. A. Soloyan, *Zh. Anal. Khim.*, 27 (1972) 1182.
4 R. L. Grob, R. J. Gondek and T. Scales, *J. Chromatogr.*, 53 (1970) 477.
5 N. H. C. Cook, E. F. Barry and B. S. Solomon, *J. Chromatogr.*, 109 (1975) 57.
6 R. P. Hirschman, H. L. Simon, L. R. Anderson and W. B. Fox, *J. Chromatogr.*, 50 (1970) 118.
7 T. Andronikashvili, C. V. Tsitsishvili and L. Laperashvili, *Chromatographia*, 8 (1975) 223.
8 W. Szczepaniak and J. Nawrocki, *J. Chromatogr.*, 168 (1979) 97.
9 W. Szczepaniak and J. Nawrocki, *Chem. Anal. (Warsaw)*, 23 (1973) 795.
10 R. A. Williams and R. A. Hamilton, *J. Amer. Chem. Soc.*, 74 (1952) 5418.
11 M. S. Vigderganz, *Gazovaya Khromatografiya tak Metod Issledovaniya Nefty*, Nauka, Moscow, 1973.
12 M. S. Vigderganz, L. V. Semenchenko, V. A. Ezrets and Yu. N. Bogoslavskii, *Kachestvennyi Gazokhromatograficheskii Analiz*, Mauka, Moscow, 1978.
13 M. Kraitr, R. Komers and F. Čuta, *Anal. Chem.*, 46 (1974) 974.
14 R. A. Hively, *Anal. Chem.*, 35 (1963) 1921.
15 J. W. Cornforth, R. H. Cornforth and K. K. Mathew, *J. Chem. Soc., London* (1959) 112.
16 S. W. Ferris, *Handbook of Hydrocarbons*, Academic Press, New York, 1955.
17 V. G. Berezkin and L. A. Shkolina, *Zh. Anal. Khim.*, 28 (1973) 1838.
18 Z. Witkiewicz, Z. Suprynowicz, J. Wójcik and R. Dąbrowski, *J. Chromatogr.*, 152 (1978) 323.
19 A. E. Habboush and A. H. Tameesh, *J. Chromatogr.*, 53 (1970) 143.
20 *Chromatography, Lipids*, Bulletin 740C, Supelco, Bellefonte ,Pa., 1974.
21 J. Nawrocki, W. Szczepaniak and W. Wasiak, *J. Chromatogr.*, 178 (1979) 91.

Journal of Chromatography, 188 (1980) 333–345

CHROM. 12,400

HIGH-PERFORMANCE GEL PERMEATION CHROMATOGRAPHY OF POLYSTYRENE WITH SILICA MICROSPHERES

JOHN V. DAWKINS and GRAHAM YEADON

Department of Chemistry, Loughborough University of Technology, Loughborough, Leicestershire LE11 3TU (Great Britain)

(First received May 2nd, 1979; revised manuscript received September 19th, 1979)

SUMMARY

Gel permeation chromatography separations have been performed with short low capacity columns containing silica microspheres (particle diameter $\approx 20\ \mu m$) having narrow particle size distributions and mean pore diameters in the range 8–120 nm. Plate height data for non-permeating polystyrene standards permitted an evaluation of chromatogram broadening due to mobile phase dispersion. These results together with plate height data for permeating polystyrene standards, tetraphenylethylene and toluene gave an assessment of chromatogram broadening due to mass transfer dispersion as a function of eluent flow-rate and solute molecular weight. It was found that mass transfer dispersion increased at higher flow-rates, the flow-rate dependence increasing as the diffusion coefficient of permeating polystyrene decreased. At very low flow-rates (0.05 $cm^3\ min^{-1}$), mobile phase dispersion is the major contributor to the chromatogram broadening of permeating polystyrene. From theoretical considerations of chromatogram broadening, a relation is derived permitting the approximate determination of the true polydispersity of a permeating polymer at very low eluent flow-rates from the plate height for that polymer and from the plate height arising from mobile phase dispersion. The results show that fast separations may be accomplished in several minutes and that the most precise determinations of polydispersity are obtained at slow eluent flow-rates with separation times of about 1 h.

INTRODUCTION

Theoretical treatments of chromatogram broadening predict that column performance depends on flow mechanisms in the mobile phase and on solute mass transfer between the stationary and mobile phases[1]. Experimental studies of the dependence of solute dispersion mechanisms on the solute diffusion coefficient D_m, on eluent flow-rate u and on the particle diameter of the column packing d_p have been performed in separations by gel permeation chromatography (GPC)[2–5]. Čoupek and Heitz[2,3] found good agreement between the theoretical treatments and experimental results for permeating and non-permeating oligomers separating on soft homo-

geneously cross-linked organic gels with $d_p \geqslant 24\ \mu m$. Billmeyer and Kelley[4,5] concluded that mobile phase dispersion was the major contributor to chromatogram broadening for cross-linked polystyrene gels with $d_p \approx 50\ \mu m$ and that mass transfer dispersion was the predominant broadening mechanism for inorganic particles with $d_p > 100\ \mu m$. This mass transfer contribution was evaluated by comparing the experimental behaviour of low polymers with non-porous and porous inorganic packings. An alternative procedure is to compare permeating and non-permeating solutes with the same column, and results for low polymers with porous glass ($d_p \approx 44$–$74\ \mu m$) have been presented by Giddings *et al.*[6].

Column performance is markedly improved by reducing d_p. Theory predicts that mobile phase dispersion due to the eddy diffusion mechanism depends on d_p and that solute dispersion due to mass transfer decreases as the depth of penetration into the stationary phase falls, and therefore as d_p is reduced[1]. Microparticulate silicas ($d_p \approx 5$–$20\ \mu m$) which produce high column efficiences for small molecules are widely used in high-performance liquid chromatography[7]. Several microparticulate silicas with wide pores have been prepared for high-performance GPC (HPGPC) separations of polymers[8–12]. Silica microspheres for chromatographic separations have been developed at AERE Harwell, and "Spherisorb" (trademark of the United Kingdom Atomic Energy Authority) has given outstanding performance in liquid chromatography[13,14]. Columns of Spherisorb contain rigid spheres with a narrow particle size distribution, so that a bed of particles has good flow properties and a low resistance to fluid flow. Previously, we showed that Spherisorb (mean pore diameter 8 nm) will separate low polymers, and that column efficiency is influenced by particle size and particle size distribution[15]. Although our earlier work demonstrated the dependence of column efficiency on d_p, our chromatographic conditions were far from optimum. We have now studied column efficiencies for Spherisorb and wide pore silicas ($d_p \approx 20\ \mu m$) having mean pore diameter in the range 30–120 nm with a much improved injection procedure and a constant-flow pump. Initial resolution results for three of the packings have been reported elsewhere[16]. In this paper column efficiency results are described for permeating and non-permeating high polymers as a function of u, in order to assess the contributions to chromatogram broadening in HPGPC arising from solute dispersion in the mobile phase and during mass transfer. These results show how the mass transfer contribution increases for high polymers as the diffusion coefficient for the solute in the stationary phase falls. These data are extremely useful in determining the optimum experimental conditions for high-speed and high-resolution GPC separations of polymers.

EXPERIMENTAL

Spherisorb silica S.20.W was supplied by Phase Separations (Queensferry, Great Britain) and three laboratory-prepared macroporous silicas designated H2, H4 and H6 were kindly provided by Dr. J. D. F. Ramsay of AERE Harwell. The silica particles were examined by scanning electron microscopy, and the particle size distribution was determined by Coulter Counter by the method described previously[17]. The silica microspheres were slurry-packed into individual 316 grade seamless stainless-steel columns (20 cm × 0.3 cm I.D.) with a balanced density of tetrabromoethane and tetrachloroethylene, as in the technique described by Majors[18]. The

packing pressures were 1800, 500 1000 and 750 p.s.i. (1 p.s.i. ≡ 6894.8 N/m^2) for S.20.W, H2, H4 and H6 columns respectively.

HPGPC separations were performed with a Perkin-Elmer Model 1220 positive displacement syringe pump (flow settings 0.05–6.00 cm^3 min^{-1}, ⩽ 3000 p.s.i., 500 cm^3 capacity). In order to ensure reproducible constant flow of the eluent, the pump was initially run at a high flow-rate until the operating pressure was achieved, and the flow-rate was then reduced to that required[19]. With the pumping system at equilibrium, the retention volume of a solute V_R was calculated from the travel of the recorder chart paper. An Applied Research Labs. ultraviolet detector (254 nm, cell volume 8 μl) was used to detect the solute in the eluent. A steady baseline free of noise and drift was obtained with the detector on maximum sensitivity. The connecting tubing between column and detector was shortened in order to minimise dead volume. A detailed description of the instrumentation requirements for HPGPC separations of polymers is given elsewhere[19,20]. Syringe injection (2 μl) through a septum into the top of a column was used for toluene (AnalaR), tetraphenylethylene (TPE) (Aldrich, Milwaukee, Wisc., U.S.A.), and polystyrene standards (Waters Assoc., Milford, Mass., U.S.A. and Pressure Chemical, Pittsburgh, Pa., U.S.A.). The GPC eluent was tetrahydrofuran (BDH, Poole, Great Britain) which was destabilised, stored over calcium hydride for 4 h, distilled from calcium hydride, and degassed by stirring under vacuum before use. Each injected solution had a solute concentration of 0.2% (w/v), except for TPE which was 0.01% (w/v). The injection procedure involved pushing the syringe needle through a porous Teflon disc so that the needle touched the centre of a stainless-steel mesh at the top of the silica packings, as described by Webber and McKerrell[21].

Plate height, H, for each solute was calculated from

$$H = L/N \tag{1}$$

where L is the column length and N is the plate number which was determined from an experimental chromatogram with the relation

$$N = 5.54\,(V_R/w_{0.5})^2 \tag{2}$$

where $w_{0.5}$ is the width of the chromatogram at half its height. Eqn. 2 assumes a symmetrical chromatogram corresponding to a normal error (or Gaussian) function. All results for H were the average of at least three injections.

THEORY

In order to interpret the experimental results for H, the dependence of H on the solute dispersion mechanisms contributing to chromatogram broadening is required. In addition, the value of H determined from an experimental chromatogram for a permeating polymer will contain a contribution from polydispersity, and an approach for relating the experimental and true values of H has been discussed[22]. Here, the expression for H considered in an earlier paper[19] will be extended to show how the true polydispersity may be derived from an experimental chromatogram. The experimental H value is given by

$$H = A + (B/u) + Cu + (\sigma_M^2/L) \tag{3}$$

in which A, B and C are coefficients depending on several parameters (see later), where term I (A) is the eddy diffusion term for solute dispersion in the mobile phase, term II (B/u) results from dispersion owing to molecular diffusion in the longitudinal direction in the mobile phase, term III (Cu) results from solute dispersion owing to mass transfer, and the fourth term contains the standard deviation σ_M for the true molecular weight distribution. For this last term by analogy with definitions for H [20], we may define σ_M (in units of length) in terms of σ_V (in units of retention volume) with

$$(\sigma_M^2/L) = (L\,\sigma_V^2/V_R^2) \tag{4}$$

where σ_V represents a contribution to the experimental chromatogram.

If it is assumed that the true molecular weight distribution of the polystyrene standards may be represented by a logarithmic normal distribution, then for a permeating polymer the true polydispersity defined as the ratio of the weight average and number average molecular weights $[\overline{M}_w/\overline{M}_n]_T$ may be calculated from

$$\ln[\overline{M}_w/\overline{M}_n]_T = \sigma_D^2 \tag{5}$$

where σ_D is the standard deviation in terms of ln molecular weight[23]. Because the experimental chromatograms for polystyrene standards are almost symmetrical and because the GPC separation gives an almost linear calibration plot of log molecular weight *versus* V_R over the permeation range, the polydispersity may be calculated from σ_V with the relation

$$\ln[\overline{M}_w/\overline{M}_n]_T = \sigma_V^2 D_2^2 \tag{6}$$

where D_2 is the slope of the GPC calibration relation between ln molecular weight and V_R. With eqns. 4 and 6 we can show that eqn. 3 gives

$$H = A + (B/u) + Cu + (L \ln[\overline{M}_w/\overline{M}_n]_T/D_2^2\,V_R^2) \tag{7}$$

Since results for permeating and non-permeating solutes as a function of polymer size will be considered, the coefficients A, B and C may be written out in full[1], giving

$$H = 2\lambda d_p + (2\gamma D_m/u) + [q\,R(1-R)d_p^2\,u/D_m] + (L \ln[\overline{M}_w/\overline{M}_n]_T/D_2^2\,V_R^2) \tag{8}$$

where λ (close to unity) is a constant characteristic of the packing, γ is a tortuosity factor, q is a configuration factor, which depends on the shape of the pores in the stationary phase, and R is the retention ratio, defined here for each solute by V_0/V_R where V_0 is the interstitial (or void) volume of the column which may be found with a non-permeating polymer.

RESULTS AND DISCUSSION

The micrographs in Fig. 1 illustrate the regularity of the silica microspheres (particle diameter $\approx 20\ \mu$m). From the particle size distribution, mean particle diameters were calculated with the equations given elsewhere[17] and are shown in Table I. These results confirm the narrow microsphere size distribution for the commercial S.20.W silica, as stated previously[15]. The laboratory-prepared silicas tend to

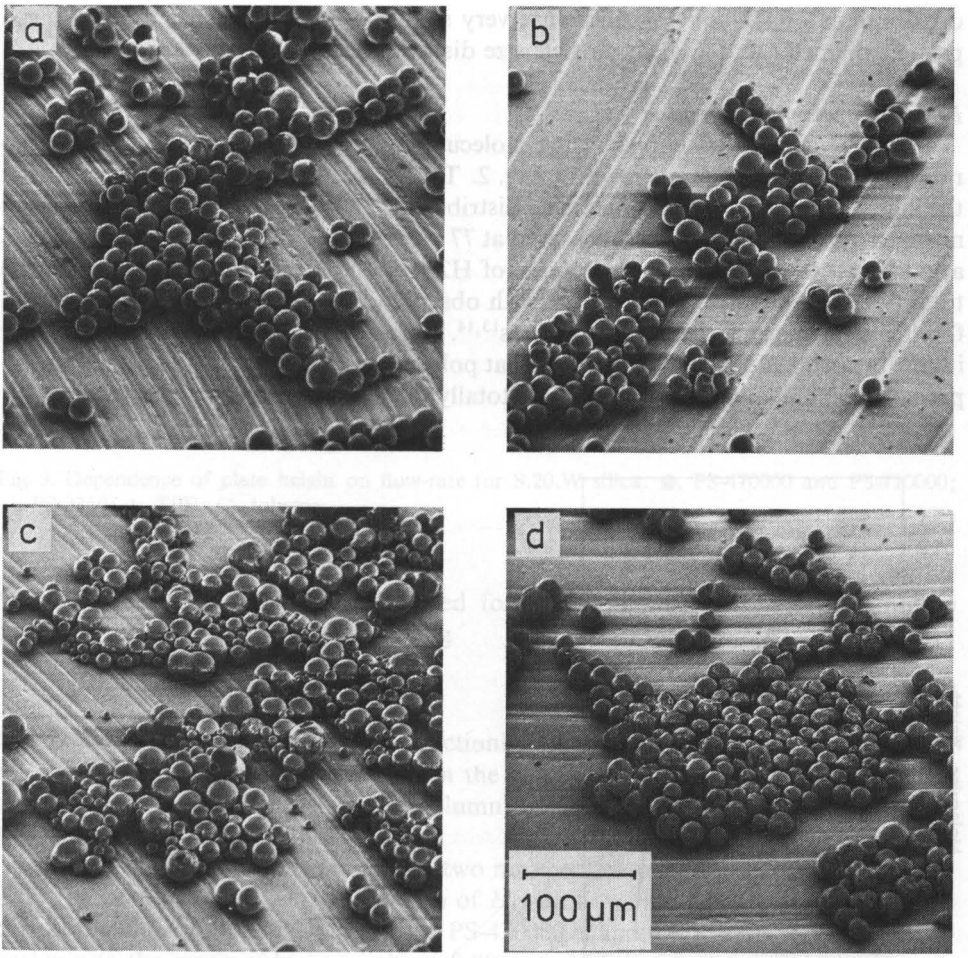


Fig. 1. Scanning electron micrographs of silicas: (a) S.20.W; (b) H4; (c) H6; (d) H2.

TABLE I

NUMBER AVERAGE AND WEIGHT AVERAGE PARTICLE DIAMETERS s_n AND s_w AND POLYDISPERSITY OF SILICA PARTICLES

Silica	s_n (μm)	s^* (μm)	s_w (μm)	s/s_n	s_w/s_n
S.20.W	16.96	17.54	18.78	1.04	1.11
H2	13.92	15.00	16.98	1.08	1.22
H4	12.76	13.74	16.59	1.08	1.30
H6	8.50	10.11	14.17	1.19	1.67

* Defined as the ratio of the second and first moments (see ref. 17).

be less regular with a wider particle size distribution. The mean particle size and width of the particle size distribution for H2 silica are similar to the characteristics of a fraction (10–15 μm) of cross-linked polystyrene gel particles[17]. The particle size

distributions of H2 and H4 silicas are very similar but H6 silica has a lower mean particle diameter and a wider particle size distribution.

Molecular weight calibration

GPC calibration curves of log molecular weight *versus* V_R at an eluent flow-rate of 0.2 cm^3 min^{-1} are shown in Fig. 2. The shapes of these curves suggest that the silicas have very narrow pore size distributions, a conclusion also reached from nitrogen adsorption isotherms measured at 77 °K (refs. 13, 14). The calibration curves also suggest that the total pore volume of H2, H4 and H6 silicas is about twice the total pore volume of S.20.W, in line with observations on adsorption isotherm data for the macroporous silica microspheres[13,14]. From the calibration curves in Fig. 2, it may be assumed for all the packings that polystyrene standard PS-1987000 is a non-permeating solute and that toluene is a totally permeating solute.

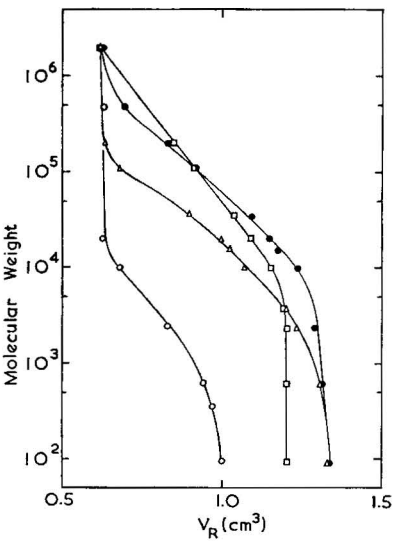


Fig. 2. GPC calibration curves for polystyrene standards, TPE and toluene; ○, S.20.W silica; △, H4 silica; ●, H6 silica; □, H2 silica.

Silica S.20.W

Curves showing the dependence of H on eluent flow-rate for S.20.W silica are given in Fig. 3. Values of H for toluene, TPE and polystyrene standard PS-110000 are about a factor of ten lower than the values reported in an earlier paper[15]. This results from the improved injection procedure, following Webber and McKerrell[21], in which the solution is injected at the central point of the column, and from the reliable slurry-packing procedure. The solute band will diverge as it progresses down the column, and with the best choice of column diameter d_c and L, the solute molecules may never reach the column wall before leaving the column. Since substantial chromatogram broadening may result from solute dispersion at the column wall, the use of "infinite diameter" columns has been advocated in liquid chromatography[24–26].

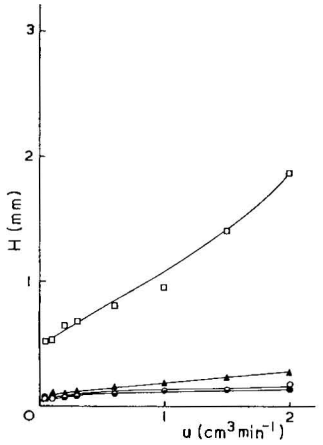


Fig. 3. Dependence of plate height on flow-rate for S.20.W silica: ●, PS-470000 and PS-110000; □, PS-2350; ▲, TPE; ○, toluene.

Several expressions have been proposed for predicting the onset of the "infinite diameter" condition, one criterion being

$$(0.4\, d_c/d_p) > (L/d_c) \tag{9}$$

Our columns are therefore close to functioning in the "infinite diameter" mode and the divergence of the solute band down the column will decrease for larger solutes and faster flow-rates. Our choice of column dimensions was mainly determined by the limited quantities of silica samples.

In Fig. 3, it is observed that the two non-permeating polystyrenes having low diffusion coefficients give little variation of H with flow-rate. For $u > 0.3$ cm^3 min^{-1} the mean value of H for PS-110000 and PS-470000 is about 0.13 mm and for $u < 0.3$ cm^3 min^{-1} the mean value was about 0.08 mm. Values of H for PS-470000 were always greater than values for PS-110000. TPE which permeates slightly less pore volume than toluene (Fig. 2), gives a much more significant rise in H as flow-rate increases than the non-permeating solutes. These observations are in agreement with the general conclusions previously reported[15]. These results may be interpreted in terms of eqns. 3, 7 and 8. For high-molecular-weight polymers with low values of D_m, term II may be neglected as discussed elsewhere[19]. For non-permeating polymers, term III and the polydispersity term do not arise, and hence H should be a constant independent of flow-rate as observed in Fig. 3. For toluene, there is no increase in H as flow-rate decreases, and term II may be neglected. The polydispersity term does not arise for toluene, and so the dependence of H for toluene on flow-rate suggests that the term for solute mass transfer between the mobile and stationary phases is not very significant. This is explained by the high value of D_m for toluene in term III in eqn. 8. Kelley and Billmeyer[4,5] have proposed that additional solute dispersion in the mobile phase may arise from a non-uniform flow velocity profile across a column, and they interpreted the much higher H values for non-permeating polystyrene than for a permeating solute as evidence for a velocity profile effect. In Fig. 3, the H values

for toluene and the two polystyrenes are very similar, suggesting that the velocity profile effect is reduced considerably. A non-uniform flow velocity effect across a column is expected with particles having a wide size range, because of variable resistance to fluid flow across a column. It is evident in Fig. 1 and Table I that S.20.W silica particles are very regular and have a narrow size distribution. Provided the column is packed carefully, a homogeneous bed of particles should result. The results for toluene and non-permeating polystyrenes with S.20.W silica are similar to H reported for cross-linked polystyrene gel particles (16–20 μm) having a spherical shape and a narrow particle size distribution, see Fig. 6 in ref. 17. All these results suggest that optimum performance requires not only a low value of d_p but a narrow particle size distribution. Small differences between H values for silica and cross-linked polystyrene gels may arise from a contribution to H from solute adsorption onto the pore surface, and Giddings *et al.*[6] have identified a small adsorption contribution for solutes in dichloroethane separating on porous glass. This effect has not been considered in eqn. 8.

In Fig. 3, the divergence of the curves for permeating solutes as flow-rate rises suggests a dependence of permeation dispersion on solute diffusion coefficient. The slope of the curve for PS-2350 is about eight times greater than the slope for TPE. This may be interpreted in terms of the mass transfer dispersion term in eqn. 8. Values for $R(1-R)$ may be calculated from Fig. 2 and are 0.19 and 0.23 for PS-2350 and TPE respectively. The diffusion coefficients, calculated as described elsewhere[19], are $3.72 \cdot 10^{-6}$ and $1.27 \cdot 10^{-5}$ cm^2 sec^{-1} respectively. Therefore, for permeating solutes the slope of a plot of H versus u increases as solute diffusion coefficient decreases, as observed previously for polystyrene standards covering a wide molecular weight range[19].

The vertical displacement of the curve for PS-2350 with respect to the curves for toluene and TPE results from the polydispersity term in eqn. 8. This term may be evaluated approximately by considering that at very low eluent flow-rates mobile phase dispersion is the major contributor to chromatogram broadening. Since the results suggest that term II is unimportant in our experiments and that the H curves for toluene and the non-permeating polystyrene standards are very close together in Fig. 3, we propose that the plate height for toluene H_t could represent the solute dispersion terms in eqn. 8 at the lowest practical flow-rate of 0.05 cm^3 min^{-1}. Consequently, we may assume that H for a permeating polystyrene standard H_{PS} at the same flow-rate of 0.05 cm^3 min^{-1} is given approximately by

$$H_{PS} = H_t + (L \ln[\overline{M}_w/\overline{M}_n]_T/D_2^2 V_R^2) \quad (10)$$

in which the second term on the right hand side is for the permeating polystyrene standard. The results in Fig. 3 suggest that term III for toluene at very low flow-rates is extremely small. For a permeating polystyrene term III will be larger because of the lower value of D_m, but provided mass transfer dispersion is less important than mobile phase dispersion at very low flow-rates, the use of toluene data for H_t will not be too unreasonable. From the difference between the H_{PS} and H_t values at the lowest flow-rate of 0.05 cm^3 min^{-1} in Fig. 3, a value of $[\overline{M}_w/\overline{M}_n]_T = 1.15$ was evaluated for PS-2350 and is given in Table II. Values of D_2 and V_R in this procedure were obtained from Fig. 2. The calculated true polydispersity is not unreasonable con-

TABLE II

POLYDISPERSITIES EVALUATED FROM PLATE HEIGHT DATA

Eluent flow-rate, 0.05 cm^3 min^{-1}

Column	*Polystyrene standard*	H_{PS} *(mm)*	H_t *(mm)*	$[\bar{M}_w/\bar{M}_n]_T$
S.20.W	PS-2350	0.52	0.062	1.16
H4	PS-9800	0.31	0.070	1.08
H4	PS-35000	0.28	0.070	1.05
H6	PS-35000	0.24	0.110	1.02
H2	PS-35000	0.12	0.110	1.00
H2	PS-200000	0.31	0.110	1.06

sidering the assumptions involved. For a polystyrene prepared by "living" anionic polymerisation, the theoretical value of $[\bar{M}_w/\bar{M}_n]_T$ for a sample of molecular weight 2350 is 1.04 (ref. 23), and a somewhat higher practical value might be expected because of the rigorous conditions required in the experimental polymerisation technique.

Silicas H4, H6 and H2

Curves showing the dependence of H on eluent flow-rate are shown in Figs. 4–6. It is observed that the non-permeating PS-1987000 and the totally permeating toluene give little or no variation of H with flow-rate, and therefore the explanations discussed for S.20.W silica also hold for the macroporous silicas. It was reported previously that plate numbers for H4 and H2 silicas were lower than for S.20.W silica[16], and this is supported by the plots in Figs. 4–6 which lie at higher values of H for toluene and PS-1987000 than the curves in Fig. 3. Since the value of H for the non-permeating polystyrene in Figs. 4–6 is higher than in Fig. 3, we may conclude from eqn. 8 that the columns of H4, H6 and H2 silicas have higher mobile phase dispersion than the S.20.W column. This follows because we may neglect, as previ-

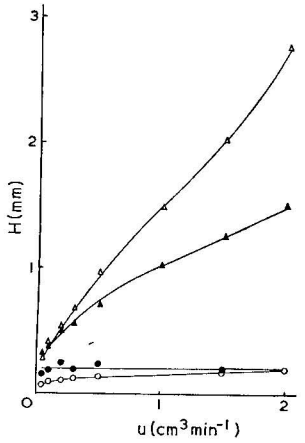
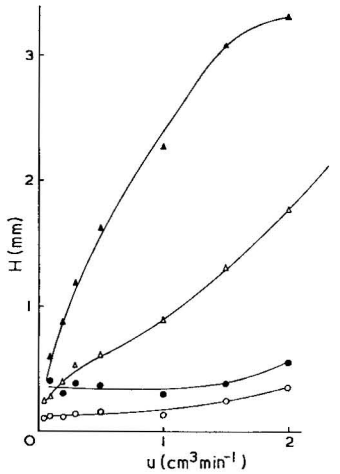


Fig. 4. Dependence of plate height on flow-rate for H4 silica: ●, PS-1987000; △, PS-35000; ▲, PS-9800; ○, toluene.



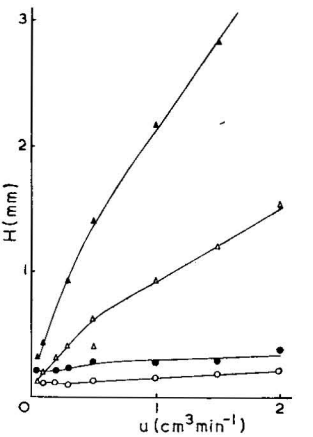


Fig. 5. Dependence of plate height on flow-rate for H6 silica: ●, PS-1987000; ▲, PS-200000; △, PS-35000; ○, toluene.

Fig. 6. Dependence of plate height on flow-rate for H2 silica: ●, PS-1987000; ▲, PS-200000; △, PS-35000; ○, toluene.

ously discussed, terms II and III and the polydispersity term when considering eqns. 3, 7 and 8 for a non-permeating polymer. This increase in mobile phase dispersion for H4, H6 and H2 silicas may be explained by the less regular particles (Fig. 1) and the wider particle size distributions (Table I) giving a heterogeneous bed of particles so that multiple non-uniform flow-paths are generated. The values of H for toluene and PS-1987000 with the H6 silica column are somewhat higher than the values for the H4 and H2 silicas, as expected from the high value of s_w/s_n in Table I. The increase in H because of the wide particle size distribution of silica H6 will tend to be balanced by a decrease in H due to the lower value of d_p.

In Fig. 2, it appears that the H6 and H2 silicas separate a similar range of molecular sizes, although the silicas may have somewhat different pore size distributions. The dependence of H on flow-rate for the permeating polystyrenes PS-35000 and PS-200000 is similar for these two silicas in Figs. 5 and 6 suggesting that the mass transfer characteristics of the two columns are the same. The slopes of these curves at $u \leqslant 1.5$ cm^3 min^{-1} again show that H increases on permeation and that permeation dispersion increases for macromolecules with lower diffusion coefficients. At the lowest flow-rate of 0.05 cm^3 min^{-1} in Figs. 5 and 6, H for PS-35000 is lower than H for PS-1987000 and not much larger than H for toluene. If the difference between H for PS-35000 and toluene is assumed to be the polydispersity term in eqns. 3, 7 and 8, then $[\overline{M}_w/\overline{M}_n]_T$ may be calculated for PS-35000 with eqn. 10. Values calculated from Figs. 5 and 6 are shown in Table II. The same procedure was used to calculate $[\overline{M}_w/\overline{M}_n]_T$ for PS-9800 and PS-200000 from Figs. 4 and 6 respectively. Fig. 6 was preferred for PS-200000 since the calibration curves in Fig. 2 suggest that H2 silica gives a better resolution of the high-molecular-weight chains and therefore a more reliable chromatogram. The average of the values of $[\overline{M}_w/\overline{M}_n]_T$ for PS-35000 in Table II may be regarded as acceptable. An even lower value of the true polydispersity

is expected for columns with smaller particles[19]. Although H_{PS} values for PS-35000 in Table II are similar at the lowest practical flow-rate of 0.05 cm^3 min^{-1} for the three silica columns, the slope of the curve of H *versus* u in Fig. 4 is higher than for H6 and H2 silicas. This cannot be explained by the value of $R(1-R)$ and may possibly be explained by differences in the internal pore structure between H4 silica and the H6 and H2 silicas. Another possibility is that D_m for PS-35000 is lower for H4 silica than for H6 and H2 silicas, since PS-35000 elutes closer to the exclusion limit with H4 silica so that there may be steric restriction as the polymer diffuses in a tight pore. We have assumed that D_m is for a polymer in free solution, but the diffusion coefficient may change depending on pore geometry and size when a polymer enters the stationary phase.

The displacement of H for PS-1987000 with respect to H for toluene in Figs. 4–6 might suggest that mobile phase dispersion is polymer size dependent, since the curves in Fig. 3 for toluene and the non-permeating polystyrenes are very close together. However, the higher values for PS-1987000 are likely to result from the practical difficulties associated with ultra-high-molecular-weight polymers which may have additional chromatogram broadening owing to experimental concentration, viscosity and degradation effects. Since the permeating polystyrenes in Figs. 4–6 have molecular weights similar to or below the molecular weights of the non-permeating polystyrenes in Fig. 3, it appears reasonable to use H data for toluene in eqn. 10 for the permeating polystyrenes in Figs. 4–6.

The values of the true polydispersity in Table II were calculated from H data with eqn. 10. This equation involves several assumptions and the determination of experimental values of H involves the standard deviation σ of the chromatogram. A much more reliable evaluation of polydispersity results from the use of all the chromatogram, as in the computer program of Pickett *et al.*[27]. This experimental polydispersity $[\overline{M}_w/\overline{M}_n]$ may be related to $[\overline{M}_w/\overline{M}_n]_T$ if it is assumed that the chromatogram and the molecular weight distribution are represented approximately by a logarithmic normal function. The experimental value of H is given by σ^2/L (ref. 1). It follows from eqns. 4, 5 and 6 that eqn. 8 may be transformed to

$$\ln[\overline{M}_w/\overline{M}_n] = \frac{D_2^2 V_R^2}{L}\{2\lambda d_p + (2\gamma D_m/u) + [qR(1-R)d_p^2 u/D_m]\} + \ln[\overline{M}_w/\overline{M}_n]_T \quad (11)$$

It follows from eqn. 10 that the chromatogram broadening terms in eqn. 11 may be evaluated from experimental H data for toluene at low u. Consequently, $[\overline{M}_w/\overline{M}_n]_T$ may be calculated from $[\overline{M}_w/\overline{M}_n]$ determined experimentally from the chromatogram of a permeating polymer at low u. The drawback of this procedure is that even at low flow-rates the mass-transfer term for a permeating polymer will be somewhat higher than for toluene. Therefore, estimates of $[\overline{M}_w/\overline{M}_n]_T$ are likely to be too high.

CONCLUSIONS

The similarity of H values for non-permeating polystyrenes and toluene suggests that mobile phase dispersion is the major cause of chromatogram broadening for small molecules. Because mass transfer dispersion is quite low for small molecules, high-resolution GPC separations of oligomers and low polymers may be performed

at fast flow-rates. High-performance will not, however, be obtained from microparticulate packings if the particles are not regular and do not have a narrow size distribution. Mass transfer dispersion becomes much more important for high polymers and increases as polymer diffusion coefficient decreases and therefore as molecular size increases. Consequently, extensive chromatogram broadening will occur for permeating high polymers at fast flow-rates. Efficient separations of high polymers, giving H similar to those of small molecules, are only obtained at extremely low flow-rates, for example below 0.1 $cm^3\ min^{-1}$. Mass transfer dispersion is then much reduced, and it is possible to calculate approximately the polydispersity of a permeating polystyrene standard from its H value and the H for toluene which is close to the H value arising from mobile phase dispersion determined with a non-permeating polystyrene standard. For the single columns investigated, separations at a low flow-rate of 0.05 $cm^3\ min^{-1}$ may be completed in under 30 min. For routine work four columns in series with samples of S.20.W, H4, H6 and H2 silicas in each column, or a single column with length 80 cm containing all four silicas, would be necessary, giving efficient separations for permeating high polymers in under 2 h. This separation time is not unreasonable, and accurate determinations of molecular weight distribution and polydispersity will then be obtained at the extremely low flow-rate. The main practical disadvantage is the determination of retention volume for short narrow columns, but the precision may be optimised with an accurately controlled constant-flow pump. Clearly, separations for a single column may be performed in about 1 min and for a series arrangement of four columns in under 5 min, but the chromatogram broadening for high polymers is extensive, giving inaccurate values for polydispersity.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. J. D. F. Ramsay for supplying the macroporous inorganic silicas and gratefully acknowledge helpful discussions with Dr. R. L. Nelson, Dr. J. D. F. Ramsay, Dr. D. C. Sammon and Mr. M. J. Holdoway of AERE Harwell. Mr. D. Pinder, Department of Chemical Engineering, and Mr. T. Stone are thanked for advice and assistance with Coulter Counter measurements and determinations of particle size distribution, and Mr. M. Hayles is thanked for the scanning electron micrographs. This research formed part of a programme funded by a grant from the Science Research Council in collaboration with the SRC supported Polymer Supply and Characterisation Centre at RAPRA, Shawbury, Shrewsbury, Great Britain. The authors thank Mr. L. J. Maisey for advice on column packing performed at the Polymer Supply and Characterisation Centre.

REFERENCES

1 J. C. Giddings, *Dynamics of Chromatography, Part I, Principles and Theory*, Marcel Dekker, New York, 1965.
2 J. Čoupek and W. Heitz, *Makromol. Chem.*, 112 (1968) 286.
3 W. Heitz and J. Čoupek, *J. Chromatogr.*, 36 (1968) 290.
4 F. W. Billmeyer, Jr. and R. N. Kelley, *J. Chromatogr.*, 34 (1968) 322.
5 R. N. Kelley and F. W. Billmeyer, *Anal. Chem.*, 41 (1969) 874; *Anal. Chem.*, 42 (1970) 399; *Separ. Sci.*, 5 (1970) 291.

6 J. C. Giddings, L. M. Bowman and M. N. Myers, *Macromolecules*, 10 (1977) 443.
7 R. E. Majors, *J. Chromatogr. Sci.*, 15 (1977) 334.
8 J. J. Kirkland, *J. Chromatogr., Sci.*, 10 (1972) 593.
9 J. J. Kirkland, *J. Chromatogr.*, 125 (1976) 231.
10 K. K. Unger, R. Kern, M. C. Ninou and K. F. Krebs, *J. Chromatogr.*, 99 (1974) 435.
11 K. Unger and R. Kern, *J. Chromatogr.*, 122 (1976) 345.
12 R. V. Vivilecchia, B. G. Lightbody, N. Z. Thimot and H. M. Quinn, *J. Chromatogr. Sci.*, 15 (1977) 424.
13 J. D. F. Ramsay, in R. Epton (Editor), *Chromatography of Synthetic and Biological Polymers, Vol. 1, Column Packings, GPC, GF, and Gradient Elution*, Ellis Horwood, Chichester, 1978, p. 339.
14 M. J. Holdoway, D. F. Rush and D. C. Sammon, *Paper presented at the 3rd International Symposium on Column Liquid Chromatography, Salzburg, September 1977.*
15 J. V. Dawkins and G. Taylor, *Polymer*, 15 (1974) 687.
16 J. V. Dawkins and G. Yeadon, *Polym. Preprints*, 18, No. 2 (1977) 227.
17 J. V. Dawkins, T. Stone and G. Yeadon, *Polymer*, 18 (1977) 1179.
18 R. E. Majors, *Anal. Chem.*, 44 (1972) 1722.
19 J. V. Dawkins and G. Yeadon, *Polymer*, 20 (1979) 981.
20 J. V. Dawkins and G. Yeadon, in J. V. Dawkins (Editor), *Developments in Polymer Characterisation—1*, Applied Science Publ., London, 1978, Ch. 3.
21 T. J. N. Webber and E. H. McKerrell, *J. Chromatogr.*, 122 (1976) 243.
22 J. H. Knox and F. McLennan, *Chromatographia*, 10 (1977) 75.
23 L. H. Peebles, *Molecular Weight Distributions in Polymers*, Wiley-Interscience, New York, 1977.
24 J. H. Knox, *Ann. Rev. Phys. Chem.*, 24 (1973) 29.
25 J. H. Knox and N. A. Parcher, *Anal. Chem.*, 41 (1969) 1599.
26 J. H. Knox, G. R. Laird and P. A. Raven, *J. Chromatogr.*, 122 (1976) 129
27 H. E. Pickett, M. J. R. Cantow and J. F. Johnson, *J. Appl. Polym. Sci.*, 10 (1966) 917.

Journal of Chromatography, 188 (1980) 347–356

CHROM. 12,408

MUTUAL DISPLACEMENT INTERACTIONS IN THE BINDING OF TWO DRUGS TO HUMAN SERUM ALBUMIN BY FRONTAL AFFINITY CHROMATOGRAPHY

NAOMI I. NAKANO, YOSHIMITSU SHIMAMORI and SHIGENORI YAMAGUCHI
Hokkaido Institute of Pharmaceutical Sciences, Otaru 047-02 (Japan)
(Received September 18th, 1979)

SUMMARY

A continuous frontal analysis chromatographic method was developed for studying the simultaneous binding of two drugs or ligands with an immobilized macromolecule. The usefulness of this method was demonstrated in the interactions of sulphamethizole and salicylic acid with human serum albumin (HSA). The mutual inhibitory effect on the binding of one drug of the presence of the other was directly shown to be due to displacement of the bound drug from HSA by the other. On the basis of a double-reciprocal plot analysis, these two drugs are interpreted as competing for the same primary binding sites.

INTRODUCTION

Of the many causes of drug interactions, the interaction thought to be caused by displacement of one drug from serum albumin by another is recognized to be clinically important in multiple dose therapy[1]. Because the concentrations of the free or unbound fraction of two drugs administered concurrently may increase by mutual displacement from albumin over those when administered alone, the consequences of this interaction can affect many aspects of drug disposition and lead to enhanced pharmacological and toxicological responses. One of the well known examples of such an interaction is that between phenylbutazone and warfarin[2,3]. A marked argumentation of hypothrombinaemia and fatal haemorrhages were reported when phenylbutazone was coadministered with warfarin[4].

The displacement interactions are interpreted as either competitive or non-competitive[5]. In competitive displacement, two drugs are considered to share the same binding sites, whereas in non-competitive displacement the binding of the displacing drug takes place at sites other than those of the displaced drug and induces structural changes in the tertiary conformation of albumin (also called allosteric mechanism[6]). Such a distinction is often made on the basis of the Scatchard plot or double-reciprocal plot and the indication of an equal number of binding sites is taken as a criterion for competitive binding or displacement[7–10]. The limitation of this approach, unless the experimental procedures permit the concentrations of the free

displacing drug to be constant, has been fully discussed[11,12]. For instance, in subtractive methods[13] such as equilibrium dialysis, it is difficult to keep the concentrations of the free displacing drug constant and experiments have to be carried out in the presence of excess amounts of the displacing drug so that this drug will not be displaced to any significant extent by the original drug. Under these conditions, however, effect of the original drug on the interaction of the displacing drug could not be observed. Only a few experimental procedures which permit the concentration of displacing drug to be set at a pre-determined value have been utilized to study the binding of two drugs with albumin. These include a continuous ultrafiltration technique[14], flow equilibrium dialysis[15] and Hummel and Dreyer's gel filtration technique[16].

We have previously demonstrated[17–19] the use of immobilized bovine and human serum albumin in the quantitative studies of serum albumin–single drug interactions by a frontal affinity chromatographic procedure and observed that the binding capacity of the albumins was retained on immobilization when a six-carbon-atom spacer was introduced between the albumin molecule and gel matrix. This study extends this technique to the interactions of two drugs with human serum albumin (HSA). The method allows a simple and direct manifestation of the mutual displacement phenomena and also facilitates the quantitative treatment of binding data of two-drug interactions by the conventional double-reciprocal plot. This is because in the present method the concentrations of the free fraction of both drugs are set at pre-determined values and the amounts of drugs bound are simultaneously determined for the two drugs.

As one of two drugs we selected salicylic acid, as its binding to serum albumin *in vitro* has been extensively studied and it is reported to be displaced by many drugs[20–22], including sulphonamides, sulphonylureas, warfarin[12] and indomethacin[15]. Further, when aspirin is administered, salicylic acid is expected to be present in plasma owing to its rapid hydrolysis. Sulphamethizole, a urinary tract antiseptic, was also chosen because of the possibility of its co-administration with aspirin and its analytical convenience.

EXPERIMENTAL

Materials

Human serum albumin (HSA) was purchased from Sigma (St. Louis, Mo., U.S.A.) (Fraction V, essentially fatty acid free, lot no. 76C-7480). It was fractionated in 0.1 *M* sodium chloride solution on Sephadex G-200 obtained from Pharmacia (Uppsala, Sweden) as described elsewhere[19]. The monomer fraction which contained over 93% of monomer was used for coupling to agarose beads. Activated CH-Sepharose 4B was purchased from Pharmacia.

Salicylic acid from Wako (Osaka, Japan) was recrystallized from hot water. Sulphamethizole from Eizai (Tokyo, Japan) was recrystallized from a mixture of water and methanol. All other chemicals were of reagent grade. Water was deionized and doubly distilled, with the second distillation performed in an all-glass apparatus.

Affinity columns

The monomeric HSA was coupled to activated CH-Sepharose 4B at pH 8

according to the procedure recommended by the manufacturer by reacting them for 1 h at room temperature[19]. The HSA-coupled gel was packed into a Pharmacia column (K16/20) with flow adaptors so that the direction of flow could be varied. Usually upwards elution was carried out by means of Pharmacia Model P-3 peristaltic pump at a rate of about 10 ml/h. The temperature of the gel was maintained at 4° with water circulated through the jacket of the column by a Haake Model FK 10 constant-temperature circulator.

Determination of drugs bound by frontal affinity chromatography for two-drug interactions

All binding experiments were carried out in 0.05 *M* Tris–hydrochloric acid buffer containing 0.1 *M* sodium chloride, ionic strength 0.142. The pH of this buffer was 7.40 ± 0.01 at 20° and 7.87 ± 0.01 at 4°. When the columns were not in use they were constantly washed with the above buffer containing 0.01 % of sodium azide. Prior to use they were pre-equilibrated with the buffer for at least 16 h.

Three types of frontal analyses (I–III) were performed and the eluate was collected in 4.4–6.1-ml fractions by means of a LKB Model 2112 Redirack fraction collector until the concentrations of both drugs applied to the column reached those of the applied solution (see Fig. 1).

In Type I (simultaneous application), a solution containing both salicylic acid and sulphamethizole at known concentrations was applied to the column. In Type II (displacement of sulphamethizole by salicylic acid), a solution containing sulphamethizole at a known concentration was applied to the column followed by a solution containing sulphamethizole at the same concentration as that of the preceding solution and salicylic acid at a known concentration. In Type III (displacement of salicylic acid by sulphamethizole), the same procedure as Type II was used except that a salicylic acid solution was applied first followed by a solution containing both.

In each instance the concentrations of sulphamethizole and salicylic acid in the eluate were determined by dual-wavelength spectrophotometry on a Shimadzu Model UV-300 double-beam spectrophotometer using either 2- or 10-mm path length cells, depending on the concentrations of the two drugs. For sulphamethizole the two wavelengths were 260.0 and 323.0 nm, and for salicylic acid 230.0 and 295.8 nm.

The amounts of drug X bound, $(D_b)_x$, by the above three procedures are indicated in Fig. 1; the areas were determined by planimetry. For S-shaped elution patterns, $(D_b)_x$ was also determined by the formula $(V - V_0) \cdot (D_f)_x$, where $(D_f)_x$ is the concentration of drug X applied to the column, V is the elution volume of drug X determined as the volume of eluate corresponding to the concentration that reached 50% of that of the applied solution and V_0 is the void volume of the column, which is the sum of the volume of gel bed available for eluting solutions and that of the tubing. The void volume was determined for each column by eluting the column with 0.1% sodium azide solution[19]. The elution volume of this solution agreed to within about 2% with the calculated value obtained by measuring the volume of gel bed and internal volume of tubing.

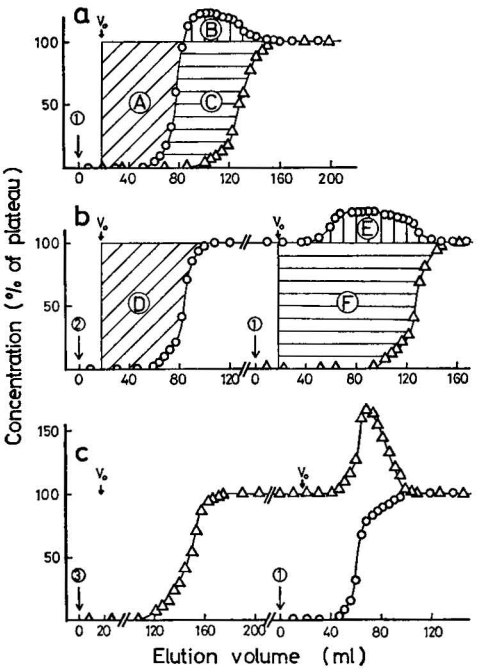


Fig. 1. Frontal analysis diagrams to illustrate mutual displacement interactions in the simultaneous binding of sulphamethizole and salicylic acid to immobilized HSA in 0.05 *M* Tris buffer containing 0.1 *M* NaCl (pH 7.87 at 4°). (a) Type I analysis. Solution 1, containing sulphamethizole ($2.94 \cdot 10^{-5}$ *M*) and salicylic acid ($2.17 \cdot 10^{-5}$ *M*), was applied to column I (see Table I for details). (b) Type II analysis. Solution 2, containing sulphamethizole ($2.94 \cdot 10^{-5}$ *M*), was applied to the column, followed by solution 1. (c) Type III analysis. Solution 3, containing salicylic acid ($2.17 \cdot 10^{-5}$ *M*), was applied to the column, followed by solution 1. ○, Elution of sulphamethizole; △, elution of salicylic acid. The amounts of sulphamethizole and salicylic acid bound were determined as follows: for Type I analysis, $(D_b)_{SMZ} = (A - B) \cdot 10^{-5} \cdot (D_f)_{SMZ}$ and $(D_b)_{SA} = (A + C) \cdot 10^{-5} \cdot (D_f)_{SA}$; for Type II analysis, $(D_b)_{SMZ} = (D - E) \cdot 10^{-5} \cdot (D_f)_{SMZ}$ and $(D_b)_{SA} = F \cdot 10^{-5} (D_f)_{SA}$; for Type III analysis, analogous to Type II analysis, where A – E denote the areas indicated and V_0 is the void volume of the column.

Continuous frontal analyses

After the column had been equilibrated with an applied solution, instead of completely washing the column with buffer to release all of the drugs bound, continuous frontal analysis was carried out by combining Types II and III.

Treatment of data

When the binding of drug 1 is inhibited by the presence of drug 2 by competitive binding to a single class of *n* binding sites, the number of moles of drugs 1 and 2 bound per mole of albumin are given by eqns. 1 and 2, respectively[23]:

$$r_1 = \frac{n K_1 (D_f)_1}{1 + K_1 (D_f)_1 + K_2' (D_f)_2} \quad (1)$$

$$r_2 = \frac{n K_2 (D_f)_2}{1 + K_1' (D_f)_1 + K_2 (D_f)_2} \quad (2)$$

where K_1 and K_2 are the binding constants of drugs 1 and 2, respectively, K_1' and K_2' are the inhibition constants of drugs 1 and 2, respectively, and $(D_f)_1$ and $(D_f)_2$ are the concentrations of free or unbound drugs 1 and 2, respectively.

Rearrangement of eqn. 1 gives eqn. 3, according to which the double-reciprocal plots were constructed for drug 1:

$$\frac{1}{r_1} = \frac{1}{n} + \frac{1 + K_2'(D_f)_2}{n K_1} \cdot \frac{1}{(D_f)_1} \tag{3}$$

The values of K_2' were determined from eqn. 4:

$$K_2' = \left(\frac{S}{S_0} - 1\right) \frac{1}{(D_f)_2} \tag{4}$$

where S and S_0 are the slopes of eqn. 3 when $(D_f)_2 \neq 0$ and $(D_f)_2 = 0$, respectively. The equation for K_1' was similarly derived from eqn. 2.

In the plots, the data points were fitted to straight lines by linear regression analysis using the values on the abscissa as the independent variable and the values on the ordinate as the dependent variable.

RESULTS AND DISCUSSION

Fig. 2 shows the binding characteristics of sulphamethizole and salicylic acid to the immobilized HSA monomer in the form of a Scatchard plot. As the pK_a values of salicylic acid and sulphamethizole are reported to be 3.0[24] and 5.4[25], respectively, both drugs exist as anions at pH 7.87. Because these plots are hyperbolic, they are likely to bind to more than one class of site. For the simulutaneous binding of these drugs, the experiments were therefore limited to low r values ($r < 1.6$), so that the plots can be considered to be linear and to represent mainly the binding to one class of high-affinity site.

The amounts of both drugs bound determined from the three frontal analysis diagrams shown in Fig. 1 corresponding to Types I–III for two pairs of fixed concentrations of both drugs are presented in Table I.

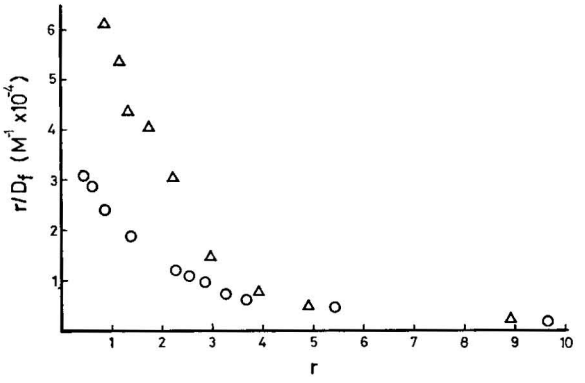


Fig. 2. Scatchard plots for single-drug interactions with immobilized HSA in 0.05 M Tris buffer containing 0.1 M NaCl (pH 7.87 at 4°). ○, Sulphamethizole; △, salicylic acid.

TABLE I

COMPARISON OF DRUGS BOUND, D_b (MOLES $\times$ 10^6), OBTAINED FROM THREE FRONTAL ANALYSIS DIAGRAMS*

D_b determined at 4° in 0.05 *M* Tris buffer containing 0.1 *M* NaCl (pH = 7.87 at 4°). SMZ and SA denote sulphamethizole and salicylic acid, respectively.

Type of frontal analysis	*Column I** $[(D_f)_{SMZ} = 2.94 \cdot 10^{-5}$ *M*, $(D_f)_{SA} = 2.17 \cdot 10^{-5}$ *M]*		*Column II** $[(D_f)_{SMZ} = 4.41 \cdot 10^{-5}$ *M*, $(D_f)_{SA} = 2.17 \cdot 10^{-5}$ *M]*	
	$(D_b)_{SMZ}$	$(D_b)_{SA}$	$(D_b)_{SMZ}$	$(D_b)_{SA}$
I, simultaneous application (Fig. 1a)	1.49	2.42	1.31	1.44
II, displacement of SMZ by SA (Fig. 1b)	1.49	2.37	1.33	1.37
III, displacement of SA by SMZ (Fig. 1c)	1.47	2.43	1.28	1.41

* The gel volumes of columns I and II were 13.7 and 9.1 ml, respectively.

Fairly good agreement of these values for each pair of concentrations of free drugs indicates a reversible nature of the binding of these drugs and the amount of the drugs bound can be determined from any of these three diagrams. Type I analysis (Fig. 1a) gives the usual frontal analysis diagram for two solutes[26] and shows that salicylic acid, with a stronger affinity, elutes behind the less strongly bound sulphamethizole, which is displaced by salicylic acid as the solution moves up the column. Therefore, this is analogous to Type II analysis (Fig. 1b), in which the displacement of sulphamethizole by salicylic acid is shown by the area indicated as E. The displacement of salicylic acid with sulphamethizole is shown in Fig. 1c by the appearance of the peak. The advantage of Types II and III over Type I is that the actual amount of drug displaced by another can be directly determined.

As the amounts of both drugs bound were independent of the three types of elution analysis, all binding studies were carried out continuously by the combination of Types II and III. Part of such a continuous analysis diagram is shown in Fig. 3. This sequence of applications of six different solutions containing either sulphamethizole or salicylic acid alone or together permitted the determinations of drugs

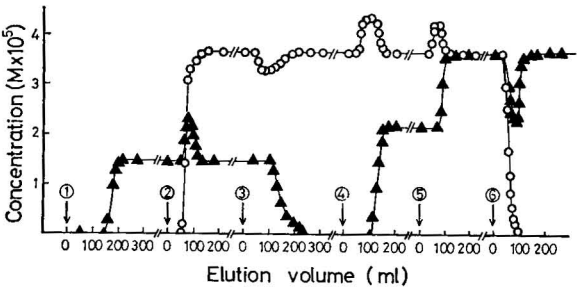


Fig. 3. Part of a continuous frontal analysis diagram from which the amounts of drugs bound were determined for both single-drug and two-drug interactions with immobilized HSA in 0.05 *M* Tris buffer containing 0.1 *M* NaCl (pH 7.87 at 4°). ○, Elution of sulphamethizole; ▲, elution of salicylic acid. The arrows indicate where solutions 1–6 were applied: 1, $1.45 \cdot 10^{-5}$ *M* salicylic acid; 2, 1 + $3.65 \cdot 10^{-5}$ *M* sulphamethizole; 3, $3.65 \cdot 10^{-5}$ *M* sulphamethizole; 4, 3 + $2.17 \cdot 10^{-5}$ *M* salicylic acid; 5, 3 + $3.62 \cdot 10^{-5}$ *M* salicylic acid; 6, $3.62 \cdot 10^{-5}$ *M* salicylic acid.

bound in the corresponding solutions. In the presence of both drugs, the concentration of free sulphamethizole was kept constant while that of salicylic acid was varied. The peak or trough around the concentration of sulphamethizole which was kept constant ($3.65 \cdot 10^{-5}$ *M*) (Fig. 3) indicates the amount of the drug released or bound, respectively, with respect to the amount of the drug bound in equilibrium with the preceding solution. Further, by interposing an application of a single drug solution between solutions containing both drugs, as shown in Fig. 3 (solution 3 or 6, the following advantages are apparent: (1) the time lag between single-drug and two-drug is shortened; this is important if the leakage of the immobilized albumin is appreciable; and (2) when a solution containing two drugs was replaced with a single-drug solution, the analysis for the drug removed allows the determination of the amount of the drug released. Thus, a check can be made on the amount of the drug bound in equilibrium with the preceding solution.

Table II gives the results of both single-drug and two-drug interactions with the immobilized HSA. As this method allows the concentrations of free drugs to be maintained constant and both drugs were analysed simultaneously, the mutual displacement interaction can be readily seen by decreasing *r* values for the drug, the concentration of which was kept constant, with increasing concentration of the other.

TABLE II

MOLES OF DRUGS BOUND PER MOLE OF IMMOBILIZED HUMAN SERUM ALBUMIN MONOMER, *r*

Determined at 4° in 0.05 *M* Tris buffer containing 0.1 *M* NaCl (pH = 7.87 at 4°). SMZ and SA denote sulphamethizole and salicylic acid, respectively. Some of these values are averages of two determinations and they were reproducible to within 5%'

$(D_f)_{SMZ}$ $(M \times 10^5)$	$(D_f)_{SA}$ $(M \times 10^5)$							
	0		1.45		2.17		3.62	
	r_{SMZ}	r_{SA}	r_{SMZ}	r_{SA}	r_{SMZ}	r_{SA}	r_{SMZ}	r_{SA}
0	—	—	—	0.891	—	1.16	—	1.57
1.46	0.450	—	0.360	0.799	0.332	1.07	0.284	1.47
2.19	0.629	—	0.495	0.760	0.457	1.03	0.401	1.42
3.65	0.876	—	0.732	0.698	0.678	0.969	0.601	1.36
7.30	1.38	—	1.19	0.604	1.13	0.829	1.03	1.19

Fig. 4 shows the double-reciprocal plots for both drugs according to eqn. 3. The binding and inhibition parameters that were calculated on the basis of these plots are summarized in Table III. All of the regression lines shown in Fig. 4 appear to show a common intercept on the ordinate and indicate that this displacement interaction is competitive. The degree of variation in the values of the intercepts is shown in Table III as the variation in the values of *n* which were obtained for each line as the reciprocals of the intercepts on the ordinate, together with the binding and inhibition constants for the two sets of data. The values of the inhibition constant of salicylic acid determined from the analyses of sulphamethizole in the presence of three different concentrations of free salicylic acid are comparable to its binding constant. The same can be said for the values of the inhibition constant of sulphamethizole determined from the salicylic acid data.

TABLE III

BINDING AND INHIBITION PARAMETERS CALCULATED FROM THE DOUBLE-RECIPROCAL PLOTS FOR INTERACTIONS OF SULPHAMETHIZOLE OR/AND SALICYLIC ACID WITH IMMOBILIZED HUMAN SERUM ALBUMIN MONOMER

Studied at 4° in 0.05 *M* Tris buffer containing 0.1 *M* NaCl (pH = 7.87 at 4°). SMZ and SA denote sulphamethizole and salicylic acid, respectively. All parameters were calculated by regression analysis.

Interaction	*Sulphamethizole*				*Salicylic acid*			
	$(D_f)_{SA}$ $(M \times 10^5)$	K_{SMZ}^* $(M^{-1} \times 10^{-4})$	$K'_{SA}{}^*$ $(M^{-1} \times 10^{-4})$	n	$(D_f)_{SMZ}$ $(M \times 10^5)$	K_{SA}^* $(M^{-1} \times 10^{-4})$	$K'_{SMZ}{}^*$ $(M^{-1} \times 10^{-4})$	n
Single-drug interaction	0	1.4	—	2.7	0	2.7	—	3.2
Two-drug interaction	1.45	—	2.1	2.6	1.46	—	1.2	3.4
	2.17	—	2.0	2.6	2.19	—	1.2	3.4
	3.62	—	2.0	2.7	3.65	—	1.2	3.8
					7.30	—	0.93	3.4

* K and K' are the binding constant and inhibition constant, respectively.

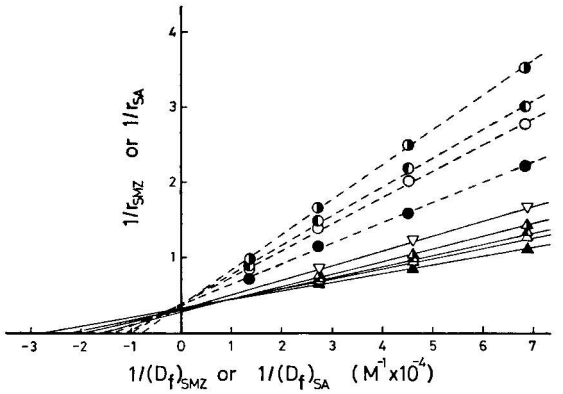


Fig. 4. Double-reciprocal plots according to eqn. 3 at low r values ($r < 1.6$) for single-drug and two-drug interactions with immobilized HSA in 0.05 M Tris buffer, containing 0.1 M NaCl (pH 7.87 at 4°). Broken lines indicate sulphamethizole data: in the absence of salicylic acid (●) and in the presence of free salicylic acid at $1.45 \cdot 10^{-5}$ M (○), $2.17 \cdot 10^{-5}$ M (◐) and $3.62 \cdot 10^{-5}$ M (◑). Solid lines indicate salicylic acid data: in the absence of sulphamethizole (▲) and in the presence of free sulphamethizole at $1.46 \cdot 10^{-5}$ M (△), $2.19 \cdot 10^{-5}$ M (◭), $3.65 \cdot 10^{-5}$ M (◮) and $7.30 \cdot 10^{-5}$ M (▽).

Therefore, this simple and fairly crude treatment of the data suggests that the mutual displacement interaction is attributable to the competitive binding of these two drugs to the same primary binding sites.

CONCLUSION

In view of potential hazards of coadministration of drugs related to mutual displacement from albumin, a simple and direct method in screening for scuh interactions is desirable at an early phase of drug development. The proposed method may be useful for this purpose as it gives a direct indication of the amount of drug displaced. The experimental procedure is very simple. Moreover, immobilized albumin can be used repeatedly.

For quantitative studies such as those presented here, the major concern is the stability of the covalent linkage between albumin and the gel matrix. All the present binding data obtained by continuous frontal analysis were obtained within 3 weeks on one column. The column remained stable during this period. However, the stability of the column varied from column to column, some being stable for several months and others for about 1 month. One of the solutions to this problem would be to shorten the analysis time further, for instance by performing the analysis under high pressure. Another possibility is to develop a method that allows corrections for the leaked albumin.

REFERENCES

1 P. D. Hansten, *Drug Interactions*, Lea and Febiger, Philadelphia, Pa., 3rd ed., 1976.
2 P. M. Aggeler, R. A. O'Reilly, L. Leong and P. E. Kowitz, *N. Engl. J. Med.*, 276 (1967) 496.
3 R. A. O'Reilly, *Ann. N.Y. Acad. Sci.*, 226 (1973) 293.
4 J. Koch-Weser and E. M. Sellers, *N. Engl. J. Med.*, 285 (1971) 487; 547.

5 J. Koch-Weser and E. M. Sellers, *N. Engl. J. Med.*, 294 (1976) 526.
6 B. J. Soltys and J. C. Hsia, *J. Biol. Chem.*, 253 (1978) 3029.
7 P. Hsu, J. K. H. Ma and L. A. Luzzi, *J. Pharm. Sci.*, 63 (1974) 27.
8 H. M. Solomon, J. J. Schrogie and D. Williams, *Biochem. Pharmacol.*, 17 (1968) 143.
9 S. Goto, T. Hara and H. Yoshitomi, *Yakugaku Zasshi*, 96 (1976) 565.
10 Y. Kaneo, A. Nishikawa, Y. Kato and S. Kiryu, *Chem. Pharm. Bull.*, 27 (1979) 1335.
11 L. P. Simonson, H. P. Bar, B. Jastorff and R. Hitkar, *Biochem. Biophys. Acta*, 379 (1975) 114.
12 L. J. Aarons, W. L. Schary and M. Rowland, *J. Pharm. Pharmacol.*, 31 (1979) 322.
13 J. Steinhardt and J. A. Reynolds, *Multiple Equilibria in Proteins*, Academic Press, New York, 1968, p. 39.
14 D. S. Champion and R. Olsen, *J. Pharm. Sci.*, 63 (1974) 249.
15 D. Hultmark, K. O. Borg, E. Elofsson and L. Palmer, *Acta Pharm. Suecica*, 12 (1975) 259.
16 B. Sebille, N. Thuaud and J.-P. Tillement, *J. Chromatogr.*, 167 (1978) 159.
17 N. I. Nakano, T. Oshio, Y. Fujimoto and T. Amiya, *J. Pharm. Sci.*, 67 (1978) 1005.
18 N. I. Nakano, T. Oshio, N. Sato, Y. Shimamori and S. Yamaguchi, *Chem. Pharm. Bull.*, 27 (1979) 2048.
19 N. I. Nakano, S. Yamaguchi, Y. Shimamori and Y. Fujimoto, *99th Annual Meeting of the Pharmacentical Society of Japan, Sapporo. 1979.*
20 M. Otagiri, and J. H. Perrin, *Biochem. Pharmacol.*, 26 (1977) 283.
21 Y. Kaneo, A. Nishikawa, Y. Kato and S. Kiryu, *Yakuhaku Zasshi*, 98 (1978) 1452.
22 A. Kober, B. Ekman and I. Sjöholm, *J. Pharm. Sci.*, 67 (1978) 107.
23 I. M. Klotz, H. Triwush and F. M. Walker, *J. Amer. Chem. Soc.*, 70 (1948) 2935.
24 A. Albert and E. P. Serjeant, *The Determination of Ionization Constants*, Chapman and Hall, London, 2nd ed., 1971, p. 89.
25 G. S. Avery (Editor), *Drug Treatment*, Adis Press, Sydney, 1976, p. 892.
26 S. Claesson, *Ann. N.Y. Acad. Sci.*, 49 (1948) 183.

Journal of Chromatography, 188 (1980) 357–362

CHROM. 12,417

DETERMINATION OF CYANIDE AND THIOCYANATE IN BIOLOGICAL FLUIDS BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

I. THOMSON* and R. A. ANDERSON**

Department of Forensic Medicine, The University, Glasgow G12 8QQ (Great Britain)

(Received September 10th, 1979)

SUMMARY

A sensitive, specific and accurate method is described for the determination of cyanide and thiocyanate in biological fluids. Potassium cyanide containing 95% ^{15}N is added to the sample as internal standard and both endogenous cyanide and standard are separated by gas-phase diffusion into sodium hydroxide solution. Cyanogen chloride is prepared by reaction with sodium *p*-toluenesulphonchloramide and extracted into *n*-heptane. Quantitative analysis is carried out by gas chromatography–mass spectrometry (GC–MS) with selective ion monitoring of the molecular ions at *m/e* 61 and *m/e* 62. Thiocyanate levels are determined by a similar procedure in which ^{15}N-labelled sodium thiocyanate is used as internal standard. The method is applicable to a wide range of biological samples and is free from interference by other sample components.

INTRODUCTION

A study of the role of smoke and fire gases in causing fire fatalities has indicated that hydrogen cyanide, produced by the pyrolysis and combustion of both natural and synthetic nitrogen-containing polymers[1–3], may be an important toxic factor in at least 5% of fire deaths[4]. The proportion of casualties due to inhalation of smoke and toxic gases has increased threefold over the last twenty years[5], in parallel with the widespread introduction of modern synthetic materials for the furnishings and construction of both domestic and industrial buildings. While no direct connection between these changes has been proved, one possible explanation of the rising mortality rate in U.K. fires lies in an increase in the toxicity of the fire atmosphere caused by the combustion of these new materials. In our study of fire deaths it was necessary to develop an analytical method for cyanide which was sensitive, accurate and specific.

* Present address: Department of the Regional Chemist, 8 Elliot Place, Glasgow G3 8EJ, Great Britain.

** To whom correspondence should be addressed.

Cyanide is a normal constituent of blood usually present at concentrations below 12.0 μmol/l although in smokers it may rise to 20.0 μmol/l[5,6]. Thiocyanate is the principal metabolite of cyanide and plasma thiocyanate may be used as a measure of transformed cyanide[7,8]. Previous methods for the determination of the two species in biological fluids used visible or ultraviolet spectrophotometry[6,7,9,10]. In general, these methods lack sensitivity and specificity, both of which can substantially be improved by using gas chromatographic procedures especially where electron capture detection is used[11–14].

A gas chromatographic–mass spectrometric (GC–MS) method is now reported for the determination of cyanide and thiocyanate in biological fluids, in which ^{15}N-labelled analogues are used as internal standards.

EXPERIMENTAL

All reagents were of analytical grade and *n*-heptane (BDH, Poole, Great Britain) was redistilled before use through a 1-m fractionating column packed with glass spirals. Sodium *p*-toluene sulphonchloramide (chloramine-T) (Hopkins and Williams, Chadwick Heath, Great Britain) was recrystallised from methanol–water. A buffered solution of chloramine-T was prepared immediately before use by mixing 3 parts 1 *M* NaH_2PO_4 and 1 part 0.25% (w/v) chloramine-T in distilled water. Potassium cyanide containing 95% ^{15}N and sodium thiocyanate containing 95.2% ^{15}N were obtained from Prochem, B.O.C., London, Great Britain. Standard cyanide solutions were prepared in 0.1 *M* NaOH solution, to minimise loss of HCN, and were determined by titration against $AgNO_3$, using *p*-dimethylaminobenzylidenerhodanine as indicator.

Methods

Blood samples were processed immediately after arrival through the first stage of microdiffusion and the resulting hydroxide solutions were stored at 4° until analysis[15]. If this was not possible the blood samples were stored at 4° until microdiffusion could be performed. Plasma samples were subjected to protein precipitation and the resulting deproteinised plasma stored at −28° until analysis.

Cyanide. Whole blood, or other biological fluid (2 ml) and standard solution of $KC^{15}N$ (10–50 μM in $KC^{15}N$, 1 ml) were placed in the outer ring of a Conway Microdiffusion dish and mixed gently. A 1-ml volume of 0.1 *M* NaOH was then placed in the inner ring and 2 ml of 3 *M* H_2SO_4 added to the outer ring. The dish was covered immediately, swirled gently and microdiffusion allowed to proceed for 2 h.

The NaOH solution was then transferred to a 5-ml septum vial containing 0.1 ml *n*-heptane and 1 ml of freshly prepared chloramine-T solution. After mixing of the contents, the vial was placed in an ice bath for 10 min. The vial was then removed and reshaken. When the layers had separated, 1–5 μl of the heptane layer containing the dissolved ClCN were used for GC–MS. Standard solutions of $KC^{14}N$ (10–50 μM) were similarly treated.

Thiocyanate. Plasma was obtained by centrifugation of whole blood at 1500 *g* for 5 min. To 1 ml of plasma was added 1 ml of a standard ^{15}N-sodium thiocyanate solution (50 μM, in distilled water). Proteins were removed by the addition of 10% (w/v) trichloroacetic acid solution (4 ml) and repeating the centrifugation. A 1-ml

aliquot of the supernatant was transferred to a septum vial containing 1 ml of freshly prepared chloramine-T solution, 0.1 ml of 0.25% (w/v) $FeCl_3$ and 0.1 ml *n*-heptane. The method then proceeded as for the cyanide analysis. Standard solutions of KSCN (50 μM, in distilled water) were treated similarly.

Gas chromatography–mass spectrometry

A Pye 104 gas chromatograph interfaced to a V.G. Micromass 16F mass spectrometer was used for the analysis. Gas–liquid chromatography (GLC) was carried out at 80° with a glass column (9 ft. × $\frac{1}{4}$ in. I.D.) packed with 7% Hallcomid M-18 on 80–100 mesh Chromosorb W AW DMCS. The helium carrier gas flow-rate was 30 ml/min. Under these conditions ClCN and solvent had retention times of 1.8 min and 4 min, respectively (Fig. 1). The column was clear for injection of the next sample after 10 min. The mass spectrometer was operated in the selective ion monitoring (SIM) mode at 70 eV ionising energy, filament current 200 μA and multiplier voltage 3.0 kV. The source and interface temperatures were 220° and 100°, respectively. A four-channel peak selection unit was used to monitor the ions at *m/e* 61 and *m/e* 62, corresponding to the molecular ions of $^{35}ClC^{14}N$ and $^{35}ClC^{15}N$, respectively, and a reference background ion at *m/e* 60. With a recorder voltage of 100 mV, a 2-ml blood sample 10 μM in CN^- would typically give a deflection of 50% of full scale on injecting 1.0 μl of heptane.

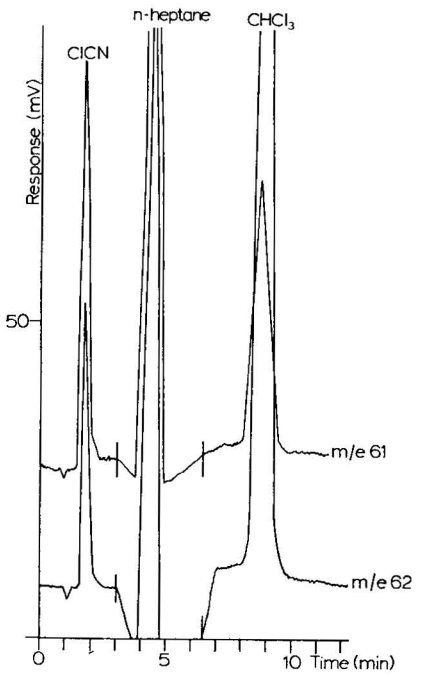


Fig. 1. Chromatograms obtained during the analysis of cyanide by SIM in a blood sample from a fire fatality. $C^{15}N^-$ is used as the internal standard and, following conversion to cyanogen chloride, the corresponding molecular ions are monitored at *m/e* 61 and *m/e* 62. The mass spectrometer dump valve is closed between 3 and 6.5 min during the elution of the solvent, *n*-heptane. Chloroform, an impurity in the solvent, elutes at 8.5 min.

RESULTS

The GC–MS method was evaluated using aqueous standards of cyanide and thiocyanate and found to be linear over a range of concentrations from 5–100 μmol/l (Figs. 2 and 3). Four replicate analyses were carried out for each of five standard solutions with concentrations in the range 5–100 μmol/l. The standard deviation was less than 3.5% for cyanide analyses or 5.4% for thiocyanate analyses. The reproducibility of the method for blood analysis was determined by analysing eight replicate samples from blood with a low cyanide level, blood with a high cyanide level and plasma with a high thiocyanate concentration. The results are summarised in Table I.

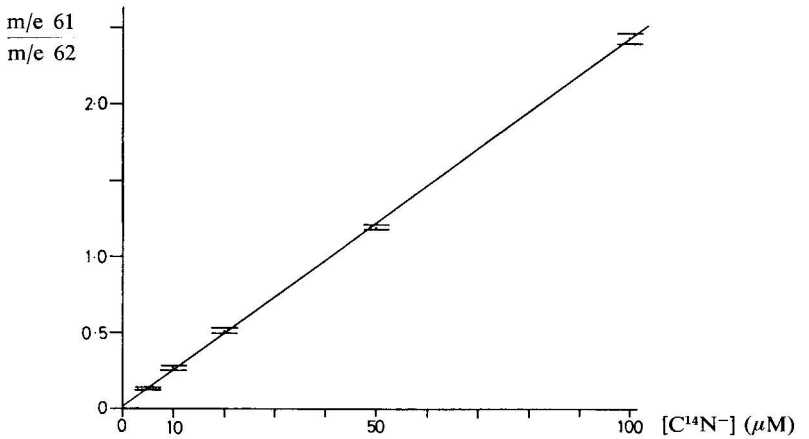


Fig. 2. Calibration curve determined with aqueous solutions of cyanide in the range 5–100 μmol/l, using a 50 μmol/l solution of $KC^{15}N$ as internal standard. The ratio of the peak heights of ClCN at *m/e* 61 and *m/z* is plotted against the $C^{14}N^-$ concentration (μmol/l).

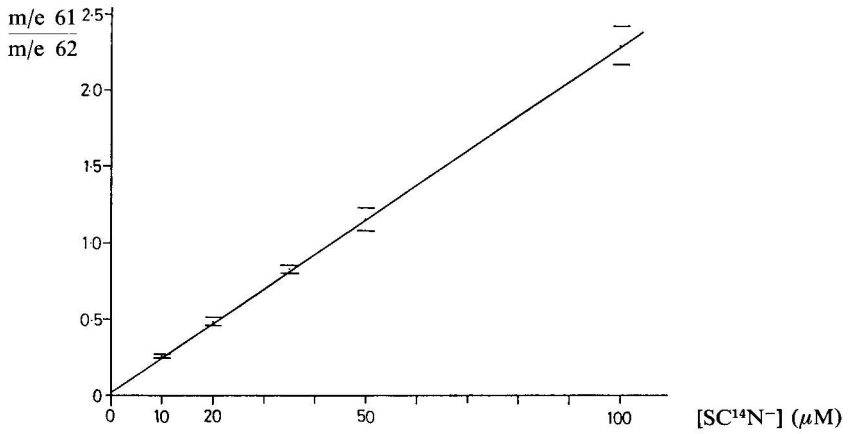


Fig. 3. Calibration curve determined with aqueous solutions of thiocyanate in the range 10–100 μmol/l using a 50 μmol/l solution of $NaSC^{15}N$ as internal standard. The ratio of the peak heights of ClCN at *m/e* 61 and *m/e* 62 is plotted against the $SC^{14}N^-$, concentration (μmol/l).

TABLE I

REPRODUCIBILITY OF THE GC–MS METHOD FOR WHOLE-BLOOD CYANIDE AND PLASMA THIOCYANATE

Number of samples: 8.

Species	*Mean level(μmol/l)*	*Range (μmol/l)*	*S.D. (μmol/l)*
CN^-	3.0	2.7– 3.3	± 0.2
CN^-	121.7	118.2–131.0	± 4.8
SCN^-	181.5	167.7–194.1	± 7.9

Addition of ^{15}N-labelled KCN to whole blood and then subsequent plasma thiocyanate analysis has shown that cyanide does not interfere in the thiocyanate method. Analyses of the cyanide content of blood to which ^{15}N-labelled NaSCN has been added have shown that, at most, about 3 % of the available thiocyanate has been converted to cyanide.

DISCUSSION

Conventional colourimetric and GLC methods have been used for some time in the Department of Forensic Medicine for the analysis of small quantities of cyanide and thiocyanate in body fluids. Both techniques involve separation of cyanide from blood by microdiffusion, with subsequent trapping in alkali, and both depend on the absence of significant quantities of cyanide in plasma to permit isolation of thiocyanate. The ratio of SCN^- to CN^- in normal human plasma is usually 80:1 (ref. 7). Colourimetric procedures have several disadvantages including a lack of sensitivity, low stability of the coloured dyestuffs formed which are usually stable over a period of a few minutes only, the involvement of carcinogenic reagents, the inapplicability of internal standards and the possibility of negative errors and interference arising from the presence of sulphide[15]. The latter problem can be extremely important in the analysis of post-mortem blood samples.

Existing chromatographic methods offer considerable advantages in terms of specificity and sensitivity. Internal standards can be used but normally these may be added only at the final stage of the analysis.

The GC–MS method reported here was developed from a GLC procedure reported by Valentour *et al.*[12], in which cyanide is converted to cyanogen chloride, a volatile gas readily detectable by electron-capture GLC. Modifications were introduced to permit the use of ^{15}N-labelled cyanide as the internal standard which is added directly to the sample, thereby compensating for incomplete recovery and conversion. Analyses are carried out by SIM using the molecular ions of $^{35}ClC^{14}N$ and $^{35}ClC^{15}N$ at *m/e* 61 and *m/e* 62. The stationary phase selected for the GLC column, Hallcomid M-18, gives a very low background at these ions and, as the solvent is eluted after the ClCN, large sample volumes up to 10 μl can be injected without interference in the analysis.

Most samples encountered in the course of our work have cyanide and thiocyanate levels in the range 0–100 μmol/l, within which the method was found to give a linear response (Figs. 2 and 3). A wide variety of biological fluids have been analysed using the method, including fresh blood from humans and experimental animals, old

blood samples, often putrified, from post-mortem dissections, plasma, serum, urine, dialysis fluid and stomach contents. No problems have been encountered as a result of interference by other sample constituents, although when interpreting the levels of cyanide observed, it must be borne in mind that artefactual changes occur during *in vitro* storage of blood samples and also, in some cases, thiocyanate may be converted to cyanide[16,17].

ACKNOWLEDGEMENTS

This work was supported by a contract from the Building Research Establishment, Fire Research Station, Borehamwood, Herts (No. FRO/28/059) and forms part of the work of the Fire Research Station. The authors gratefully acknowledge the assistance of Professor W. A. Harland, Dr. A. A. Watson and Dr. I. S. Symington in providing blood samples. It is contributed by permission of the Director of Building Research Station.

REFERENCES

1 W. D. Woolley, *Brit. Polym. J.*, 4 (1972) 27.
2 D. N. Napier, *Med. Sci. Law*, 17 (1977) 83.
3 E. Urbas and E. Küllik, *J. Chromatogr.*, 137 (1977) 210.
4 I. S. Symington, R. A. Anderson, I. Thomson, J. S. Oliver, W. A. Harland and J. W. Kerr, *Lancet*, 2 (1978) 91.
5 P. C. Bowes, *Med. Sci. Law*, 16 (1976) 104.
6 A. R. Pettigrew and G. S. Fell, *Clin. Chem.*, 19 (1973) 466.
7 A. R. Pettigrew and G. S. Fell, *Clin. Chem.*, 18 (1972) 996.
8 M. Ansell and F. A. S. Lewis, *J. Forensic Med.*, 17 (1970) 148.
9 S. Nagashima, *Anal. Chim. Acta*, 91 (1977) 303.
10 P. A. F. Pranitis and A. Stolman, *J. Forensic Sci.*, 22 (1977) 443.
11 R. E. Isbell, *Anal. Chem.*, 35 (1963) 255.
12 J. C. Valentour, V. Aggarwal and I. Sunshine, *Anal. Chem.*, 46 (1974) 924.
13 R. Altman, *The Microdetermination of Cyanide in Fire Fatalities, Ph.D. Dissertation*, University of Maryland, Md., 1976.
14 H. F. de Brabander and R. Verbeke, *J. Chromatogr.*, 138 (1977) 131.
15 F. L. Rodkey and R. F. Robertson, *Clin. Chem.*, 24 (1978) 2184.
16 S. Nagashima, *Anal. Chim. Acta*, 99 (1978) 197.
17 C. J. Vesey and J. Wilson, *J. Pharm. Pharmacol.*, 30 (1978) 20.

Journal of Chromatography, 188 (1980) 363–375

CHROM. 12,424

QUANTITATIVE DETERMINATION OF PTERINS IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

BALDASSARRE STEA, RICHARD M. HALPERN, BARBARA C. HALPERN and ROBERTS A. SMITH*

Departments of Chemistry, Medicine, and the Molecular Biology Institute, University of California, Los Angeles, Calif. 90024 (U.S.A.)

(Received October 3rd, 1979)

SUMMARY

During our continuing study of pteridine metabolism, the need arose for a more rapid and quantitative determination of pterins in biological fluids. By adopting and modifying previously developed techniques, we have obtained a rapid and sensitive method that allows the simultaneous determination of eight different pterins in human urine and blood. When examined over a 10-day period, the levels of pterins excreted by a normal individual averaged the following values expressed in picomoles per mg of creatinine: biopterin, 9104; neopterin, 6018; xanthopterin, 6561; pterin, 1136; isoxanthopterin, 636; pterin-6-carboxylate, 483; and 6-hydroxymethylpterin, 315. Moreover, 6-hydroxymethylpterin and pterin-6-carboxaldehyde were detected for the first time in the blood of normal individuals.

INTRODUCTION

The rapid and quantitative measurement of pterins in urine, blood and other biological fluids has assumed a major importance because of reports describing the occurrence of elevated levels of certain pteridines in tissue culture media of malignant cells[1], in the urines[2] and in the blood of cancer patients[3], as well as in the urines of mice carrying the Ehrlich ascites tumor[4].

In order to establish the significance of such observations, and to further the study of pterin metabolism in normal as well as pathological conditions, a simple, rapid and sensitive method of analysis is required. We have recently shown that separation of all the major pterins known to occur in human urines can be accomplished by high-performance liquid chromatography (HPLC) on a strong cation-exchange column[5]. This method has also been shown to be highly sensitive, since, when coupled to fluorescence detection, it permits the detection of pterins in the picomolar range. The sensitivity of the measurements, however, is strictly dependent on the state of

* To whom correspondence should be addressed.

oxidation of the pterins, since they are known to yield their greatest fluorescence in the fully oxidized state. We have thus examined a variety of methods for the oxidation of reduced pterins present in urines. Since a purification step is needed before analysis by HPLC, we have modified some existing methods so that eight different pterins could be readily purified from urines and subsequently analyzed by HPLC[5].

In this communication, we also report an HPLC system for the exclusive determination of pterin-6-carboxylic acid. Finally, the method so developed was applied to the quantitative determination of unconjugated pterins excreted in the urine of a healthy subject and to the study of the diurnal variation in the excretion of these compounds.

MATERIALS AND METHODS

Collection of specimens

Urines were collected in brown-glass bottles or darkened containers to protect pterins from photo-oxidation and then frozen at −20°. When aliquots were needed for analysis, a specimen was melted in a water-bath at 40–45° with vigorous mixing and a 10 ml aliquot removed. Creatinine levels were determined by the method of Jaffe (as described in ref. 6) on aliquots that had been acidified to pH 1 with 6 *N* HCl. We found that warming of the urine to about 40° and acidification to pH 1 were necessary to obtain reproducible results. Bloods were collected in heparinized tubes and the plasma was separated from the cells by centrifugation in an International desk-top clinical centrifuge. These specimens were also stored at −20° until used. All of the following operations were carried out in dim light.

Oxidation of reduced pterins

Biopterin, neopterin[7,8] and xanthopterin[9] have been reported to occur in urine as their dihydro- or tetrahydro-derivatives. The same has also been reported for biopterin present in blood[3,8]. Since our detection method strictly depends on the fluorescence efficiency of the compound, it became necessary to adopt an oxidation method that would convert the non-fluorescent hydrogenated forms of pterins into the highly fluorescent, fully oxidized forms. Three different procedures were considered and the results compared. (i) It has been reported[10] that dihydro- and tetrahydrobiopterin could be easily converted to their oxidized form by incubation of an acidic solution (0.05 *N* HCl) of the compounds for 20 min in a boiling water-bath. Ten different urines were thus incubated as described above after partial purification by a two-step ion-exchange procedure described later. (ii) The same set of urines were also subjected to an oxidation procedure adopted from a recently published method[8]. In short, oxidation was accomplished by addition to the acidified (pH 1) urine of a solution of 0.5% I_2–1% KI in 0.1 *N* HCl to a final concentration of 0.05% I_2–0.1% KI or until excess I_2 persisted in solution. We found that biological fluids differ widely from one another in the content of compounds capable of reducing I_2 to I^-. Thus, the amount of I_2–KI solution needed to obtain an excess of I_2 differed from sample to sample. Furthermore, it has been reported[8] that incubation of the sample in the presence of I_2 for 15 min would be sufficient to oxidize fully all the reduced pterins. We found this to be correct in the majority of the cases, but occasionally we encountered some urines that required a longer incubation time in the presence of I_2.

TABLE I

OXIDATION OF REDUCED PTERINS IN URINE BY I_2 AS A FUNCTION OF TIME

Aliquots (0.87 ml) of a urine were exposed to 0.05% I_2–0.1% KI (final concentration) for the length of time indicated. After each incubation, the remaining I_2 was reduced with ascorbic acid and then the samples were purified and analyzed by HPLC. Results are expressed in picomoles/mg creatinine.

Incubation time (min)	*Pterins excreted (pmoles/mg creatinine)*					*Total output*
	Xanthopterin	*Neopterin**	*Biopterin*	*Pt-6-CH_2OH*	*Pterin*	
15	996	2000	3855	134	583	7568
30	7056	3741	10,310	247	1109	22,463
45	10,759	3963	12,069	288	1438	28,517
60	9352	3593	10,552	280	1219	24,436

* Neopterin levels represent the sum of both the D-*erythro* and the L-*threo* isomers.

An example is reported in Table I where four aliquots of the same urine were subjected to I_2 oxidation for different lengths of time. Clearly, in this case, an incubation for 45 min was necessary to maximize the yield of pterins that could be detected. We thus adopted a 45 min incubation time as the standard operating procedure. In order to ensure that excess I_2 was present in solution for the entire incubation period, the biological fluid being oxidized was tested periodically with starch which imparts an intense blue color to solutions containing the triiodide ion. At the end of the incubation time, the excess I_2 was removed by addition of a few drops of 1% ascorbic acid until the starch test was negative. (iii) Finally, a third method was attempted for oxidizing reduced pterins present in urines. This consisted of addition of H_2O_2 to a final concentration of 1% and incubation at room temperature for 15 min. In this case, too, the oxidation was stopped by addition of a small amount of an appropriate ascorbic acid solution. When three different aliquots of the same set of urines were oxidized in the three different ways and the results obtained were compared, we found that the I_2 and the H_2O_2 procedure gave consistently better results than the incubation in acidic medium in a boiling water-bath. The I_2 and the H_2O_2 method appeared to give consistent and comparable results. However, we adopted the I_2 method because it had already been extensively used in other laboratories and proven to be very effective in the oxidation of tetrahydro-, 7,8-dihydro- and quinoid dihydrobiopterin[8].

Purification of samples before analysis by HPLC

In order to analyze biological materials by HPLC, a preliminary purification step is often necessary[11], because these samples contain salts, compounds and high-molecular-weight molecules that would otherwise interfere with the separation as well as with the detection of the compounds of interest. Furthermore, if the purification step were omitted, the microparticulate column used for the separation in HPLC would irreversibly degenerate with a few chromatographic runs, thus making repetitive analysis of biological samples prohibitively expensive. For these reasons, all the biological materials analyzed by HPLC were purified as follows. A certain volume of the biological fluid (for urines, the volume containing 2 mg of creatinine) was first acidified to pH 1 with 6 *N* HCl. Then 100 μl of a 30 μM solution of internal standard were quantitatively added to the sample. The use of an internal standard is necessary

to account for any possible day-to-day variation in the purification procedure, as well as in the injection volume and in the performance of the detector. In choosing an internal standard, availability, stability, solubility, detection and non-interference with the compounds of interest were considered. We chose 6,7-dimethylptherin (6,7-DMP) as it is commercially available, highly fluorescent, and therefore easy to detect, very stable to ring oxidation and, more importantly, it chromatographed on a strong cation exchange column with a retention time longer than any other fluorescent compound present in urines. The sample to be analyzed was then subjected to an oxidation step with I_2 as described above and finally purified by a modification of a method previously described[12]. The sample is applied to a 6 × 20 mm Dowex 50-X8 column (H^+, 100–200 mesh), followed by 15 ml of double-distilled water. This wash is collected and analyzed for isoxanthopterin, which is not retained on the cation exchange column because of its low pK_a [13]. The Dowex 50 column is then stripped with 6 ml of 1 *N* NH_4OH which is directly applied to a 5 × 8 mm Dowex 1-X8 column (OH^-, 200–400 mesh). This column is washed with 6 ml of a solution of NH_4OH adjusted to pH 9 and then with 0.5 ml of water. Both of these washes are discarded. Finally, the Dowex 1 column is eluted successively with 2 ml of 1 *N* acetic acid–7% methanol–5% acetonitrile followed by 2 ml of 0.5 *N* HCl–7% methanol–5% acetonitrile. The following pterins are eluted in the acetic acid fraction: xanthopterin, D-*erythro*-neopterin, D-*threo*-neopterin, biopterin, pterin, pterin-6-carboxaldehyde (Pt-6-CHO), 6-hydroxymethylpterin (Pt-6-CH_2OH) and the internal standard, 6,7-DMP. The HCl eluate contains pterin-6-carboxylic acid (Pt-6-COOH) which binds to the anion exchange column rather strongly because of the ionizable carboxylic group. The 15 ml of water-wash containing isoxanthopterin are alkalinized with concentrated NH_4OH to pH 11, and then the entire solution is applied to another 5 × 8 mm Dowex 1-X8 column (OH^-, 200–400 mesh); this column is washed in the same way as described above with 6 ml NH_4OH pH 9 and 0.5 ml water, then eluted with 3 ml of 0.5 *N* HCl–7% methanol–5% acetonitrile. Methanol and acetonitrile were added to the eluting buffer to overcome hydrophobic interactions of the pterin ring with the Dowex backbone. The purification scheme is summarized in Fig. 1. The recovery of each pterin from urine purified by this procedure is shown in Table II. With the exception of Pt-6-CHO, all the other pterins were recovered in yields that ranged between 77% and 100%. Only 35–40% of Pt-6-CHO was recovered when added to urine and subjected to the purification procedure. Nearly 100% recovery was obtained instead when Pt-6-CHO was subjected to the purification scheme in the absence of urine. Thus Pt-6-CHO reacts with some component(s) in the urine and is not recovered quantitatively.

Blood and other biological materials were first deproteinized by addition of isopropanol to a final concentration of 70% and incubation at 4° for at least 2 h; denatured proteins were removed by centrifugation and isopropanol by extraction with six volumes of toluene. The aqueous extract was first acidified to pH 1 and then subjected to I_2 oxidation as described previously for urines; finally, the solution was purified by the same two-step ion-exchange method.

Analysis of pterins present in biological fluids by HPLC

The acetic acid eluate from the Dowex 1 column was analyzed by HPLC on a Partisil 10-SCX (Whatman, Clifton, N.J., U.S.A.) column as described previously[5].

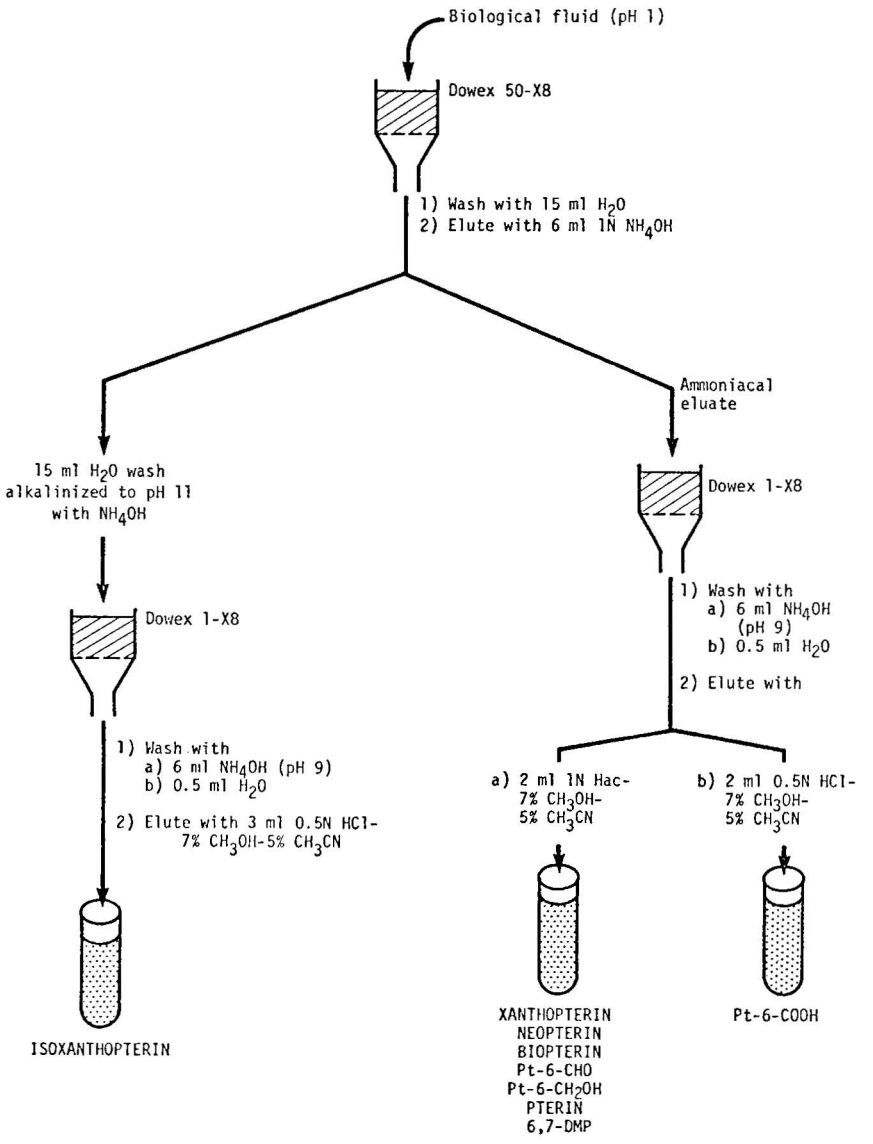


Fig. 1. Purification scheme for pterins.

Because of its exceedingly high retention time, the internal standard was eluted from the strong cation-exchange column by increasing column temperature to 55° and flow-rate to 2.5 ml/min after all the compounds of interest had been eluted at a flow of 1 ml/min and at room temperature.

The HCl eluate containing isoxanthopterin was chromatographed on two Partisol SCX columns joined in tandem, and also equipped with a 5-cm guard column. Chromatography was performed with the same solvent system used for analysis of the acetic acid eluate (1 m*M* $NH_4H_2PO_4$ pH 2.8–7% methanol–5% acetonitrile) at

TABLE II

RECOVERIES OF PTERINS FROM URINE PURIFIED BY THE DOWEX 50 AND DOWEX 1 PROCEDURE

A solution containing approximately 3 nmoles of each of the pterins listed below was added to a 500-μl aliquot of urine. After acidification to pH 1, the resulting solution was purified through the Dowex 50 and 1 columns, as described in Materials and methods, and the eluates analyzed by HPLC. Recoveries were calculated by taking the ratios of the areas under each peak obtained after purification to those obtained when an identical solution of the same standards was injected without prior purification.

Compound	*Recovery (%)*
Pt-6-CHO	40
Pt-6-COOH	77
Xanthopterin	80
Isoxanthopterin	89
Pt-6-CH_2OH	91
Pterin	96
Neopterin	99
Biopterin	100
6,7-DMP	100

1 ml/min and 4°. This low temperature is necessary in order to separate the isoxanthopterin peak from other early eluting unknown peaks which would otherwise comigrate with isoxanthopterin at room temperature.

Finally, for analysis of Pt-6-COOH, the HCl eluate containing this compound is first lyophilized to remove the HCl as the column used to analyze this fraction is sensitive to the acid. The residue so obtained is dissolved in 1 ml of the same buffer used for HPLC. Chromatographic analysis is performed in this case with a Partisil 10-SAX column (Whatman) equipped with 5-cm precolumn containing the same packing as the main column. Isocratic elution is performed with 20 m*M* KH_2PO_4 (adjusted to pH 3.3 with H_3PO_4)–5 % *n*-propanol at 1.5 ml/min and room temperature.

Detection was performed in every case with a Farrand-A4 fluorometer (Farrand Optical, Valhalla, N.Y., U.S.A.) equipped with an excitation filter centered at 360 nm and a narrow band emission filter at 450 nm. The aperture of the fluorometer was set at a value of 5 and the range varied between the values of 0.3 and 10 depending on the concentration of the samples injected.

Quantitation of pterins

Quantitative determination of pterins present in urines was accomplished by means of linear calibration curves constructed by adding different amounts of each pterin along with the same amount of internal standard to 0.5 ml urine aliquots. These aliquots were subjected to the purification scheme described above (Fig. 1) and to HPLC. The peak area ratios of each pterin peak to the 6,7-DMP peak were plotted against the corresponding amount of standard present in the aliquot injected (100 μl). Concentration values of xanthopterin, neopterin, biopterin, pterin, Pt-6-CHO and Pt-6-CH_2OH present in urines were obtained through this type of standard curve. An example is shown only for neopterin (Fig. 2). Pt-6-COOH and isoxanthopterin concentrations instead were determined from linear standard curves obtained by

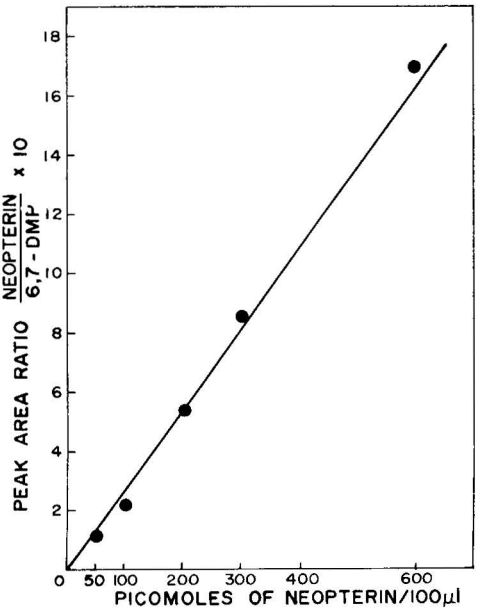


Fig. 2. Standard curve for neopterin. Increasing amounts of authentic D-*erythro*-neopterin were added to five different urine aliquots (0.5 ml) together with a constant amount of 6,7-DMP (3 nmoles). The samples were subjected to I_2 oxidation and purification by the two-step ion exchange method; then the fractions obtained by eluting the Dowex 1 columns with 1 *N* acetic acid–7% methanol–5% acetonitrile were analyzed by HPLC on a Partisil SCX column. Peak area ratios of neopterin to 6,7-DMP were plotted *vs.* the amount of neopterin present in the aliquot analyzed by HPLC (100 μl). Detection was performed with a Farrand A_4 fluorometer with aperture set at a value of 5 and range at 3.

simply plotting areas *vs.* the respective amount of standard present in the aliquot injected (100 μl). Area ratios of the compound under study to internal standard could not be obtained for Pt-6-COOH and isoxanthopterin since 6,7-DMP is eluted in the acetic acid fraction (see Fig. 1).

Chemicals

Standard pterins were obtained as described in ref. 5; 6,7-DMP was purchased from Regis (Morton Grove, Ill., U.S.A.). Salts used for HPLC buffers were of the highest grade available, and the organic solvents used as components of these buffers were of the HPLC grade (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.).

RESULTS

During our initial study on the excretion of unconjugated pterins in human urines, we observed that if urines were not oxidized, and no precautions were taken to keep pterins in the reduced state, increasing amounts of biopterin, neopterin and xanthopterin would be detected as a function of time when the acetic acid fraction of a purified urine was analyzed by HPLC. This indicated to us that the non-fluorescent reduced forms of those pterins were being spontaneously oxidized while

standing at room temperature in acetic acid thus becoming fluorescent and detectable at 450 nm. This confirmed previously published data on the occurrence of reduced pterins in urines[7,8]. We also found that incubation for 20 min of the acetic acid fraction containing reduced pterins in a boiling water-bath would immediately increase the level of detectable pterins. The levels obtained this way, however, were always lower than those obtained when the urine had been oxidized either by the I_2 or the H_2O_2 method before purification. Fig. 3 shows a typical chromatographic profile obtained when the acetic acid fraction of a purified urine from a normal individual was analyzed by HPLC. Peak assignments were made strictly by retention times which remain relatively constant until the column starts to deteriorate. When this happens, we found that by simply decreasing by a few degrees the temperature at which the chromatographic run is performed, we could restore the original retention times. This is possible because migration of pterins on a Partisil 10-SCX column is very sensitive to temperature[5]. It is evident from Fig. 3 that pterins occur in human urines at different concentrations, as it was necessary to change the sensitivity of the fluorometer over a ten-fold range to obtain a good signal/noise ratio for some peaks. A peak having the same mobility as authentic Pt-6-CH_2OH was consistently seen in all the urines examined. This compound was present in very minute amounts when compared with more abundant pterins, such as neopterin and biopterin (see Table I or III).

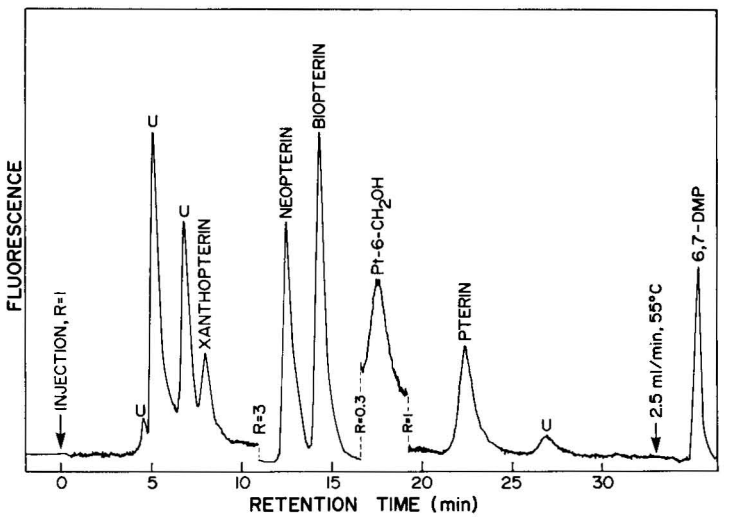


Fig. 3. Elution profile of urinary pterins in a healthy individual. The volume of urine containing 2 mg of creatinine (1 ml) was oxidized and purified as described in Methods. Chromatographic analysis of the acetic acid fraction (100 μl) was performed by HPLC on a Partisil SCX column with 1 m*M* $NH_4H_2PO_4$ pH 2.8–7% methanol–5% acetonitrile as the solvent at 1 ml/min and 20° for the first 32 min. Then the internal standard was eluted by placing the column in a 55° water-bath and by increasing the flow-rate to 2.5 ml/min. Detection was performed fluorometrically as described in Methods; the aperture of the fluorometer was set at a value of 5 and the range (R) varied as shown. Unidentified peaks present in the chromatogram were labeled U. The peak labeled as neopterins represents the combination of both D-*erythro*-neopterin and L-*threo*-neopterin. Because the latter is present in lower amounts than the D-*erythro*-isomer, it appears as a shoulder on the descending limb of the D-*erythro*-neopterin peak.

TABLE III

DAILY URINARY EXCRETION OF PTERINS IN A NORMAL INDIVIDUAL DURING A 10-DAY PERIOD

The volume of urine containing 2 mg of creatinine was oxidized by the I_2 method and purified as described in Methods. The different fractions obtained were analyzed by HPLC and the results expressed in picomoles/mg of creatinine.

Days	*Neopterin***	*Biopterin*	*Pt-6-CH_2OH*	*Pterin*	*Xanthopterin*	*Isoxanthopterin*	*Pt-6-COOH*
1	5592	6758	264	969	6110	ND**	408
2	6370	11,448	292	1156	9073	831	372
3	5963	7414	461	855	9258	719	520
4	4848	9424	177	994	5830	ND	349
5	6370	8241	306	1312	6234	744	563
6	6630	9655	373	1139	6025	837	596
7	5852	11,138	293	1290	5376	575	562
8	8444	9828	458	1461	6357	462	612
9	5222	9690	299	1225	5796	494	510
10	4888	7448	269	956	5555	425	341

* Neopterin levels represent the sum of both the D-*erythro* and the L-*threo* isomers.
** ND = not determined.

Though only identified by its retention time on HPLC, to our knowledge, this is the first report describing the occurrence of Pt-6-CH_2OH in human urine. Its presence in very small amounts has prevented its positive identification by other means. The pterins purified from urines and present in the acetic acid fraction appear to be stable indefinitely when kept at $-20°$ and in the dark, with the single exception of xanthopterin which slowly decreases with time over a period of several days.

The separation of peaks present in the HCl fraction containing Pt-6-COOH and the identification of this pterin are shown in Fig. 4. As can be seen from the chromatographic profile, this pterin is also present in small amounts in the urine of this normal individual. Fig. 5 depicts the chromatographic profile of the HCl fraction containing isoxanthopterin. Many other peaks were present in this and the other two fractions, but no attempts were made to identify them.

Peaks attributed to pterins were identified either directly by comigration with authentic standards or indirectly by adsorption on charcoal with concomitant disappearance of the peaks attributed to pterins, a criterion previously used by other investigators to verify the identity of certain peaks[12].

In order to gather data on the urinary levels of pterins in normal individuals and obtain clues on factors controlling their excretion, ten consecutive urines were collected daily from a young healthy adult and analyzed for their content of pterins. Table III shows the levels of pterins present in these ten urines. The mean output for each pterin as well as the maximum and minimum levels excreted are shown graphically in Fig. 6. It is clear that urinary excretion levels of pterins vary less than two-fold during the 10-day period. The reason for this variation is not known and requires further investigation. However, the values reported in Table III for the individual under study are typical for people in the same age group as shown elsewhere[14]. In addition to studying the daily variation in the urinary excretion levels of pterins, we also followed excretion during a 24-h period by examining timed urines collected

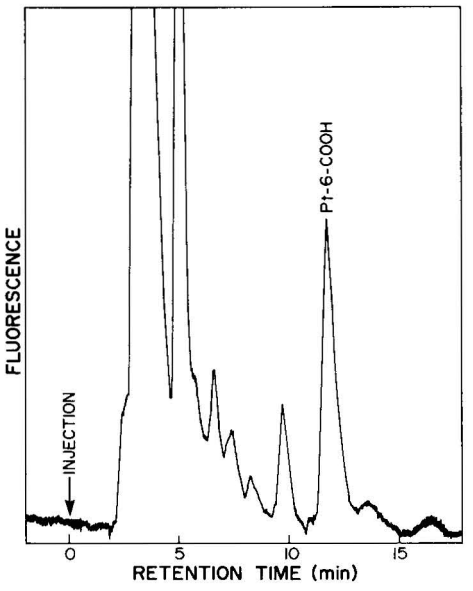


Fig. 4. Elution profile of the HCl fraction containing Pt-6-COOH from a urine of a healthy individual. The volume of urine containing 2 mg of a creatinine (1 ml) was oxidized and purified as described in the legend of Fig. 3. The HCl fraction obtained after elution with acetic acid was lyophilized to remove the HCl and the residue redissolved in 1 ml of 20 m*M* KH_2PO_4 pH 3.3–5% *n*-propanol. Then 50 μl of this solution were analyzed by HPLC on a Partisil SAX column which was eluted isocratically with the same buffer used to redissolve the residue obtained after lyophilization. Flow-rate, 1.5 ml/min; temperature, 22°. Detection was performed fluorometrically with the aperture of the fluorometer set at 5 and the range at 0.3. No attempts were made to identify all the other peaks present in this fraction.

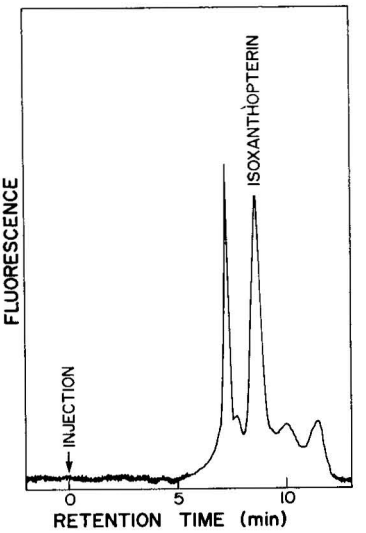


Fig. 5. Elution profile of the HCl fraction containing isoxanthopterin from a urine of a healthy individual. The HCl eluate containing isoxanthopterin (see Fig. 1) obtained from the urine of a normal individual was analyzed by HPLC on two Partisil SCX columns joined in tandem; 100 μl were injected. Isocratic elution was performed with 1 m*M* $NH_4H_2PO_4$ pH 2.8–7% methanol–5% acetonitrile at 1 ml/min and in a water-bath containing melting ice (4°). Detection was performed fluorometrically as described in Methods with an aperture setting of 5 and range of 1. Unknown peaks present in the chromatogram were not identified.

every 6 h. The results of this study are shown in Fig. 7. With the possible exception of xanthopterin, all the other pterins examined were excreted in constant amounts during the 24-h period. Xanthopterin levels instead appeared to decrease between the morning and the night specimen; this change, however, is not as great as the day-to-day variation (Table III). These results validate our practice of collecting urine specimens at any time of the day, since pterins' excretion appears to remain relatively constant during a 24-h period.

Finally, the method described in this paper can also be applied to quantitate the levels of pterins present in blood. Fig. 8 shows the typical pattern obtained for the plasma fraction of the blood of a normal individual after I_2 oxidation and purification as described in Methods. From the profile shown in Fig. 8, it is apparent that peaks having the same chromatographic mobility as Pt-6-CHO and Pt-6-CH_2OH are present in the blood of this normal individual in addition to neopterin, biopterin,

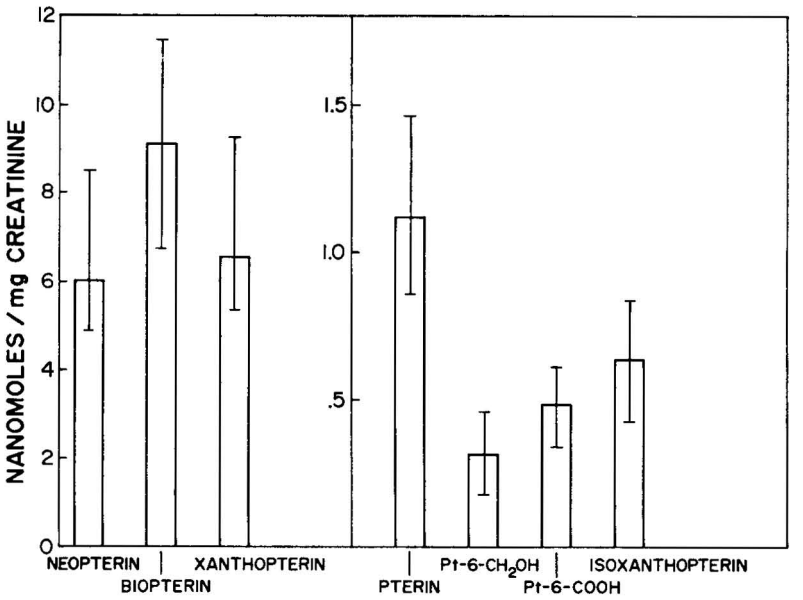


Fig. 6. Mean urinary excretion levels of pterins in a healthy individual over a 10-day period. The volume of urine containing 2 mg of creatinine was processed in each case as described in Table III. The bars indicate the average daily excretion level while the brackets indicate the lowest and the highest level excreted during the period of study. Neopterin levels represent the sum of both the D-*erythro* and the L-*threo* isomers.

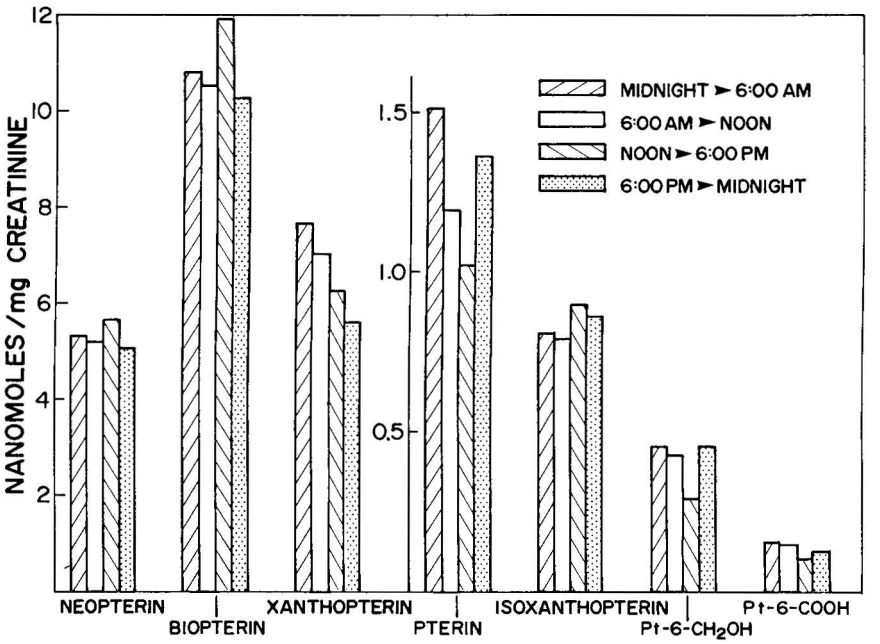


Fig. 7. Diurnal variation in the urinary excretion levels of pterins in a healthy individual. Timed urines were collected every 6 h as shown. The volume of urine equivalent to 2 mg of creatinine was processed for each specimen. Analysis was performed by HPLC as described in the legend of Figs. 3–5. Neopterin levels represent the sum of both the D-*erythro* and the L-*threo* isomers.

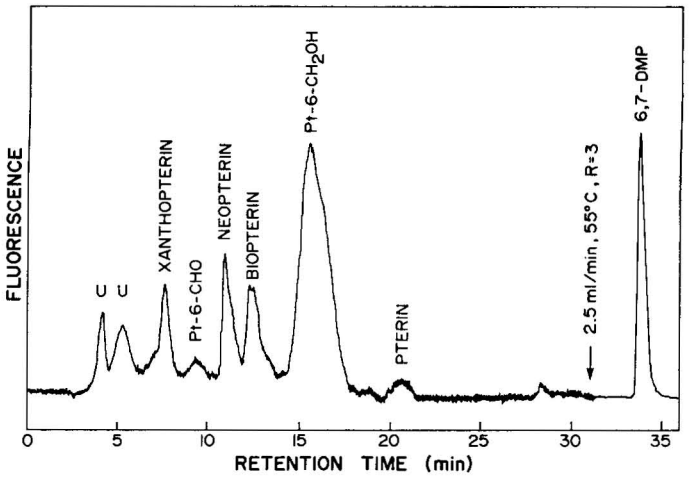


Fig. 8. Elution profile of plasma pterins of a healthy individual. Blood was collected from a healthy male individual (60 years old). The plasma was separated from the cellular fraction by centrifugation and then processed as described in Methods. After purification (Fig. 1), 300 μl of the acetic acid fraction were analyzed by HPLC in the same way described for urines in Fig. 3. The range of the fluorometer was set at a value of 0.3 and the aperture at 5. Peaks were identified by coelution with authentic standards.

xanthopterin and pterin. Moreover, the relative level of Pt-6-CH_2OH appear to be much greater in the blood than in the urine. Although it seems unlikely that other compounds could copurify and comigrate on HPLC with Pt-6-CHO or Pt-6-CH_2OH and also fluoresce at 450 nm, we feel that a more positive identification is needed for these two new pterins found in the blood of normal individuals. Pt-6-CCOH and small amounts of isoxanthopterin were also found in the plasma of the individual under study (results not shown).

DISCUSSION

The method described in this publication is a rapid, highly sensitive and relatively inexpensive technique to analyze and quantitate all the major pterins present in human urine, blood and other biological fluids. Although other rapid methods utilizing gas chromatography–mass spectrometry[10,12] or reversed-phase HPLC[15] have previously been described for the quantitation of pterins, none, however, addresses the question of the simultaneous determination of eight different pterins from a single specimen. Most of the existing methods concern themselves with the quantitation of only one or two pterins. Our experience in the detection and measurement of pterins in urines of cancer patients has shown us that quantitation of all the known pterins can be of great diagnostic importance[14]. It is our hope that because of its simplicity and reproducibility, this method will find wide application in the study of folate and pterin metabolism in the normal as well as the pathological state.

ACKNOWLEDGEMENTS

This work was supported by the Julius and Dorothy Fried Research Foundation and by the Cellular and Molecular Biology Training Grant No. GM 07185-04.

REFERENCES

1 B. Stea, P. S. Backlund, Jr., P. B. Berkey, A. K. Cho, B. C. Halpern, R. M. Halpern and R. A. Smith, *Cancer Res.*, 38 (1978) 2378.
2 R. M. Halpern, B. C. Halpern, B. Stea, A. Dunlap, K. Conklin, B. Clark, H. Ashe, L. Sperling J. A. Halpern, D. Hardy and R. A. Smith, *Proc. Nat. Acad. Sci. U.S.*, 74 (1977) 587.
3 N. Kokolis and I. Ziegler, *Cancer Biochem. Biophys.*, 2 (1977) 79.
4 H. Wachter, K. Brassmayr and A. Hausen, *Cancer Lett.*, 6 (1979) 61.
5 B. Stea, R. M. Halpern and R. A. Smith, *J. Chromatogr.*, 168 (1979) 385.
6 R. J. Henry, K. C. Cannon and J. W. Winkleman, *Clinical Chemistry: Principles and Techniques*, Harper and Row, Hagerstown, Md., 1974, pp. 54*H*–552.
7 T. Fukushima and T. Shiota, *J. Biol. Chem.*, 247 (1972) 4549.
8 T. Fukushima, K. Kobayashi, I. Eto and T. Shiota, *Anal. Biochem.*, 89 (1978) 71.
9 P. Schlesinger, B. M. Watson, R. G. H. Cotton and D. M. Danks, *Clin. Chim. Acta*, 92 (1979) 187.
10 E. M. Gál and A. D. Sherman, *Prep. Biochem.*, 7 (1977) 155.
11 A. Bye and M. E. Brown, *J. Chromatogr. Sci.*, 15 (1977) 365.
12 R. Röthler and M. Karobath, *Clin. Chim. Acta*, 69 (1976) 457.
13 R. C. Blakley, *The Biochemistry of Folic Acid and Related Pteridines*, North Holland, Amsterdam, 1969, pp. 61–65.
14 B. Stea, R. M. Halpern, B. C. Halpern and R. A. Smith, in preparation.
15 T. Fukushima and J. C. Nixon, in R. L. Kisliuk and G. M. Brown (Editors), *Chemistry and Biology of Pteridines*, Elsevier/North Holland, New York, 1979, pp. 35–36.

Journal of Chromatography, 188 (1980) 377–382

CHROM. 12,418

FRACTIONNEMENT D'UNE PRÉPARATION CELLULASIQUE DE *TRICHODERMA VIRIDE* PAR CHROMATOGRAPHIE D'AFFINITÉ SUR CELLULOSE RÉTICULÉE

M. WEBER, M. J. FOGLIETTI et F. PERCHERON

Laboratoire de Chimie Biologique, Faculté des Sciences Pharmaceutiques et Biologiques, 4 Avenue de l'Observatoire, 75006 Paris (France)

(Reçu le 6 juillet 1979)

SUMMARY

Affinity chromatography of a cellulase complex from Trichoderma viride on cross-linked cellulose

A procedure involving affinity chromatography on cross-linked cellulose was developed for separating enzymatic components of a cellulase complex from *Trichoderma viride*. The exoglucanases [β-(1 → 4)-D-glucane cellobiohydrolase and cellobiase] passed through the column in the equilibrating buffer, whereas the endoglucanases were fixed in the same conditions. Five endo-β(1 → 4)-glucanases were fractionated by elution with stepwise increasing concentrations of carboxymethylcellulose added to the buffer. These endoglucanases were differentiated by their substrate specificities and by their reaction pattern toward carboxymethylcellulose, cellulose, insoluble cellodextrins and oligosaccharides.

INTRODUCTION

La dégradation de la cellulose est réalisée par des systèmes enzymatiques hétérogènes comprenant un certain nombre d'enzymes cellulolytiques agissant en synergie: endo-β-(1 → 4)-glucanases ou 1 → 4-β-D-glucane 4-glucanohydrolases (EC 3.2.1.4), exo-β-(1 → 4)-glucanases ou 1 → 4-β-D-glucane glucohydrolases (EC 3.2.1.74) et 1 → 4-β-D-glucane cellobiohydrolase[1–4]. Le plus souvent, ces cellulases sont associées à une cellobiase (EC 3.2.1.21). La grande hétérogénéité des préparations cellulasiques rend complexe le fractionnement de leurs différents composants et la plupart des techniques nécessitent la mise en oeuvre de nombreuses étapes faisant appel, soit à la filtration sur gel, soit à la chromatographie sur échangeurs d'ions.

Au cours de précédents travaux, nous avons décrit la séparation de deux types de polysaccharidases par chromatographie d'affinité sur leur substrat réticulé: endo-polygalacturonase[5] et α-amylases[6]. Nous décrivons ici l'application de cette technique au fractionnement d'une préparation cellulasique de *Trichoderma viride*.

PARTIE EXPÉRIMENTALE

Préparation enzymatique

Cellulase de *Trichoderma viride* (Boehringer, Mannheim, R.F.A.).

Substrats

Cellulose microcristalline en poudre (Whatman, Maidstone, Grande Bretagne); Carboxyméthylcellulose très polymérisée et peu substituée (Blanose R 195, Novacel, France); D-(+)-cellobiose (Sigma, St-Louis, Mo., États-Unis).

Cellodextrines insolubles et solubles

Après acétolyse de la cellulose pendant une nuit à la température du laboratoire selon le procédé de Hess et Dziengel[7] et désacétylation par le méthylate de sodium, les oligosaccharides de degré de polymérisation élevé sont précipités par l'éthanol. Les oligosaccharides solubles sont fractionnés par chromatographie sur colonne de Séphadex G-15.

Détermination de l'activité enzymatique

Une solution de carboxyméthylcellulose à 0.2% ou de cellobiose 0.05 *M* en tampon acétate 0.02 *M*, pH 4.6, est utilisée comme substrat. L'activité enzymatique est déterminée, après incubation à 30°, par dosage des sucres réducteurs libérés (exprimés en glucose) au moyen de la technique de Nelson et Somogyi[8,9]. L'activité enzymatique est exprimée en unités correspondant à la quantité d'enzyme libérant 1 μmole de glucose/min.

L'estimation du glucose libéré est effectuée par la méthode à la glucose-oxydase[10].

Lorsqu'un substrat insoluble est utilisé (cellulose microcristalline, cellodextrines insolubles), 5 mg de substrat sont ajoutés au milieu et l'incubation est effectuée sous agitation à 30°.

Identification des produits d'hydrolyse enzymatique

La séparation des produits d'hydrolyse enzymatique est effectuée par chromatographie sur couche mince de silice, à l'aide du solvant: *n*-propanol–acétate d'éthyle–éthanol absolu–acide acétique–pyridine–eau (7:3:3:2:2:4). La révélation est effectuée par chauffage après pulvérisation d'acétone sulfurique à 5%.

Support chromatographique

La réticulation de la cellulose est effectuée par action de l'épichlorhydrine en milieu alcalin suivant une modification de la technique de Kuniak et Marchessault[11].

Le degré de réticulation de la cellulose, dont dépendent les qualités du support chromatographique, est conditionné par les quantités de cellulose, de soude et d'épichlorhydrine présentes dans le milieu réactionnel ainsi que par la température et la durée de la réaction. Les conditions opératoires retenues doivent permettre d'obtenir un polysaccharide suffisament réticulé pour ne pas être hydrolysé par les endo-β-(1 $\rightarrow$ 4)-glucanases mais possédant encore des séquences de résidus glycopyranosyles non substitués, susceptibles d'être reconnus par ces enzymes qui se fixeront spécifiquement sur le support insoluble.

Différentes préparations se sont révélées efficaces quant à la fixation de l'activité endocellulasique. Pour des raisons pratiques (gonflement, vitesse d'écoulement), nous avons retenu le protocole décrit ci-dessous.

La cellulose microcristalline est traitée par de la soude en solution aqueuse à 10% (rapport molaire soude/cellulose = 0.6, calculé d'après la concentration en anhydroglucose) et par de l'épichlorhydrine (rapport molaire épichlorhydrine/cellulose = 5). Le mélange est maintenu sous agitation continue 15 min à la température du laboratoire, puis 1 h à 50°. Le milieu réactionnel est ensuite neutralisé par de l'acide acétique. Le polysaccharide réticulé est ensuite lavé à l'eau puis déshydraté par l'acétone et pulvérisé en fines particules.

Fractionnement du mélange cellulasique

Une colonne (28 cm × 2 cm) constituée par 10 g de cellulose réticulée est équilibrée avec un tampon acétate 0.02 *M*, pH 4.6 à 4°. 1 ml de solution tamponnée contenant 50 mg de préparation enzymatique et 5 mg de sérum albumine destinée à simuler des protéines contaminantes, est adsorbé sur la colonne. Un lavage de la colonne est réalisé avec le tampon d'équilibrage jusqu'à ce que l'éluat ne présente plus d'absorption à 280 nm.

Le fractionnement des activités endo-β-(1 → 4)-glucanasiques est ensuite réalisé grâce à une élution par palier avec des solutions de carboxyméthylcellulose de concentrations croissantes = 0.5, 1.75, 2 $g \cdot l^{-1}$ dans le tampon d'équilibrage de la colonne (pH 4.6) et 3 $g \cdot l^{-1}$ dans un tampon pH 5.6 correspondant au pH optimum de l'activité enzymatique retrouvée dans cette fraction.

RÉSULTATS

La chromatographie de la préparation cellulasique de *Trichoderma viride* sur cellulose réticulée (Fig. 1) nous a permis: (1) de séparer les activités exo- et endoglucanasiques; (2) de fractionner les activités endoglucanasiques retenues sur la colonne.

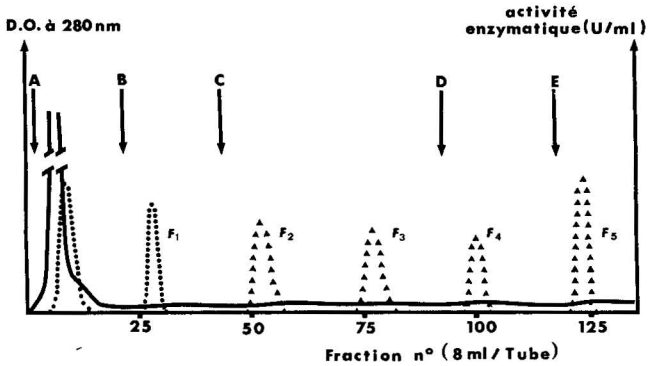


Fig. 1. Chromatographie d'une préparation cellulasique de *Trichoderma viride* sur cellulose réticulée. Colonne de 28 × 2 cm. Elution par Tampon d'équilibrage (A) et par solution tamponnée de carboxyméthylcellulose à: 0.5 $g \cdot l^{-1}$ (B); 1.75 $g \cdot l^{-1}$ (C); 2 $g \cdot l^{-1}$ (D) et 3 $g \cdot l^{-1}$ (E). ▬ = Densité optique (D.O.) à 280 nm, ● = pouvoir réducteur apparu après incubation sur carboxyméthylcellulose; ▲ = pouvoir réducteur apparu après incubation sur cellulose.

Séparation des activités exo- et endoglucanasiques

La fixation de l'activité endo-β-(1 $\rightarrow$ 4)-glucanasique est maximale à pH 4.6, pH voisin du pH optimum des endocellulases.

La cellulose réticulée constitue un support spécifique pour la fixation des endo-β-(1 $\rightarrow$ 4)-glucanases. En effet, les activités exo-β-(1 $\rightarrow$ 4)-glucanasique et cellobiasique présentes dans la préparation de *Trichoderma viride* sont éluées lors du lavage de la colonne par le tampon d'équilibrage, en même temps que les protéines contaminantes.

Ceci a pu être vérifié par incubation sur cellulose, cellodextrines et cellobiose en présence du premier pic d'élution, incubation suivie de l'estimation du pouvoir réducteur apparu, de celle du glucose libéré et d'une chromatographie des milieux réactionnels.

Cette première fraction conduit avec chacun des substrats à la seule libération de glucose. Ce type d'hydrolyse pouvant être obtenu, soit par action d'une β-(1 $\rightarrow$ 4)-D-glucane glucohydrolase, soit par action d'une β-(1 $\rightarrow$ 4)-D-glucane cellobiohydrolase et d'une cellobiase associées, nous avons soumis cette première fraction à une filtration sur Ultrogel AcA-34 (LKB, Stockholm, Suède). Ceci nous a permis de séparer une glucane cellobiohydrolase, hydrolysant la cellulose avec libération de cellobiose, et une cellobiase; l'action simultanée de ces deux enzymes conduisant à la libération de glucose.

Fractionnement des activités endo-β-(1 $\rightarrow$ 4)-glucanasiques

Différents essais d'élution des activités endo-β-(1 $\rightarrow$ 4)-glucanasiques par modification du pH (pH 3 à pH 7.5) ou par augmentation de la force ionique de l'éluant (tampons additionnés de KCl 0 à 2 *M*) se sont révélés inefficaces.

Lors d'un fractionnement d'une préparation cellulasique de *Trichoderma koningii* par chromatographie d'affinité sur colonne de cellulose, Halliwell et Griffin[12] ont pu utiliser avec succès une diminution de la force ionique de l'éluant (0.02 à 0 *M*). Ce protocole, appliqué pour l'élution des activités endo-β-(1 $\rightarrow$ 4)-glucanasiques fixées sur la cellulose réticulée s'est avéré inefficace.

L'utilisation de solutions tamponnées de cellobiose (2 *M*) permet l'élution de l'activité endo-β-(1 $\rightarrow$ 4)-glucanasique, mais en un pic très étalé sans séparation des diverses enzymes.

Des essais d'élution par des solutions tamponnées d'hydroxyéthylcellulose se sont également révélés négatifs.

Par contre, le fractionnement de cinq activités endo-β-(1 $\rightarrow$ 4)-glucanasiques a pu être réalisé grâce à une élution par palier avec des solutions tamponnées de carboxyméthylcellulose de concentrations croissantes: 0.5, 1.75, 2 et 3 $g \cdot l^{-1}$ (Fig. 1). Au cours de cette élution, l'enzyme attaque partiellement la carboxyméthylcellulose et les oligosaccharides libérés doivent être séparés de la protéine enzymatique par passage de l'éluat sur une colonne de Séphadex G-25. Tous les pics élués par des solutions de carboxyméthylcellulose correspondent à des activités endo-β-(1 $\rightarrow$ 4)-glucanasiques. En effet, chacun d'eux conduit à partir de la cellulose, de la carboxyméthylcellulose ou des cellodextrines, à la libération d'oligosaccharides de degré de polymérisation varié, dégradation caractéristique de celle réalisée par des endoglucanases.

Différenciation des activités endo-β-(1 → 4)-glucanasiques

Afin de différencier les cinq activités (F_1 à F_5) éluées par les solutions tamponnées de carboxyméthylcellulose, nous avons déterminé leur pH optimum d'action, leur spécificité de substrat et tenté de préciser leur mode d'action sur carboxyméthylcellulose, cellulose, cellodextrines insolubles ou solubles (degré de polymérisation de 2 à 5).

pH optimum. À l'exception de l'enzyme F_5 qui possède un pH optimum de 5.6, les quatre autres enzymes (F_1 à F_4) ont le même pH optimum, égal à 5.

Dégradation de la carboxyméthylcellulose. Seule la fraction F_1, éluée par la plus faible concentration en carboxyméthylcellulose, attaque ce substrat avec libération d'oligosaccharides de degré de polymérisation supérieur ou égal à 2. Cette fraction correspondrait donc à la "carboxyméthylcellulase" identifiée dans les préparations de *Trichoderma viride* par Okada[13].

Dégradation de la cellulose microcristalline. La fraction F_1 ne présente aucune activité vis-à-vis de ce substrat.

Les fractions F_2 à F_5 hydrolysent la cellulose insoluble avec libération de cellobiose et d'oligosaccharides de degré de polymérisation plus élevé. Lors d'hydrolyses prolongées, on note la présence presque exclusive de cellobiose dans les hydrolysats obtenus avec les fractions F_2 et F_3.

Dégradation des cellodextrines insolubles. L'hydrolyse des cellodextrines insolubles par les cinq fractions enzymatiques conduit à la libération préférentielle de cellobiose pour des temps d'hydrolyse élevés, mais l'étude de la cinétique de l'hydrolyse permet de signaler quelques différences dans le mode d'attaque de ces enzymes.

Les fractions F_1, F_2 et F_3 entraînent la disparition rapide des oligosaccharides de degré de polymérisation élevé (supérieur à 6), alors que les fractions F_4 et F_5 ne les hydrolysent que très lentement.

Dégradation des cellodextrines solubles. Toutes les enzymes hydrolysent le cellopentaose avec libération de cellotriose. Les fractions F_2 et F_3 se révèlent les plus actives vis-à-vis de ce substrat alors que la fraction F_5 n'a qu'une faible activité. De plus, F_2 et F_3 se différencient par le fait que dans les hydrolysats obtenus en présence de F_2, il y a apparition de cellotétraose, accompagné d'une faible quantité de glucose, quantité inférieure à celle du tétramère. Ceci pourrait s'expliquer par la mise en jeu, parallèlement à la réaction d'hydrolyse, d'une réaction de transfert. Le même type d'action peut également être attribué à la fraction F_4. Ces deux enzymes F_2 et F_4 seraient identiques aux cellulases IIA et IIB isolées par Okada[13] à partir de préparations cellulasiques de *Trichoderma viride* et catalysant la synthèse de cellotétraose à partir de cellobiose.

Le cellotétraose est hydrolysé par les fractions F_1, F_2 et F_3 avec attaque préférentielle de la liaison centrale et libération de cellobiose comme produit majeur d'hydrolyse. L'hydrolyse de ce substrat par les fractions F_4 et F_5 est nulle.

Aucune de ces enzymes n'hydrolyse le cellobiose.

L'ensemble de ces expériences est en faveur de l'existence d'entités enzymatiques différant par leur mode d'action. Les expériences d'électrofocalisation qui ont été tentées n'ont pas permis la détermination du point isoélectrique des protéines. Après l'élution de la colonne d'affinité par la carboxyméthylcellulose, une partie de ce polysaccharide reste adsorbé sur la protéine et la filtration sur gel de l'ensemble ne

permet pas de dissocier le complexe. Ceci ne paraît pas gêner les activités enzymatiques mais entrave les migrations électrophorétiques.

DISCUSSION

L'isolement des composants des complexes cellulasiques d'origines diverses nécessite en général une méthodologie longue et laborieuse. A notre connaissance, une seule technique de fractionnement d'activités cellulasiques par chromatographie d'affinité sur cellulose a été mise au point par Halliwell et Griffin[12]. Cependant cette technique ne permet la séparation de l'activité endoglucanasique de *Trichoderma koningii* qu'en deux fractions, une carboxyméthylcellulase et une endoglucanase, alors que ce complexe cellulasique semble contenir au moins quatre activité endoglucanasiques vraies[15].

La chromatographie d'affinité des complexes cellulasiques sur cellulose réticulée constitue un moyen simple et rapide pour, d'une part, séparer les activités exo- et endocellulasiques associées et, d'autre part, fractionner les diverses activités endocellulasiques le plus souvent présentes dans les préparations, afin d'en étudier le mode d'action.

REMERCIEMENTS

Ce travail a bénéficié d'une aide de la D.G.R.S.T. (No. 75 70 538 01).

RÉSUMÉ

Un procédé de chromatographie d'affinité sur cellulose réticulée est mis en oeuvre pour séparer les différents composés enzymatiques d'un complexe cellulasique de *Trichoderma viride*. Les exoglucanases [β-(1 $\rightarrow$ 4)-D-glucane cellobiohydrolase et une cellobiase] sont éluées par le tampon d'équilibrage alors que les endoglucanases sont retenues par le support. Cinq endo-β-(1 $\rightarrow$ 4)-glucanases sont fractionnées grâce à une élution par palier avec des solution tamponnées de carboxyméthylcellulose de concentrations croissantes. Afin de différencier les activités endoglucanasiques, nous étudions leur spécificité de substrat et tentons de préciser leur mode d'action sur carboxyméthylcellulose, cellulose, cellodextrines insolubles et oligosaccharides solubles.

BIBLIOGRAPHIE

1 T. M. Wood et S. I. McCrae, *Biochem. J.*, 128 (1972) 1183.
2 G. Halliwell et M. Griffin, *Biochem. J.*, 135 (1973) 587.
3 M. Streamer, K. E. Eriksson et B. Petersson, *Eur. J. Biochem.*, 59 (1975) 607.
4 T. M. Wood et S. I. McCrae, *Carbohyd. Res.*, 57 (1977) 117.
5 M. J. Foglietti, M. Girerd et F. Percheron, *Biochimie*, 57 (1975) 667.
6 M. Weber, M. J. Foglietti et F. Percheron, *Biochimie*, 58 (1976) 1299.
7 K. Hess et K. Dziengel, *Ber. Deut. Chem. Ges. B*, 68 (1935) 1594.
8 N. Nelson, *J. Biol. Chem.*, 153 (1944) 375.
9 M. Somogyi, *J. Biol. Chem.*, 160 (1945) 61.
10 J. B. Lloyd et W. J. Whelan, *Anal. Biochem.*, 30 (1968) 467.
11 L. Kuniak et R. H. Marchessault, *Starke*, 4 (1972) 110.
12 G. Halliwell et M. Griffin, *Biochem. J.*, 169 (1978) 713.
13 G. Okada, *J. Biochem. (Tokyo)*, 80 (1976) 913.
14 G. Okada, *J. Biochem. (Tokyo)*, 77 (1975) 33.
15 T. M. Wood et S. I. McCrae, *Biochem. J.*, 171 (1978) 61.

Journal of Chromatography, 188 (1980) 383–390

CHROM. 12,410

ASSAY METHOD FOR UROKINASE ACTIVITY BY CAPILLARY-TUBE ISOTACHOPHORESIS USING A SYNTHETIC SUBSTRATE

KAZUO KATOH and HIROSHI MIYAZAKI*

Research Laboratories of Pharmaceutical Division, Nippon Kayaku Co., 3-31 Shimo, Kita-ku, Tokyo 115 (Japan)

(First received March 23rd, 1979; revised manuscript received September 20th, 1979)

SUMMARY

Urokinase activity was determined by capillary-tube isotachophoresis using N-α-acetyl-L-lysine methyl ester as a synthetic substrate. The resulting N-α-acetyl-L-lysine was separated and detected isotachophoretically using methanolic potassium hydroxide solution adjusted to pH 5.32 by adding α-ketoglutaric acid as a leading electrolyte and methanolic betaine hydrochloride solution as a terminating electrolyte.

The enzymatic reaction was stopped by addition of tannic acid and the resulting supernatant solution was injected into an isotachophoretic analyser. Linearity was observed at urokinase activities in the range 1–30 I.U. The urokinase activities in commercial products determined by the method were in good agreement with those determined by Walton's modified plate method.

The coefficient of variation of the method was less than 3.4%.

INTRODUCTION

The physiological role of urokinase is to convert plasminogen into plasmin, and the resulting plasmin lyses the clots of fibrin[1,2]. Conventional assay methods such as the test-tube method[3] and the fibrin plate method[4] are based on this mechanism. However, these two methods have the disadvantage that the analytical error may be increased owing to the subjective judgement of the end-point by the analyst.

On the other hand, spectrophotometric methods based on esterase activity of urokinase have been reported by various investigators. Of these synthetic substrates, N-α-acetyl-L-lysine methyl ester is the most specific substrate for the determination of urokinase activity, and the assay methods using this substrate were developed by Sherry *et al.*[5] and Hamberg and Savolainen[6]. However, these methods require tedious and time-consuming procedures for the determination of the reaction products.

Capillary-tube isotachophoresis[7,8] has been used as a rapid and specific method for the simultaneous qualitative and quantitative analyses of a mixture of peptides[9] and amino acids[10].

* To whom correspondence should be addressed.

This paper describes the isotachophoresis of N-α-acetyl-L-lysine, the separation of which has been thought to be difficult, and a rapid method for the determination of urokinase activity by isotachophoresis.

EXPERIMENTALS

Reagents

N-α-Acetyl-L-lysine methyl ester monohydrochloride and N-α-acetyl-L-lysine monohydrochloride were obtained from Sigma (St. Louis, Mo., U.S.A.). Tannic acid and tris(hydroxymethyl)aminomethane (Tris) were purchased from E. Merck (Darmstadt, G.F.R.). Potassium hydroxide, α-ketoglutaric acid, 2-amino-2-methyl-1,3-propanediol (Ammediol) tetra-*n*-butyl-ammonium chloride and betaine hydrochloride were purchased from Tokyo Kasei Co. (Tokyo, Japan). Barium hydroxide was of extra-pure reagent grade and purchased from Wako (Osaka, Japan). Methanol was of reagent grade and purchased from Kanto Chemical Co. (Tokyo, Japan). Absolute ethanol was purchased from Amakasu Chemical Co. (Tokyo, Japan). Gelatin was obtained from Difco Labs. (Detroit, Mich., U.S.A.).

The substrate solution for enzymatic reactions was prepared by dissolving 5.5 mg of N-α-acetyl-L-lysine methyl ester monohydrochloride in 10 ml of 0.01 *M* phosphate buffer containing 0.01% of gelatin (buffer solution A).

Measurement of urokinase activity using plasminogen

The modified method of Walton[4] was used as a representative method with plasminogen as a substrate. The content of protein in urokinase solution was determined by the Folin method[12].

Incubation procedure for determination of urokinase activity

Urokinase preparations were dissolved in 6 ml of 0.06 *M* Tris–hydrochloric acid buffer (pH 7.2) containing 0.09 *M* sodium chloride and 0.1% of gelatin.

Urokinases with different specific activities were prepared as solutions containing 0.025 *M* phosphate buffer (pH 7.5) containing 1.5% of sodium chloride.

A 100-μl volume of the substrate solution and an aliquot of the sample solution containing urokinase activities in the range 1–10 I.U. were placed in a test-tube and the total volume of the mixture was made up to 130 μl with buffer solution A. The test-tube was shaken gently for 60 min at 37.5°. After 20 μl of a 0.2% solution of tannic acid in buffer solution A had been added to the reaction mixture, the solution was cooled for 5 min in an ice-bath to stop completely the reaction and the precipitate was removed by centrifugation. An aliquot of the resulting supernatant was injected into the isotachophoretic analyser.

Determination of minimum amount of tannic acid for cessation of the enzyme reaction

A 10-μl volume of urokinase in the buffer solution A (1000 I.U./ml), 100 μl of the substrate solution and 20 μl of buffer solution A were used as the incubation mixture for determination of urokinase activity. After the first enzymatic reaction for 30 min at 37.5°, 18 μl of the solutions with various concentrations of tannic acid were added to the reaction mixture and the solution was cooled for 5 min in an

ice-bath. The solution was incubated again for 30 min at 37.5° and finally 2 μl of 2.0% tannic acid solution in water were added to stop completely the reaction.

Isotachophoretic conditions for determination of urokinase activity

The isotachophoresis was carried out with a Shimadzu isotachophoretic analyser, Model IP-1B and PGD-1, equipped with a capillary of length 150 mm and I.D. 0.57 mm.

As the leading and terminating electrolytes for isotachophoresis, 0.01 *N* methanolic potassium hydroxide solution adjusted to pH 5.32 by adding α-ketoglutaric acid and 0.01 *M* methanolic betaine hydrochloride solution respectively, were used after deaeration with an aspirator. The starting voltage was 2.2 kV and the final voltage was 5.1 kV, maintaining a migration current of 50 μA at 25°.

RESULTS AND DISCUSSION

N-α-Acetyl-L-lysine methyl ester exhibits basicity due to the α-amino group of lysine and its pK_a value is 10.8. However, the basicity of N-α-acetyl-L-lysine obtained by incubation of the substrate with urokinase decreases owing to the influence of the carboxyl group at the α-position in the molecule, and the pK_a value of the resulting product is 3.5.

Isotachophoretic conditions for the migration of this product towards the anode in alkaline solution of pH 8.0, 8.5, 9.0, 9.5 and 10.0 were investigated by adding ammediol or Tris solution as the leading electrolyte and aqueous solutions of β-alanine(pH 10.9), phenol (pH 10.0), γ-aminobutyric acid (pH 9.7), δ-aminocaproic acid (pH 9.4) and ω-aminocapric acid (pH 9.8), their pH values being adjusted with barium hydroxide solution, as the terminating electrolyte. However, the product did not migrate towards the anode under these conditions. Therefore, the isotachophoretic conditions for the migration of this compound as a cation were investigated. Potassium and barium ions have generally been used as the leading electrolytes for the migration of amino acids as cations.

Kopwillem *et al.*[15] reported the separation of basic amino acids such as lysine, arginine and histidine using an aqueous barium solution adjusted to pH 9.9 by adding valine as a leading electrolyte and Tris buffer adjusted to pH 8.0 with hydrochloric acid as the terminating electrolyte. Although the isotachophoresis of N-α-acetyl-L-lysine and its methyl ester was carried out under the above conditions, these compounds in acidic solution were tried again using aqueous potassium hydroxide solution adjusted to pH 3.0, 4.0, 5.0 and 6.0 by adding α-ketoglutaric acid, cacodylic acid or caproic acid as the leading electrolyte, and Tris–hydrochloric acid buffer (pH 8.0) as the terminating electrolyte.

N-α-Acetyl-L-lysine was not detected because its mobility was lower than that of the terminating ion under the above condition. As shown in Fig. 1, only its methyl ester migrated when aqueous potassium hydroxide solution adjusted to pH 5.4 by adding cacodylic acid and aqueous tetra-*n*-butylammonium chloride solution were used as the leading and terminating electrolytes, respectively.

Thus, urokinase activity could be determined indirectly by measuring the residual amounts of this substrate in the incubation mixture[13]. A disadvantage of this method is the relatively low sensitivity: at least 4 I.U. of urokinase are required.

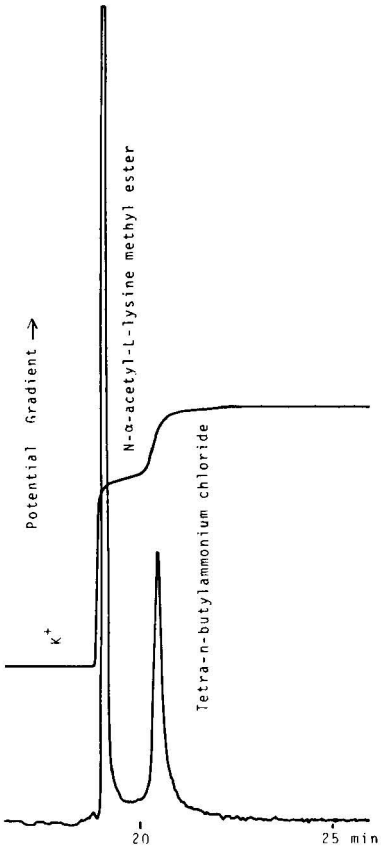


Fig. 1. Isotachopherogram of N-α-acetyl-L-lysine methyl ester.

Therefore, it is necessary to determine directly the N-α-acetyl-L-lysine in order to enhance the sensitivity of isotachophoresis. However, it was very difficult to make this compound migrate in an aqueous solution. Consequently, the use of organic solvents[14] was tried in order to make the lysine analogues migrate. Contrary to our expectations, the lysine analogues were not detected when methanolic potassium hydroxide solution adjusted to pH 3.0–6.0 was used as the leading electrolyte and methanolic Tris solution (pH 8.0) as the terminating electrolyte. This suggests that the mobilities of the ions of interest may be equal to or lower than that of the terminating ion. Thus, it was found that migration of N-α-acetyl-L-lysine was observed on the isotachopherogram when 0.01 *N* methanolic potassium hydroxide solution adjusted to pH 5.32 by adding α-ketoglutaric acid was used as the leading electrolyte and 0.01 *M* methanolic betaine hydrochloride solution as the terminating electrolyte.

Fig. 2a shows the isotachophoretic separation of N-α-acetyl-L-lysine and its methyl ester. Fig. 2b shows a representative isotachopherogram of the incubation mixture for the assay of urokinase activity. The potential unit (P.U.) values[11] of the two compounds were found to be 0.256 and 0.121, respectively. Betaine hydrochloride was very useful as a terminating ion in organic solvents, especially absolute ethanol.

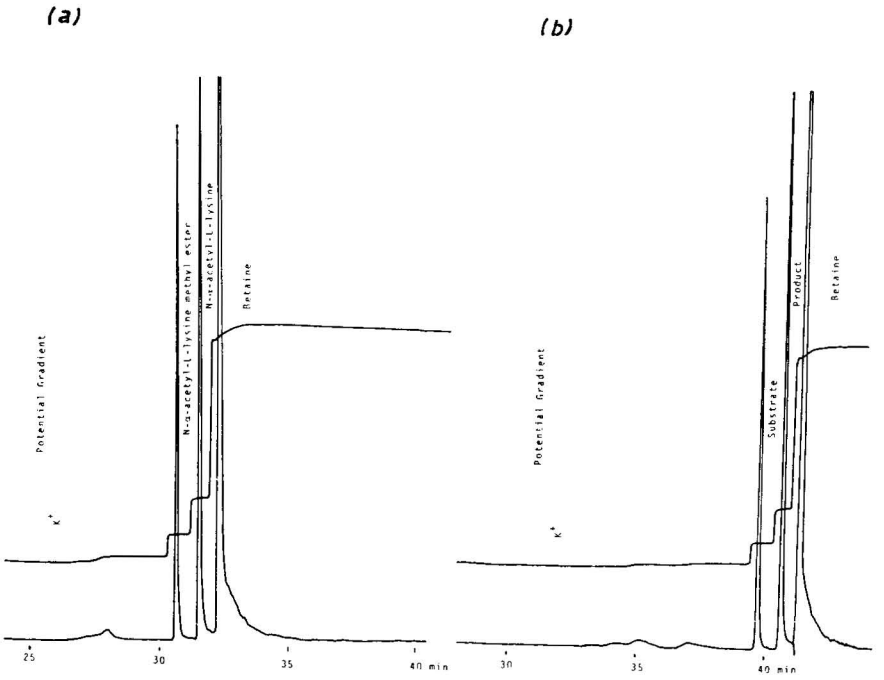


Fig. 2. (a) Isotachophoretic separation of N-α-acetyl-L-lysine and its methyl ester and (b) representative isotachopherogram of the reaction mixture. A 10-μl volume of urokinase solution (690 I.U./ml of 0.025 *M* phosphate buffer containing 1.5% of sodium chloride buffer, pH 7.5) was used.

As shown in Fig. 3, the calibration graph for N-α-acetyl-L-lysine was a straight line.

The presence of large amounts of inorganic salts in the incubation mixture is undesirable in isotachophoresis because of the time required for migration of these inorganic ions because the mobilities of the salts were greater than that of the

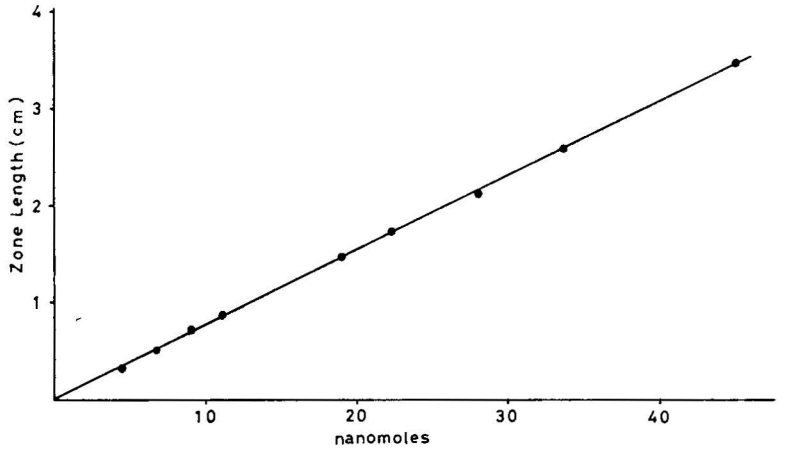


Fig. 3. Calibration graph for quantification of N-α-acetyl-L-lysine.

product. Therefore, the concentration of the buffer in the incubation mixture should be kept as low as possible, and phosphate buffer solution containing 0.1% of gelatin, which assists in the stabilization of urokinase, can be used as the incubation mixture. The optimum concentration of the substrate for this assay was then investigated.

Fig. 4a shows the titration curve for substrate solutions in the concentration range $4.27 \cdot 10^{-4}$–$3.43 \cdot 10^{-2}$ *M*. The results indicate that the enzymatic reaction attains a maximum at a concentration of $1.4 \cdot 10^{-2}$ *M* and subsequently 100 μl of $2.22 \cdot 10^{-2}$ *M* substrate solution were used in the experiment. Fig. 4b shows the zone length of N-α-acetyl-L-lysine produced at various pH values. The optimum pH was 7.5. Fig. 4c shows the relationship between the zone length of N-α-acetyl-L-lysine and the incubation time. Good linearity was obtained from 0.5 to 60 min. The incubation time was subsequently fixed at 60 min.

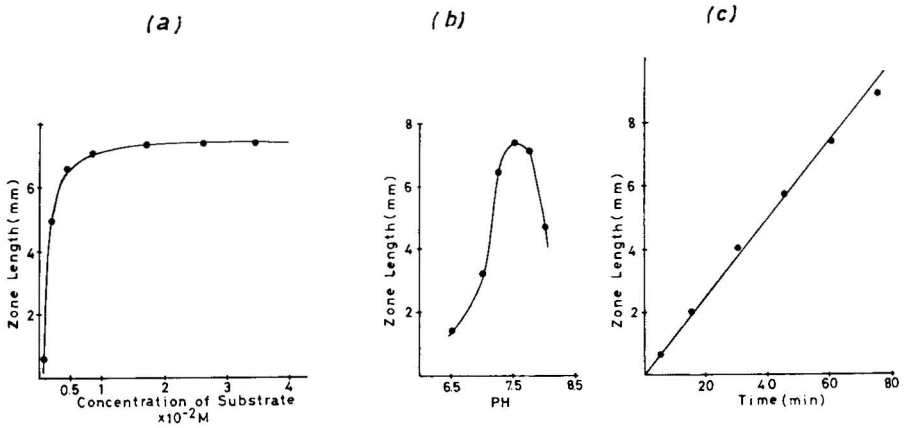


Fig. 4. (a) Subtrate titration curve; (b) relationship between zone length and pH of the reaction mixture; and (c) relationship between zone length and incubation time. A 10-μl volume of urokinase solution (1000 I.U./ml of buffer A) was used in each experiment. Each zone length was obtained by subtracting that in a blank run without urokinase from that of the incubation mixture.

In general, strong acids such as trichloroacetic or perchloric acid have been used as the terminator of the enzymatic reaction. However, these acids are unsuitable for isotachophoretic analysis because a long period is required for the migration of large amounts of these acids. In contrast, it was found that tannic acid is useful not only as a terminator but also as deproteinizing agent for enzymatic mixtures.

Fig. 5 shows the minimum amount of tannic acid required for terminating the reaction. The enzymatic reaction could be stopped completely by adding more than a 30-fold excess of tannic acid over the amount of protein in the urokinase solution.

Fig. 6 shows the calibration graph for the determination of urokinase, constructed by subtracting the zone length in a blank run without urokinase from that of the incubation mixture. A straight line was obtained in the range from 1 to 30 I.U. Then, the urokinase activities in commercially available preparations and active ingredients with various specific activities were determined by the present method and Walton's modified plate method.

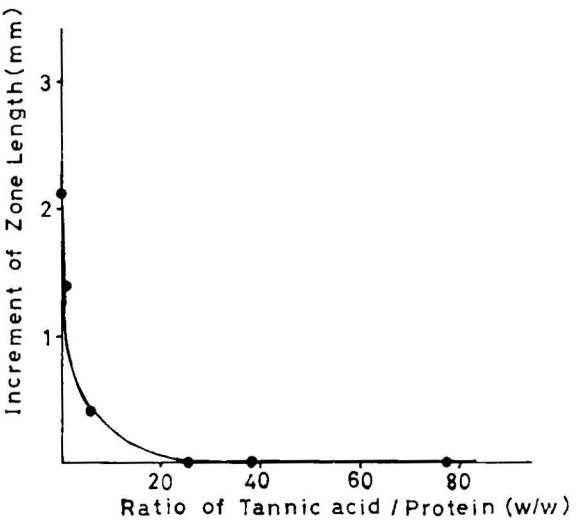


Fig. 5. Minimum amount of tannic acid for terminating the enzymatic reaction. The minimum amount was obtained when there was no increase in the zone length in the first and second incubations.

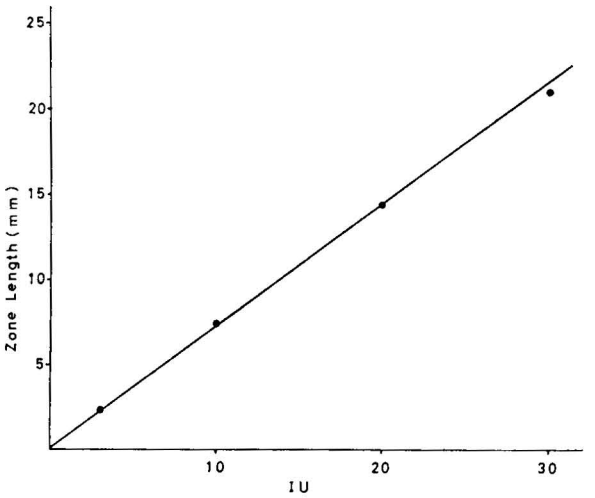


Fig. 6. Calibration graph for determination of urokinase activity.

Table I shows a comparison between the urokinase activities of the same samples determined by the two methods. Both results were in good agreement with each other.

The coefficient of variation of the isotachophoretic method was 3.4% ($n = 5$), indicating that it may be a convenient method for the determination of urokinase activity.

TABLE I

COMPARISON BETWEEN WALTON'S MODIFIED PLATE METHOD AND PRESENT METHOD FOR UROKINASE ACTIVITIES IN COMMERCIALLY AVAILABLE PREPARATIONS AND ACTIVE INGREDIENTS WITH VARIOUS SPECIFIC ACTIVITIES

Sample	*Sample No.*	*Specific activity (I.U./mg.)*	*Walton's modified method (I.U./vial)*	*Present method (I.U./vial)*
Commercially available preparation	1	—	6401	6240
	2	—	6483	6600
	3	—	6746	6960
	4	—	6528	6720
	5	—	6328	6360
Active ingredients	1	811	10,685	10,200
	2	14,350	36,167	34,560
	3	13,848	36,143	35,640
	4	15,890	28,765	28,200
	5	37,270	125,706	122,400

ACKNOWLEDGEMENTS

The authors are grateful to Dr. W. Tanaka, Research Laboratories, Nippon Kayaku Co., for his encouragement and useful suggestions throughout this work. They thank Mr. S. Arai and Mrs. T. Kyochi, Research Laboratories, Nippon Kayaku Co., for their cooperation in measuremening the urokinase activities by Walton's modified plate method.

REFERENCES

1 J. R. B. Wiliiams, *Brit. J. Exp. Pathol.*, 32 (1951) 530.
2 T. Astrup and I. Sterndorff, *Proc. Soc. Exp. Med.*, 81 (1952) 675.
3 J. Ploug, *Biochem. Biophys. Acta*, 24 (1957) 278.
4 P. L. Walton, *Clin. Chim. Acta*, 13 (1966) 680.
5 S. Sherry, N. Alkjaersig and A. P. Fletcher, *J. Lab. Clin. Med.*, 64 (1964) 145.
6 U. Hamberg and M.-L. Savolainen, *Acta Chem. Scand.*, 22 (1968) 1452.
7 H. Haglund, *Sci. Tools*, 17 (1970) 2.
8 F. M. Everaerts, J. L. Beckers and P. E. M. Verheggen, *Isotachophoresis—Theory, Instrumentation and Applications*, Elsevier, Amsterdam, Oxford, New York, 1976.
9 A. Kopwillem, F. Chillemi, A. B. Bosisio-Righetti and P. Righetti, *Protides Biol. Fluids, Proc. Colloq.*, 21 (1973) 657.
10 F. M. Everaerts and A. J. M. Vander Put, *J. Chromatogr.*, 52 (1970) 415.
11 H. Miyazaki and K. Katoh, *J. Chromatogr.*, 119 (1976) 369.
12 O. H. Lowry, J. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 67 (1959) 166.
13 H. Miyazaki, *Review Symposium on Analytical Isotachophoresis and Ion-Exchange Resin Chromatography, Sussex Univ., Brighton, Great Britain, 1976.*
14 J. L. Beckers and F. M. Everaerts, *J. Chromatogr.*, 51 (1970) 339.
15 A. Kopwillem, U. Moberg, G. W. Sjödahl, R. Lundin and H. Sievertsson, *Anal. Biochem.*, 67 (1975) 166.

Journal of Chromatography, 188 (1980) 391–399

CHROM. 12,396

CHROMATOGRAPHIC SEPARATION OF METAL IONS ON MACRORETICULAR ANION-EXCHANGE RESINS OF A LOW CAPACITY

D. T. GJERDE and J. S. FRITZ

Ames Laboratory-USDOE and Department of Chemistry, Iowa State University, Ames, Iowa 50011 (U.S.A.)

(Received September 18th, 1979)

SUMMARY

The capacity of anion-exchange resins is a useful variable in devising practical analytical separations. Metal ions that form anionic chloro complexes are more weakly retained on resins of very low exchange capacity. Palladium(II), platinum(IV), gold(III), bismuth(III) and mercury(II) are retained on a column of 0.21 mequiv./g XAD-4 anion-exchange resin from 0.2 *M* hydrochloric acid and base metals pass through. The metals are then selectively eluted by various higher concentrations of hydrochloric acid. Batch distribution data are used to show the effect of capacity and to predict what separations are possible.

INTRODUCTION

The separation of metals in the form of chloro complexes from aqueous hydrochloric acid solutions using anion exchange columns is a well established analytical technique[1–7]. Hydrochloric acid has been a useful medium because it can be used to dissolve a large number of metals. Equally important, it is a useful reagent for the quantitative spectrophotometric determination[8] of many metals that may be automated with the use of a flow cell[9,10]. The very strong sorption of chloro complexes of several metal ions on conventional anion-exchange resins makes it difficult or impossible to elute these metal ions. In some cases elution has been accomplished by mixing perchloric acid with the hydrochloric acid eluent[10].

Recently it was shown that the distribution coefficients of simple inorganic anions decrease with decreasing capacity of the anion-exchange resin[11]. Fritz and Story[12,13] have reported a similar effect for cations on cation-exchange resins of lower capacity. In the present investigation a series of macroporous anion-exchange resins of very low capacity was prepared and the exchange behavior of anionic metal chloride complexes studied. Distribution coefficients on the new anion-exchange resins are significantly lower, and many analytical separations of metal ions have been obtained that would be impossible to achieve on conventional resins of high capacity.

EXPERIMENTAL

Apparatus

Most of the work was done with a liquid chromatograph that has been described previously[14]. The system employs automatic detection with a Tracor 970 variable-wavelength detector. All detection was done at 225 nm unless otherwise stated. Various concentrations of hydrochloric acid were used as the dye. Flow-rates of the eluent and dye were 1.5 ml/min. Sample loop size was 200 μl. A 80 $\times$ 2 mm column was used for the platinum–gold and palladium–gold separations. For the bismuth–mercury and for the tin separations a 25 $\times$ 2 mm column with 20 $\times$ 2 mm ends was used. Both columns used Altex 200-28 glass connectors.

The copper, palladium and platinum separation required a longer column. The resulting higher back pressure made the liquid chromatograph described above unsuitable. The chromatograph used for this separation is outlined in Fig. 1. This chromatograph was designed to allow only glass, PTFE, Kel-F or Tefzel plastic to come into contact with the mobile phase.

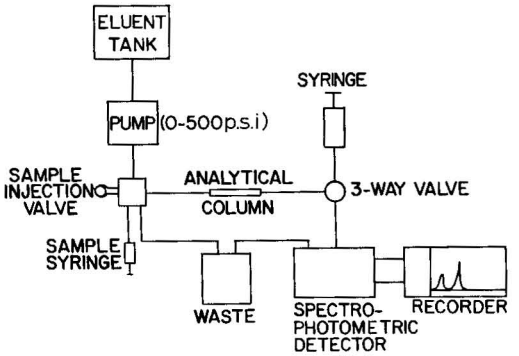


Fig. 1. Schematic diagram of the liquid chromatograph.

The eluent tank was a 500 ml glass reagent bottle. The mobile phase was pumped from the eluent tank by a CMP-2 Cheminert metering pump. The pump is rated 0–500 p.s.i. It employs a three piston design to reduce pressure and flow pulsation. The pump can be adjusted to flow-rates of 2.4–120 ml/h. All experiments were done at a flow-rate of 60 ml/h. Flow control is provided by pneumatically actuated three-way valves which control the filling and draining of each piston. The sample injection system is a three-way and four-way valve modeled after an Altex 201-56 sample injection valve. All work was performed using a 56.3 μl sample loop. The three-way valve between the column and detector allowed the removal of bubbles in the flow cell by rapidly forcing water through the system with a syringe. Two 500 $\times$ 2 mm glass columns were connected in series. The columns were individually slurry packed with a syringe. The resin was 0.04 mequiv./g XAD-1 (150–160 mesh), a strong base anion-exchange resin. The detector used was the same as for the other separations.

Measurement of distribution coefficients

Approximately 0.15 g of dried resin was accurately weighed into a 50 ml glass stoppered flask. The metal salt–hydrochloric acid solution was pipetted into the flask and the mixture was left for 6–12 h, with occasional shaking. Generally 15 ml of solution were used, however smaller and larger amounts of solution were used for extremely small and large distribution coefficients. The resin was filtered off and the metal content of the solution was determined by the absorbance at an appropriate wavelength. Distribution coefficients, D, were calculated by assuming all the metal gone from the solution was sorbed on on the resin where D is defined as

$$D = \frac{\text{amount of metal per g of dry resin}}{\text{amount of metal per ml of solution}}$$

Reagents

The copolymer material used for the synthesis of low capacity anion-exchange resins was macroreticular poly(styrene–divinylbenzene)beads. XAD-1 and XAD-4 were obtained from Rohm & Haas (Philadelphia, Pa., U.S.A.). The synthesis has been described in another paper[11]. The beads are highly cross-linked and have excellent chemical and mechanical stability.

Reagent grade metal chlorides were used when available. The stock silver solution was made from silver perchlorate. Platinic chloride solutions were made by diluting a 10% stock solution obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.) with the appropriate concentration of hydrochloric acid.

RESULTS

The distribution coefficients for several metal ions were determined as a function of aqueous hydrochloric acid concentration for low capacity anion-exchange resins. The metal ions chosen for study form chloro complexes that are tenaciously held by conventional anion-exchange resins. The results in Fig. 2 show distribution coefficients that are significantly lower for the new resins than for conventional anion-exchange resins of high capacity. Furthermore, the distribution coefficients tend to decrease more rapidly at the very low capacities, as shown by the behaviour of platinum(IV) in Fig. 2.

The distribution coefficients in Fig. 2 suggest that by suitable selection of hydrochloric acid concentration, column separations of some of the metal ions in Fig. 2 from other metals and from each other should be possible. Several actual separations are reported below using the chromatographic and detection systems outlined in Experimental.

Separation of platinum(IV) or palladium(II), gold(III) and base metals

A separation of chromium(III), platinum(IV) and gold(III) on 0.21 mequiv./g XAD-4 is shown in Fig. 3. The sample is dissolved in *ca.* 0.2 *M* hydrochloric acid and injected on the column with eluent 0.2 *M* hydrochloric acid. Chromium(III) and other base metals are eluted under these conditions. Platinum(IV) is removed with 3.5 *M* hydrochloric acid. Gold(III) is eluted with 1.0 *M* hydrochloric acid in methanol. A

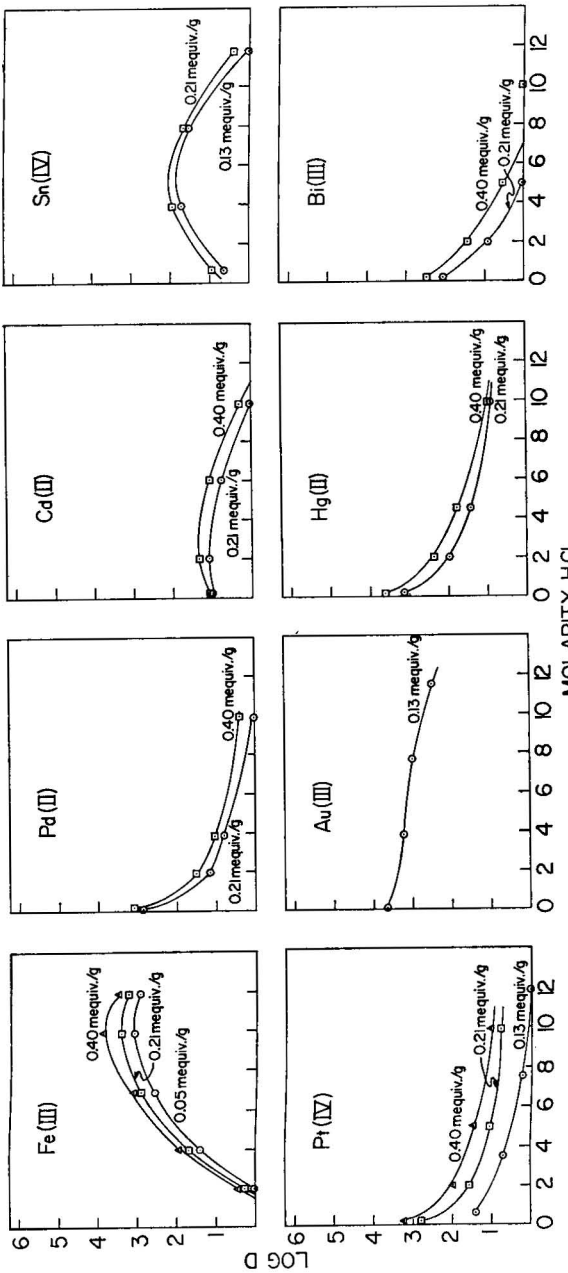
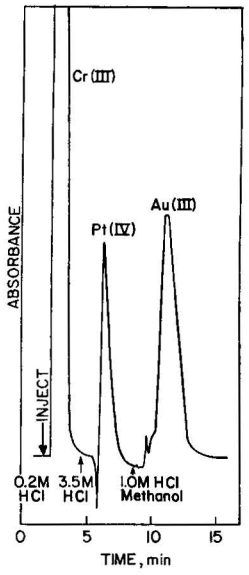


Fig. 2. XAD-4 anion-exchange distribution coefficients (D) of various metals as a function of HCl concentration and capacity.



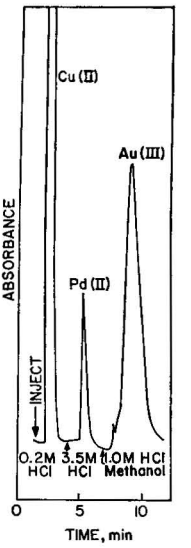


Fig. 3. Separation of 2.0 mg chromium(III), 39 μg platinum(IV) and 7.8 μg gold (III).
Fig. 4. Separation of 1.3 mg copper(II), 2.1 μg palladium(II) and 9.8 μg gold(III).

separation of copper(II), palladium(II) and gold(III) on 0.21 mequiv./g XAD-4 is shown in Fig. 4. The same procedure is used to carry out the separation.

The negative peak directly preceding platinum(IV) in Fig. 3 is a solvent change peak and is typical in a step gradient elution from a low concentration to a high concentration of hydrochloric acid. The small peak right ahead of the gold(III) peak is also a solvent change peak. None of the solvent change peaks interfered with the peak heights of the metals analyzed. Standard solutions of platinum, palladium and gold of various concentrations gave linear calibration curves of peak height *vs.* μg of metal ion.

TABLE I

DETERMINATION OF PLATINUM AND GOLD IN THE PRESENCE OF FOREIGN METALS

Foreign metal	*Pt (IV)*		*Au(III)*	
	Molar excess foreign metal	*Recovery % (av. of 2)*	*Molar excess foreign metal*	*Recovery (%) (av. of 2)*
Mn(II)	200	100.8	1000	99.1
Fe(III)	200	99.2	1000	79.0
Fe(III)	—	—	500	98.2
Cr(III)	200	96.9	1000	99.8
Ni(II)	200	97.1	1000	99.0
Cu(II)	200	97.0	1000	98.7
Sr(II)	200	98.3	1000	99.2
Co(II)	200	97.7	1000	99.6
	Average range 2.3%		Average range 1.1%	

TABLE II

DETERMINATION OF PALLADIUM AND GOLD IN THE PRESENCE OF FOREIGN METALS

Foreign metal	*Pd(II)*		*Au(III)*	
	Molar excess foreign metal	*Recovery (%) (av. of 3)*	*Molar excess foreign metal*	*Recovery (%) (av. of 3)*
Mn(II)	1000	96.5	400	99.7
Fe(III)	1000	96.6	400	99.6
Cr(III)	1000	98.0	400	99.8
Ni(II)	1000	96.0	400	99.6
Cu(II)	1000	97.6	400	99.7
Sr(II)	1000	96.7	400	99.8
Co(II)	1000	98.8	400	100.0
	Average range 1.7%		Average range 0.4%	

Tables I and II show there is little or no interference with the foreign metals tested.

Separation of bismuth(III) and mercury(II) from other metals

Bismuth(III) and mercury(II) also form stable anion complexes but are somewhat difficult to elute with aqueous hydrochloric acid from ordinary anion-exchange resins. In the chromatogram shown in Fig. 5 bismuth(III) and mercury(II) are separated from each other and iron(III) on 0.21 mequiv./g XAD-4. The sample is injected onto the column with an eluent of 0.2 *M* hydrochloric acid. After the major metal ion [iron(III)] has eluted, bismuth(III) is eluted with 2.2 *M* hydrochloric acid. Mercury(II) is eluted with 8.5 *M* hydrochloric acid. A larger column was used for

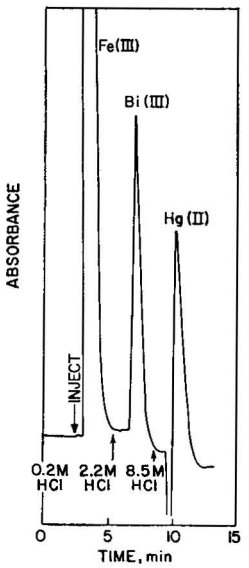


Fig. 5. Separation of 2.2 mg iron(III), 4.2 μg bismuth(III) and 4.0 μg mercury(II).

TABLE III

DETERMINATION OF BISMUTH AND MERCURY IN THE PRESENCE OF FOREIGN METALS

Foreign metal	*Bi(III)*		*Hg(II)*	
	Molar excess foreign metal	*Recovery (%) (av. of 3)*	*Molar excess foreign metal*	*Recovery (%) (av. of 3)*
Cu(II)	1000	99.7	1000	99.8
Cr(III)	1000	100.1	1000	100.6
Fe(III)	1000	101.9	1000	100.0
Mn(II)	1000	99.2	1000	100.2
Sr(II)	1000	99.3	1000	99.6
Ni(II)	1000	100.0	1000	100.9
Co(II)	1000	100.5	1000	100.7
Zn(II)	1000	100.1	1000	102.7
Cd(II)	1000	99.7	1000	101.1
Sn(IV)	100	98.8	100	101.9
As(III)	100	98.9	100	100.2
Pb(II)	20	101.2	20	101.3
Sb(III)	20	100.6	20	104.4
Ag(I) $\lambda = 225$	1	>150	1	99.9
Ag(I) $\lambda = 327$	1	99.0	1	–
	Average range 1.3%		Average range 1.5%	

this separation, which permitted samples to be dissolved in up to 2 *M* hydrochloric acid with no premature bleeding of the bismuth(III).

The results in Table III show the method is quite selective. Samples containing antimony(III) and tin(IV) have a different baseline shift which is caused by bleeding of the residue metals. However, this effect is not serious and recovery is still good. Silver(I) interferes with bismuth(II) when detection is at 225 nm. Changing to 327 nm eliminates this interference because silver(I) no longer absorbs.

Separation of tin(IV) from other metals

Satisfactory separations of tin(IV) and nickel(II) have been obtained on 0.21 mequiv./g XAD-4. The sample is dissolved in *ca.* 5 *M* hydrochloric acid and is

TABLE IV

DETERMINATION OF TIN IN THE PRESENCE OF FOREIGN METALS

Foreign metal	*Sn(IV)*	
	Molar excess foreign metal	*Recovery (%) (av. of 3)*
Cu(II)	100	100.9
Cr(III)	100	100.5
Ni(II)	100	100.6
Zn(II)	100	99.7
As(III)	100	interfers
Sb(III)	100	interfers
	Average range 1.3%	

injected on the column. Nickel(II) and other metals are eluted with 5.0 *M* hydrochloric acid. Tin(IV) is eluted with 1.0 *M* hydrochloric acid. There is a solvent change peak preceding the tin(IV) peak, but a linear calibration curve peak height *vs.* μg of tin(IV) was obtained nevertheless. Data for determination of tin(IV) in the presence of other metal ions are given in Table IV.

Separation of copper(II), palladium(II) and platinum(IV)

When a column with higher performance is used, palladium(II) and platinum(IV) can be easily separated from each other and a non-retained metal such as copper(II). The chromatogram in Fig. 6 shows this separation on 0.04 mequiv./g XAD-1. The peaks given by platinum represent two different complexes of platinum(IV).

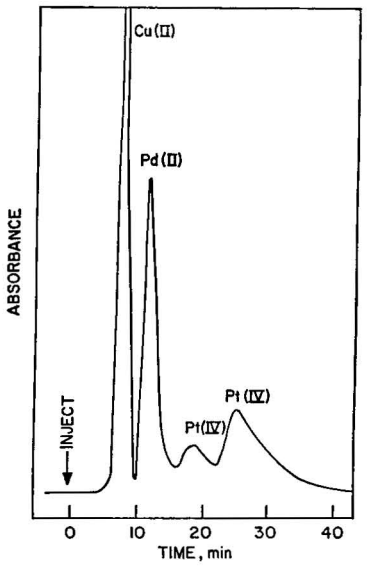


Fig. 6. Separation of copper(II), palladium(II) and platinum(IV).

CONCLUSION

Ion-exchange resin capacity can be easily varied and a parameter to be considered when devising analytical separations. Low capacity anion-exchange resins permit elution of many metal chloro complexes that previously were difficult or impossible to elute. XAD anion-exchange resins have excellent mechanical and chemical stability and lend themselves well to a forced flow system with automatic detection.

ACKNOWLEDGEMENTS

This work was supported by the U.S. Department of Energy, contract No. W-7405-eng-82, Division of Chemical Sciences, budget code EE-03-02-05. The authors also express their appreciation to Rohm & Haas Co. and to Dr. R. L. Albright for providing the starting resin used in this work.

REFERENCES

1 K. A. Kraus and F. Nelson, *Symposium on Ion Exchange and Chromatography in Analytical Chemistry*, ASTM Special Publication No. 195, American Society for Testing Materials, Philadelphia, Pa., 1958, pp. 27–57.
2 K. A. Kraus and F. Nelson, *Proc. Int. Conf. Peaceful Uses of Atomic Energy*, Vol. 7, United Nations, 1956, pp. 113, 131.
3 K. A. Kraus and G. E. Moore, *J. Amer. Chem. Soc.*, 75 (1953) 1460.
4 G. E. Moore and K. A. Kraus, *J. Amer. Chem. Soc.*, 74 (1952) 843.
5 D. H. Wilkins and L. E. Hibbs, *Anal. Chim. Acta*, 20 (1959) 271.
6 D. H. Wilkins, *Anal. Chim. Acta*, 20 (1959) 271.
7 D. H. Wilkins, *Anal. Chim. Acta*, 18 (1958) 372.
8 J. L. Goodkin, M. D. Seymour and J. S. Fritz, *Talanta*, 22 (1975) 245.
9 M. D. Seymour, J. P. Sickafoose and J. S. Fritz, *Anal. Chem.*, 43 (1971) 1734.
10 M. D. Seymour and J. S. Fritz, *Anal. Chem.*, 45 (1973) 1394.
11 D. T. Gjerde and J. S. Fritz, *J. Chromatogr.*, 176 (1979) 199.
12 J. S. Fritz and J. N. Story, *J. Chromatogr.*, 90 (1974) 267.
13 J. S. Fritz and J. N. Story, *Anal. Chem.*, 46 (1974) 825.
14 J. N. King and J. S. Fritz, *J. Chromatogr.*, 153 (1978) 507.

Journal of Chromatography, 188 (1980) 400–404

CHROM. 12,394

Note

Improved gas chromatographic method for measuring phenylethylene glycol

Application to the determination of styrene monooxygenase and epoxide hydrase activities

GIULIANO GAZZOTTI, ENRICO GARATTINI and MARIO SALMONA*

Laboratory for Enzyme Research, Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157-Milan (Italy)

(First received June 7th, 1979; revised manuscript received September 20th, 1979)

We have previously described a gas chromatographic (GC) method for the simultaneous determination of styrene monooxygenase and styrene oxide hydrase[1]. This method determined both activities by measuring only phenylethylene glycol (diol), which was quantitated after derivatization with *n*-butylboronic acid and detected with a flame-ionization detector. In this paper we present a more sensitive method which measures picomoles of phenylethylene glycol after its esterification with trifluoroacetic anhydride, using a gas chromatograph equipped with an electron-capture detector. We applied the method to the quantitative measurement of nuclear or microsomal styrene monooxygenase and styrene epoxide hydrase activities.

EXPERIMENTAL

Chemicals

Styrene (Carlo Erba, Milan, Italy) phenylethylene glycol (Merck, Darmstadt, G.F.R.), trifluoroacetic anhydride (Pierce, Rockford, Ill., U.S.A.), trimethylamine (Carlo Erba), 1-bromo-2-phenylethane (Merck), NADH (Boehringer, Mannheim, G.F.R.), NADPH (Boehringer) and styrene oxide (Merck) were used.

Apparatus

A Carlo Erba gas chromatograph with a nickel-63 electron-capture detector was used. The column was a glass tube (2 m × 4 mm I.D.) packed with 3% OV-17 on 100–120-mesh Gas-chrom Q (Supelco, Bellefonte, Pa., U.S.A.). The column temperature was 140°, the injector port temperature 250° and the detector temperature 275°. The carrier gas was nitrogen at a flow-rate of 30 ml/min and the chart speed was 1 cm/min.

Animals

Male CD-COBS rats (200 ± 20 g) were obtained from Charles River (Calco, Como, Italy). The rats were given a commercial laboratory feed and water *ad libitum*

* To whom correspondence should be addressed.

and kept in air-conditioned quarters with 12-h light–dark cycles. The rats were fasted for 12 h, then killed. The livers were removed immediately and used for the isolation of microsomes and nuclei.

Isolation of microsomes and nuclei

Rat liver microsomes were isolated as described previously[2], except for the buffer, to which sucrose was added to a final concentration of 0.25 *M*. Rat liver nuclear preparations were purified by means of a discontinuous sucrose gradient according to the method of Berezney *et al.*[3]. The protein concentration was determined by the method of Lowry *et al.*[4].

Assay of styrene monooxygenase

Intact nuclear or microsomal preparations were suspended in 50 mM phosphate buffer containing 150 m*M* potassium chloride, 5 m*M* magnesium chloride and 0.25 *M* sucrose to obtain a final protein concentration of about 1–2 mg/ml. To 1 ml of the suspension, 50 μl of a 20 mg/ml NADPH buffer solution were added. After 5 min of pre-incubation the reaction was started by adding 10 μl of a 250 m*M* methanolic solution of styrene. After 10 min of incubation at 37° in a Dubnoff incubator, the reaction was stopped with 0.4 ml of 0.6 *N* sulphuric acid and the preparation was left overnight. The samples were made alkaline with 0.8 ml of 0.6 *N* sodium hydroxide solution, then extracted twice with 3 ml of ethyl acetate. Acidification of the styrene resulted in more than 95% transformation into the diol. The combined extracts were dried under a gentle stream of nitrogen in a thermostatic bath at 37° and the phenylethylene glycol was determined as described below. The recovery of phenylethylene glycol under these conditions was 96%.

For all experiments a series of blanks was prepared, consisting either of boiled nuclei or microsomes or of the buffer solution alone, containing the amounts of NADPH and styrene used for fresh nuclei or microsomes.

Assay of styrene epoxide hydrase

The activity of this enzyme was determined as described above for styrene monooxygenase with the following exceptions: (a) 42 m*M* methanol solution of styrene oxide instead of styrene was used as substrate; (b) the NADPH was omitted; and (c) the reaction was stopped by adding 0.4 ml of 0.6 *N* sodium hydroxide solution.

In the experiments to determine nuclear hydrase activity, as spontaneous hydrolysis may be quantitatively important, a series of blanks was always prepared, consisting of boiled nuclei or buffer solution alone. In experiments to determine microsomal hydrase activity, the same kind of blanks were prepared but, as microsomal is much greater than nuclear activity, they are less important. The enzymatic activity was calculated by subtracting the blank value from that for the fresh nuclei; for the nuclear preparations we took into account only activity values that were at least twice as high as the blank values.

Derivative formation

The extraction residue was dissolved in 1 ml of toluene and 200 μl of 0.05 *M* trimethylamine in toluene and 100 μl of pure trifluoroacetic anhydride were added. Derivatization was carried out by holding the samples at 60° for 30 min. They were

then left to cool at room temperature; 1 ml of distilled water was added to the organic phase and, after 1 min, 1 ml of 5% ammonia solution was added. The samples were shaken for 5 min and centrifuged at 3500 *g*. To 50 μl of the organic phase, 50 μl of a 28 μg/ml toluene solution of 1-bromo-1-phenylethane used as internal standard were added. A 1-μl volume of this solution was injected into the gas chromatograph.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram, where peak 1 corresponds to the trifluoroacetyl derivative of phenylethylene glycol and peak 2 to the internal standard, 1-bromo-2-phenylethane. The overall sensitivity of the method is 5 ng/ml, but more reliable values were above 100 ng/ml and all the experiments were carried out in a linearity range from 125 to 2000 ng/ml. The calibration graph for phenylethylene glycol is shown in Fig. 2.

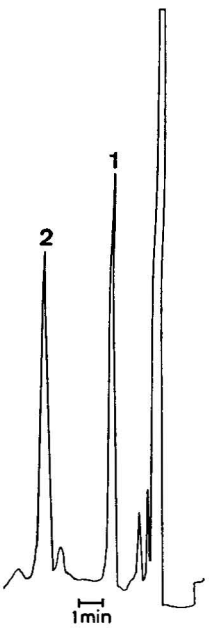


Fig. 1. Gas chromatogram of phenylethylene glycol trifluoroacetyl derivative. Peak 1 corresponds to the derivatized diol and peak 2 to 1-bromo-2-phenylethane (internal standard).

The trifluoroacetylderivative of phenylethylene glycol was identified by mass spectrometry (MS) and GC–MS. The mass spectrum of the glycol ester with trifluoroacetic anhydride is shown in Fig. 3. The amounts of glycol formed during the enzymatic reaction catalysed by monooxygenase or hydratase are linear up to incubation times of 15 and 30 min, respectively, and in the ranges of nuclear protein from 0.5 to 2.5 mg/ml and 1 to 2.5 mg/ml, respectively. The coefficient of variation of the method, calculated for two concentrations, is about 7% for the lower concentration (125 ng/ml) and about 11% for the higher concentration (1500 ng/ml).

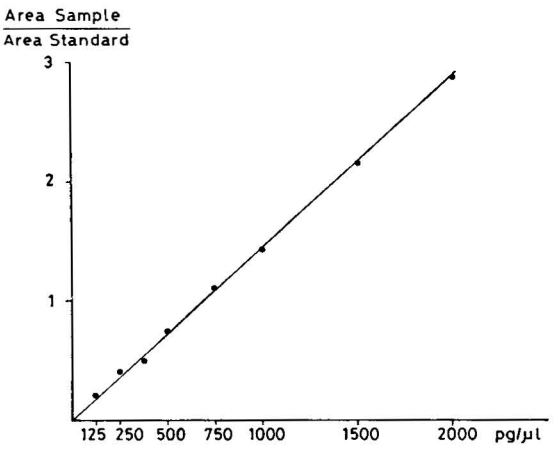


Fig. 2. Calibration graph for derivatized phenylethylene glycol.

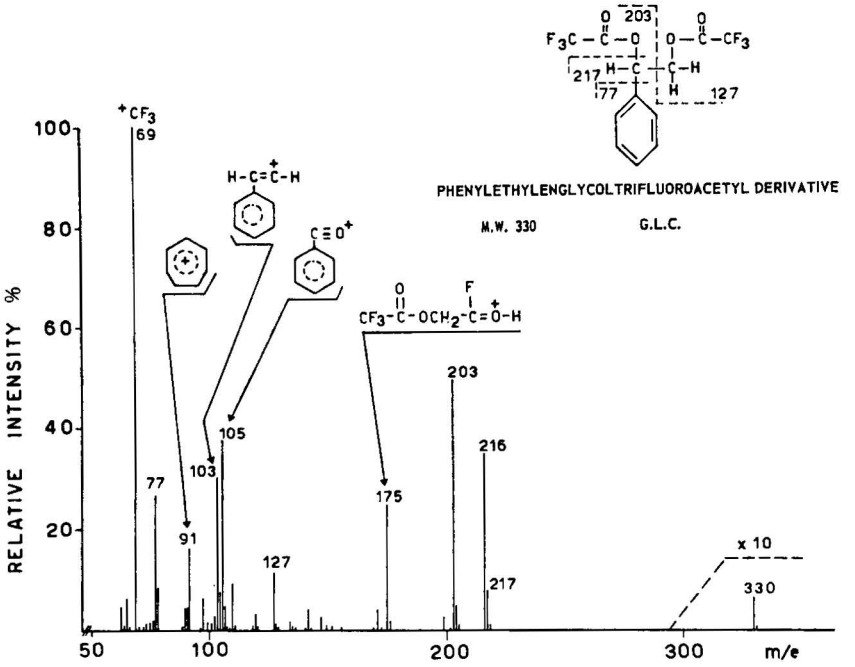


Fig. 3. Mass spectrum of phenylethylene glycol trifluoroacetyl derivative. Analysis was carried out by using an LKB 2091-051 instrument operated in the electron-impact mode at 70 eV.

Table I shows monooxygenase and hydrase activity values in nuclei and microsomal preparations of liver.

The GC method described here measures concentrations of phenylethylene glycol at the picomole level and enabled us to determine for the first time the presence on the nuclear envelope of an enzymatic activity capable of oxidizing the aliphatic

TABLE I

STYRENE MONOOXYGENASE AND EPOXIDE HYDRASE ACTIVITIES IN MICROSOMAL AND NUCLEAR PREPARATIONS

Both activities are expressed as pmole/min/mg protein. Each figure is the mean value ± standard error of at least 10 determinations.

Preparation	*Styrene monooxygenase*	*Styrene epoxide hydrase*
Microsomes	1950 ± 270	8660 ± 1640
Nuclei	128 ± 3	303 ± 36

double bond of styrene. We also found epoxide hydrase values in good agreement with the data available in literature, mainly based on radioactive studies[5].

The method described may be useful for investigations involving the determination of styrene monooxygenase and epoxide hydrase in small samples of tissues or in nuclei, monocytes and platelets which contain only limited amounts of such enzymatic activities[6–9].

ACKNOWLEDGEMENT

This work was supported by C.N.R. (National Research Council) Contract No. 78.02864.96, within the Programma Finalizzato "Control of Cancer Growth".

REFERENCES

1 G. Belvedere, J. Pachecka, L. Cantoni, E. Mussini and M. Salmona, *J. Chromatogr.*, 118 (1976) 387.
2 M. Salmona, J. Pachecka, L. Cantoni, G. Belvedere, E. Mussini and S. Garattini, *Xenobiotica*, 6 (1976) 585.
3 R. Berezney, L. K. Macaulay and F. L. Crane, *J. Biol. Chem.*, 247 (1972) 5549.
4 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
5 H. Mukhtar, T. H. Elmamlouk and J. R. Bend, *Chem. Biol. Interactions*, 22 (1978) 125.
6 A. R. Boobis, S. A. Atlas and D. W. Nebert, *Pharmacology*, 17 (1978) 241.
7 W. E. Fahl, C. R. Jefcoate and C. B. Kasper, *J. Biol. Chem.*, 253 (1978) 3106.
8 E. G. Rogan, P. Mailander and E. Cavalieri, *Proc. Nat. Acad. Sci. U.S.*, 73 (1976) 457.
9 E. Bresnick, J. B. Vaught, A. H. L. Chuang, T. A. Stoming, D. Bockman and H. Mukhtar, *Arch. Biochem. Biophys.*, 181 (1977) 257.

Journal of Chromatography, 188 (1980) 405–407

CHROM. 12,421

Note

Binding of zinc and copper to some gel filtration media

PHYLLIS E. JOHNSON and GARY W. EVANS

United States Department of Agriculture, Science and Education Administration, Human Nutrition Laboratory, Grand Forks, N.D. 58202 (U.S.A.)

(Received October 1st, 1979)

Gel filtration chromatography is a widely used technique. Many of the substances purified by this method are metalloenzymes or other metalloproteins. In addition to its use as a method of purification, gel filtration has been used to determine the stability constants of various metal–ligand or metal–protein complexes[1–5].

Chromatography of metal-containing substances is complicated by two problems. The first is that complexes of some metals such as zinc are labile, and dissociation of the complex may occur during chromatography. This can be overcome by the use of a modified gel filtration technique[1,6] in which the eluting buffer contains a constant level of the metal in question. A second complication is that many commonly used gels are themselves avid metal binders. Morgan *et al.*[7] reported in 1972 that some Sephadex gels bind zinc; Sephadex G-15 binds as much as 108 μg Zn/g gel, depending on the buffer used[6].

This paper is a short report of the zinc- and copper-binding capacities of various types of gel chromatographic media.

EXPERIMENTAL

Zinc and copper binding to the following gels was measured*: Sephadex G-10, G-15, G-25 and G-75; Biogel P-2, P-10, P-100; LKB Ultragel AcA-54 and Biogel A-5 m. Sephadex is a cross-linked dextran gel. Biogel P gels are polyacrylamide gels. Biogel A-5 m is an agarose gel and LKB Ultragel AcA-54 is a mixture of agarose and acrylamide. Biogel A-5 m and LKB Ultragel AcA-54 were washed with water before use to remove sodium azide, Tris, and EDTA.

Known weights of each gel were swollen in distilled deionized water and packed into 30 × 1.5 cm glass columns. The void volume of each gel column was determined with blue dextran and the total volume was defined as the elution volume of tritiated water (^3H_2O), which was measured by liquid scintillation counting.

Each gel was washed with a solution containing 10 ppm (1.54×10^{-4} *M*) Zn as $Zn(NO_3)_2$ and either no buffer, 10 m*M* acetate buffer pH 5.5, or 10 m*M* Tris–acetate

* Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

buffer pH 7.4. Fractions of 0.8 ml were collected and the zinc or copper content was determined by atomic absorption spectrophotometry.

The metal-binding capacity of the gels was calculated as follows: μg metal/g gel = (elution volume in ml required to reach equilibrium — elution volume of ^3H_2O [V_T])/g of dry gel. Two gels, LKB AcA-54 and Biogel A-5 m, are not available in a dry form. Zinc binding to these gels was expressed as μg Zn/ml settled bed volume of gel. For comparison, binding of zinc to other gels was expressed in the same fashion.

RESULTS AND DISCUSSION

The amounts of zinc and copper bound by each of the gels tested is listed in Table I. For the dextran and polyacrylamide gels, the amount of metal bound tended to increase with increased degrees of cross-linking in the gel; thus Sephadex G-10 and Biogel P-2 are the most avid binders. The two agarose-containing gels are not highly cross-linked, having exclusion limits of 5,000,000 (Biogel A-5 m) and 70,000 (LKB AcA-54) daltons, but they bind more zinc than any of the other gels examined except Biogel P-2. Sepharose 4B, another agarose gel, also binds zinc, as shown by the fact that ^{65}Zn applied to a column of Sepharose 4B cannot be eluted from the column.

TABLE I

BINDING OF ZINC AND COPPER TO VARIOUS GELS

	μg Zn/g gel			*μg Zn/ml bed vol.*	*μg Cu/g gel*	
	No buffer	*10 mM Tris, pH 7.4*	*10 mM Ac, pH 5.5*	*No buffer*	*No buffer*	*10 mM Ac, pH 5.5*
Sephadex						
G-10	154	43		46	143	44
G-15 (ref. 6)	108	29				
G-25	144	32	9	20	123	21
G-75	45	8		2	33	3
Biogel						
P-2	569	210		164	337	337
P-10	403	30		31	275	36
P-100	57	11		3	34	26
LKB AcA-54				85		
Biogel A-5 m				83		

Increasing buffer strength decreased the amount of metal bound, except for copper binding to Biogel P-2. Increasing buffer strength to 50 m*M* (ref. 6) further decreased zinc binding to Sephadex to G-15.

The zinc experiments were originally done at pH 7.4 because many biochemists prefer to operate at physiological pH; because of solubility limitations, it was more practical to use pH 5.5 for copper. Copper is generally a better ligand than zinc if conditions are equal. At pH 7.4 for zinc and pH 5.5 for copper, the amount bound is similar for zinc and copper. Sephadex G-25 bound about three times as much zinc at pH 7.4 as at pH 5.5. It seems likely that at pH 7.4, copper binding to these gels would be higher than copper binding at pH 5.5, and would exceed that of zinc at pH 7.4.

When the experiments were completed and the metal-saturated gels dried for storage, all of the copper-containing gels were distinctly blue. Biogel P-2 was noticeably blue even in the swollen state at the end of the experiment.

Examination of the binding of every metal to every possible gel would be an endless project. This sampling of common gels shows they are good binders of zinc and copper. Other metals, such as calcium, magnesium, sodium, potassium, and lithium are known to displace zinc from Sephadex gels[6]. It seems reasonable to expect that most transition metals would bind to these gels, in varying degrees, depending upon the experimental conditions.

Biochemists who work with both metalloproteins and low-molecular-weight metal compounds would do well to check the metal-binding properties of the gels they use, particularly if the metal in question is one (such as zinc) that forms labile complexes that may dissociate during chromatography. The agarose gels and the highly cross-linked gels appear to have the greatest potential for causing problems. In the determination of stability constants[1,3–5], a constant level of metal is passed through the column in the buffer, which eliminates any problem that might arise from metal binding by the gel. However, purification procedures may benefit considerably if metal binding by the gels is taken into account.

REFERENCES

1 N. Yoza, *J. Chem. Educ.*, 54 (1977) 284–287.
2 C. S. Sato and F. Gyorkey, *J. Biochem.*, 80 (1976) 883–886.
3 E. Breslow and A. W. Girotti, *J. Biol. Chem.*, 245 (1970) 1527–1536.
4 P. Cuatrecasas, S. Fuchs and C. B. Anfinsen, *J. Biol. Chem.*, 242 (1967) 3063–3067.
5 P. A. Price, *J. Biol. Chem.*, 247 (1972) 2895–2899.
6 G. W. Evans, P. E. Johnson, J. G. Brushmiller and R. W. Ames, *Anal. Chem.*, 51 (1979) 839–843.
7 R. S. Morgan, N. H. Morgan and R. A. Guinvan, *Anal. Biochem.*, 45 (1972) 668–669.

Journal of Chromatography, 188 (1980) 408–411

CHROM. 12,427

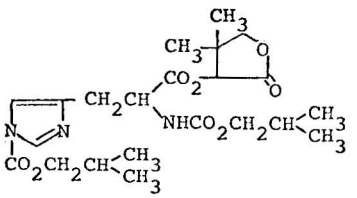
Note

Resolution of histidine diastereomers by gas chromatography

MASAMI MAKITA, YASUHIKO OHKARU and SHIGEO YAMAMOTO
Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700 (Japan)
(First received September 7th, 1979; revised manuscript received October 1st, 1979)

Although the gas chromatographic (GC) separation of diastereomeric histidine derivatives on a capillary column has recently been accomplished by König and co-workers[1], to our knowledge the separation of histidine diastereomers on a conventionally packed column with an optically inactive stationary phase has so far not been reported.

This paper describes a method for the separation and determination of enantiomeric histidines as their N-isobutyloxycarbonyl (isoBOC) L-(+)-pantoyl lactone esters (I). The derivatization procedure is based on N-isobutyloxycarbonylation in aqueous alkaline medium[2], followed by esterification with L-(+)-pantoyl lactone using N,N-carbonyldiimidazole (CDI) as a condensation reagent[3]. GC separation of the resulting diastereomers was carried out on a conventionally packed column.



(I)

EXPERIMENTAL

Reagents

Isobutyl chloroformate stabilized with calcium carbonate was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.) and used without further purification. L-(+)-Pantoyl lactone of *ca.* 99.5% optical purity was obtained from Tokyo Kasei (Tokyo, Japan) and dried under vacuum overnight. CDI was obtained from Merck (Darmstadt, G.F.R.) and stored in a desiccator. DL-Histidine monohydrochloride was obtained from Nakarai Chemicals (Kyoto, Japan), and D-histidine monohydrochloride, L-histidine monohydrochloride and arachidic acid as an internal standard from Sigma (St. Louis, Mo., U.S.A.). Triethylamine was obtained from Nakarai Chemicals and dried over sodium hydroxide. All other reagents and solvents were reagent grade purity and used as received from commercial sources.

Derivatization

N-Isobutyloxycarbonylation of histidine was carried out by the method previously reported[2]. To the ethereal extracts containing N-isoBOC-histidine was added 10 μg of arachidic acid and the solvent was evaporated to dryness. After addition to the residue of 0.1 ml of freshly prepared 1.0 *M* CDI in dichloromethane, the mixture was left to stand for 10 min at room temperature and subsequently 0.1 ml of a dichloromethane solution containing 50% triethylamine and 30% L-(+)-pantoyl lactone was added. After standing for 10 min at 40°, 4 ml of water saturated with sodium chloride was added and then the resulting N-isoBOC-L-(+)-pantoyl lactone ester of histidine was extracted three times with 3 ml of *n*-hexane. The combined *n*-hexane extracts were evaporated to dryness and the residue was dissolved in 0.1 ml of ethyl acetate. A 2–4 μl volume of the resulting solution was injected on to the gas chromatograph.

Gas chromatography

A Shimadzu Model 4CM gas chromatograph equipped with a hydrogen flame ionization detector, and an on-column injection port was used. The operating conditions are given in Fig. 1. The liquid phase, OV-17, and the support, Uniport HP (100–120 mesh) were purchased from Gasukuro Kogyo (Tokyo, Japan). The glass column (1 m) and quartz-wool plugs placed in each end of the column used throughout this work were silanized with dimethyldichlorosilane vapour. The column packing, 3% OV-17 on Uniport HP, was prepared by the "solution coating" technique[4].

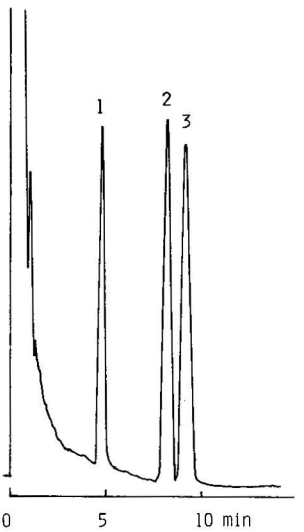


Fig. 1. Gas chromatogram of N-isoBOC-DL-histidine L-(+)-pantoyl lactone esters. Conditions: column; 3% OV-17 on Uniport HP (100–120 mesh), 1 m × 3 mm I.D., glass; column temperature, 280°; nitrogen flow-rate, 40 ml/min. Peaks: 1 = arachidic acid (internal standard); 2 = D-histidine derivative; 3 = L-histidine derivative.

RESULTS AND DISCUSSION

The two-step process involving N-isobutyloxycarbonylation and esterification was used to prepare the derivatives. N-Isobutyloxycarbonylation was performed in the first step exactly according to our previous method[2]. This reaction is extremely useful because it proceeds simply and rapidly in aqueous alkaline medium at room temperature. Subsequently, esterification for the introduction of a second chirality into N-isoBOC-histidine with an optically active alcohol using CDI as a condensation reagent was carried out with modifications according to the method of Ko and Royer[3]. From the results of preliminary experiments on separation and GC properties, L-(+)-pantoyl lactone was found to be the most suitable as a resolving reagent among various optically active alcohols tested. This alcohol is commercially available at relatively low cost.

The structures of the derivatives of D- and L-histidine prepared by the procedure described above were elucidated by the use of a Shimadzu LKB 9000 gas chromatograph–mass spectrometer. Each of derivatives gave a molecular ion peak with a *m/e* value of 467 and the same fragment pattern, and this suggests that they have the structure of (I).

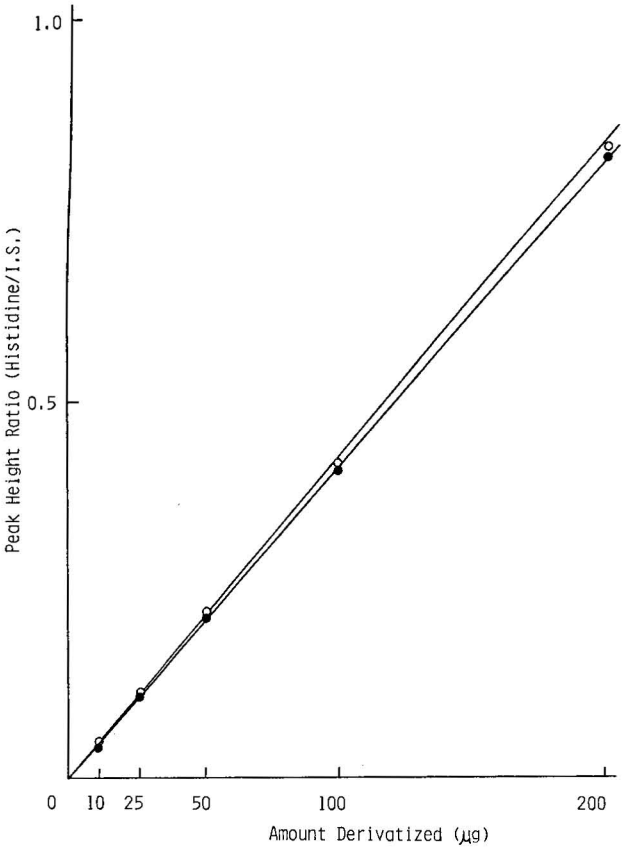


Fig. 2. Calibration curves for enantiomeric histidine. ○, D-Histidine; ●, L-histidine; internal standard, arachidic acid (10 μg).

For the separation of the diastereomeric histidine derivatives a 1-m column packed with 3% OV-17 on Uniport HP was found to be sufficient. The chromatogram of the histidine derivatives is illustrated in Fig. 1, showing the separation. The retention time of the D-L compound is shorter than that of the L-L diastereomer, and each gave a single and symmetrical peak. Throughout this derivatization procedure no racemization was observed. The separation factor, which was calculated by the method of Nambara and co-workers[5], is 1.00, and this indicates that the separation of the histidine diastereomers was complete. The linearity of the calibration curve for each enantiomer in the range studied (10–200 μg) was found to be satisfactory (Fig. 2). In order to examine the quantitative reliability of this method, mixtures with various known proportions of D- and L-histidine were derivatized and analysed. The results are shown in Fig. 3. The peak height ratios of N-isoBOC-L-(+)-pantoyl lactone esters of D- and L-histidine show a linear relationship with the ratio of enantiomers present. Therefore, it can be seen that the method would be suitable for the determination of enantiomeric percentages of histidine in biological samples.

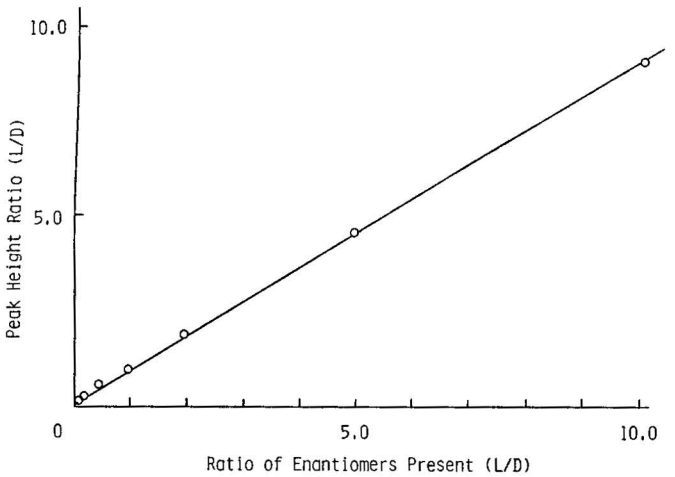


Fig. 3. Relationship between peak height ratio and enatiomeric composition of mixtures of D- and L-histidine.

This method is simple and convenient because a conventionally packed column with a thermostable phase such as OV-17 can be used, and derivatization can be performed within 40 min without requiring an elaborate procedure. Moreover, the resulting derivatives are very stable towards moisture.

Application of the technique to other amino acids is in progress.

REFERENCES

1 W. A. König, W. Rahn and J. Eyem, *J. Chromatogr.*, 133 (1977) 141.
2 M. Makita, S. Yamamoto and M. Kono, *J. Chromatogr.*, 120 (1976) 129.
3 H. Ko and M. E. Royer, *J. Chromatogr.*, 88 (1974) 253.
4 E. C. Horning, W. J. A. VandenHeuvel and B. G. Creech, *Methods Biochem. Anal.*, 11 (1963) 69.
5 T. Nambara, J. Goto, K. Taguchi and T. Iwata, *J. Chromatogr.*, 100 (1974) 180.

Journal of Chromatography, 188 (1980) 412–416

CHROM. 12,409

Note

Determination of halogenated anilines in urine by high-performance liquid chromatography with an electrochemical detector

E. M. LORES, F. C. MEEKINS and R. F. MOSEMAN

Environmental Toxicology Division, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, N.C. 27711 (U.S.A.)

(Received September 17th, 1979)

This paper describes a method for the determination of halogenated anilines in urine using high-performance liquid chromatography (HPLC) and an electrochemical detector. The electrochemical detector provides the sensitivity needed to determine anilines at subnanogram levels and HPLC provides a method of chromatography that does not require derivatization. This method is based on a previous publication describing the chromatography of anilines, with the addition of a few modifications to eliminate some of the interferences found in urine[1].

There are several classes of pesticides that are metabolized or degraded to anilines, such as substituted ureas and carbamates. Anilines that are ingested or formed in the body by metabolism are generally excreted from the body in the urine. The analysis of anilines in the urine can provide an index of exposure to many of the pesticides in the environment. Pesticides, however, are not the only source of halogenated anilines in the environment and this index of exposure can only be used as a guide to possible sources. Also, there are several pesticides that can be metabolized to the same aniline. These include linuron, diuron and propanil, all of which are metabolized to 3,4-dichloroaniline when ingested.

The only existing method that could be found in the literature for the analysis of halogenated anilines in urine requires derivatization, silica gel cleanup and gas chromatography[2]. HPLC eliminates the need for derivatization and makes the cleanup easier. The existing method did not investigate the need for acid hydrolysis of the urine on biologically incorporated anilines. As suggested by Edgerton and Moseman[3], the use of urine in which the compounds of interest are biologically incorporated can give significantly different results when compared to urine which has simply been fortified in the laboratory. In this paper, we investigated the effect of acid hydrolysis on urine which contained biologically incorporated 3,4-dichloroaniline to determine the need for acid hydrolysis.

The anilines used in this study are by no means a complete list of anilines that can be found in urine. Other anilines can be added and the chromatography can be adjusted to provide the separation as needed[4]. The factors affecting separation will be discussed.

EXPERIMENTAL

A Tracor Model 995 isochromatographic pump with a Model CV-6-UHPa-HC Valco valve and a Model LC-2A electrochemical detector from Bioanalytical Systems[5,6] made up the chromatographic system. A 15-cm Zorbax® ODS column was used throughout the study. Two different mobile phases were used in this study because gradient elution cannot be used with the electrochemical detector. The anilines used in this study were divided into two groups depending on which mobile phase was required. The mobile phase used for aniline, *p*-chloroaniline and *p*-bromoaniline was a mixture of 80% 0.1 *M* phosphate buffer adjusted to a pH of 3.0 and 20% acetonitrile. The mobile phase mixture used for *m*-chloroaniline, *o*-chloroaniline and 3,4-dichloroaniline was 60% 0.15 *M* phosphate buffer adjusted to a pH of 2.1 and 40% acetonitrile. The electrochemical detector was operated at an oxidative potential of +1.1 V with a CP-W graphite paste electrode.

The standards used in this study were recrystallized or distilled under nitrogen from technical grade materials.

Cleanup of urine

The samples were hydrolyzed by adding 2 ml of 6 *N* HCl to 10 ml of urine in a screw cap culture tube. The tubes were then heated in a boiling water-bath for 1 h. The samples were removed from the water-bath, cooled to room temperature and washed four times with 5-ml portions of hexane which were discarded. A 3-ml volume of 10 *N* NaOH was added to neutralize the samples and the anilines were then extracted with three successive 4-ml portions of hexane. To return the anilines to an aqueous phase, the combined hexane fractions were successively extracted with 5, 3 and 2 ml of 5% phosphoric acid extracts were then combined, adjusted to final volume and injected directly into the liquid chromatograph.

RESULTS AND DISCUSSION

Urine samples fortified with known amounts of anilines were used to test the recovery of anilines from urine. Using simple acid–base–organic partitioning, the recoveries appeared to be good. However, as can be seen in Fig. 1, when urine containing biologically incorporated 3,4-dichloroaniline from rats fed linuron or diuron was analyzed and the results compared to the same urine which had been acid hydrolyzed, the amount of 3,4-dichloroaniline found in the hydrolyzed urine was at least ten times that found in the unhydrolyzed urine. As suggested by Edgerton and Moseman, this again points out the need for using biologically incorporated residues when developing methodology, especially methodology for polar residues. While it can be shown that the acid hydrolysis step increased the apparent level of 3,4-dichloroaniline, there is still no way of knowing if the hydrolysis releases all the anilines from the urine. Therefore, the levels found in urine should be considered a minimum level.

An attempt was made to develop a method for the analysis of anilines in soil, but even with acid hydrolysis the recovery from some fortified soils was as low as 50%.

The anilines appear to be stable to acid hydrolysis since the recovery of anilines

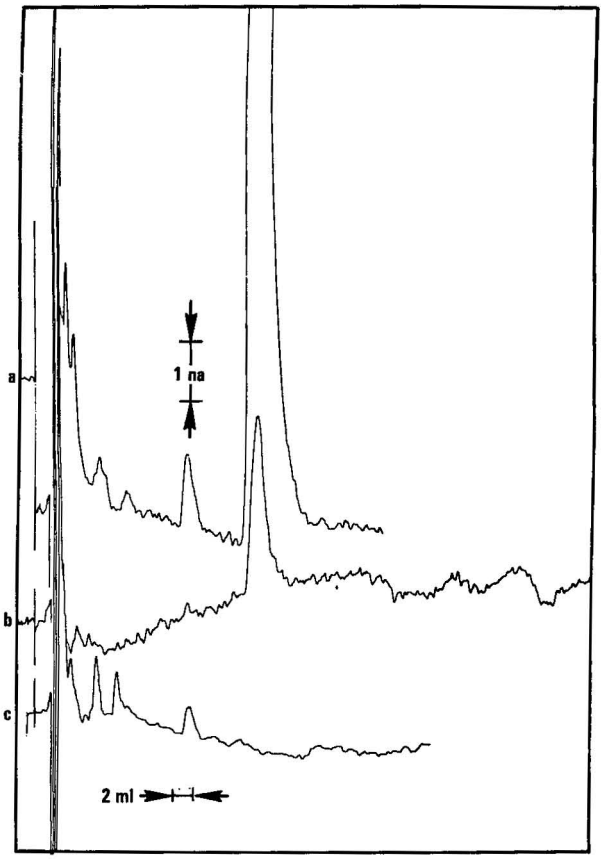


Fig. 1. Chromatograms of urine samples from rats fed linuron containing 3,4-dichloroaniline: a, acid hydrolyzed urine from rat fed linuron; b, unhydrolyzed urine from rat fed linuron; c, acid hydrolyzed control urine. na = nanoampère.

TABLE I

RECOVERY OF ANILINES FROM URINE

Compound	*Fortification level (ppb)*	*Recovery (%)*	
		Before hydrolysis	*After hydrolysis*
p-Chloroaniline	20	$\bar{x}_6$** 83	$\bar{x}_3$ 104
	50	$\bar{x}_6$ 86	
	100	$\bar{x}_6$ 81	$\bar{x}_5$ 93
p-Bromoaniline	20	$\bar{x}_6$ 98	ND* (interference)
	50	$\bar{x}_6$ 84	—
	100	$\bar{x}$ 86	$\bar{x}_5$ 94
m-Chloroaniline	20	$\bar{x}_9$ 82	
	50	$\bar{x}_9$ 80	$\bar{x}_3$ 100
o-Chloroaniline	20	$\bar{x}_9$ 80	
	50	$\bar{x}_9$ 80	$\bar{x}_3$ 81
3,4-Dichloroaniline	20	$\bar{x}_9$ 90	
	50	$\bar{x}_9$ 93	$\bar{x}_3$ 93

* ND = not determined.

** $\bar{x}_6$ = average of 6 determinations.

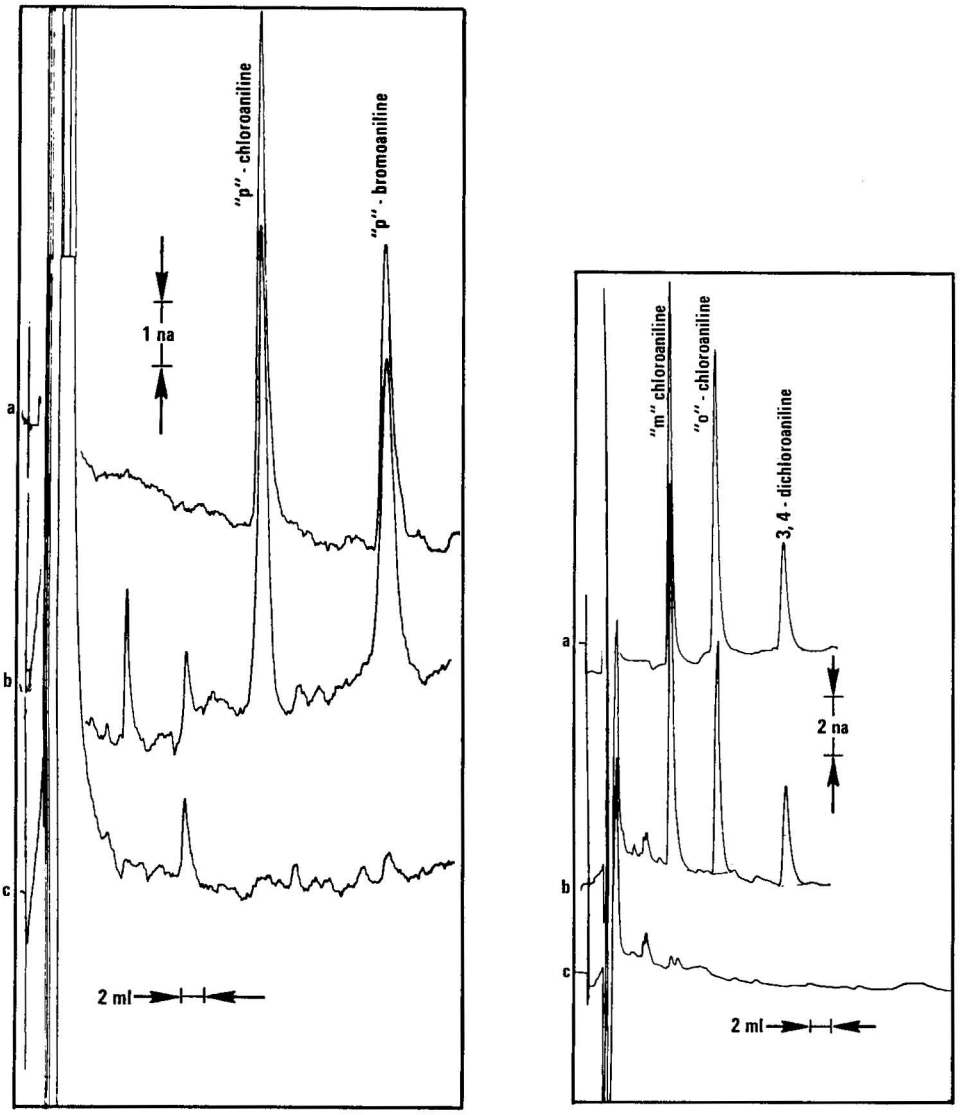


Fig. 2. Chromatograms of anilines on 15-cm C_{18} column with 80% pH 3 phosphate buffer and 20% acetonitrile: a, standard, 0.05 ppm; b, fortified urine sample, 0.05 ppm; c, control human urine.

Fig. 3. Chromatograms of anilines on 15-cm C_{18} column with 60% pH 2.1 phosphate buffer and 40% acetonitrile. Details as in Fig. 2.

from fortified urine samples with hydrolysis was greater than 80% for all the anilines tested. Table I shows the results of the recovery studies. Since the acid hydrolysis increases the interferences in the urine, the chromatography of one group had to be changed and an additional cleanup step had to be added to remove the interference. The hexane wash of the acid hydrolysate seemed to remove most of the interference. In this study, it was found that the older the urine sample was, the higher the level of

interference. No studies were made on the shelf life of the urine samples, but based on the increase in interference and the possibility of biological degradation of the anilines in the sample, it is believed the urine samples should be analyzed as soon as possible after collection.

The pH of the mobile phase used for *p*-chloroaniline and *p*-bromoaniline is higher than the pH reported in the previous paper[1]. This change in the pH was needed to separate the anilines from an interfering peak in hydrolyzed urine. Using the higher pH mobile phase the interfering peaks are completely separated as can be seen in Fig. 2. A chromatogram of the Group B anilines is shown in Fig. 3.

The mobile phase is circulated through the pumping system and detector 24 h/day, 7 days/week. This allows the detector to purge the mobile phase of any oxidizable compounds and eliminates the long warmup periods required for high sensitivity operation.

The limits of detection for this method will depend on the sample size and the noise level of the detector. In this study, with an injection volume of 70 μl and without concentration of the sample, levels below 5 ppb can be detected. Unsubstituted aniline cannot be detected using either of these mobile phases, since it is obscured by peaks eluting with the solvent front.

REFERENCES

1 E. M. Lores, D. W. Bristol and R. F. Moseman, *J. Chromatogr. Sci.*, 16 (1978) 358.
2 E. E. Bradway and T. Shafik, *J. Chromatogr. Sci.*, 15 (1977) 322.
3 T. R. Edgerton and R. F. Moseman, *J. Agr. Food Chem.*, 27 (1979) 197.
4 R. B. Sleight, *Chromatographia*, 6(1) (1973) 3.
5 P. T. Kissinger, C. Refshauge, R. Dreiling and R. N. Adams, *Anal. Lett.*, 6 (1973) 465.
6 P. T. Kissinger, *Anal. Chem.*, 49 (1977) 477.

Journal of Chromatography, 188 (1980) 417–419

CHROM. 12,411

Note

Differential fluorimetric determination of picogram levels of thiamine, thiamine monophosphate, diphosphate and triphosphate using high-performance liquid chromatography

MIEKO KIMURA
Department of Hygiene, Faculty of Medicine, Kyoto University, Kyoto 606 (Japan)
TOMIO FUJITA
Analytical Application Laboratory, Shimadzu Corporation, Kyoto 604 (Japan)
and
SHIGEKI NISHIDA and YOSHINORI ITOKAWA
Department of Hygiene, Faculty of Medicine, Kyoto University, Kyoto 606 (Japan)
(First received July 31st, 1979; revised manuscript received October 4th, 1979)

Free thiamine and thiamine monophosphate (TMP), diphosphate (TDP) and triphosphate (TTP) are found in animal tissues, and column chromatographic[1–3] and electrophoretic[4,5] methods have been used for their determination.

There is evidence for two independent roles of thiamine[6–8], as a cofactor in intermediate carbohydrate metabolism and in a physiological role in the nerve excitation process. The above-mentioned methods have not been utilized to determine thiamine phosphates in small nervous tissues in animals (*e.g.*, pheripheral nerve in the rat). Recently, Roser *et al.*[9] established a sensitive high-performance liquid chromatographic (HPLC) method for the determination of urinary thiamine. We have developed an HPLC method capable of determining 0.05 pmole of thiamine phosphates.

EXPERIMENTAL

Apparatus

The following were used: LC-3A pump for liquid chromatograph; SIL-1A injector; Shimadzu ISA-07/S2504 LC column (25 mm × 0.4 mm I.D.); CTO-2A column oven (35°); stainless-steel mixing coil (100 mm × 0.1 mm I.D.; CRB-1B incubator box (35°); PRR-1A proportioning pump (flow-rate 0.5 ml/min); RF 500-LCA spectrofluorimetric detector (excitation 375 nm, emission 435 nm); square-shaped flow cell (12 μl); and strip-chart recorder (chart speed 2.5 mm/min). All of the equipment was purchased from Shimadzu (Kyoto, Japan).

Reagents

Thiamine was obtained from Wako (Osaka, Japan), TMP and TDP from Sigma (St. Louis, Mo., U.S.A.), and TTP was donated by the Central Research Division of Takeda Chemical Co. (Osaka, Japan). All other chemicals were of the best grade

commercially available. For the mobile phase 0.7 *M* sodium acetate solution was used. To convert thiamine and thiamine phosphates into fluorophores, a mixture of 15% sodium hydroxide and 0.02% potassium hexocyanoferrate(III) solution was used.

Procedure

For HPLC, the mobile phase was pumped at a flow-rate of 0.5 ml/min. A 20-μl volume of a solution containing thiamine and its phosphate esters (10^{-6}–10^{-7} *M*) was loaded on the sample loop and then injected on to the column, the zero time being marked. Potassium hexacyanoferrate(III)–sodium hydroxide solution was applied at 0.5 ml/min by a proportioning pump and mixed with the column effluent to convert thiamine phosphates into fluorophores. The fluorophores were measured with the spectrofluorimeter connected to the chromatograph and recorded graphically. The peak-height method was used for quantitation. Fig. 1 shows a schematic diagram of the method.

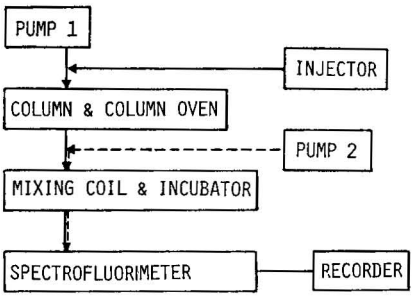


Fig. 1. Schematic diagram of the high-performance liquid chromatograph equipped with a fluorescence detector, and using potassium hexacyanoferrate(III) and sodium hydroxide as reagents.

RESULTS AND DISCUSSION

Using the proposed method, thiamine phosphates were eluted in the order thiamine, TMP, TDP and TTP. A typical chromatogram obtained with a solution containing 1 pmole each of thiamine, TMP, TDP and TTP in 20 μl is shown in Fig. 2.

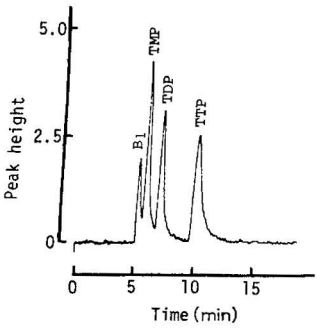


Fig. 2. Separation of thiamine (B_1), TMP, TDP and TTP by HPLC.

Fig. 3 illustrates the relationships between peak heights and concentrations of thiamine, TMP, TDP and TTP. A linear relationship was observed for each compound in the concentration range 0.05–1.5 pmoles.

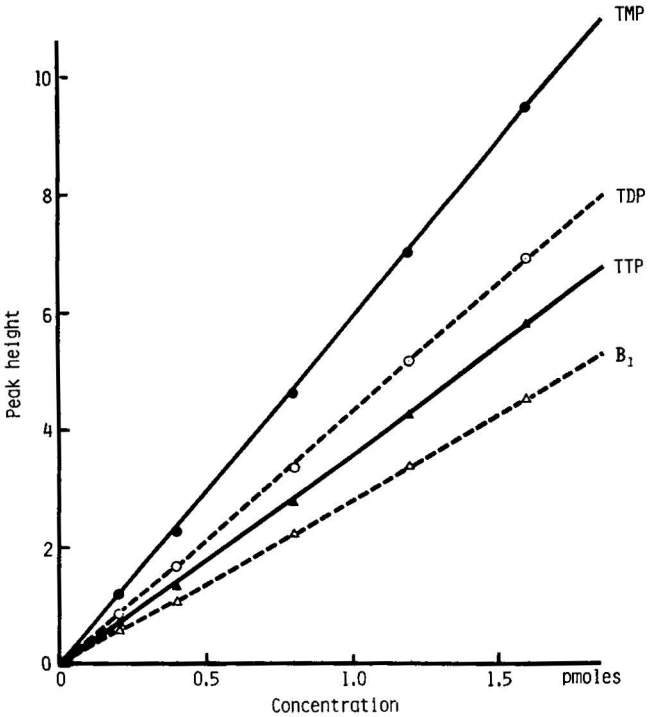


Fig. 3. Calibration graph obtained for thiamine, TMP, TDP and TTP.

This method is simple, reproducible and rapid. The sensitivity is sufficient for application to the determination of thiamine and its phosphate esters in a small amount of nervous tissues in animals.

ACKNOWLEDGEMENTS

We thank Emeritus Professor M. Fujiwara for encouragement throughout this work and Mr. T. Yamamoto and Mr. I. Tanaka, Shimadzu Corporation, for technical assistance. Thanks are also due to M. Ohara for advice on the manuscript.

REFERENCES

1 D. J. Hennessy and L. R. Cerecedo, *J. Amer. Chem. Soc.*, 61 (1939) 179.
2 L. de Giuseppe and G. Rindi, *J. Chromatogr.*, 1 (1958) 545.
3 Y. Itokawa and K. Inoue, *Vitamins*, 36 (1967) 544.
4 Y. Itokawa and J. R. Cooper, *Methods Enzymol.*, 18A (1970) 91.
5 H. K. Penttinen, *Acta Chem. Scand.*, 32 (1978) 609.
6 A von Muralt, *Vitam. Horm. (N.Y.)*, 5 (1941) 93.
7 Y. Itokawa and J. R. Cooper, *Science*, 166 (1969) 759.
8 Y. Itokawa, *J. Appl. Nutr.*, 29 (1977) 5.
9 R. L. Roser, A. H. Andrist, W. H. Harrington, H. K. Naito and D. Lonsdale, *J. Chromatogr.*, 146 (1978) 43.

Journal of Chromatography, 188 (1980) 420–425

CHROM. 12,407

Note

Amperometric high-performance liquid chromatographic method for narcotic alkaloids

ROBERT G. PETERSON

University of Colorado Health Sciences Center, Denver, Colo. 80262 (U.S.A.)

BARRY H. RUMACK

University of Colorado Health Sciences Center, Director, Rocky Mountain Poison Center, Denver General Hospital, Denver, Colo. 80262 (U.S.A.)

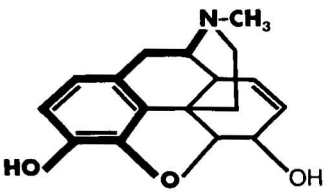
and

JOHN B. SULLIVAN, Jr. and ANNE MAKOWSKI

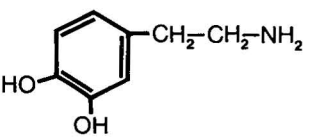
University of Colorado Health Sciences Center, Denver, Colo. 80262 (U.S.A.)

(Received September 17th, 1979)

Phenanthrene alkaloids of opium have structural similarities to the catecholamines (Fig. 1). Since the catecholamines have been shown to participate in an oxidation reaction in a low potential field, thus affording their detection by amperometric means, we investigated the same method for quantitation of the alkaloid opiates: morphine, oxymorphone and codeine as well as the closely related narcotic antagonists: naloxone, naltrexone, nalorphine and pentazocine.



MORPHINE (a)



DOPAMINE

Fig. 1. Similarity between the opiate alkaloids and catecholamines. Heavy lines superimposed on the morphine structure coincide with the general structure of the catecholamine shown on the right.

We have established conditions whereby a rapid reversed-phase chromatographic separation of several of these substances can be made and found that amperometry detected only those alkaloids with a "catechol" structure.

METHODS

A Waters Assoc. (Milford, Mass., U.S.A.) high-pressure liquid chromatograph consisting of a Model 6000 pump, U6K loop injector and a Model 450 ultraviolet detector (254 nm) in series with an amperometric detector (Model LC-2A electronic controller, Model TL-3 electrochemical cell; Bioanalytic Systems, West Lafayette, Ind., U.S.A.) afforded separation using a reversed-phase μBondapak C_{18} column (Waters Assoc.) with dimensions of 30 cm $\times$ 4 mm. The column eluent was methanol–water (20:80) containing 50 m*M* tetramethylammonium hydroxide and pH adjusted to 6.1 with H_3PO_4.

An isocratic elution at 2.0 ml/min in ambient room temperature resulted in a back pressure of 13.8 MPa (2000 p.s.i.). Standards of morphine, codeine, nalorphine (Applied Science Labs., State College, Pa., U.S.A.), oxymorphone, naltrexone and naloxone (Endo Labs, Garden City, N.Y., U.S.A.) and pentazocine (Sterling Winthrop Labs., New York, N.Y., U.S.A.) were made by dissolving the hydrochloride salts in methanol.

The amperometric method was compared with a gas chromatographic procedure. Samples were prepared by adding known amounts of morphine to human plasma. To 1.0-ml aliquots were added 20 μg nalorphine as an internal standard, 500 μl carbonate buffer (1 *M*, pH 9) and 5 ml benzene. After extraction with shaking, the samples were centrifuged. The organic layer was removed and evaporated to dryness. A 20-μg amount of Meth Elute® (Supelco, Bellefonte, Pa., U.S.A.) was added, the sample vortexed and again evaporated to dryness. The residue was taken up in 25 μl methanol, of which 2–4 μl were injected into the gas chromatograph (Perkin-Elmer Sigma 3, with a nitrogen–phosphorus detector). The column (6 ft. $\times$ $^1/_4$ in.) was packed with 3% OV-17, operated at 250°.

The same samples were assayed amperometrically, using an injection volume of 1 μl. Both assays were run in duplicate.

RESULTS

Fig. 2 shows the chromatographic separation with detection by amperometry of a standard mixture of oxymorphone (OXY), morphine (MOR), naltrexone (NT), naloxone (NX) and nalorphine (NL). The substances were present in equimolar quantities of 0.3 nmoles with the exception of oxymorphone which was present at 0.15 nmoles. Morphine was observed to give the best signal at a potential across the electrochemical cell of +0.8 V. Table I gives the peak height ratios of each of these substances as compared to morphine. An example of the lower limits for detection of morphine and naloxone is shown in Fig. 3. Increased sensitivities of the detector (5–20 nA/V) indicated an ability to detect morphine down to 100 pg and naloxone down to 1000 pg. As shown on the right in Fig. 3, the signal-to-noise ratio of morphine deteriorates below 1 ng. Nevertheless, the ability to quantitate morphine to 1 ng and naloxone to 5 ng is demonstrated by the linear response when peak height is plotted *versus* nanograms injected in Figs. 4 and 5.

The comparison of the high-performance liquid chromatographic (HPLC)–amperometric method for morphine to the gas chromatographic method is shown in Fig. 6. As can be seen, the comparison resulted in a line giving a correlation coefficient of 0.994 (slope, 14.7; and *y*-intercept, −0.044).

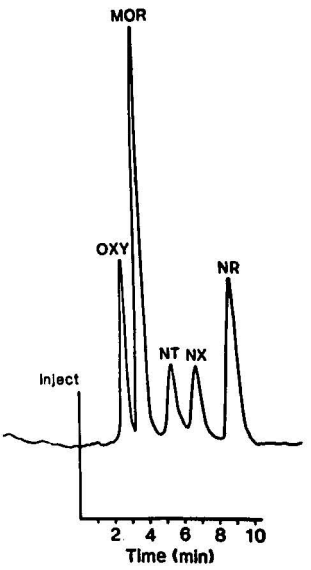


Fig. 2. Chromatographic separation of oxymorphone (OXY), morphine (MOR), naltrexone (NT), naloxone (NX) and nalorphine (NR) under conditions given in text. Detection is by amperometry.

TABLE I

COMPARISON OF AMPEROMETRIC RESPONSE OF OTHER ALKALOIDS TO MORPHINE

Alkaloids	*Ratio (A:M)*
Morphine	1
Oxymorphone	0.70
Nalorphine	0.41
Naltrexone	0.28
Naloxone	0.26

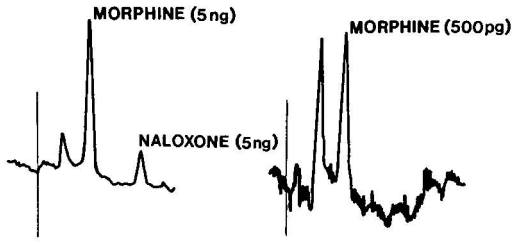


Fig. 3. Amperometric detection of standards of morphine and naloxone. Numbers in parentheses indicate quantities injected.

We next investigated the necessity for a catechol-like structure of the alkaloid for the appearance of an amperometric response. The measured response to codeine by both ultraviolet (UV) absorption and amperometry (both detectors in series following the reversed-phase column) is shown in Fig. 7. A 200-ng amount of codeine

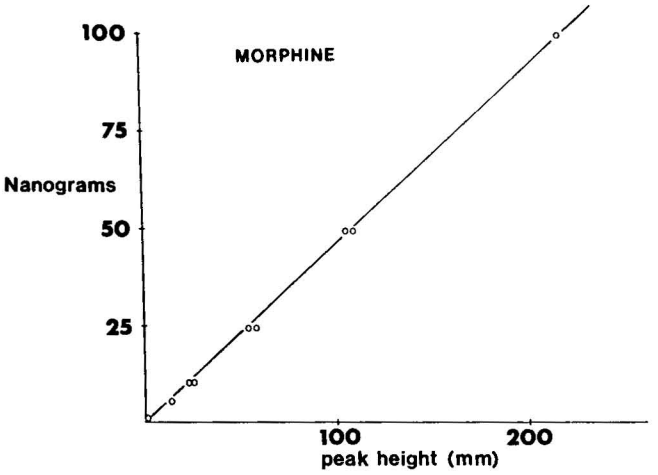


Fig. 4. Plot of amperometric response for standard solutions of morphine. Ordinate represents quantity injected.

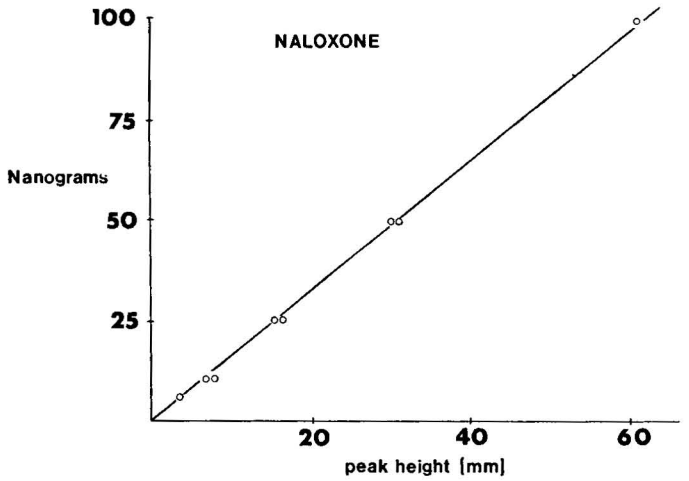


Fig. 5. Plot of amperometric response for standard solutions of naloxone. Ordinate represents quantity injected.

was injected in order to detect the material by UV absorption. The same sensitivity of the amperometric detector as allowed detection of 500 pg of morphine was used. As can be seen, there was no response by amperometry to codeine.

One further compound investigated was pentazocine, the structure and response shown in Fig. 8. Here, once again, there was no measured response by amperometry.

DISCUSSION

Standard methods for narcotics and narcotic antagonists have been reported using gas chromatography with a nitrogen or an electron capture detector[1–4]. In

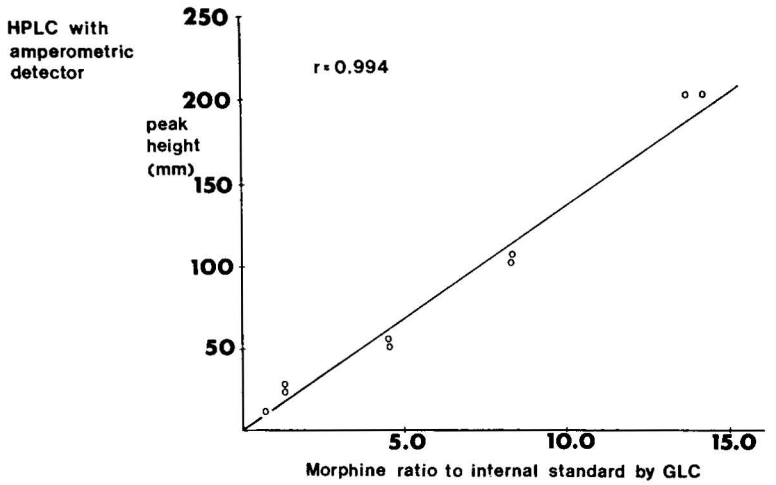


Fig. 6. Comparison of HPLC–amperometric method for morphine to a standard gas chromatographic (GLC) procedure.

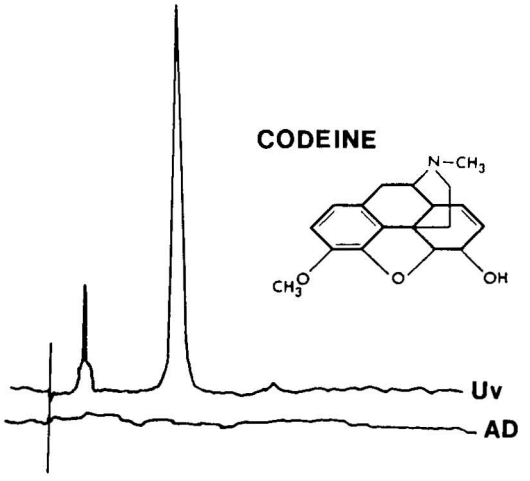


Fig. 7. Structure–response relationship for codeine and amperometry. Position of codeine in column elution demonstrated by ultraviolet (Uv) peak. No amperometric response was observed.

addition, radioimmunoassay methods have been reported for morphine[5,6] and for naloxone[7]. We have utilized the structural similarities between the alkaloid opiates and the catecholamines to allow a more rapid analytical method not requiring the derivitizations necessary for electron capture gas chromatography nor the long incubation and counting times for radioimmunoassay. The method allows separation of complex mixtures of this class of compounds and detection limits which are comparable to the alternate methods discussed above.

Evaluation of the analyses of various alkaloids did show striking differences in the amperometric response based upon structural alterations as compared to morphine, the compound showing the best signal-to-noise ratio (Fig. 2, Table I). Sub-

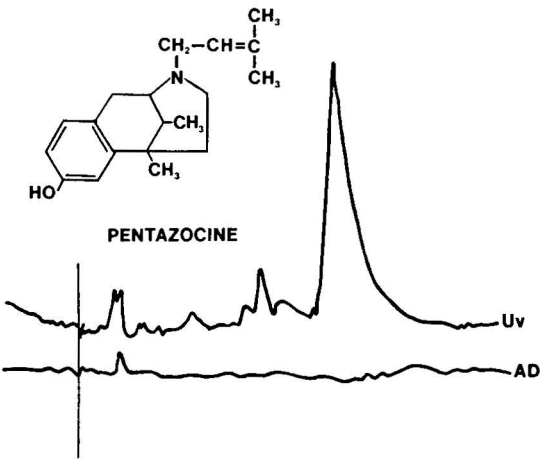


Fig. 8. Structure–response relationship for pentazocine. Broad ultraviolet (Uv) peak not seen by amperometry.

stitutions of various alkyl groups on the alkaloid ring nitrogen did decrease the amperometric signal as could be seen with naloxone, naltrexone and nalorphine, but not with oxymorphone, a compound with the same N-methyl structure as morphine. Alteration of the structure by incorporation of a hydroxyl group on the 14 position of morphine as is the case in oxymorphone did not have a large effect upon the amperometric signal, and therefore, is probably not related to the low response seen for naloxone and naltrexone, the other alkaloids with this structural feature.

Very marked alterations in response was seen for those compounds where the "catechol" structure was altered. No signal was seen for codeine (3-methoxymorphine) and this most likely reflects the necessity for the 3-hydroxyl group of the alkaloid opiates for oxidation in the electrochemical field. In addition, the necessity for the second "catechol" oxygen between the 4 and 5 positions of morphine was demonstrated by analysis of pentazocine, the other compound without an amperometric response. It must be noted that there is a bulky substitution of the analogous nitrogen in pentazocine which could have lowered the signal, but such a large excess of the substance was injected in order to obtain the UV response that there appeared to be a total loss of the response by amperometry.

REFERENCES

1 J. E. Wallace, H. E. Hamilton, K. Blum and C. Petty, *Anal. Chem.*, 46 (1974) 2107.
2 B. Dahlstrom, L. Paalzow and P. O. Edlund, *Acta Pharmacol. Toxicol.*, 41 (1977) 273.
3 R. C. Baselt and C. B. Stewart, *J. Anal. Toxicol.*, 2 (1978) 107.
4 S. H. Weinstein, M. Alteras and J. Gaylord, *J. Pharm. Sci.*, 67 (1978) 547.
5 S. Spector and C. W. Parker, *Science* 168 (1970) 1347.
6 S. Spector, *J. Pharmacol. Exp. Ther.*, 178 (1971) 253.
7 B. A. Berkowitz, S. H. Ngai, J. Hempstead and S. Spector, *J. Pharmacol. Exp. Ther.*, 195 (1975) 499.

Journal of Chromatography, 188 (1980) 426–430

CHROM. 12,395

Note

Liquid chrom. ɔgraphic identification of clorazepate in pharmaceutical products

C. RANDALL C ARK

Department of Pharmacal Sciences, School of Pharmacy, Auburn University, Auburn, Ala. 36830 (U.S.A.)

and

F. TAYLOR NOGGLE, Jr.

Alabama Department of Forensic Sciences, Wire Road, Auburn, Ala. 36830 (U.S.A.)

(First received August 3rd, 1979; revised manuscript received September 17th, 1979)

Clorazepate is a relatively new benzodiazepine available in oral dosage forms as Tranxene and Azene. Tranxene is the dipotassium salt of clorazepate and Azene is the monopotassium salt. Product information[1,2] indicates that aqueous solutions of clorazepate are unstable and undergo rapid decarboxylation. The decarboxylation product and primary metabolite, N-desmethyldiazepam (nordiazepam), quickly appears in the blood stream following ingestion of clorazepate. Clorazepate in its original form is not normally detected in the blood and the pharmacological activity of clorazepate is essentially that of N-desmethyldiazepam. Detailed pharmacological data on N-desmethyldiazepam is available[3,4].

Recent reports[5,6] have indicated difficulties in the analysis of clorazepate. Clorazepate salts are highly water soluble with little or no organic solvent solubility. Aqueous solutions of clorazepate salts are basic and upon acidification the clorazepate rapidly decarboxylates to yield N-desmethyldiazepam. Extraction and analysis of the decarboxylation product would serve only as an indication of the presence of clorazepate and could not be used as positive identification. Direct mass spectral analysis[6] of clorazepate salts by solid probe yields ions at *m/e* values identical to those observed for N-desmethyldiazepam. This is attributed to a loss of CO_2 due to thermal decomposition on the solid probe. Infrared spectral data has been used to identify clorazepate salts[6]. This paper reports the results of our efforts to develop a liquid chromatographic method of analysis for clorazepate in pharmaceutical dosage forms.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a Waters Model 6000 solvent pump, Model U6K injector equipped with a 2-ml loop, a Model 440 UV detector and a Varian A-25 recorder. Ultraviolet absorption spectra were measured using a Hitachi Model 60 or a Perkin-Elmer Model 200 spectrophotometer. A Beckman Model 3500 pH meter was used to make all pH measurements.

Reagents

All reagents were of ACS reagent-grade quality and were used as purchased without further purification. HPLC grade methanol was obtained from Fisher Scientific (Atlanta, Ga., U.S.A.). Clorazepate dipotassium was obtained from Abbott Labs. (Chicago, Ill., U.S.A.) and N-desmethyldiazepam was supplied by Hoffmann-La Roche (Nutley, N.J., U.S.A.). The pH 8.04 buffer was prepared by mixing 5.0 ml of a stock solution of 9.2 g/l of NaH_2PO_4 and 95.0 ml of a stock solution of 17.86 g/l of Na_2HPO_4. All aqueous solutions were prepared in double distilled water.

Chromatographic procedures

Separation was accomplished using a 3.9 mm I.D. by 30 cm μBondapak C_{18} column (Waters Assoc., Milford, Mass., U.S.A.). The mobile phases described were made up of pH 8.04 phosphate buffer and HPLC grade methanol. Paired-ion chromatographic procedures were accomplished by adding sufficient tetrabutylammonium phosphate to the mobile phase to produce a 0.005 *M* solution.

Analysis of dosage forms

Samples of Tranxene and Azene were added to 10 ml of pH 8.04 phosphate buffer and the resulting suspension agitated for 5 min. The solution was filtered and a 3 μl sample of the solution analyzed by liquid chromatography.

RESULTS AND DISCUSSION

The results of our initial attempts at the analysis of clorazepate by liquid chromatography are shown in Fig. 1. A sample of clorazepate salt was dissolved in water and aliquots of the solution were injected into the liquid chromatograph. The mobile phase was water–methanol (3:7) adjusted to various acidic pH values. Chromatograms A and B in Fig. 1 were obtained by injecting 3 μl of the clorazepate

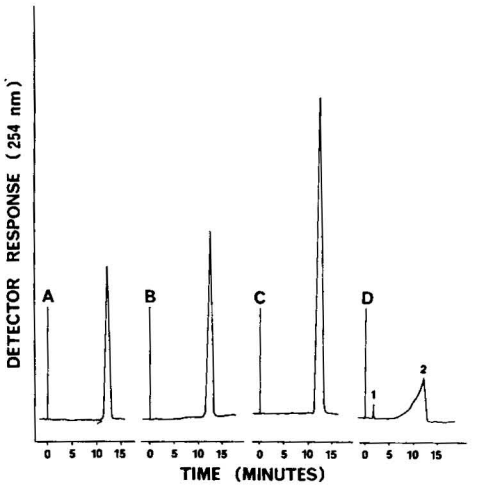


Fig. 1. Effects of mobile phase pH on clorazepate decarboxylation. Mobile phase: methanol–water (7:3) at 1.5 ml/min. Chromatograms: A, clorazepate injection at mobile phase pH 4.0; B, clorazepate injection at mobile phase pH 4.6; C, N-desmethyldiazepam injection at mobile phase pH 4.6; D, clorazepate injection at mobile phase pH 5.6 (peaks: 1 = clorazepate; 2 = N-desmethyldiazepam).

solution at a mobile phase pH of 4.0 and 4.6 respectively. Chromatogram C resulted from an injection of a known sample of N-desmethyldiazepam. As the mobile phase pH was increased to 5.6, an injection of the clorazepate solution showed two peaks (Fig. 1D). The first peak occured at a much shorter retention time than N-desmethyldiazepam and the second peak which eluted at approximately the same retention time as N-desmethyldiazepam exhibited a fronting effect. Further increases in mobile phase pH resulted in an increase in the area of peak 1 and a symmetrical shape for peak 2. This series of experiments shows that at pH levels of 4.6 and less the clorazepate sample underwent complete decarboxylation in the injector and was chromatographed as N-desmethyldiazepam. At a mobile phase pH of about 5.6 the rate of decarboxylation is slower allowing a small amount of clorazepate to elute through the octadecylsilane column to produce peak 1 and contribute to the fronting effect for the N-desmethyldiazepam peak. The fronting on the second peak is most likely produced by the decarboxylation of portions of the clorazepate sample at various points along the column. A sample of clorazepate which undergoes on-column decarboxylation would cause the elution of the resulting N-desmethyldiazepam at a shorter retention time than normal thus the fronting effect is produced. N-desmethyldiazepam was not detected in a pH 8 clorazepate solution stored at ambient temperature for 1 week. Thus, by maintaining the mobile phase at pH 8 the clorazepate decarboxylation can be prevented. Further increases in mobile phase pH must be avoided due to the dissolution of the silica based stationary phase in basic solvents of greater than pH 8[7].

The effects of pH on clorazepate salts are shown in Fig. 2. The structures shown suggest that clorazepate is at least partially ionized at pH 8 and thus ionized under the chromatographic conditions. The results of some attempts at paired-ion chromatography with clorazepate further support this conclusion. A solution of

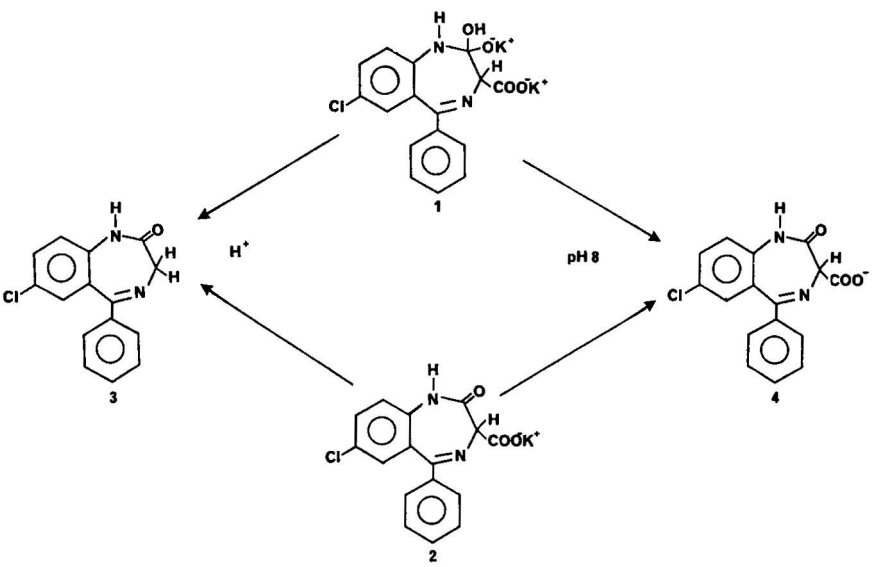


Fig. 2. The effects of pH on clorazepate stability. Structures: 1 = clorazepate dipotassium (Tranxene); 2 = clorazepate monopotassium (Azene); 3 = N-desmethyldiazepam; 4 = clorazepate anion.

clorazepate and N-desmethyldiazepam was chromatographed in methanol–pH 8 phosphate buffer (2:1) and in 0.005 *M* tetrabutylammonium phosphate prepared in the same mobile phase. The addition of the tetrabutylammonium ion produced an increase in the retention time of the clorazepate peak while the retention of the non-ionic N-desmethyldiazepam was not altered. The retention of the clorazepate–tetrabutylammonium ion-pair was only slightly greater than that observed for free clorazepate. This perhaps indicates that the octadecylsilane stationary phase has some affinity for the ionized clorazepate. The retention of organic ions by hydrocarbonaceous stationary phases has been reported in other work[8]. The mechanism of retention in reversed-phase chromatography in the presence of solvophobic ions is the topic of a great deal of current research[9]. One mechanism postulated for ion-pair chromatography suggests the retention of the organic ion-pairing agent by the stationary phase to form a reversible ion-exchange column[10]. Thus, the retention of organic ions by an octadecylsilane column is not a new concept. Further adjustments showed that an increase in the water content of the mobile phase produced the desired increase in retention for clorazepate. Due to the hydrophobic nature of the clorazepate anion the ion-pairing was not necessary for the adjustment of chromatographic conditions.

The separation of clorazepate from other commonly encountered benzodiazepines was accomplished using the octadecylsilane column and a methanol–pH 8 phosphate buffer (4:3) mobile phase. The separation is shown in Fig. 3. Dosage forms of Tranxene and Azene when dissolved in pH 8 buffer and injected into the

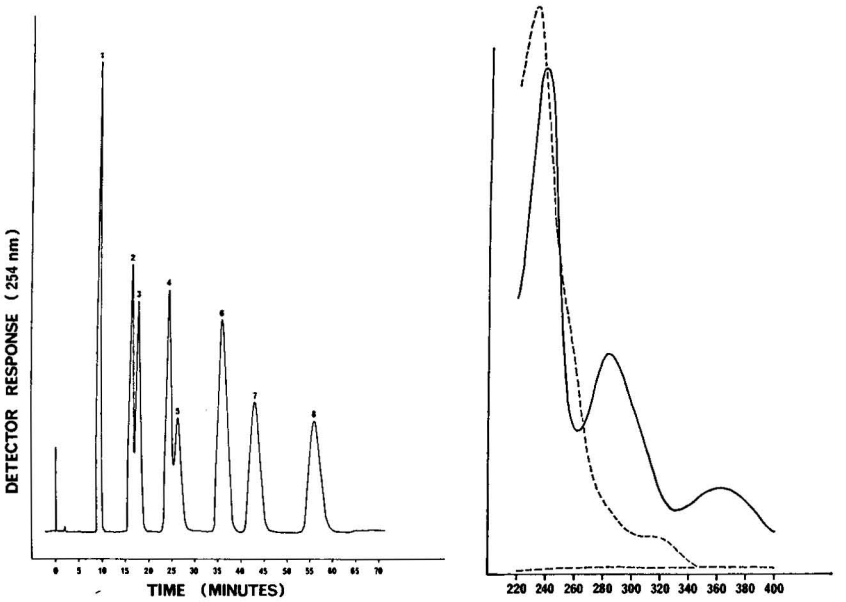


Fig. 3. Liquid chromatographic separation of benzodiazepines. Mobile phase: methanol–pH 8 phosphate buffer (4:3) at 1.5 ml/min. Peaks: 1 = clorazepate; 2 = nitrazepam; 3 = clonazepam; 4 = oxazepam; 5 = lorazepam; 6 = chlordiazepoxide; 7 = N-desmethyldiazepam; 8 = diazepam.

Fig. 4. Ultraviolet absorption spectrum of the clorazepate peak collected from the liquid chromatographic effluent. Dotted line is the spectrum obtained in the mobile phase at pH 8; solid line represents the spectrum obtained upon acidification of the mobile phase solvent system with 0.5 *N* H_2SO_4.

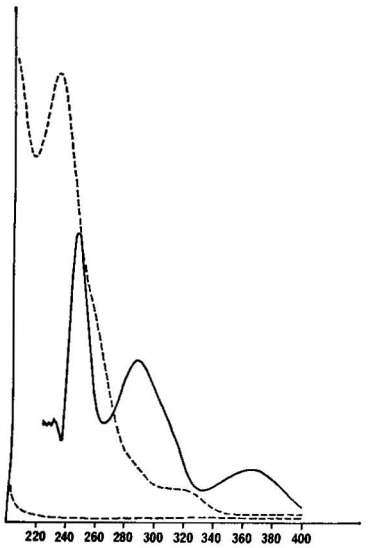


Fig. 5. Ultraviolet absorption spectrum of clorazepate standard in the liquid chromatographic mobile phase solvent system. Dotted line is the spectrum obtained at pH 8; solid line is the spectrum obtained upon acidification with 0.5 *N* H_2SO_4.

liquid chromatograph under the described conditions showed identical retention times. Thus, the two salt forms produce equivalent species in solution at pH 8 (Fig. 2).

Further analytical data can be obtained on unknown samples of clorazepate following liquid chromatographic analysis. Fig. 4 shows the UV spectrum obtained by collection of the clorazepate peak from the liquid chromatographic effluent. The spectrum was obtained first in basic solution followed by acidification of the solution which resulted in the production of the UV spectrum of N-desmethyldiazepam. Fig. 5 shows the UV spectrum for a standard sample of clorazepate obtained in basic and acidic mobile phase solvent. These spectra are consistent with UV spectra determined in aqueous acid and base[11]. Thus, clorazepate can be analyzed in its original form by liquid chromatography followed by peak collection for positive identification by UV analysis and other confirming tests. The procedure described in this paper can be used for the positive identification of clorazepate in its original form and the method is applicable to a large group of benzodiazepines.

REFERENCES

1 *Physicians' Desk Reference*, Medical Economics Company, Oradell, N.J., 30th ed., 1976, pp. 548–549.
2 R. Raveux and M. Briot, *Chim. Ther.*, 4 (1969) 303.
3 H. Dasberg, *Psychopharmacologia (Berlin)*, 43 (1975) 191.
4 S. H. Curry, *Clin. Pharmacol. Ther.*, 16 (1974) 192.
5 K. E. Cockerham, personal communication, September 1978.
6 J. H. Siefert, *Microgram*, 10 (1977) 136.
7 A. Wehrli, J. C. Hildenbrand, H. P. Keller, R. Stampfli and R. W. Frei, *J. Chromatogr.*, 149 (1978) 199.
8 N. E. Hoffman and J. C. Liao, *Anal. Chem.*, 49 (1977) 2231.
9 C. Horvath, W. Melander, I. Molnar and P. Molnar, *Anal. Chem.*, 49 (1977) 2295.
10 P. T. Kissinger, *Anal. Chem.*, 49 (1977) 833.
11 F. T. Noggle, Jr. and C. R. Clark, *J. Ass. Offic. Anal. Chem.*, 62 (1979) 799.

Journal of Chromatography, 188 (1980) 431–434

CHROM. 12,429

Note

Separation of iodinated compounds of L-tyrosyl-L-tyrosine from iodothyronines by Biogel P-2 column chromatography

J. MICHELOT, D. GODENECHE and J. C. MAURIZIS

INSERM U 71, B.P. 184, Rue Montalambert, 63005 Clermont-Ferrand (France)

and

G. MEYNIEL

Laboratoire de Biophysique Médicale, Faculté de Médecine, 28, Place Henri Dunant, 63001 Clermont-Ferrand Cedex (France)

(First received July 11th, 1979; revised manuscript received October 1st, 1979)

Previous investigations conducted in our laboratory have shown that the sequence L-tyrosyl-L-tyrosine is part of the primary structure of thyroglobulin. The dipeptide 3,5,3′,5′-tetraiodo-L-tyrosyl-L-tyrosine (I_2Tyr-I_2Tyr) extracted from trypsic digests of bovine thyroglobulin[1] has demonstrated the presence of this iodinated sequence under physiological conditions. *In vitro* experiments showed that this dipeptide is a better substrate than diiodotyrosine for the synthesis of iodothyronines[2], and we proposed a mechanism of this synthesis which appealed to a cyclic agent without breaking the peptide bond[3,4]. During these *in vitro* experiments, we developed a new technique for the separation of the iodinated compounds of tyrosyl-tyrosine from the known iodoamino acids. Various methods for the separation of iodo compounds have already been described: paper chromatography, thin-layer chromatography, ion-exchange column chromatography, Sephadex gel filtration and gas–liquid chromatography[5–10]. Up to 1975 no procedure allowing a complete separation of all the known iodoamino acids was available. Since 1975, following the work of Thomopoulos[11], we have been using a single Biogel P-2 column which separates L-monoiodohistidine, L-diiodohistidine, L-monoiodotyrosine, L-diiodotyrosine, L-triiodothyronine and L-thyroxine. But this technique does not separate the different iodinated compounds of L-tyrosyl-L-tyrosine. To obtain such a separation we performed a systematic study of the relative role of the different constituents, in particular the gel and the elution buffer.

This paper presents a new method for the separation of the iodinated tyrosines, tyrosyl tyrosines and thyronines on the basis of the pK_b value and of the iodine content.

EXPERIMENTAL

The iodination of L-tyrosyl-L-tyrosine leads to eight different iodinated compounds: ITyr-Tyr, Tyr-ITyr, I_2Tyr-Tyr, Tyr-I_2Tyr, ITyr-ITyr, I_2Tyr-ITyr, ITyr-I_2Tyr and I_2Tyr-I_2Tyr.

The synthesis and the labelling with ^{125}I or ^{131}I of these compounds were

carried out by peptidic coupling of iodotyrosines and then by isotopic exchange with radioactive iodine. A mixture of iodinated compounds of L-tyrosyl-L-tyrosine was obtained by labelling with peroxidase[12] and with a 75% iodine deficiency. I_2Tyr-I_2Tyr labelling was carried out by the chloramine T method[13]. Purification was performed on a Dowex 50-X4 column (200–400 mesh). The specific activity was 1000–1200 Ci/g. The radiochemical purity was 98%. Biogel P-2 polyacrylamide gel (200–400 mesh) (Bio-Rad Labs., Richmond, Calif., U.S.A.) was allowed to swell for 24 h at 20° in the elution buffer. The gel was then poured into plastic columns (50 × 0.9 cm I.D.) (Pharmacia, Uppsala, Sweden) and equilibrated with at least three total column volumes of the elution buffer.

We investigated the behaviour of the eight iodinated compounds of L-tyrosyl-L-tyrosine under the conditions described by Thomopoulos. Each ^{125}I- or ^{131}I-labelled product was deposited at the top of the column. Fractions of 1 ml were collected with a Gilson fraction collector. The radioactivity content for ^{125}I or ^{131}I of each fraction was measured in a dual channel Nuclear Chicago Autogamma Spectrometer with appropriate corrections for ^{131}I counts appearing in the ^{125}I channel, when needed.

Simultaneously, for each compound ^{127}I was detected on a Technicon auto-analyser by the method of Block and Mandl[7], as modified by Aquaron[14].

RESULTS AND DISCUSSION

Analysis of the results obtained with the Thomopoulos method shows that the bonding of the iodinated compounds of L-tyrosyl-L-tyrosine to a gel with an acid pH decreases with the rate of iodination. As Biogel P-2 possesses weak cationic exchange properties at alkaline pH, we can assume that the low degree of absorption of iodinated compounds is due to a reduction in their basicity compared with their less iodinated homologues. Therefore, it was logical to consider that the triiodinated compounds are less firmly bonded than the tetraiodinated ones. Hence the desorption pH of the triiodinated compounds must be lower than the desorption pH of the tetraiodinated ones. From these observations we were led to modify two constituents of the Thomopoulos method.

(1) A Biogel P-2 (minus 400) was used instead of the Biogel P-2 (200–400) in order to obtain a higher resolution of the gel filtration by the use of smaller particles.

(2) The replacement of the second eluent with pH 9.0 by a linear pH gradient was expected to produce a better separation of the iodinated compounds of I_2Tyr-I_2Tyr because of the difference in pK values for each compound. To obtain a good separation of all the iodinated compounds we used a linear pH gradient starting with a 0.05 *M* Tris–maleate buffer (pH 5.3) and ending with a 0.05 *M* Tris–HCl buffer (pH 9.0), at a 6 ml/h flow-rate for 24 h.

As can be seen from the elution curve (Fig. 1) and from Table I, a complete separation of five iodinated compounds of L-tyrosyl-L-tyrosine is achieved with this technique. Using the classical ion-exchange column chromatography these five compounds were eluted as a single component[1]. The method described by Sorimachi[9,10] allows a separation of most but not of all the compounds and metabolites related to LT_3. As mentioned earlier, the Thomopoulos method separates only two iodohistidines and two iodotyrosines from the thyronines[11]. None of these methods provided the separation needed in our specific case, *i.e.* the separation of iodinated compounds

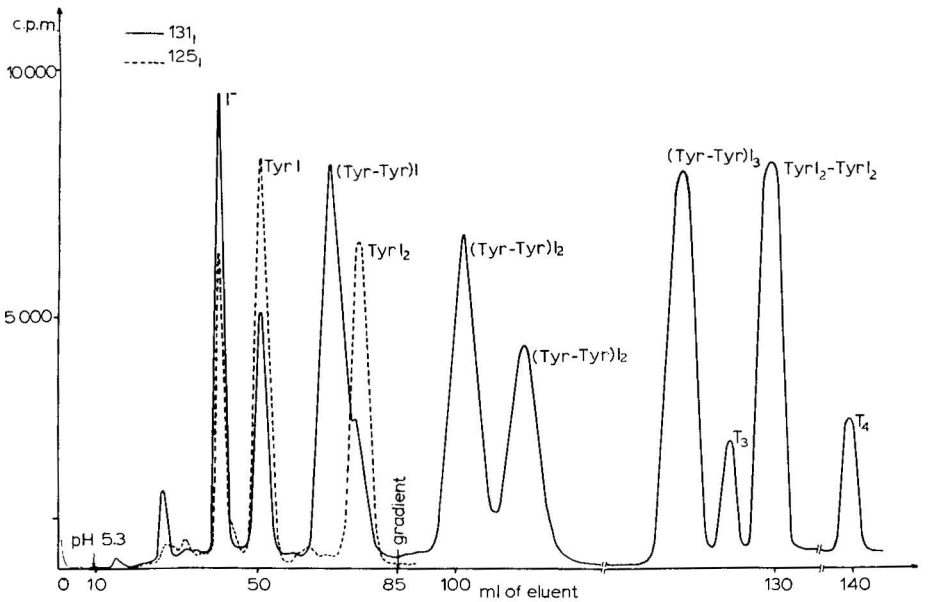


Fig. 1. Elution pattern of radioactive iodoamino acids on a Biogel P-2 (minus 400) column equilibrated and eluted at a flow-rate of 6 ml/h for 24 h with 0.05 *M* Tris–maleic acid–NaOH at pH 5.3 followed (arrow) by a linear pH gradient starting with a 0.05 *M* Tris–maleate buffer (pH 5.3) and ending with a 0.05 *M* Tris–HCl buffer (pH 9).

of L-tyrosyl-L-tyrosine present in the digests obtained from *in vitro* iodination both chemically and enzymatically.

Our new procedure should be helpful to predict tentatively the structure of unknown metabolites of I_2Tyr-I_2Tyr by comparing their R_F values to those of authentic iodotyrosines, iodotyrosyl-tyrosines and iodothyronines. It can also provide information on the structure–mobility relationship in gel filtration column chromatography. This technique has also been adapted to the problem of identification and purification of minor iodinated components in thyroglobulin digests, in the study of iodothyronine biosynthesis from I_2Tyr-I_2Tyr, and in *in vitro* iodination experiments performed in our laboratory.

TABLE I

ELUTION VOLUME/VOID VOLUME RATIOS AND pH DATA FOR EIGHT IODINATED COMPOUNDS

Iodinated compounds	V_e/V_0	*pH*
I_2Tyr-Tyr	1.8	5.9
Tyr-I_2Tyr	2.3	6.1
ITyr-ITyr	3.5	6.6
I_2Tyr-ITyr + ITyr-I_2Tyr	8.9	8.5
T_3	10	8.9
I_2Tyr-I_2Tyr	10.7	9
T_4	11.4	9

ACKNOWLEDGEMENTS

We thank Prof. Didier Isabelle for his critical reading of the manuscript. This work was supported by an INSERM grant (C.R.L. No. 78.5.047.3).

REFERENCES

1 J. Michelot, J. C. Madelmont and G. Meyniel, *C.R. Acad. Sci.*, 276 (1973) 1357.
2 J. C. Maurizis, D. Godeneche, J. Michelot and G. Meyniel, *Biochim. Biophys. Acta*, 404 (1975) 188.
3 J. Michelot, J. C. Maurizis, C. Nicolas and G. Meyniel, *Biochim. Biophys. Acta*, 540 (1978)463.
4 J. C. Maurizis, J. Michelot and G. Meyniel, *Biochim. Biophys. Acta*, 540 (1978) 472.
5 I. Radichevich and E. M. Volpert, in V. Stole (Editor), *Endocrinologica Experimentalis, Vol. II, Estimation of Iodo Compounds in Biological Material*, Publishing House of the Slovak Academy of Sciences, Bratislava, 1966, p. 105.
6 H. J. Cahnmann, in J. E. Rall and J. Jopin (Editors), *Methods in Investigative and Diagnostic Endocrinology, Vol. I, The Thyroid and Biogenic Amines*, North Holland, Amsterdam, 1972, p. 27.
7 R. J. Block and R. H. Mandl, *Ann. N.Y. Acad. Sci.*, 102 (1962) 87.
8 M. Rolland, M. F. Montfort, L. Valenta and S. Lissitzky, *Anal. Biochem.*, 33 (1970) 307.
9 K. Sorimachi and N. Ui, *J. Biochem.*, 76 (1974) 39.
10 K. Sorimachi, *Anal. Biochem.*, 93 (1978) 31.
11 P. Thomopoulos, *Anal. Biochem.*, 65 (1965) 600.
12 G. S. Bayse, A. W. Michaels and M. Morrison, *Biochim. Biophys. Acta*, 284 (1972) 30.
13 F. C. Greenwood, W. M. Hunter and J. S. Glover, *Biochem. J.*, 89 (1963) 114.
14 R. Aquaron, *Thèse Doct. État Med.*, Marseille, 1969.

Journal of Chromatography, 188 (1980) 435–438

CHROM. 12,430

Note

Isolement de glycoprotéines végétales hydrosolubles par chromatographie d'affinité sur concanavaline A immobilisée

C. AKIKI, R. BOURBOUZE, C. LUPORSI et F. PERCHERON

Laboratoire de Chimie Biologique, Faculté des Sciences Pharmaceutiques et Biologiques, 4 av. de l'Observatoire, 75006 Paris (France)

(Reçu le 13 juin 1979; manuscrit modifié reçu le 3 octobre 1979)

Les plantes, et notamment les graines, renferment des teneurs non négligeables en glycoprotéines; cependant, les glycoprotéines végétales ont donné lieu à peu d'investigations, et seules quelques-unes d'entre-elles ont été caractérisées[1]. Des données récentes semblent néanmoins indiquer que de nombreuses glycoprotéines végétales possèdent une structure oligomannosidique, et sont donc susceptibles d'interagir spécifiquement avec la concanavaline A (Con A)[2–5].

Nous décrivons une méthode adaptée à l'isolement et à la caractérisation de glycoprotéines hydrosolubles affines de la concanavaline A, et ceci à partir du broyat d'un matériel végétal (graines de sarrasin, *Fagopyrum esculentum* Polygonacées). Nous avons utilisé la concanavaline A sous forme immobilisée comme support d'affinité; l'étude de l'influence de divers paramètres (essais en cuve et en colonne, concentration en méthyl-α-D-mannoside, température, adjuvants) nous a permis de sélectionner un protocole expérimental assurant un rendement optimal, et de préciser la spécificité de l'interaction glycoprotéines végétales–concanavaline A.

PARTIE EXPÉRIMENTALE

Méthodes analytiques

La concentration en protéines des éluats chromatographiques est évaluée par mesure de l'absorption différentielle à 260 et 280 nm selon Warburg et Christian[6]. La teneur en oses neutres des glycoprotéines est déterminée selon la méthode de Dubois *et al.*[7], en utilisant le mannose comme témoin interne.

Extraction des protéines hydrosolubles

La fraction soluble après dialyse contre de l'eau distillée du broyat de graines sèches de sarrasin (20 g) est adsorbée sur une colonne (25 × 150 mm) d'hydroxylapatite (HA-Ultrogel) équilibrée avec un tampon phosphate 0.001 *M* pH 6. Après lavage avec le tampon d'équilibrage, les protéines fixées sur le support sont éluées par un tampon phosphate 0.5 *M*, pH 7. Cette étape chromatographique s'effectue avec un débit de 25 ml/h et des fractions d'environ 10 ml sont recueillies.

Isolement des glycoprotéines affines de la concanavaline A

L'éluat de l'HA-Ultrogel est dialysé et lyophilisé. Le lyophilisat protéique (100 mg) est remis en solution dans du tampon acétate 0.1 *M* (pH 6)–NaCl *M*, et chromatographié sur colonne (20 × 170 mm) de Con A–Sepharose (Pharmacia, Uppsala, Suède) équilibrée avec le même tampon, à 20° et avec un débit de 10 ml/h. Après passage de la fraction protéique non affine de la lectine et lavage de la colonne avec le tampon d'origine, l'élution des glycoprotéines fixées sur Con A–Sepharose s'effectue par du méthyl-α-D-mannoside 0.5 *M* après équilibrage de la colonne à 37°. Après passage d'une quantité de tampon renfermant le compétiteur correspondant à une fois et demi le volume du gel, la chromatographie est interrompue et la colonne portée dans une enceinte thermostatée à 37° pendant 2 h. L'élution des glycoprotéines affines de la Con A s'effectue ensuite avec un débit de 60 ml/h et recueil de fractions de 5 ml.

La fraction glycoprotéique, dialysée et concentrée par lyophilisation ou ultrafiltration sur membrane Diaflo PM 30 (Amicon, Lexington, Mass., É.U.), est chromatographiée sur colonne (25 × 360 mm) de Sephadex G25 (Pharmacia) avec un débit de 15 ml/h et recueil de fractions de 3 ml (rendement en glycoprotéines 10 mg).

En l'absence de précisions contraires dans le texte, toutes les opérations d'extraction et les étapes chromatographiques sont réalisées à 4°, et les tampons renferment 0.02% d'azoture de sodium.

Electrofocalisation

Les expériences d'électrofocalisation ont été réalisées à l'aide d'un appareillage "Ampholine PAGplate" (LKB, Stockholm, Suède), avec des gels de 5% en acrylamide et pour un gradient d'ampholytes de pH 3.5–9.5. Les protéines sont révélées par le bleu de coomassie. La coloration des glycoprotéines par le réactif de Schiff ne s'est pas montrée assez sensible; individuellement, chaque glycoprotéine au sein d'un échantillon se trouve au-dessous du seuil minimal de sensibilité[8].

RÉSULTATS ET DISCUSSION

Les conditions opératoires décrites permettent d'obtenir en un nombre limité d'étapes une quantité appréciable de glycoprotéines (10 mg de glycoprotéines dans le cas d'une préparation type à partir de 100 mg de protéines totales éluées de l'HA-Ultrogel et correspondant à 20 g de graines de sarrasin) dont la teneur globale en oses neutres est de 11%.

L'étape chromatographique sur HA-Ultrogel permet d'isoler la totalité des protéines de l'extrait végétal aqueux, et d'éliminer les oses libres, polysaccharides et pigments du broyat, non adsorbables par l'hydroxylapatite dans ces conditions opératoires[9].

Sous forme immobilisée, la Con A constitue un outil de choix pour l'isolement d'une population de glycoprotéines. Des tentatives préliminaires d'élution fractionnée sur colonne de Con A–Sepharose à l'aide de gradients de concentration continu ou discontinu en méthyl-α-D-mannoside se sont révélés de faible rendement. L'élution globale des glycoprotéines fixées sur le support d'affinité est fonction de la concentration en compétiteur et de la température; en protocole final, nous avons retenu leur élution par un tampon méthyl-α-D-mannoside 0.5 *M* après équilibrage de la colonne de Con A–Sepharose à 37°.

L'analyse par électrofocalisation de cette fraction glycoprotéique affine de la Con A révèle une forte hétérogénéité; on identifie une population de bandes protéiques se répartissant dans toute la gamme du gradient (pH 3.5–9.5), avec néanmoins quelques bandes majeures qui focalisent entre pH 4 et 6 (Fig. 1C).

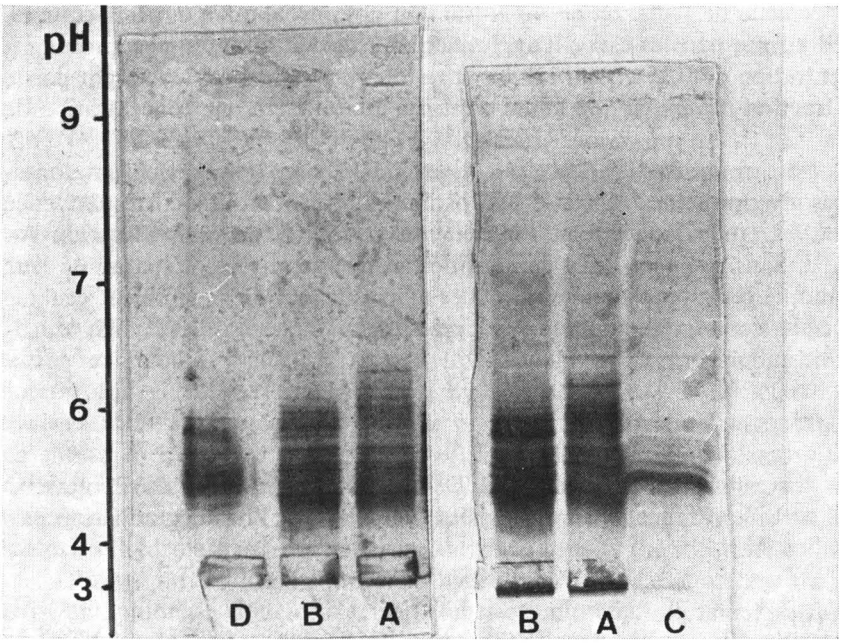


Fig. 1. Electrofocalisation en plaques d'acrylamide à 5% et gradient d'ampholytes pH 3.5–9.5. A = Protéines hydrosolubles: éluat HA-Ultrogel; B = protéines non fixées par la colonne de Con A–Sepharose; C = glycoprotéines affines de la Con A; éluat méthyl-α-D-mannoside, 0.5 *M* à 37°; D = glycoprotéines affines de la Con A; éluat méthyl-α-D-mannoside, 0.02 *M* à 20°.

Les conditions nécessaires à l'élution des glycoprotéines de la colonne de Con A–Sepharose sont le reflet d'une forte interaction entre la majorité d'entre-elles et la Con A. Néanmoins, une élution préférentielle, sinon sélective, de glycoprotéines de faible affinité peut être envisagée pour une concentration de 0.02 *M* en méthyl-α-D-mannoside à 20°; on identifie au niveau de cette fraction une concentration notable des glycoprotéines qui focalisent entre pH 4 et 6 par rapport à la fraction glycoprotéique globale (Fig. 1D).

Nous n'avons pu déceler la présence éventuelle d'interactions non spécifiques ou irréversibles entre certaines protéines et le gel de Con A–Sepharose. La présence de NaCl *M* dans le tampon de fixation prévient les interactions ioniques[10,11]. Après lavage par le tampon méthyl-α-D-mannoside 0.5 *M* (cinq fois le volume de la colonne), l'addition à ce tampon d'éthylène glycol (50%), ou le passage d'un tampon borate 0.1 *M*, pH 6, n'entraînent pas l'élution d'une fraction protéique, indice respectivement de l'absence d'interactions de type hydrophobe[12] ou polysaccharide–lectine[13]. L'absence d'interactions irréversibles est illustrée par le passage de solutions d'HCl 0.1 *M* ou de SDS 0.1% sur des gels ayant servi à plusieurs chromatographies successives. Ces adjuvants provoquent la dissociation de la Con A et la perte de son activité

biologique[14]; l'éluat protéique correspondant à ce traitement ne contient pas de matériel glucidique. La fraction protéique non fixée par passage sur colonne de Con A–Sepharose, et la fraction glycoprotéique affine de la lectine sont rechromatographiées selon les mêmes conditions expérimentales. Par recyclage, chacune de ces fractions présente à nouveau le même comportement chromatographique.

La spécificité de l'interaction de la fraction glycoprotéique pour la lectine est également confirmée par des essais d'agglutination. Seule la fraction glycoprotéique, à une concentration de 0.25 mg/ml inhibe l'agglutination d'hématies de lapin par la Con A. La fraction protéique non affine n'est pas inhibitrice à une concentration de 1 mg/ml.

Dans cette approche, les données concernant le comportement chromatographique de ces glycoprotéines végétales hydrosolubles présentent une correspondance certaine avec les études concernant l'interaction glycoprotéines animales–Con A–Sepharose[15–17]. Sans préjuger de la composition et de l'arrangement spatial de leur partie glycanique, ces glycoprotéines végétales affines de la Con A semblent génératrices, avec cette lectine, d'interaction spécifiques comparables à celles des glycoprotéines d'orgine animale.

RÉMERCIEMENTS

Nous remercions vivement Mr. H. Debray, du Laboratoire du Professeur J. Montreuil de Lille (France), pour les essais d'inhibition de l'hémagglutination par la concanavaline A.

BIBLIOGRAPHIE

1 N. Sharon, dans J. B. Pridham (Rédacteur), *Plant Carbohydrate Biochemistry*, Academic Press, New York, London, 1974, p. 236.
2 W. T. Forsee, G. Valkovich et A. D. Elbein, *Arch. Biochem. Biophys.*, 174 (1976) 469.
3 P. A. Gleeson et M. A. Jermyn, *Aust. J. Plant Physiol.*, 4 (1977) 25.
4 S. Bouquelet et G. Spick, *Eur. J. Biochem.*, 63 (1978) 331.
5 H. Lis et N. Sharon, *J. Biol. Chem.*, 153 (1978) 3468.
6 O. Warburg et W. Christian, *Biochem. Z.*, 310 (1941) 384.
7 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers et F. Smith, *Anal. Chem.*, 28 (1956) 350.
8 R. A. Kapitany et E. J. Zebrowsky, *Anal. Biochem.*, 56 (1973) 361.
9 A. Tiselius, S. J. Hjerten et O. Levin, *Arch. Biochem. Biophys.*, 65 (1956) 132.
10 R. J. Doyle, E. E. Woodside et C. N. Fishel, *Biochem. J.*, 106 (1968) 35.
11 Y. Matsushina et T. Mega, *J. Biochem.*, 81 (1977) 571.
12 I. J. Goldstein et C. E. Hayes, *Adv. Carbohydr. Chem. Biochem.*, 35 (1978) 127.
13 K. O. Lloyd, dans H. Bittiger et H. P. Schnebli (Rédacteurs), *Concanavalin A as a Tool*, Wiley, New York, 1976, p. 323.
14 R. Lotan, G. Beattie, W. Hubbel et G. L. Nicolson, *Biochemistry*, 15 (1977) 1787.
15 M. B. Fiddler, Y. Ben-Yoseph et H. L. Nadler, *Biochem. J.*, 177 (1979) 175.
16 G. W. Norden et J. S. O Brien, *Biochem. Biophys. Res. Comm.*, 56 (1974) 193.
17 M. G. Brattain, P. M. Kimball, T. G. Pratlow et M. E. Marks, *Biochem. J.*, 163 (1977) 247.

Journal of Chromatography, 188 (1980) 439–441

CHROM. 12,397

Note

Purification of phlorizin by column chromatography on Sephadex LH-20 with aqueous propan-2-ol

JONATHAN R. GREEN and JOCELYNE COUSINEAU

Department of Gastroenterology, University Children's Hospital, Freiburgstrasse 15, 3010 Bern (Switzerland)

(Received September 21st, 1979)

In several mammals, including man, the brush-border membrane of the small intestine exhibits a β-glucosidase activity capable of hydrolysing phlorizin* to phloretin and glucose[1]. This "phlorizin hydrolase" activity is associated with the lactase (neutral β-galactosidase) enzyme of the small intestinal mucosa[2], and has been shown to hydrolyse a wide range of glucosylceramides and lactosylceramides occurring in milk[3]. The high cost of pure glycosylceramides, and their low solubility in detergent-free aqueous solutions, precludes their regular use for the measurement of phlorizin hydrolase activity and so phlorizin is usually the substrate employed.

During our studies on phlorizin hydrolase it became apparent that the free glucose and phloretin content of some batches of phlorizin interfered, presumably by product inhibition, with the kinetic analysis of enzymatic activity. As recrystallization from aqueous ethanol gave no marked increase in purity, column chromatography on Sephadex LH-20 with aqueous solutions of propan-2-ol was investigated and found to be a suitable purification method.

MATERIALS AND METHODS

Phlorizin, phloretin (phlorizin aglucon), phloroglucinol (1,3,5-trihydroxybenzene), 2,4,6-trihydroxybenzoic acid and 3-(4-hydroxyphenyl)propionic acid were purchased from commercial suppliers. D-[U-^{14}C]Glucose was obtained from the Radiochemical Centre (Amersham, Great Britain). A bed of Sephadex LH-20 (Pharmacia, Uppsala, Sweden), 33 × 2.5 cm, was packed in a Pharmacia SR25/45 column in accordance with the manufacturers instructions. Preliminary experiments indicated that a mixture of redistilled propan-2-ol (35–50%) and water was suitable as eluent. The column was eluted upwards with a peristaltic pump at a flow-rate of 0.4 ml/min and 7.5 ml fractions were collected. Fluctuations in temperature were observed to affect markedly the elution volume of each compound so the entire operation was performed in a cold room at 4°, and care was taken to protect the column and collected samples from strong light. Standard marker solutions of

* Phlorizin = 4,6-dihydroxy-2-(β-D-glucosido)-β-(*p*-hydroxyphenyl)propiophenone; also known as phlorhizin, phloridzin or phlorrhizen.

phlorizin and its related component molecules were made at a concentration of 1 mg/ml in a mixture of propan-2-ol and water corresponding to the column eluent. All samples were labelled with *ca.* 10^5 cpm of [^{14}C]glucose before being loaded onto the column. Aliquots (0.5 ml) of each eluted fraction were mixed with 10 ml Aquasure (NEN Chemicals, Dreieich, G.F.R.) and the ^{14}C-content measured in a liquid scintillation counter. The elution of aromatic compounds off the column was followed by measuring the UV absorption of the eluate at 220 nm. Each peak was identified by comparison of its elution volume and UV spectrum with those of standard marker compounds.

The free glucose content of phlorizin before and after chromatography was measured at 365 nm with the glucose–fructose UV test kit produced by Boehringer (Mannheim, G.F.R.).

RESULTS AND DISCUSSION

Chromatography on Sephadex LH-20 using propan-2-ol[4] or acetone[5] as solvent has been shown to be an effective method for the separation of aromatic and cyclic compounds by reversible adsorption. Our initial experiments with Sephadex LH-20 and solvent mixtures of 35–50% propanol-2-ol and water indicated that phlorizin retention was inversely related to propan-2-ol concentration whereas glucose always eluted in the void volume. In the solvent mixture finally chosen, 38% propan-2-ol in water, an excellent separation of phlorizin from glucose and phloretin (peaks E, A and F respectively in Fig. 1) was achieved, and in addition the system

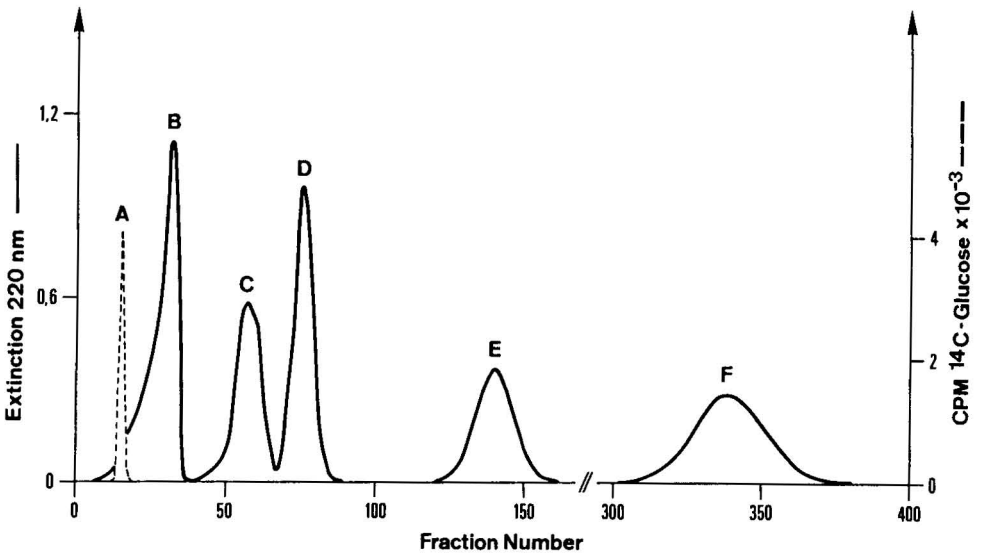


Fig. 1. The chromatographic separation on Sephadex LH-20 of a mixture of 1 mg glucose containing 10^5 cpm [^{14}C]glucose (A), 0.5 mg 2,4,6-trihydroxybenzoic acid (B), 1 mg 3-(4-hydroxyphenyl)-propionic acid (C), 1 mg phloroglucin (D), 1 mg phlorizin (E) and 1 mg phloretin (F). The column, 33 × 2.5 cm, was eluted upwards at 4° with 3 l of 38% propan-2-ol in water at a flow-rate of 0.4 ml/min. Fractions of 7.5 ml volume were collected every 18.8 min. The elution profile was measured at 220 nm; the broken line represents radioactivity.

resolved 2,4,6-trihydroxybenzoic acid, 3-(4-hydroxyphenyl)propionic acid and phloroglucin (peaks B, C and D respectively in Fig. 1). With higher concentrations of propan-2-ol the phlorizin eluted earlier but too close to other components to be of use as a preparative method, although still suitable for analytical work.

For the routine purification of phlorizin the column was loaded with 100 mg phlorizin (containing 0.1–9% free glucose as an impurity) in 1.5 ml of solvent and eluted with 3 l of 38% propan-2-ol at 4°. Those eluate fractions containing phlorizin were pooled and lyophilized to give a dry phlorizin powder in which there was no detectable free glucose. The same column has been used continuously for more than twenty such runs over a period of 6 months without any alteration in the elution profile.

ACKNOWLEDGEMENTS

This work was supported by grant No. 3.855-0.77 from the Swiss National Science Foundation. J.C. was in receipt of a fellowship from "Le Conseil de la recherche en Santé du Québéc", Canada.

REFERENCES

1 H. Lorenz-Meyer, A. L. Blum, H. P. Haemmerli and G. Semenza, *Eur. J. Clin. Invest.*, 2 (1972) 326.
2 V. Colombo, H. Lorenz-Meyer and G. Semenza, *Biochim. Biophys. Acta*, 327 (1973) 412.
3 H. J. Leese and G. Semenza, *J. Biol. Chem.*, 248 (1973) 8170.
4 M. Wilk, J. Rochlitz and H. Bende, *J. Chromatogr.*, 24 (1966) 414.
5 B. J. Mair, P. T. R. Hwang and R. G. Ruberto, *Anal. Chem.*, 39 (1967) 838.

Journal of Chromatography, 188 (1980) 442–444

CHROM, 12,415

Note

Separation of S-sulphocysteine and related compounds by anion-exchange chromatography and electrophoresis

TOSHIHIKO UBUKA, MASAHIRO KINUTA, REIKO AKAGI and SHOZO KIGUCHI
Department of Biochemistry, Okayama University Medical School, Okayama, 700 (Japan)
(Received August 27th, 1979)

Sulphite is an intermediate in the sulphur metabolism of cysteine in animals. It has been reported that "cystine disulphoxide" reacts quantitatively with sulphite to produce S-sulphocysteine (2-amino-2-carboxyethylsulphosulphane)[1]. It has also been found that "cystine disulphoxide" reacts with thiosulphate to form S-sulphothiocysteine (2-amino-2-carboxyethylsulphodisulphane). Attempts have been made to separate these S-sulpho derivatives of cysteine by Dowex 1 column chromatography

EXPERIMENTAL

S-Sulphocysteine[2] and S-sulphothiocysteine[3,4] were prepared according to reported methods. These amino acids were also synthesized from "cystine disulphoxide" and sulphite or thiosulphate, respectively, in high yields. The details of the methods will be reported elsewhere. S-Sulphoglutathione was prepared according to Eriksson and Rundfeldt[5]. L-Cysteic acid and taurine were obtained from Sigma (St. Louis, Mo., U.S.A.).

Dowex 1-X8 (Cl^-) (200–400 mesh) was packed in a 550 × 10 mm glass tube and washed with water until the washings were neutral. A 1-ml volume of 1 *M* acetic acid containing L-cysteic acid (3 μmol), taurine (3 μmol), S-sulphocysteine (5 μmol) and S-sulphothiocysteine (7 μmol) was placed on the top of the column. Elution was carried out at room temperature with a linear gradient of sodium chloride in 1 *M* acetic acid prepared from 150 ml of 1 *M* acetic acid placed in a mixing chamber and 150 ml of 1 *M* acetic acid containing 2 *M* sodium chloride placed in a reservoir. The flow-rate was regulated at 41 ml/h with a peristaltic pump and 2-ml fractions were collected. Amino acids in the fractions were determined with ninhydrin reagent[6], and with Gaitonde's acidic ninhydrin reagent 2 after treatment with dithiothreitol[7].

High-voltage paper electrophoresis was performed in pyridine–acetic acid–water (0.5:10.0:79.5, pH 3.1)[8] for 45 min. Amino acids were detected with 1% ninhydrin–2% pyridine in acetone.

RESULTS AND DISCUSSION

Fig. 1 shows the elution profile of taurine, cysteic acid, S-sulphocysteine and S-sulphothiocysteine with the Dowex 1-X8 column. These four amino acids were

clearly separated. Purdie *et al.*[9] reported the chromatography of sulphonic and sulphinic acids on a long Dowex 1-X8 (chloroacetate) column, in which S-sulphocysteine and S-sulphothiocysteine were eluted as broad peaks. By using the present method these amino acids were eluted as sharp peaks in a shorter time, and their quantitative determination became possible.

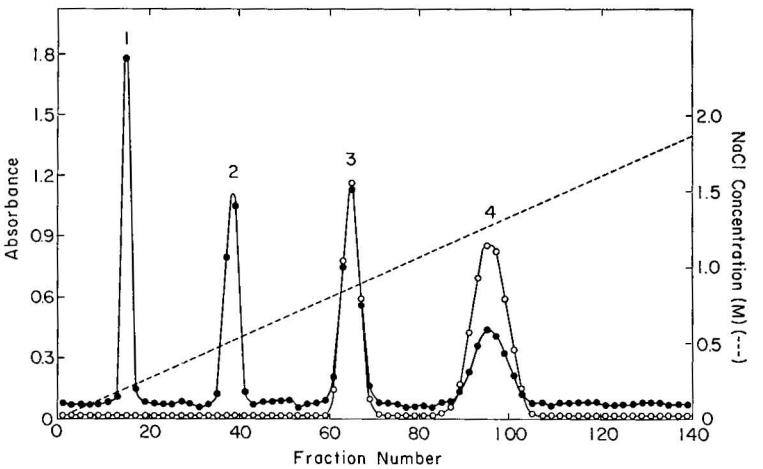


Fig. 1. Chromatography of S-sulphocysteine and related compounds on Dowex 1-X8 column (550 × 10 mm). Elution was carried out with a linear gradient of sodium chloride in 1 *M* acetic acid; flow-rate, 41 ml/h. Fractions of 2 ml were collected. Eluates were checked with ninhydrin reagent (absorbance at 570 nm, ●) and acidic ninhydrin reagent 2 (absorbance at 560 nm, ○). Peaks: 1 = taurine; 2 = cysteic acid; 3 = S-sulphocysteine; 4 = S-sulphothiocysteine.

The colour in the ninhydrin reaction of S-sulphothiocysteine is brownish and its colour value is low, as shown in Table I. However, this was improved by use of Gaitonde's acidic ninhydrin reagent 2, which is equally sensitive to cysteine and cysteine-producing amino acids following treatment with dithiothreitol.

TABLE I

NINHYDRIN COLOUR VALUES OF S-SULPHOCYSTEINE (SSC) AND S-SULPHOTHIOCYSTEINE (SSTC)

Amino acid (0.2 μmol) in 0.5 ml of 1 *M* acetic acid containing 1 *M* NaCl was reacted with 0.5 ml of ninhydrin reagent for the manual method[6] or with 0.5 ml of Gaitonde's acidic ninhydrin reagent 2 after dithiothreitol treatment[7] in standard procedures. The absorbance at 570 nm of the usual ninhydrin reaction of leucine was taken as 100%.

Reaction	*Leucine*	*Cysteine*	*SSC*	*SSTC*
Usual ninhydrin reaction	100	23	80	47
Acidic ninhydrin reaction	0	104	89	91

Fig. 2 shows the paper electrophoretic separation of cysteic acid, S-sulphocysteine, S-sulphothiocysteine, S-sulphoglutathione, aspartic acid and taurine. Although S-sulphocysteine and S-sulphoglutathione were not separated by the present column chromatographic method, they could be separated by electrophoresis.

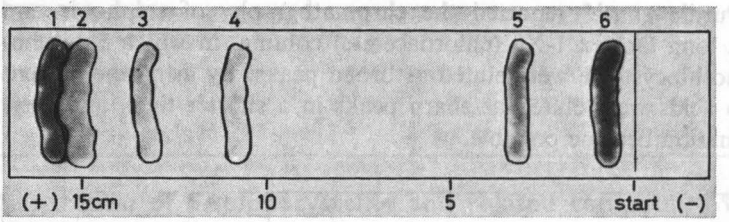


Fig. 2. Paper electrophoresis of S-sulphocysteine and related compounds. Electrophoresis was performed in pyridine–acetic acid–water (0.5:10.0:79.5, pH 3.1) at 85 V/cm for 45 min. Bands: 1 = cysteic acid; 2 = S-sulphocysteine; 3 = S-sulphothiocysteine; 4 = S-sulphoglutathione; 5 = aspartic acid; 6 = taurine.

The present method will be useful for the study of the metabolism of sulphur-containing amino acids.

ACKNOWLEDGEMENT

The authors are grateful to Prof. Dr. Shunzi Mizuhara for his valuable advice.

REFERENCES

1 T. Ubuka, S. Uuasa, M. Kinuta and R. Akagi, *Physiol. Chem. Phys.*, 11 (1979) in press.
2 T. Nakamura and R. Sato, *Biochem. J.*, 86 (1963) 328.
3 T. W. Szczepkowski, *Nature (London)*, 182 (1958) 934.
4 T. W. Szczepkowski, *Rocz. Chem.*, 35 (1961) 563; *C.A.*, 55 (1961) 23359h.
5 B. Eriksson and M. Rundfelt, *Acta Chem. Scand.*, 22 (1968) 562.
6 S. Moore, *J. Biol. Chem.*, 243 (1968) 627.
7 M. K. Gaitonde, *Biochem. J.*, 104 (1967) 627.
8 T. Ubuka, *J. Biochem.*, 52 (1962) 440.
9 J. W. Purdie, J. P. Farant and R. A. Gravelle, *J. Chromatogr.*, 23 (1966) 242.

Journal of Chromatography, 188 (1980) 445–447

CHROM. 12,413

Note

Fractionation of steroid digitonides by thin-layer and column chromatography on silica gel

FRANCOIS-X. GARNEAU, JEAN CÔTÉ, ODETTE HARVEY and JEAN-LUC SIMARD

Département des Sciences Pures, Université du Québec à Chicoutimi, Chicoutimi, Québec G7H 2B1 (Canada)

(First received June 13th, 1979; revised manuscript received October 4th, 1979)

The quantitative determination of cholesterol has often been accomplished by its isolation as an insoluble complex with digitonin[1,2] followed by fractionation of the digitonide in order to recover the cholesterol. The same complex-forming reaction has been used to selectively precipitate sea cucumber saponins (holothurins) using cholesterol as the complexing reagent[3,4]. In the course of our investigations on the selectivity and efficiency of this complex formation as a means of isolating holothurins, we observed that steroid digitonides could be separated into their constituents by thin-layer (TLC) and column chromatography on silica gel. The digitonides are most often decomposed using pyridine[3,5] or dimethyl sulphoxide (DMSO)[6] under reflux followed by extraction with solvents. Dextran gel column chromatography has been successfully used[7], although digitonin and stigmasterol are eluted fairly close to one other. The TLC method described in this report has the usual advantages associated with this technique, as well as resulting in very different R_F values for the constituents (cholesterol–digitonin and stigmasterol–digitonin) and also in a partial separation of the saponin mixture itself. As expected, column chromatography also permitted a very good fractionation of larger quantities of the digitonides as well as resulting in highly different migration rates for the constituents and a partial separation of the commercial digitonin mixture.

EXPERIMENTAL

Materials and reagents

Digitonin (ICN Pharmaceuticals, Plainview, N.Y., U.S.A.), m.p.: 240–245° (d), stigmasterol (Sigma, St. Louis, Mo., U.S.A.), m.p. 164–167°, and cholesterol (J. T. Baker, Phillispburg, N.J., U.S.A.) m.p. 144–146° were used as received. Benzene, chloroform, methanol and diethyl ether were from Fisher Scientific (certified ACS grade; Fair Lawn, N.J., U.S.A.). The diethyl ether was fractionally distilled. Silica gel 7G (TLC reagent) and silica gel (60–200 mesh, analyzed) were purchased from J. T. Baker. Polygram SIL G (Brinkmann, Westbury, N.Y., U.S.A.) precoated plastic sheets were used for qualitative analysis.

Preparation of digitonides

The method for digitonide formation was that described by Fernholz[8] and involves adding the same volume of a 1% solution of digitonin in ethanol to a hot solution of the sterol in 90% ethanol. The precipitated digitonide was washed several times with diethyl ether (benzene and chloroform with the stigmasterol digitonide) and methanol to remove all traces of uncomplexed sterol and digitonin respectively.

Thin-layer chromatography

The preparative TLC was carried out by spreading a uniform layer (1 mm) of silica gel 7G on 20 × 20 cm glass plates with a Desaga/Brinkmann standard adjustable applicator. The plates were then placed in an oven at 110° for 1 h. Great care was taken in placing the complexes (a suspension in benzene–methanol, 1:1) as a uniform band on the plates. Development was carried out in a glass chamber with the lower phase of the solvent system chloroform–methanol–water (65:35:10) (solvent I). Visualization was achieved on a similar plate using the Liebermann–Buchard detection reagent followed by heating in an oven at 110° for 5 min. Cholesterol and digitonin were extracted exhaustively from the silica gel using diethyl ether and methanol respectively. The purity of the sterols and the digitonin was checked on pre-coated plastic sheets (3 × 8 cm) of silica gel and they were identified by a comparison of their melting points and/or their R_F values with authentic material. Cholesterol ($R_F = 0.79$) and stigmasterol ($R_F = 0.81$) each appeared as a single pink spot and digitonin as two light brown spots ($R_F = 0.19, 0.26$) with the aforementioned detection reagent.

Column chromatography

A 37.5-g amount of silica gel was introduced as a slurry with chloroform–methanol–water (60:15:2) into a glass column (2 × 37.5 cm) fitted with a PTFE stopcock. The complexes were placed on the column as a partially soluble mixture with benzene–methanol (1:1). Elution was achieved with chloroform–methanol–water (60:15:2 and 60:30:4) (solvents II and III). The fractions were examined by TLC and the compounds were identified by comparison of melting points and/or R_F values with authentic material.

RESULTS AND DISCUSSION

The insoluble complex formed from digitonin and cholesterol in alcohol was separated into its constituents when placed on an analytical pre-coated plastic sheet of silica gel and developed with solvent I. It was subsequently observed that the efficiency of fractionation depended on the quantities of digitonide relative to the thickness of the silica gel layer. A too high ratio of amount of digitonide to layer thickness resulted in a black spot at the point of application (presumably intact complex) as well as the spots characteristic of digitonin and the sterol. On a preparative scale, a quantity $\geqslant$ 16 mg of digitonide on 1 mm of silica gel resulted in a poor fractionation. An ideal situation was obtained with 10 mg of digitonide on a 1 mm layer of silica gel. The bands resulting from the fractionation of the cholesterol digitonide were extracted and the purity and identity of the two constituents were confirmed by analytical TLC. 90% of the digitonin was recovered based on a 1:1

complex. Although the stigmasterol digitonide was not fractionated on a preparative scale by TLC, a purified sample of the complex was separated into its constituents in the same manner on an analytical plate.

Larger quantities of the digitonides were fractionated by column chromatography on silica gel. Ratios of adsorbent to digitonide of 1000:1 and 1500:1 resulted in complete fractionation whereas the presence of both cholesterol and digitonin was observed (TLC) in the initial fractions when a ratio $\leqslant$ 750:1 was used. In this latter case, the column was prepared and eluted with solvent I. In a typical run, 37.5 mg of the cholesterol digitonide was placed on a column containing 37.5 g of silica gel. Elution was carried out at first with 200 ml of solvent II followed by solvent III (5-ml fractions). Cholesterol was obtained in fractions 13–40 and the digitonin was collected as follows (monitored by TLC): fractions 54–61, component 1 ($R_F = 0.26$); fractions 62–67, components 1 and 2 ($R_F = 0.26, 0.19$); and fractions 68–87, component 2 ($R_F = 0.19$). An essentially quantitative yield was obtained of cholesterol (m.p. 140–143° after one recrystallization) and digitonin (combined fractions) based on a 1:1 complex.

In the same manner, 37.5 mg of the stigmasterol digitonide were fractionated. Stigmasterol was collected in fractions 9–32 (5 ml each) using solvent II. At fraction 37 elution was continued with solvent III (10 ml fractions). The two components of digitonin were again separated as follows: fractions 49–57, component 1; fractions 58–64, components 1 and 2; and fractions 65–86, component 2. Here again an essentially quantitative yield of stigmasterol was obtained as well as a yield of $\approx$85% digitonin.

This method is particularly useful when isolating saponins as insoluble complexes with cholesterol as it allows an efficient fractionation of the complex under conditions which also favor a separation of the constituents of saponin mixtures in a single chromatography operation. The method may be applicable to the quantitative determination of sterols which precipitate with digitonin.

REFERENCES

1 A. Windaus, *Hoppe-Seyler's Z. Physiol. Chem.*, 65 (1910) 110.
2 R. Schöenheimer and W. M. Sperry, *J. Biol. Chem.*, 106 (1934) 745.
3 J. D. Chanley, R. Ledeen, J. Wax, R. F. Nigrelli and H. Sobotka, *J. Amer. Chem. Soc.*, 81 (1959) 5180.
4 G. B. Elyakov, V. A. Stonik, E. V. Levina, V. P. Slanke, T. A. Kuznetsova and V. S. Levin, *Comp. Biochem. Physiol.*, 44B (1973) 325.
5 R. Schöenheimer and H. Dam, *Hoppe-Seyler's Z. Physiol. Chem.*, 215 (1933) 59.
6 C. H. Issidorides, I. Kitagawa and E. Mosettig, *J. Org. Chem.*, 27 (1962) 4693.
7 M. M. El-Olemy and S. J. Stohs, *J. Pharm. Sci.*, 64 (1975) 161.
8 E. Fernholz, *Hoppe-Seyler's Z. Physiol. Chem.*, 232 (1935) 97.

Journal of Chromatography, 188 (1980) 448–451

CHROM. 12,425

Note

Dünnschichtchromatographische Untersuchungen von Nitrobenzylarenen

D. KLEMM und E. KLEMM

Friedrich-Schiller-Universität Jena, Sektion Chemie, Humboldtstrasse 10, DDR-69 Jena (D.D.R.)

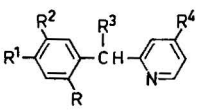
(Eingegangen am 4. Oktober 1979)

Nitroarene sind als Zwischen- und Endprodukte der organischen Synthese von grosser allgemeiner Bedeutung und besitzen als π-Elektronenakzeptoren sowie aufgrund ihrer photochemischen Reaktivität wachsendes Interesse. Sie werden als Sensibilisatoren/Elektronenakzeptoren in organischen Photoleiterschichten[1–3] und als Initiatoren für die Photopolymerisation[4,5] eingesetzt. Nitrobenzylverbindungen zeigen darüber hinaus photochrome Eigenschaften[6–8] und sind als photoempfindliche Schutzgruppen für Amino-, Hydroxyl- und Carboxylfunktionen bei der Peptid- und Nucleinsäuresynthese von grossem Wert[9–12].

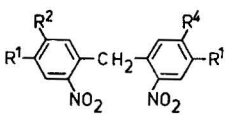
Zur schnellen Reinheitskontrolle der häufig nur in geringer Menge benötigten Verbindungen sowie für Aussagen zum Verlauf der Photoreaktionen ist die Dünnschichtchromatographie sehr gut geeignet. Die vorliegende Arbeit beinhaltet dünnschichtchromatographische Untersuchungen von Nitrobenzylarenen.

EXPERIMENTELLES

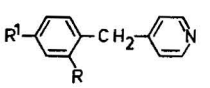
Die untersuchten 20 Nitrobenzylarene aus der Reihe der 2-Nitrobenzylpyridine (1–12) und 4-Nitrobenzylpyridine (13–14) sowie Nitrodiphenylmethane (15–20) wurden durch Nitrieren entsprechender Benzylpyridine bzw. durch Umsetzungen am 2-(2′,4′-Dinitrobenzyl)pyridin (3)[9–12] sowie durch Nitrieren der substituierten Diphenylmethane[13] synthetisiert. Alle Verbindungen waren elementaranalytisch rein. Ihre Struktur ist durch Infrarot (IR)- und magnetische Kernresonanz (NMR)-spektroskopische Untersuchungen gesichert.



1–12



15–20



13–14

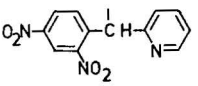
R–R⁴ s. Tabelle I

Formel A.

Zur Dünnschichtchromatographie fanden handelsübliche Platten Silufol-UV_{254}* Verwendung. Die Platten wurden 60 min auf 120° erwärmt und anschliessend im Exsikkator aufbewahrt. Die Probelösungen wurden durch Einwaage von 10 mg der Nitrobenzylarene in einem Masskolben und Auffüllen mit Aceton zu 5 ml hergestellt. Von diesen Lösungen wurden 50 μl in einer Entfernung von 1.5 cm vom unteren Plattenrand in Startpunkten auf die Platten aufgebracht. Als Fliessmittel fanden Benzol und Gemische aus Benzol und 96 prozentigem Ethanol Verwendung, die zwei Stunden vor Beginn der Chromatographie in die Kammer eingebracht wurden. Die Laufzeit der Platten betrug bei aufsteigender Arbeitsweise und einem Anstellwinkel von 60° 50–60 min, die Länge der Trennstrecke 15 cm. Der Nachweis der chromatographierten Verbindungen erfolgte nach Verdunsten des Fliessmittels bei Raumtemperatur durch Belichten mit einer UV-Lampe bzw. durch Besprühen mit einer Lösung von Jod in Chloroform. Durch Verwendung von Chloroform als Lösungsmittel wird eine störende Verfärbung des stärkegebundenen Trägermaterials unterdückt. Die Nitrodiphenylmethane wurden spezifisch durch Behandeln mit Ammoniak sichtbar gemacht.

ERGEBNISSE UND DISKUSSION

Die Untersuchungsergebnisse bei Verwendung verschiedener Fliessmittelverhältnisse sind in Tabelle I zusammengestellt. Die Detektion mit Jod in Chloroform ergab bei Raumtemperatur bei allen untersuchten Substanzen braune Flecken. Bei UV-Belichtung gelang der Nachweis mit Hilfe des in der Schicht enthaltenen UV-Indikators. Darüber hinaus färbten sich bei längerer Belichtung bzw. bei gleichzeitiger UV- und IR-Bestrahlung in wenigen Minuten alle Substanzflecken aufgrund verschiedenartiger Photoreaktionen gelb bis braun. Durch Behandeln mit Ammoniak nahmen die Flecken der Nitrodiphenylmethane eine intensiv blaue Farbe an. Diese Farbreaktion ist sehr wahrscheinlich auf die Bildung der durch Protonenablösung aus den Nitrodiphenylmethanen erhältlichen Polymethine vom Diazaoxonol-Typ zurückzuführen, wie wir sie in Lösung beim Belichten bzw. der Basenbehandlung nachweisen konnten[7,8].


Formel B.

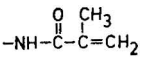

Formel C.

Tabelle I zeigt, dass 4-Nitrobenzylpyridine stärker adsorbiert werden als 2-Nitrobenzylpyridine, und diese wiederum stärker als vergleichbare Verbindungen aus der Reihe der Nitrodiphenylmethane. Hieraus ist die für die Adsorption erforderliche möglichst uneingeschränkte Verfügbarkeit des Pyridinstickstoffs ersichtlich.

* Durch Stärke gebundenes Silikagel auf Aluminiumfolie mit Fluoreszenzindikator (Kavalier, Sklárny, Czechoslovakia).

TABELLE I

R_F-WERTE DER NITROBENZYLARENE BEI VERSCHIEDENEN FLIESSMITTEL VERHÄLTNISSEN

Fliessmittel: Benzol–Ethanol; A = (100:0); B = (50:1); C = (5:1).

Nitrobenzylarene	*R*	R^1	R^2	R^3	R^4	R_F-*Werte*		
						A	*B*	*C*
1 2-Pyridine	H	NO_2	H	H	H	*	0.23	0.70
2 2-Pyridine	H	NO_2	H	H	CH_3	*	0.13	0.65
3 2-Pyridine	NO_2	NO_2	H	H	H	*	0.42	0.85
4 2-Pyridine	NO_2	NO_2	CH_3	H	H	*	0.38	0.84
5 2-Pyridine	NO_2	NO_2	H	CH_3	H	0.10	0.65	0.89
6 2-Pyridine	NO_2	NO_2	H	H	CH_3	*	0.27	0.87
7 2-Pyridine	NO_2	NO_2	CH_3	H	CH_3	*	0.36	0.85
8 2-Pyridine	NO_2	NO_2	H	Br	H	0.20	0.80	0.90
9 2-Pyridine	NO_2	NO_2	H	**	H	0.11	0.84	0.92
10 2-Pyridine	NO_2	NO_2	H	**	H	0.45	0.89	0.93
11 2-Pyridine	NO_2	NH_2	H	H	H	*	*	0.37
12 2-Pyridine	NO_2	***	H	H	H	*	*	0.41
13 4-Pyridine	H	NO_2	—	—	—	*	0.10	0.37
14 4-Pyridine	NO_2	NO_2	—	—	—	*	0.10	0.36
15 Diphenylmethane	—	NO_2	H	—	H	0.27	0.80	0.90
16 Diphenylmethane	—	NO_2	CH_3	—	H	0.27	0.82	0.91
17 Diphenylmethane	—	NO_2	CH_3	—	CH_3	0.29	0.83	0.90
18 Diphenylmethane	—	NH_2	H	—	H	*	0.10	0.34
19 Diphenylmethane	—	NH_2	CH_3	—	CH_3	*	0.17	0.61
20 Diphenylmethane	—	CHO	H	—	H	*	0.56	0.89

* R_F-Werte für praktische Auswertung zu niedrig.
** Formel B.
*** Formel C.

Aus Tabelle I ist auch zu entnehmen, dass die in 2-(2′,4′-Dinitrobenzyl)pyridin (3) eingeführten funktionellen Gruppen einen deutlichen Einfluss auf die Adsorption an die stationäre Phase ausüben. Sie nimmt deutlich zu, wenn in 4′-Stellung eine primäre Amino- bzw. Carbonsäureamind-Gruppe fixiert ist (11, 12). Zusätzliche Methyl-Gruppen im Phenyl- bzw. Pyridin-Ring (4, 6, 7) von 3 erhöhen geringfügig die Adsorption, eine Substitution der *ortho*-Nitrogruppe durch Wasserstoff wie in 1 bewirkt den gleichen Effekt. Grossvolumige Gruppen in Nachbarschaft des Pyridinstickstoffs von 3 (α-Substitution, vgl. 5, 8–10) führen zu einer deutlichen Verringerung der Adsorption.

In der Reihe der untersuchten Tetranitrodiphenylmethane zeigen Amino-Gruppen den gleichen Effekt abnehmender Adsorption an die stationäre Phase, für Methyl-Gruppen ist kein Einfluss erkennbar.

Aus Tabelle I ist darüber hinaus ersichtlich, dass diastereomere *ortho*-Nitrobenzylarene wie Verbindung 9, 10 unter den gewälten Bedingungen gut aufgetrennt werden. Sie sind auf diesem Wege präparativ in reiner Form isolierbar[14].

Dünnschichtchromatographische Untersuchungen der photochromen *ortho*-Nitrobenzylarene sind auch als Schnellmethode für durch Licht ausgelöste Ermüdungsreaktionen von Bedeutung und mit Erfolg angewendet worden[15]. Für diesen

Zweck werden die Substanzflecke nach Auftragen belichtet und die gebildeten Photolyseprodukte mit Hilfe der beschriebenen Chromatographie aufgetrennt und zugeordnet bzw. anschliessend analysiert.

LITERATUR

1 J. W. Weigl, *Photochem. Photobiol.*, 16 (1978) 291.
2 P. J. Reucroft, *Polymer-Plast., Technol. Eng.*, 5 (1975) 199.
3 J. Rochlitz, *Chem. Ztg.*, 96 (1972) 561.
4 S. Tazuke, *Adv. Polymer Sci.*, 6 (1969) 321.
5 *Houben-Weyl, Methoden der organischen Chemie*, Vol. 4, Part 5b, G. Thieme Verlag, Stuttgart 1975, p. 1499.
6 G. H. Brown, *Photochromism*, Wiley, New York 1971, p. 558.
7 E. Klemm und D. Klemm, *J. Prakt. Chem.*, 321 (1979) 406.
8 D. Klemm, E. Klemm, A. Graness und J. Kleinschmidt, *Chem. Phys. Lett.*, 55 (1978) 113,503.
9 A. Patschornik, B. Amit und R. B. Woodward, *J. Amer. Chem. Soc.*, 92 (1970) 6333.
10 J. A. Barltrop, P. J. Plant und P. Schofield, *Chem. Commun.*, (1966) 822.
11 D. Klemm, E. Klemm, S. Winkelmann und H.-H. Hörhold, *J. Prakt. Chem.*, 317 (1975) 761.
12 C. Mercier und J. Dubosc., *Bull. Soc. Chim. France*, (1969) 4425.
13 D. Klemm, E. Klemm, H. Jerusel, G. Drefahl und H.-H. Hörhold, *J. Prakt. Chem.*, 320 (1978) 767.
14 E. Klemm, D. Klemm und H.-H. Hörhold, *Synthesis*, (1977) 342.
15 D. Klemm und E. Klemm, *J. Prakt. Chem.*, 320 (1978) 551.

Journal of Chromatography, 188 (1980) 452–456

CHROM. 12,412

Note

Eine neue Methode zur quantitativen Bestimmung von Galanthamin in Drogenextrakten von *Leucojum aestivum*

FRIEDRICH WURST, THEODOR PREY, LEOPOLD PUCHINGER und ENGELBERT BANCHER

Institut für Botanik, Technische Mikroskopie und Organische Rohstofflehre, Technische Universität Wien, Getreidemarkt 9, A-1060 Wien (Österreich)

(Eingegangen am 14. August 1979; geänderte Fassung eingegangen am 4. Oktober 1979)

Die quantitative Bestimmung von Galanthamin und verwandten Alkaloiden in Drogenextrakten erfolgt neben der Ionenaustauschchromatographie[1], Säulenchromatographie[2], Elektrophorese[3] und Kolorimetrie[4] auch mit Hilfe der Dünnschichtchromatographie (DC)[5–8]. Sandberg und Michel[7] benutzen die zweidimensionale Dünnschichtchromatographie zu phytochemischen Studien der Alkaloidzusammensetzung in den einzelnen Organen von *Pancratium maritimum* L. Die quantitative Auswertung erfolgt nach Sprühen mit Dragendorff-Reagens, bei Sandberg und Michel[7] mit Platin(IV)chlorwasserstoffsäure. Bei Verwendung von Dragendorff-Reagens konnte durch zusätzliche Behandlung mit verdünnter Schwefelsäurelösung die Empfindlichkeit des Reagens im hohen Mass gesteigert werden. Mit Hilfe der Fleckengrösse ist eine annähernd quantitative Schätzung möglich. In letzter Zeit werden für die Bestimmung der Amaryllidaceenalkaloide gaschromatographische Methoden benutzt[9].

Im folgenden soll ein Verfahren beschrieben werden, das eine rasche und genaue quantitative Bestimmung von Galanthamin in Drogenextrakten ermöglicht. Durch die Messung der Remissionsminderung bei 288 nm auf der DC-Platte entfällt das Sprühen des Chromatogramms und damit auch die sich daran anschliessenden Schwierigkeiten bei der quantitativen Auswertung.

MATERIAL UND METHODIK

Drogenextraktion: 3 g getrocknete und in einem Mixer mit 100 ml Äthanol zerkleinerte Blätter von *Leucojum aestivum* werden mit zusätzlichen 70 ml Äthanol versetzt und bei 40° unter Vakuum mit einem Rotationsverdampfer bis zur Trockene eingedampft. Der Rückstand wird mit 250 ml 1%iger Salzsäure in einen mit Rührer und Kühler ausgestatteten Kolben gebracht und mit 100 ml Petroläther (120°) 30 min bei 40° gerührt. Danach wird filtriert, die Petroleumbenzinphase abgetrennt, die wässrige Phase mit 25%iger Ammoniaklösung auf pH 11 eingestellt und mehrmals mit Chloroform ausgeschüttelt oder perforiert. Die Chloroformphase wird zur Trockene eingeengt und der Rückstand in 25 ml Chloroform aufgenommen; diese Lösung wird zur DC verwendet.

Die DC erfolgt auf Kieselgel G 60 Fertigplatten (Merck, Darmstadt, B.R.D.) im Laufmittelsystem Diäthyläther–Methanol–Diäthylamin (80:15:5) bei Kammersättigung. Pro Platte werden mit einer Hamilton-Spritze vier Punkte zu je 10 μl einer Standardreihe, ebenso vier Punkte zu je 10 μl einer Verdünnungsreihe des Drogenextraktes aufgetragen. Die Platte wird im Laufmittelsystem einmal entwickelt.

Die getrockneten DC-Platten werden auf dem Chromatogrammspektralphotometer der Fa. Zeiss in der Messanordnung Lichtquelle–Monochromator–Probe–Empfänger vermessen und die Remissionsortskurven mit Hilfe eines angeschlossenen Potentiometerschreibers (Servogor RE 511) aufgezeichnet. Folgende Einstellgrössen am Gerät haben sich als günstig erwiesen: Wellenlänge: 288 nm; Spalthöhe: 6 mm; Spaltbreite: *ca.* 0.5 mm; Vorschubgeschwindigkeit des Kreuztisches und des Schreiberpapiers: 120 mm/min.

Die Messung erfolgt in Laufrichtung des Chromatogramms; dies bringt den Vorteil, dass Untergrundstörungen bei Drogen- und Frischpflanzenextrakten besser beurteilt werden können und dass eine "durchhängende" Laufmittelfront nicht stört. Ebenso kann man die Abtrennung der entsprechenden Substanz von Verunreinigungen genau verfolgen und danach am Papierstreifen die entsprechende Auswertung vornehmen; diese erfolgt durch Ausmessen und Berechnen der Peakflächen unter Verwendung einer unter gleichen Bedingungen erstellten Eichkurve.

ERGEBNISSE UND DISKUSSION

Die Fig. 1 und 2 zeigen die Chromatogramm-Messkurven für die Reinsubstanz Galanthamin und einen Drogenextrakt, die eine für die quantitative Auswertung befriedigende Trennung erkennen lassen. Der der Reinsubstanz benachbarte niedrige Peak mit kleinerem R_F-Wert resultiert aus einer Verunreinigung der käuflichen Rein-

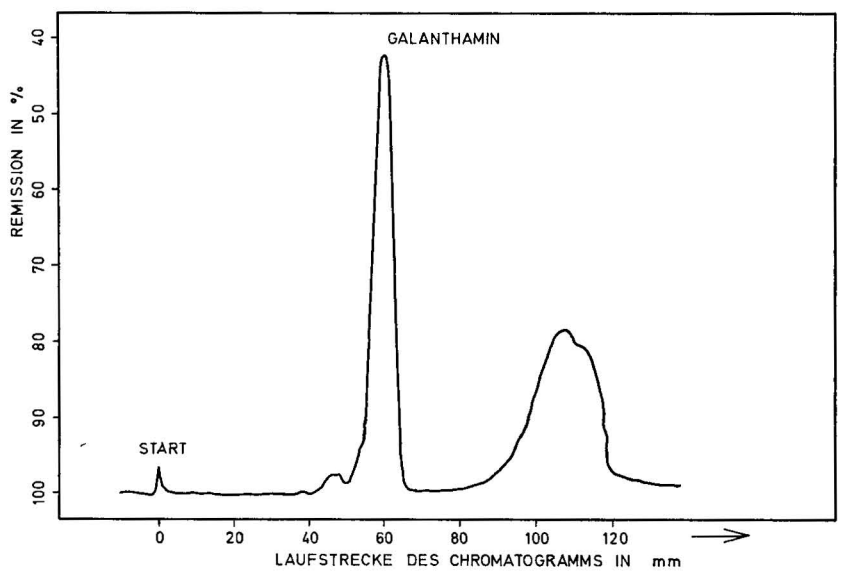


Fig. 1. Ortsremissionskurve der Reinsubstanz Galanthamin auf der DC-Platte.

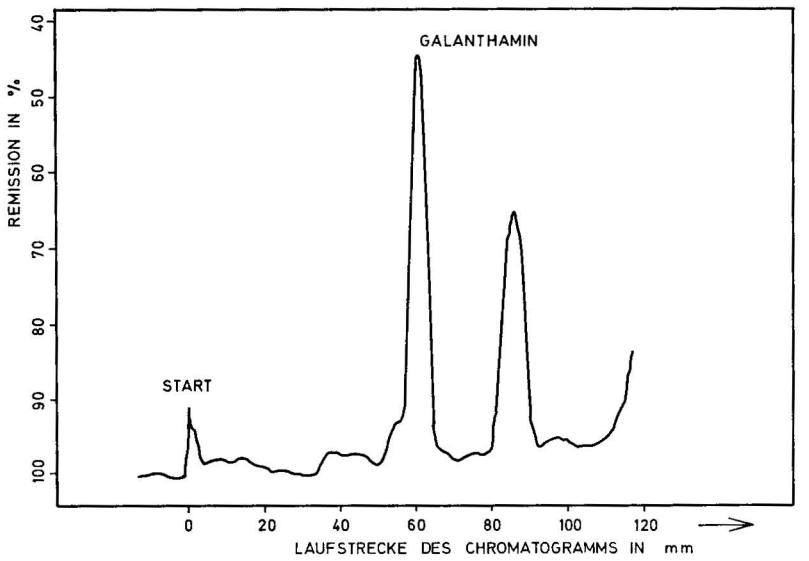


Fig. 2. Ortsremissionskurve eines chromatographierten Drogenextraktes.

substanz. Der breite Peak unterhalb der Laufmittelfront wird durch die Bindezusätze des Schichtmaterials verursacht, ist jedoch der Auswertung des Chromatogramms nicht weiter hinderlich. Der R_F-Wert der Reinsubstanz beträgt 0.50, die Laufzeit des Chromatogrammes 45 min. Zur Auffindung der günstigsten Messwellenlänge wurde die Remissionsortskurve von 20 μg Galanthamin im Wellenlängenbereich von 210 bis 305 nm aufgezeichnet und deren jeweilige Fläche berechnet (Fig. 3). Aus

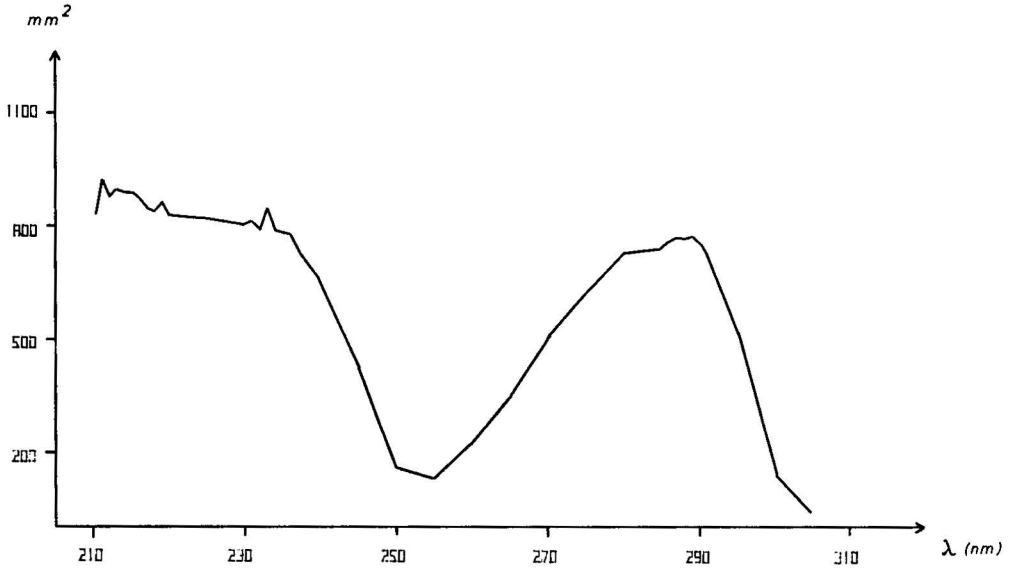


Fig. 3. Fläche der Ortsremissionskurve von Galanthamin auf der DC-Platte in Abhängigkeit von der Wellenlänge, punktförmig vermessen.

dem Ergebnis ist ersichtlich, dass die geeignete Messwellenlänge bei 288 nm liegt, da hier einerseits die Substanz ein Absorptionsmaximum aufweist, andererseits auch genügend Energie durch die Lampe abgestrahlt wird, die keine allzugrosse Verstärkung des Messsignals bzw. Öffnung der Spaltbreite bedingt.

Das Auswerten der Remissionskurven der Galanthamin-Standardlösungen führt zu einer gut reproduzierbaren Eichkurve, Tabelle I (Fig. 4), wobei die untere Grenze der sicher auswertbaren Flächen bei 20 mm^2 liegt. Das entspricht in der obigen Messanordnung einer Substanzmenge von etwa 0.2 μg. Diese Methode wurde zur Untersuchung mehrerer Drogenextrakte angewandt, wobei einzelne Extrakte wiederholt an getrennten Platten chromatographiert wurden. Die Auswertung (eine Auswahl ist in Tabelle II zusammengestellt) zeigt eine gute Reproduzierbarkeit der Ergebnisse.

TABELLE I

AUFTRAGMENGE UND PEAKFLÄCHE FÜR GALANTHAMIN BEI GLEICHBLEIBENDEM AUFTRAGVOLUMEN VON 5 μl

Verdünnung der Standardlösung	*Galanthamin (μg)*	*Peakfläche (mm^2)*
1:1	5	320.3
1:1.2	4.17	288.1
1:1.3	3.85	265.5
1:1.5	3.33	237.7
1:1.8	2.78	191.7
1:2.5	2.0	145.0
1:4	1.25	94.4

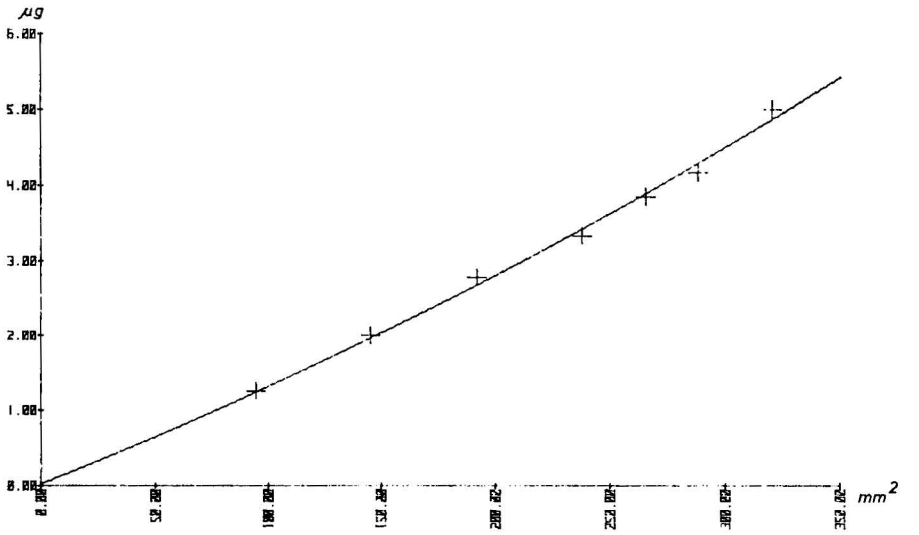


Fig. 4. Eichkurve für Galanthamin nach DC-Trennung, wobei das Auftragvolumen mit 5 μl konstant gehalten wurde.

Damit bietet sich die Möglichkeit, dieses Bestimmungsverfahren für umfangreicher angelegte Studien zum Vorkommen und zur Produktion von Galanthamin in Pflanzen einzusetzen. Inwieweit auch andere wichtige Amaryllidaceen-Alkaloide in

TABELLE II

ANALYSENERGEBNISSE VERSCHIEDENER DROGENEXTRAKTE IN EINER WIEDERHOLUNG

Angabe in % Galanthamin bezogen auf die Trockendroge.

Drogenprobe	*Galanthamin (%)*
Probe 1 - 26B	1.112
	1.087
Probe 2 - 26B	1.000
	1.007
Probe 3 - 26B	0.508
	0.527

Drogenextrakten mit Hilfe der direkten Auswertung auf der DC-Platte bestimmbar sind, ist Gegenstand einer noch laufenden Untersuchung. Über Ergebnisse dieser Versuche wird demnächst berichtet.

LITERATUR

1 T. Yoshino und M. Sugihara, *Kagatu To Kogyo (Osaka)*, 31 (1957) 96; *C.A.*, 51 (1957) 11659f.
2 S. Kori, K. Shibata und I. Nishimura, *Yakugaku Zasshi*, 81 (1961) 1042; *C.A.*, 55 (1961) 26368b.
3 A. Gheorghiu, A. Constantinescu und E. Ionescu-Matiu, *Rev. Med. (Tirgu-Mures)*, 8 (1962) 54; *C.A.*, 57 (1962) 12903h.
4 V. I. Kuznetsov, N. S. Volkova und V. A. Morozova, *Farmatsiya (Moscow)*, 18 (1969) 39; *C.A.*, 71 (1969) 33488j.
5 S. Laiho und M. Fales, *J. Amer. Chem. Soc.*, 86 (1964) 4434.
6 E. Stahl (Herausgeber), *Dünnschicht-Chromatographie*, Springer, Berlin, 2. Aufl., 1967, s. 427.
7 F. Sandberg und K.-H. Michel, *Lloydia*, 26 (1963) 78.
8 I. D. Kalashmikov, *Issled. Obl. Lek. Sredstv.*, (1968) 228; *C.A.*, 74 (1969) 136420x.
9 D. S. Millington, D. E. Games und A. H. Jackson, in A. Frigerio (Herausgeber), *Proceedings of the International Symposium on Gas Chromatography Mass Spectrometry, Isle of Elba, Italy, 1971*, s. 276–288.

Journal of Chromatography, 188 (1980) 457–458

CHROM. 12,349

Book Review

Thin-layer chromatography, by J. G. Kirchner, Wiley-Interscience, New York, 2nd ed., 1978, Chichester, Brisbane, Toronto, XXI + 1137 pp., price £ 42.50, ISBN 0-471-93264-7.

This book, written by Justus G. Kirchner, and edited by Edmond S. Perry, is Volume XIV in the series *Techniques of Chemistry* (edited by A. Weissberger), which is the successor to the *Techniques of Organic Chemistry* and *Techniques of Inorganic Chemistry* series. The first edition appeared in 1967 and its approximately 800-page size has now been increased by some 40%, while the price (at least in Dutch guilders) has almost doubled. Part I deals with the techniques of thin-layer chromatography (TLC), which are discussed in 12 chapters. These include well known topics such as preparation of the plates, development of the chromatogram, and detection and quantitation. Part II, the major part of the book, deals with applications. In 21 chapters, the TLC of the same number of classes of compounds is thoroughly discussed; in terms of length, special attention has been devoted to lipids, pharmaceutical products and steroids. Two appendices (reagents and addresses of commercial firms) and two indexes (compounds and subjects) complete the book.

Dr. Kirchner is to be complimented for writing an excellent, up-to-date and comprehensive treatment of one of the major branches of separation science. It is his, and my, opinion that owing to its low cost and flexibility and the inherent simplicity of the method, TLC will remain for a long time an important tool for many analytical chemists, both in the routine laboratory and for screening studies. This point of view is corroborated by the fact that in the decade 1967–1977 some 10,000 papers have been added to the about 5000 publications on TLC available in 1967. However, a major problem may well arise here. It will not be long before a much enlarged *third* edition of Kirchner's book has to be published. To take only one example, high-performance TLC, programmed multiple development and the use of plates with concentrating zones were hardly known in 1976 (the last year for which the literature has been covered in the present book), whereas today already well over 100 papers have been devoted to these topics. In addition, although more than 6000 references are quoted out of an estimated 15,000–20,000, which is a major achievement, the reader is still left more or less in the dark: what are the contents of the other 10,000–15,000 papers and how does he locate them? In summary, should the third edition be a large two-volume book? To this, my tentative answer is "no". Possibly, a preferred solution would be not to increase the size of the book but, instead, to limit oneself to about the same number of pages and write a thoroughly revised text; as for details on all separate topics, the inclusion of a sufficient number of references to reviews should make extensive literature searches superfluous. The familiarity of the author with the experimental aspects of TLC and his thorough knowledge of the existing literature will, hopefully, enable him to master even that task!

For the rest, although the title to the present series is *Techniques* of chemistry, I should welcome the inclusion of a 10–15-page chapter on the theoretical aspects of TLC. Today, too many chemists believe TLC to be a technique without a theory, and this point of view should not be stimulated by the absence of a basic theoretical discussion from Kirchner's widely read book.

Amsterdam (The Netherlands) U. A. Th. BRINKMAN

Journal of Chromatography, 188 (1980) 459

CHROM. 12,350

Book Review

Aflatoxins: chemical and biological aspects, by J. G. Heathcote and J. R. Hibbert, Elsevier, Amsterdam, Oxford, New York, 1978, IX + 212 pp., price Dfl. 120.00, US$ 53.50, ISBN 0-444-41686-2.

The aim of this book, as stated in the Preface, is "to bring together various aspects of the aflatoxins in a comprehensive account of their chemistry and biology". The monograph is divided into nine chapters.

Chapter 1 deals with the historical aspects of the discoveries of aflatoxins and Chapter 2 covers their production. The chemistry of aflatoxins is described in Chapter 3. Although some chromatographic separations of individual aflatoxins are mentioned in Chapters 1 and 3, the most important physico-chemical and biological assays are described in Chapter 4. Several techniques used for the preparation of samples of aflatoxins from cultivation media, mycelia and natural commodities are given in detail. Thin-layer and liquid chromatography are the most commonly used analytical methods and various chromatographic techniques are included in this chapter. Rapid screening methods and confirmatory tests are also presented. Miscellaneous bioassays with various model systems (embryological tests, tests with microorganisms, invertebrate and vertebrate animals) are described in the closing section of the chapter.

Chapters 5 and 6 deal with pathological and biochemical effects of aflatoxins, biotransformations of aflatoxins by microorganisms and animals are dealt with in Chapter 7 and Chapter 8 is devoted to early and more recent schemes for the biosynthesis of aflatoxins from their precursors and intermediates. In Chapter 9 methods for the control of aflatoxins (prevention of moulding and detoxification of foods) can be found.

Each chapter has a separate list of references. In the Appendix are presented tables and figures containing some physico-chemical properties of the aflatoxins, mass spectral data, and ultraviolet and infrared spectra.

The authors and the publishers are to be congratulated on this valuable book, which can be recommended to those working in theoretical and applied fields dealing with mycotoxins as a serious environmental hazard.

Bratislava (Czechoslovakia) VLADIMÍR BETINA

Author Index

Erratum

J. Chromatogr., 178 (1979) 105–116

Page 108, section *TLC on silica gel G*, 1st line, "μl" should read "μm".

Page 109, Table I, section *Glyceryl-1-alkyl ethers*, delete the following: "Glyceryl-1-(batyl alcohol), Glyceryl-1-(chimyl alcohol) and Glyceryl-1-(selachyl alcohol)"; and delete "CH_2Cl_2" in the column *Eluting solvents sequence (μl)* (3rd line).

Page 111, Fig. 2F, peaks 1–6 are, respectively, myristyl; palmityl; palmitelaidyl plus palmitoleyl; stearyl; vaccenyl; linoelaidyl plus linoleyl.

Page 112, 1st line, "μg/ml" should read "μg/mg".

Page 112, section *Fatty acid methyl esters*, 1st line, "fatty and" should read "fatty acid"; 7th and 8th lines, "*trans*-octadecenoic" should read: "*trans*-3-octadecenoic".

Page 113, section *Fatty alcohols*, 3rd line, "2-position C-2", should read: "in the 2-position".

Page 114, legend to Fig. 3, 2nd line, "ester" should read "esters"; 3rd line, "linoleate" should read "linoleate, linoelaidate".

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

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

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

Title. The title of the paper should be concise and informative. Since titles are widely used in information retrieval systems, care should be taken to include the key words. The title should be followed by the authors' full names, academic or professional affiliations, and the address of the laboratory where the work was carried out. If the present address of an author is different from that mentioned, it should be given in a footnote. Acknowledgements of financial support are *not* to be made in a footnote to the title or name of the author, but should be included in the Acknowledgements at the end of the paper.

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

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. One original and two photocopies are required. Attention should be given to any lettering (which should be kept to a minimum) and to spacing on axes of graphs in order to ensure that numbers etc. remain legible after reduction. Axes of a graph should be clearly labelled. The figures should preferably be of such a size that the same degree of reduction can be applied to all of them. Photographs should have good contrast and intensity. Sharp, glossy photographs are required to obtain good halftones. References to the illustrations should be included in appropriate places in the text using arabic numerals. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate* sheet. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the authors' expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. The numbers should appear in the text at the appropriate places in square brackets. In the reference list, periodicals [1], books [2], multi-author books [3] and proceedings [4] should be cited in accordance with the following examples:

1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication". The *Journal of Chromatography; Journal of Chromatography, Biomedical Applications* and *Chromatographic Reviews* should be cited as *J. Chromatogr.*

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints of Full-length papers, Short communications and Notes will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

News. News releases of new products and developments, and information leaflets of meetings should be addressed to: The Editor of the News Section, Journal of Chromatography/Journal of Chromatography, Biomedical Applications, Elsevier Scientific Publishing Company, P.O. Box 330, 1000 AH Amsterdam, The Netherlands.

Advertisements. Advertisement rates are available from the publisher on request. The Editors of the journal accept no responsibility for the contents of the advertisements.

ELECTROPHORESIS

A SURVEY OF TECHNIQUES AND APPLICATIONS

Part A: Techniques

Z. DEYL, Czechoslovak Academy of Sciences, Prague (editor)
F. M. EVERAERTS, Z. PRUSÍK, and P. J. SVENDSEN (co-editors)

JOURNAL OF CHROMATOGRAPHY LIBRARY 18

This first volume in a two part set, deals with the principles, theory and instru ntation of modern electromigration methods. The second volume will be concer with details of applications of electromigration methods to diverse categor of compounds, although a few applications are already discussed in Part

Some electromigration methods have become standard procedures because their extensive use in analytical and preparative separations. These are discus together with newer developments in the field. Hints are included to help reader to overcome difficulties frequently arising from the lack of suita equipment. Adequate theoretical background of the individual techniques is cluded. A theoretical approach to the deteriorative processes is presen in order to facilitate further development of a particular technique and its ap cation to a special problem.

In each chapter practical realizations of different techniques are discus and examples are presented to demonstrate the limits of each method. T mathematical and physicochemical background is arranged so as to make it coherent as possible for both non-professionals such as p st-graduate stude and experts using electromigration techniques.

1979 xvi + 492 pages US $83.00/Dfl. 170.00 ISBN: 0-444-41721-4

ELSEVIER

P.O. Box 211,
1000 AE Amsterdam
The Netherlands

52 Vanderbilt Ave
New York, N.Y. 10017

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