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VOL. 118 NO. 3 APRIL 7, 1976

THIS ISSUE COMPLETES VOL. 118

NAL OF HROMATOGRAPHY

RNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
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CONTENTS

Pharmaceutical applications of Thin-Layer and Paper chromatography

edited by **KAREL MACEK,** *Medical Faculty, Charles University, Prague*

1972, xvi + 744 pages, Dfl. 250.00 (ca. $78.25)
ISBN 0-444-40939-4

Chromatography is the most widely used modern procedure in analytical chemistry today. With an increasing awareness of the importance of the applications of paper and thin-layer chromatography in the fields of pharmaceutical research, production and control, it has become necessary to survey the possibilities of these methods with their associated literature, and to present this information in a useful form.
With this in mind, the editor provides an introduction to the techniques, the evaluation, and the applications of paper and thin-layer chromatography. Selected procedures such as the preparation of samples, detection methods, choice of solvent systems and sorbents, and the principles of quantitative analysis, are discussed generally and the book is richly tabulated. An appendix outlines the preparation of more than 150 detection reagents. On the basis of these data, readers with an understanding of the principles of the techniques described can solve any analytical problems they may encounter in drug analysis.

CONTENTS: Introduction. Techniques of paper and thin-layer chromatography. Radioactive compounds. Combination of TLC and PC with other chromatographic techniques. Combination of PC and TLC with some spectroscopic methods. Identification of organic compounds by PC and TLC. Documentation of chromatograms. Laboratory for PC and TLC. The tasks of paper and thin-layer chromatography. Synthetic drugs. Steroids. Cardiac glycosides and their genins. Saponins. Peptide and protein hormones. Alkaloids. Vitamins. Antibiotics. Plant extracts. Auxiliary compounds. Investigation of the fate of drugs. Detection reagents. Author index. Subject index. List of substances chromatographed.

CONTRIBUTORS: V. Betina, J. Davídek, I. M. Hais, K. Hiller, J. Janák, G. Katsui, B. P. Lisboa, M. Luckner, K. Macek, L. Nover, V. Rábek, G. Székely, H. D. Woitke.

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CHROMATOGRAPHY

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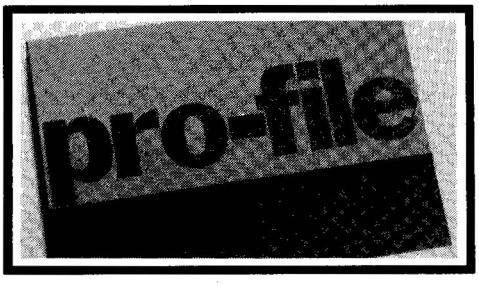

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DEUTERIUM AND HEAVY WATER

A Selected Bibliography

by **GHEORGHE VÅSARU, DANIEL URSU, ALEXANDRU MIHÅILÅ** and **PAUL SZENTGYÖRGYI,** Institute of Stable Isotopes, Cluj, Romania.

1975. 418 pages. US $41.75/Dfl. 100.00. ISBN 0-444-41321-9

Possibly the most complete and accurate bibliography on deuterium and heavy water available in a single source, this volume lists the authors, titles and standard references of 3763 publications that appeared from 1932 to May 1974.

References are arranged chronologically and then alphabetically according to first author, and all titles have been translated into English. Particularly valuable to those interested in the properties, analysis and production of deuterium and heavy water, and the behaviour of heavy water as moderator, this comprehensive bibliography will serve as an indispensable reference tool to all interested in the results achieved by other contributors in the field.

CONTENTS: Sources of Information. References. Author Index. Subject Index. Abundance of Deuterium. Catalysts. Catalytic Exchange. Chemical Equilibria, Chemical Kinetics. Deuterium and Heavy Water Analysis. Deuterium and Heavy Water Properties. Deuterium and Heavy Water Separation. Exchange Reactions. General and Review. Heavy Water as Moderator. Isotope Effects. Synthesis of Deuterium Compounds.

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METASTABLE IONS

By **R. G. COOKS, J. H. BEYNON** and **R. M. CAPRIOLI,** Purdue University, West Lafayette, Indiana, U.S.A., and **G. R. LESTER,** University of Salford, Lancashire, England.

1973. 305 pages. US$31.25/Dfl. 75.00. ISBN 0-444-41119-4

This text draws together information scattered throughout the literature concerning metastable ions in mass spectrometry. It also deals with high energy ion/molecule reactions because these lead to the observation of collision induced peaks very similar to the metastable peaks that arise from the unimolecular fragmentations of ions in the field-free regions of a mass spectrometer. Starting from extremely simple considerations it brings out the important factors that enable metastable peaks to be focused, observed with high sensitivity and measured with the greatest possible accuracy. It includes the results of research carried out in the authors' laboratories until the latter half of 1972. This includes development of new techniques (IKES and MIKES) based upon the measurement of ion kinetic energies and the discussion of the ways in which these can contribute to our understanding of the structures of metastable ions and the mechanisms, energetics and kinetics of the fragmentation of these ions. The methods by which excited states can be studied and by which basic thermodynamic information such as double ionization potentials and electron affinities can be obtained are stressed. Energy partitioning between internal and translational modes during fragmentation is discussed in detail. Recent applications in physics and in physical, organic, biological and analytical chemistry are given.

As well as practising mass spectroscopists, a wider audience composed of those involved in the study of excited states and mechanisms of energy transfer and the physics and chemistry of ionic and electronic impact phenomena will also find much to interest them.

CONTENTS:

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CHROMATOGRAPHY OF ENVIRONMENTAL HAZARDS

Volume III: Pesticides

by **LAWRENCE FISHBEIN,** Chemistry Division, National Center for Toxicological Research, Jefferson, Adjunct Professor of Chemistry, University of Arkansas, Little Rock, Arkansas, U.S.A.

1975. 830 pages. US $108.50/Dfl. 260.00. ISBN 0-444-41158-5

Pesticides represent a major area of increasing environmental concern both in terms of their potential ubiquity as well as toxicity. The main objective of this third volume in the series Chromatography of Environmental Hazards is to provide the analytical chemist with a practical text as well as a literature reference source of selected descriptive chromatographic procedures. Information is provided wherever possible concerning the basic equipment, operating parameters, sensitivity, and interferences encountered in the analysis of a particular pesticide and/or group of related contaminants.
The unique feature of the book is the blending of relevant information regarding the synthesis, areas of utility, degradation and metabolic fate of pesticide toxicants, thus presenting as thorough and cohesive a picture as possible of the specific environmental hazard.

CONTENTS: Introduction. DDT and metabolites. Cyclodienes: Dieldrin, Aldrin and Endrin. Chlordane, Heptachlor and Heptachlor epoxide. Miscellaneous organochlorine pesticides Perthane, Methoxychlor, Endosulfan and Toxaphene. Benzene hexachloride and hexachlorobenzene. Multiple organochlorine analyses. 2,4-D and its esters. Pentachlorophenol. Miscellaneous herbicides and acaricides (bipyridylium salts, Dinoseb, Trifluralin and Cycocel). Parathion, Methyl Parathion and Malathion. Bidrin, Azodrin, Diazinon, Dursban and Dasanit. Disyston, Dimethoate, Phorate. Multiple organophosphorus pesticide analysis. Carbamates. Ureas. Triazines. Pesticidal synergists. Index.

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Sublethal Effects of Toxic Chemicals on Aquatic Animals

Proceedings of the Swedish-Netherlands Symposium, Wageningen, The Netherlands, September 2-5, 1975

edited by J.H. KOEMAN *and* J.J.T.W.A. STRIK, *Department of Toxicology, Agricultural University, Wageningen, The Netherlands.*

1975. 240 pages. US $25.95/Dfl. 62.00. ISBN 0-444-41399-5

Published within three months of the Swedish-Netherlands Symposium, this book is one of the first publications to deal explicity with the problems of investigating sublethal effects of chemicals on aquatic animals. It covers descriptions of methods, test systems and test organisms as well as papers on laboratory and field studies concerning effects of various metals (such as cadmium, chromium, zinc, mercury and lead) and other chemicals (including polychlorinated biphenyls, hexachlorobutadiene and dieldrin) on fish, crustaceans and other aquatic animals. As the Symposium itself showed, a multi-disciplinary approach can contribute much to the validity of future studies in this field. The Proceedings, therefore, should be of widespread interest and value to research workers.

Selection of papers:
Vertebral damage in fish induced by pollutants. Toxicity of chromium (VI) in fish, with special reference to organoweights, liver and plasma enzyme activities, blood parameters and histological alterations. Chronic toxic effects of the water of the river Rhine upon rainbow trouts. Toxicity of hexachlorobutadiene in aquatic organisms. Metabolic effects of PCB (polychlorinated biphenyls) in the European eel, *Anguilla anguilla L.*

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Journal of Chromatography Library

A series of books devoted to chromatographic techniques and their applications. Although complementary to the Journal of Chromatography, each volume in the library series is an important and independent contribution in the field of chromatography. It should be stressed that the library contains no material reprinted from the journal itself.

Volume 1

CHROMATOGRAPHY OF ANTIBIOTICS

by G. H. Wagman and M. J. Weinstein.

1973 ix + 238 pages
Price: US $26.95 / Dfl. 65.00
ISBN 0-444-41106-2

At the present time thousands of antibiotics are known, yet the systematic chromatographic classification of these substances is extremely difficult. This book has been written to aid the identification of very similar compounds by use of specific chromatographic techniques. It contains detailed data on paper and thin-layer chromatography, electrophoresis, counter-current distribution and gas chromatographic systems for over 1,200 antibiotics and their derivatives, and provides information on chromatographic media, solvents, detection methodology and mobility of the antibiotics. Complete references are given for all methods.

CONTENTS: Chromatographic classification of antibiotics. Detection of antibiotics on chromatograms. Comments on the use of this index. Abbreviations. Index - chromatography of antibiotics. Index by compound.

Volume 2

EXTRACTION CHROMATOGRAPHY

edited by T. Braun and G. Ghersini.

1975 xviii + 566 pages
Price: US $54.25 / Dfl. 130.00
ISBN 0-444-99878-0

This volume is the result of the collective work of many specialists, each responsible for a chapter in which a definite aspect of column extraction chromatography is thoroughly presented and discussed. Subjects presented include the basic and technical aspects of the method, the organic stationary phases and supports, the separation of elements with particular reference to radiochemical problems, the separation of lanthanides, actinides and fission products, radiotoxicological separations and the preconcentration of trace elements in various materials prior to their determination.

Author and subject indices are included.

Volume 3

LIQUID COLUMN CHROMATOGRAPHY

A survey of modern techniques and applications.

edited by Z. Deyl, K. Macek and J. Janák.

1975 xxii + 1176 pages
Price: US $120.95 / Dfl. 290.00
ISBN 0-444-41156-9

This book provides an up-to-date account of liquid column chromatography for the specialist and non-specialist. The main attention is focussed on techniques developed or widely used during the past 10 years. Both classical and modern techniques of chromatographic separation are treated in detail, thus providing a clear reflection of the present situation in the field.
The wide selection of applications in various fields of chemistry and biochemistry, written by specialists in the area, makes this volume a necessary reference work for those involved in chromatographic investigations.

CONTENTS: Theoretical Aspects of Liquid Chromatography. Techniques of Liquid Cromatography. Practice of Liquid Cromatography. Applications. Subject index. List of compounds chromatographed.

Volume 4

DETECTORS IN GAS CHROMATOGRAPHY

by J. Ševčík.

1976 192 pages
Price: US $24.00 / Dfl. 60.00
ISBN 0-444-99857-8

This publication is devoted to the function and optimal working conditions of gas chromatographic detectors. The first systematic treatment of gas chromatographic detection techniques, it devotes special attention to so-called specific detectors and working conditions which strongly influence results (e.g. gas flow, effect of additives in gases, working temperature, detector form and dimensions). Anomalous detector responses are explained and the form and size of response for various working conditions are indicated. The problems presented are illustrated by experimental data which are summarized in numerous tables and figures. The book should be of interest to all who use gas chromatography in research and who would like to explore the possibilities and working conditions of different detector systems.

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Journal of Chromatography, 118 (1976) 283–294

CHROM. 8797

ÉTUDE PAR CHROMATOGRAPHIE LIQUIDE HAUTE PRESSION DE 17-CÉTOSTÉROÏDES LIBRES ET CONJUGUÉS*

MICHEL LAFOSSE, GÉRARD KÉRAVIS et MARC HENRI DURAND

Laboratoire de Chimie Organique Physique, U.E.R. Sciences, Université d'Orléans, 45045 Orléans CEDEX (France)

(Reçu le 15 juillet 1975; manuscrit modifié reçu le 10 octobre 1975)

SUMMARY

High-pressure liquid chromatography of free and conjugated 17-ketosteroids

High-pressure liquid chromatography (HPLC) should be used more frequently in biomedical research because heavy molecules can be analysed at ambient temperature without derivatisation to volatile compounds. In this paper the separation of four free 17-ketosteroids, their ester sulfates and glucuronides by reversed-phase HPLC on a Micropak CH column is described. In order to improve the separation several parameters have been studied. Retention data allow comparison of the hydrophilic behaviour of free and conjugated steroids.

INTRODUCTION

L'analyse des stéroïdes à des fins médicales ou scientifiques est réalisée principalement par chromatographie en phase gazeuse (CPG). Les stéroïdes, extraits des liquides biologiques, sont soumis à une hydrolyse nécessaire pour obtenir le stéroïde libre à partir de sa forme conjuguée. Ils sont souvent silylés et analysés à température élevée (200–250°). Les travaux les plus complexes sont réalisés sur colonnes capillaires et en couplant l'appareil de CPG à un spectrographe de masse[1–5].

Depuis peu, certains stéroïdes hormonaux ont été analysés par chromatographie liquide haute pression (CLHP)[6–10]. Cette technique permet d'éviter les opérations chimiques de silylation et d'étudier les composés, en particulier les stéroïdes conjugués, tels qu'ils se trouvent dans les liquides biologiques[11,12].

Nous étudions dans le présent travail la séparation par chromatographie de partage avec phases inversées de quatre stéroïdes libres et de leurs formes conjuguées, sulfates et glucuronides: androstérone (A), épiandrostérone (EA), étiocholanolone (E), déhydroépiandrostérone (DHEA) (Fig. 1). Nous examinerons l'action des différents paramètres sur la séparation en vue de l'améliorer.

Ce travail est réalisé dans le cadre de la Thèse de G. Kéravis.

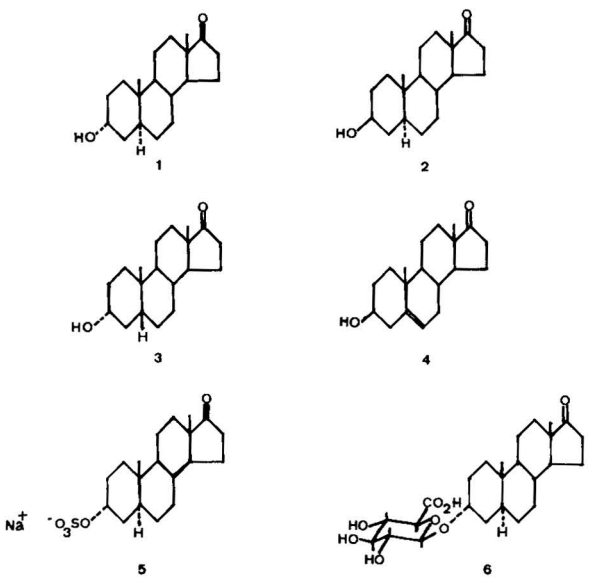

Fig. 1. Représentation des différentes molécules étudiées. 1 = Androstérone (A) ou 3α-hydroxy 5α-androstane 17-one; 2 = épiandrostérone (EA) ou 3β-hydroxy 5α-androstane 17-one; 3 = étiocholanolone (E) ou 3α-hydroxy 5β-androstane 17-one; 4 = déhydroépiandrostérone (DHEA) ou 3β-hydroxy androstène-5 17-one; 5 = sulfate de sodium de l'androstérone (AS): représente les différents sulfates de stéroides étudiés; 6 = glucuronide de l'androstérone sous forme acide et non de sel de sodium (AG): représente les différents glucuronides étudiés.

PARTIE EXPÉRIMENTALE

L'appareil utilisé, réalisé au laboratoire, est schématisé (Fig. 2). L'éluant eau–méthanol est dégazé par ébullition. La pompe Orlita DMP 1515, protégée par un filtre Nupro, assure la circulation du liquide sous des pressions allant de quelques bars à 100 bars environ. Les pulsations créées par cette pompe à membrane sont rendues négligeables par l'amortisseur de Halász.

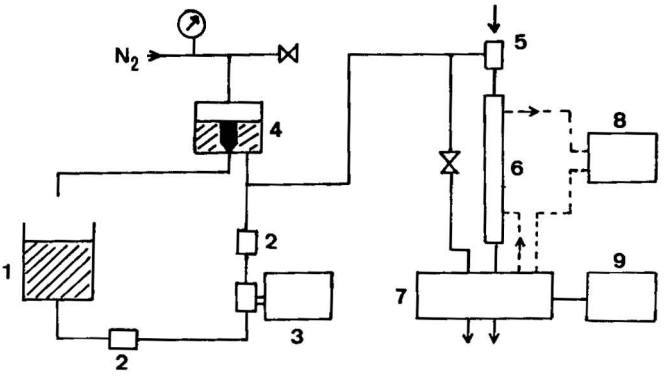

Fig. 2. Schéma de l'appareil CLHP. 1 = Réservoir de solvant; 2 = filtres; 3 = pompe; 4 = amortisseur de pulsations Halász; 5 = injecteur; 6 = colonne thermostatée; 7 = détecteur réfractométrique; 8 = bain thermostatique; 9 = enregistreur.

Les solutions méthanoliques de stéroïdes (1 g/l) sont introduites par injection de 2–5 μl à l'aide de seringues Hamilton HP 305, 7105 N ou SGE 10 B LRD-3. Les essais réalisés avec cette dernière se sont révélés peu satisfaisants. L'injecteur (Fig. 3) réalisé au laboratoire reprend un système connu utilisant deux septa maintenus par des disques d'acier. Nous ajoutons un guide pour le corps de la seringue; ceci permet d'injecter toujours dans le même trou ce qui facilite le travail de la seringue et augmente la durée de vie du septum[13].

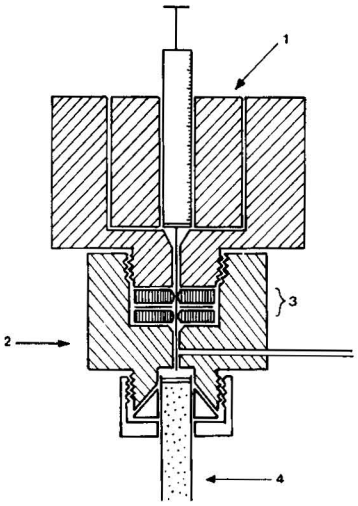

Fig. 3. Injecteur. 1 = Guide du corps de seringue; 2 = guide de l'aiguille; 3 = septa séparés par disques d'acier; 4 = colonne.

Le guide permet avec un seul septum de faire deux à trois fois plus d'injections qu'avec un injecteur sans guidage sous une pression pouvant aller jusqu'à 100 bars. Au delà de 100 bars il faut mettre deux septa.

La phase stationnaire de la colonne (longueur 25 cm, diamètre extérieur $3^3/_8$ mm) est une chaîne alkyle en C_{18} greffée sur des grains de silice de 10 μm (Micropak CH; Varian).

Les produits élués sont détectés par le réfractomètre différentiel Varian. La colonne et le détecteur sont maintenus à température constante. Cette précaution est importante pour obtenir la stabilité de la ligne de base. L'enregistrement est fait avec un appareil Hitachi utilisé sur la sensibilité 1 mV.

L'eau et le méthanol sont purifiés par distillation.

Les produits étudiés ont été fournis par Sigma: les quatre stéroïdes libres (A, E, EA et DHEA), les esters sulfates de sodium correspondants (AS, ES, EAS, DHEAS) (5 dans Fig. 1), les glucuronides de l'androstérone (AG), de l'étiocholanolone (EG) et de la DHEA (DHEAG) sous forme acide (6, Fig. 1).

Les Tableaux I et II donnent en fonction de la nature de l'éluant les volumes de rétention réduits V'_{R_t} de chaque stéroïde libre et conjugué pris séparément à 25°. Les valeurs sont calculées à partir de la distance de sortie de l'eau non retenue sur la phase stationnaire.

TABLEAU I

VOLUMES DE RÉTENTION RÉDUITS V'_R DES STÉROÏDES LIBRES À 25° EN FONCTION DE LA NATURE DE L'ÉLUANT EAU–MÉTHANOL

Eau (%)		δ_M	V'_R *(ml)*			
En masse	*En volume**		*DHEA*	*EA*	*E*	*A*
20.14	16.6	14.25	0.38	0.44	0.46	0.51
27.45	23.0	14.8	0.68	0.81	0.85	0.96
36.21	31.0	15.4	1.24	1.54	1.63	1.85
45.98	40.2	16.2	3.00	3.82	4.30	5.00
49.43	43.6	16.4	3.87	4.98	6.05	7.03
50.60	44.8	16.5	3.96	5.08	6.08	7.06
55.77	49.8	16.95	10.9	15.6	19.6	23.2

* Avant le mélange qui entraîne une réduction de volume.

TABLEAU II

VOLUMES DE RÉTENTION RÉDUITS V'_R DES STÉROÏDES CONJUGUÉS À 25° EN FONCTION DE LA NATURE DE L'ÉLUANT EAU–MÉTHANOL

Eau (%)		δ_M	V'_R *(ml)*						
En masse	*En volume**		*DHEAS*	*EAS*	*ES*	*AS*	*DHEAG*	*EG*	*AG*
70.0	64.8	18.1	2.04	2.84	3.49	4.20	–	–	–
75.0	70.3	18.6	2.72	3.70	4.24	4.92	1.80	4.08	4.88
80.0	75.9	19.0	3.66	4.40	4.92	5.75	2.16	5.36	6.30

* Avant le mélange qui entraîne une réduction de volume.

OPTIMISATION DE LA SÉPARATION

Le pouvoir de résolution R de la colonne pour deux composés en quantités sensiblement égales s'exprime en fonction du nombre de plateaux N, du facteur de sélectivité α et du facteur de capacité k' par la relation classique:

$$R = \frac{\sqrt{N}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k'}{1 + k'}$$

N et k' sont relatifs au deuxième composé. Si l'on est en présence de plusieurs isomères dont la rétention est du même ordre de grandeur, ce sont les valeurs optimales de N et k' du premier et du dernier pic qui encadreront celles de l'ensemble.

Recherchons quelles sont les valeurs de N, α et k' qui entraînent un pouvoir de résolution au moins égal à l'unité.

Influence du facteur de capacité k'

Pour qu'on puisse au moins envisager l'étude d'un problème de séparation il faut que les distances de rétention des pics ne soient ni trop petites ni trop grandes. Cela se traduit par des valeurs du facteur $k' = V'_K/V_M$* que l'on considère habituellement devoir être comprises entre 1 et 10. Nous avons cherché quelles sont les compositions de l'éluant eau–méthanol satisfaisant à cette condition. Le Tableau III montre

* V_M = volume mort de la colonne.

TABLEAU III

FACTEUR DE CAPACITÉ k' DE LA DEHYDROÉPIANDROSTÉRONE ET DE L'ANDROSTÉRONE EN FONCTION DE LA NATURE DE L'ÉLUANT EAU–MÉTHANOL

Eau en masse (%)	k'_{DHEA}	k'_A
20.14	0.5	0.7
27.45	0.9	1.3
36.21	2.0	2.5
45.98	3.6	5.9
49.43	5.3	9.8
50.60	5.7	10.4
55.77	14.5	30.9

que k' croît rapidement avec le taux d'eau dans le mélange car k' est lié au coefficient de partage K qui est lui-même fonction exponentielle de ce taux comme nous le rappelons ci-après.

On voit qu'il faut éliminer les éluants à 20.14% et à 27.45% d'eau car les échanges entre phases sont trops courts et l'éluant à 55.77% d'eau qui provoque une trop forte rétention.

Influence du facteur de sélectivité α

La séparation exige $\alpha > 1$ ce qui correspond à des coefficients de partage différents puisque $\alpha = K_i/K_j$. Le seul paramètre est ici encore la composition de l'éluant.

Le coefficient de partage K_i d'un soluté i entre deux phases liquides stationnaire S et mobile M non miscibles est lié à la "polarité" ou "paramètre de solubilité δ de Hildebrand" de chacun de ces trois éléments respectivement δ_i, δ_S et δ_M par la relation suivante[14]:

$$\ln K_i = \frac{\overline{V}_i}{RT}\left[(\delta_i - \delta_M)^2 - (\delta_i - \delta_S)^2\right]$$

où $\overline{V}_i$ est le volume molaire du soluté i. Nous étudions les systèmes isocratiques eau–méthanol donc δ_M est seul variable:

$$\ln K_i = \frac{\overline{V}_i}{RT}\left(\delta_M{}^2 - 2\delta_i\delta_M + 2\delta_i\delta_S - \delta_S{}^2\right)$$

Le volume de rétention réduit est lié au coefficient de distribution K_i par la relation fondamentale: $V'_{R_i} = K_i V_S$ (V_S = volume de phase stationnaire) et s'exprimera en régime isocratique comme une fonction exponentielle de la "polarité" de l'éluant δ_M

$$V'_{R_i} = V_S \exp\frac{\overline{V}_i}{RT}\left(\delta_M{}^2 - 2\delta_i\delta_M + 2\delta_i\delta_S - \delta_S{}^2\right)$$

La polarité de l'éluant δ_M est calculée à partir de celles du méthanol ($\delta = 12.9$) et de l'eau ($\delta = 21$) selon la loi linéaire des mélanges[14]:

$$\delta_M = \frac{(21 - 12.9)\,x}{100} + 12.9$$

x étant le pourcentage en volume de l'eau qui va constituer le mélange.

Ainsi le diagramme de la Fig. 4 représentant les volumes de rétention en fonction du pourcentage d'eau exprime la variation des coefficients de partage de chaque stéroïde en fonction de la polarité de l'éluant. Cette variation suit une loi exponentielle.

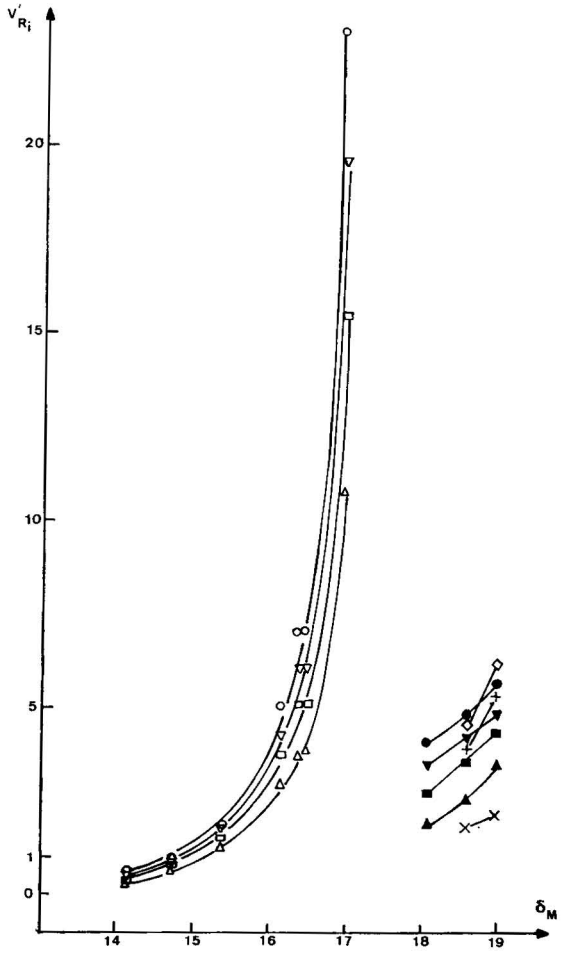

Fig. 4. Volume de rétention réduit V'_R en fonction de la polarité de l'éluant δ_M. △ = DHEA, □ = EA, ▽ = E, ○ = A, ▲ = DHEAS, ■ = EAS, ▼ = ES, ● = AS, × = DHEAG, + = EG, ◇ = AG.

Le facteur de sélectivité α, donné au Tableau IV pour chaque couple de composés voisins, montre que l'influence de δ_M est différente selon les stéroïdes libres et les stéroïdes conjugués (Fig. 5). En effet l'augmentation de δ_M provoque une amélioration de la séparation des stéroïdes libres et l'on choisira un mélange d'environ 50% en masse d'eau.

Par contre si l'on veut obtenir $\alpha_{i,j} > 1.2$ pour les conjugués il faut travailler avec un éluant à 70% d'eau en masse. En effet à 60% les solutés sortent dans la traînée du solvant avec un facteur de capacité inférieur à 1, alors qu'à 70% ce facteur de capa-

TABLEAU IV

FACTEUR DE SÉLECTIVITÉ $\alpha_{i,j}$ DE DEUX COMPOSÉS CONSÉCUTIFS EN FONCTION DE LA POLARITÉ δ_M DE LA PHASE MOBILE

1 = DHEA; 2 = EA; 3 = E; 4 = A; 5 = DHEAS; 6 = EAS; 7 = ES; 8 = AS; 9 = DHEAG; 10 = EG; 11 = AG.

δ_M	$\alpha_{1,2}$	$\alpha_{2,3}$	$\alpha_{3,4}$	$\alpha_{5,6}$	$\alpha_{6,7}$	$\alpha_{7,8}$	$\alpha_{9,10}$	$\alpha_{10,11}$
14.25	1.16	1.04	1.11					
14.8	1.20	1.04	1.13					
15.4	1.24	1.06	1.13					
16.2	1.27	1.13	1.16					
16.4	1.29	1.21	1.16					
16.5	1.28	1.20	1.16					
16.95	1.43	1.26	1.18					
18.1				1.39	1.23	1.20		
18.6				1.36	1.14	1.16	2.26	1.20
19.0				1.20	1.12	1.17	2.48	1.17

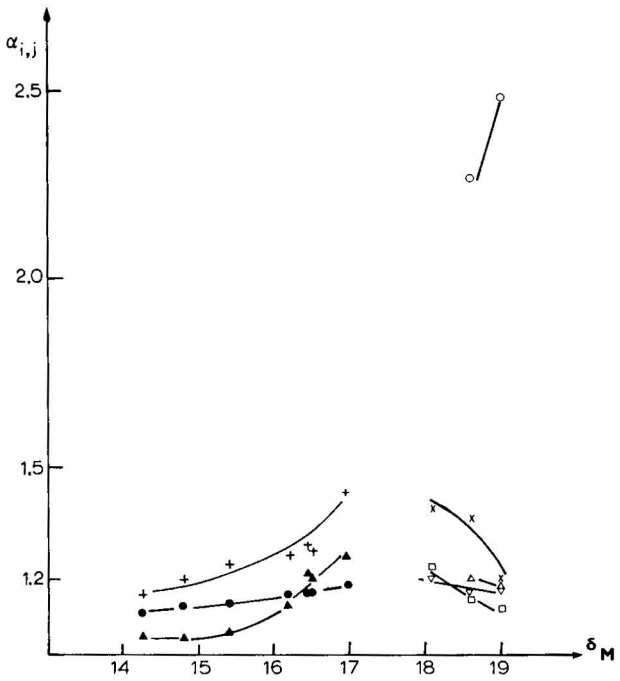

Fig. 5. Variation de $\alpha_{i,j}$ de deux stéroïdes consécutifs en fonction de la polarité de l'éluant. Notation voir Tableau IV. + = $\alpha_{1,2}$; ▲ = $\alpha_{2,3}$; ● = $\alpha_{3,4}$; × = $\alpha_{5,6}$; □ = $\alpha_{6,7}$; ▽ = $\alpha_{7,8}$; ○ = $\alpha_{9,10}$; △ = $\alpha_{10,11}$.

cité est compris dans les limites raisonnables de 2–5 et de plus le facteur de sélectivité α est d'environ 1.2. Un taux plus élevé d'eau diminue α et augmente la viscosité de l'éluant. Il est curieux de noter que le glucuronide de DHEA a un comportement différent des deux autres composés qui le suivent et l'augmentation de δ_M améliore sa séparation par rapport à ces deux composés.

Selon les grandeurs relatives des volumes molaires $\overline{V}_i$ et $\overline{V}_j$, la variation exponentielle de $\alpha_{i,j}$ peut être différente:

$$\alpha_{i,j} = \exp \frac{1}{RT} [(\overline{V}_j - \overline{V}_i)\,\delta_M{}^2 + (\overline{V}_j\delta_j - \overline{V}_i\delta_i)\,2(\delta_S - \delta_M) - (\overline{V}_j - \overline{V}_i)\delta_S{}^2]$$

Pour un éluant de polarité donnée δ_M, la séparation de deux composés de structures proches et de polarité semblable ($\delta_j \approx \delta_i$) est régie par leur différence de volume molaire selon une loi simple (A = facteur constant)

$$\alpha_{i,j} = \exp \frac{A}{RT} (\overline{V}_j - \overline{V}_i)$$

De même si leurs volumes molaires sont voisins ($V_j \approx V_i$), la séparation est due à leur différence de polarité (B = facteur constant):

$$\alpha_{i,j} = \exp \frac{B}{RT} (\delta_j - \delta_i)$$

Influence de l'efficacité

Le pouvoir de résolution R d'une colonne donnée pour deux produits dépend également de l'aptitude qu'a cette colonne à délivrer des pics fins.

L'étude des paragraphes précédents montre que des taux d'eau de l'ordre de 50% et 70% sont respectivement nécessaires pour séparer les stéroïdes libres et les stéroïdes conjugués entre eux.

La relation exprimant la résolution en fonction du nombre de plateaux effectifs N_{eff} est de la sélectivité α

$$R = \frac{\sqrt{N_{\text{eff}}}}{4} \cdot \frac{\alpha - 1}{\alpha}$$

permet de connaître l'efficacité N_{eff} suffisante pour avoir $R = 1$ et $\alpha = 1.2$. Dans le cas présent il faut au moins 576 plateaux soit 2300 plateaux au mètre. Cette efficacité nécessaire n'est pas considérable car la valeur choisie pour α est grande[15]. Mais on remarque d'après le Tableau V que l'efficacité N_{eff} relative à la DHEA ne dépasse guère 2500 plateaux au mètre, cette valeur étant sensiblement la même pour les autres stéroïdes libres. C'est pourquoi pour avoir les meilleurs conditions de séparation en un temps minimum, on doit travailler à un débit d'environ 0.40 ml/min pour une efficacité suffisante.

TABLEAU V

NOMBRE DE PLATEAUX EFFECTIFS PAR MÈTRE DE COLONNE MICROPAK CH RELATIFS À LA DHEA EN FONCTION DE LA COMPOSITION DE L'ÉLUANT EAU-MÉTHANOL ET DU DÉBIT À 25°

Eau en masse (%)	*Débit (ml/min)*	*N_{eff}/m (rel. à la DHEA)*
49.43	0.33	2700
	0.49	1600
50.60	0.49	1680

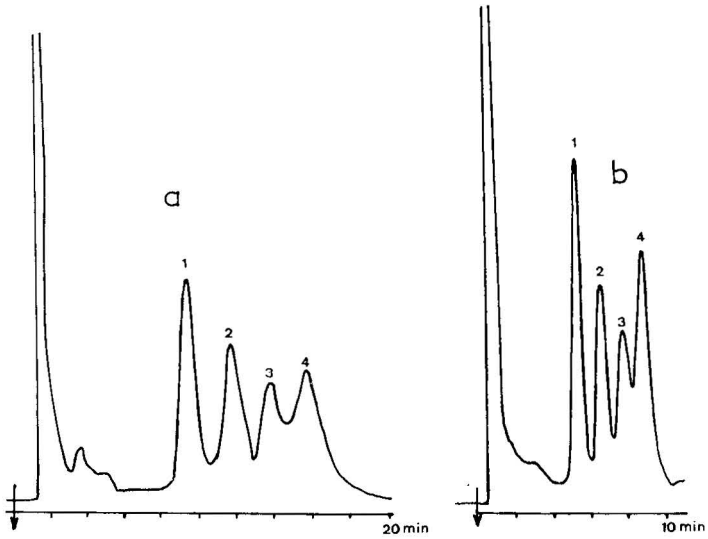

Fig. 6. Chromatogrammes des quatre stéroïdes libres à 25° (a) et à 45° (b). Colonne, Micropak CH (25 cm × $3^3/_8$ mm). Éluant, eau–méthanol (50.6% eau en masse). Débit, 0.49 ml/min à 25° et 0.55 ml/min à 45°. Volume injecté, 4 μl. 1 = DHEA, 2 = EA, 3 = E, 4 = A.

Les chromatogrammes de la Fig. 6a représentent la séparation optimale des stéroïdes libres.

La séparation des sulfates et des glucuronides entre eux est effectuée avec un éluant à 70–80% d'eau (Figs. 7a et 7c). Signalons que pour un tel éluant de viscosité élevée pour la colonne Micropak, il faut opérer à des pressions de 150 bars pour de faibles débits.

TABLEAU VI

VARIATION AVEC LA TEMPÉRATURE DES GRANDEURS DE RÉTENTION DES STÉROÏDES LIBRES

Température (°C)	*Composé*	*Polarité de l'éluant*			
		16.5		*16.95*	
		V'_R	$\alpha_{DHEA,i}$	V'_R	$\alpha_{DHEA,i}$
25	DHEA	3.96	1	10.9	1
	EA	5.08	1.28	15.6	1.43
	E	6.08	1.54	19.6	1.80
	A	7.06	1.78	23.2	2.13
45	DHEA	2.58	1	6.12	1
	EA	3.28	1.27	8.52	1.39
	E	3.92	1.51	11.40	1.86
	A	4.48	1.73	13.68	2.23

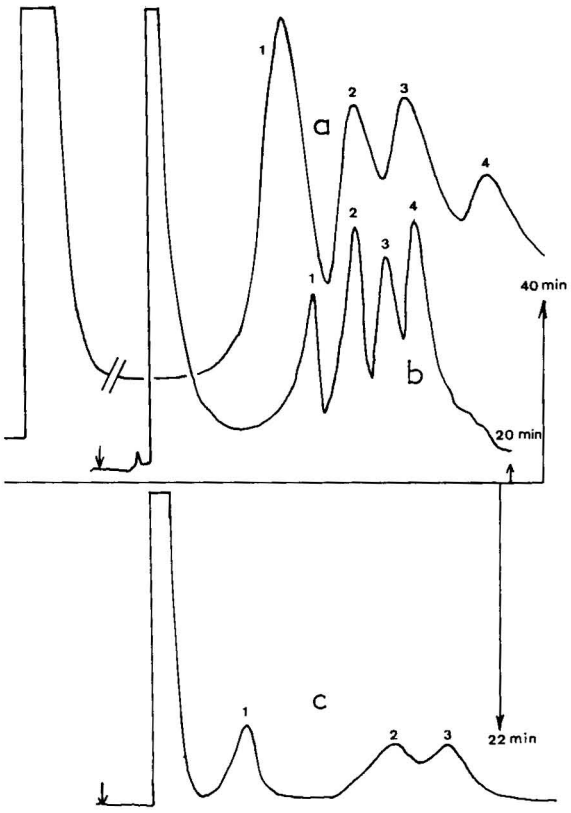

Fig. 7. Chromatogrammes des stéroïdes conjugués esters sulfates (a) et (b), et glucuronides (c). Colonne, Micropak CH (25 cm × $3^3/_8$ mm). Éluant, eau–méthanol (a et b 80% d'eau; c 70% d'eau). Débit, a 0.20 ml/min; b et c 0.30 ml/min. Volume injecté, a et b, 4 μl; c, 1 μl. Température, a et c, 25°; b, 45°. Pics, a et b: 1 = DHEAS, 2 = EAS, 3 = ES, 4 = AS, c: 1 = DHEAG, 2 = EG, 3 = AG.

Influence de la température

Ses effets sont différents et plus complexes que ceux observés en CPG. En CPG, le rôle de la tension de vapeur des solutés fait que très souvent l'augmentation de la température provoque un abaissement de la résolution et conduit à un temps de rétention plus court. En CLHP, la température a une influence à la fois sur la viscosité du fluide mais aussi sur la dissolution dans la phase mobile et dans la phase stationnaire[7,16]. Nous avons réalisé la séparation des quatre stéroïdes libres et des quatre esters sulfates à 45°.

Le volume chassé de la pompe à chaque pulsation est conservé. Comme la viscosité de l'éluant est plus faible à 45° qu'à 25°, ce volume constant va se répartir de façon plus importante dans la colonne et moindre dans l'amortisseur Halász si l'on conserve la pression d'azote constante. Ceci explique que l'augmentation de température provoque dans notre système une vitesse ou un débit de l'éluant supérieur en sortie de colonne, tous les autres réglages étant maintenus identiques.

Le Tableau VI exprime la variation du volume de rétention de chaque stéroïde libre avec la température. Lorsque la température augmente on constate que V'_R diminue ainsi que le coefficient de partage lié par la relation:

$$V'_R = K_i V_S$$

où V_S est le volume constant de phase stationnaire.

Par contre la rétention relative à la DHEA ne change guère. La température a donc pour effet de diminuer pour chaque composé la dissolution dans la phase stationnaire par rapport à celle dans la phase mobile. Mais cet effet étant sensiblement identique pour tous les composés on observe une aussi bonne séparation en un temps plus court (Figs. 6b et 7b), car l'efficacité relative à la DHEA est très peu abaissée (N_{eff}/m (25°) = 1680, N_{eff}/m (45°) = 1252).

COMPARAISON DU CARACTÈRE HYDROPHILE–LIPOPHILE DES STÉROÏDES LIBRES ET CONJUGUÉS

Le graphique de la Fig. 4 illustrant les Tableaux I et II conduit aux remarques suivantes:

Le coefficient de partage des stéroïdes conjugués est inférieur à celui des stéroïdes libres puisque leur volume de rétention réduit est inférieur. En conséquence les conjugaisons sulfate et glucuronide confèrent à la molécule de stéroïde une moins grande solubilité dans la phase stationnaire. Les stéroïdes conjugués sont plus hydrophiles.

L'augmentation du taux d'eau dans la phase mobile eau–méthanol accentue la dissolution des conjugués dans la phase stationnaire. Le méthanol se présente donc comme un bon solvant de ces molécules.

Lorsque l'éluant est très riche en eau on constate que la nature du stéroïde et de la conjugaison donnent des résultats différents. Aussi le glucuronide de DHEA est plus hydrophile que l'ester-sulfate correspondant. C'est l'inverse, mais avec moins de différence pour l'étiocholanolone et l'androstérone.

En général l'ordre croissant du caractère lipophile (K_i augmentant) est le même pour les stéroïdes libres que pour les conjugués DHEA, épiandrostérone, étiocholanolone et androstérone.

CONCLUSIONS

La chromatographie liquide–liquide en phases inversées a permis de séparer des stéroïdes isomères sans transformations chimiques. La séparation de chaque série de composés conjugués ou libres a été effectuée séparément. Pour séparer tous les composés ensemble il faut d'abord considérer que les sulfates sont des sels de sodium et les glucuronides sont sous forme acide organique. Or dans le corps humain, les composés sont des sels de sodium et il a été prouvé que le sel de sodium du glucuronide est plus soluble dans le butanol que l'acide libre[17], ce qui laisse à penser que le sel de sodium du glucuronide serait plus soluble en milieu hydroalcoolique que le glucuronide. Pour les sels de sodium on aurait une courbe $V'_R = f(\delta_M)$ située en dessous de celle des acides. Un gradient d'élution serait sans doute possible avec des calculs

comme ceux de Snyder relatifs à l'adsorption[18] mais nous n'avons pu l'essayer à cause de l'amortisseur Halász et du détecteur réfractométrique. Notons qu'en système à phases inversées le gradient d'élution va souvent du mélange méthanol–eau ($12.9 < \delta_M < 21$) à méthanol pur ($\delta_M = 12.9$) c'est à dire en baissant la "polarité" de l'éluant.

RÉSUMÉ

La chromatographie liquide haute pression (CLHP) mérite sans doute d'être plus utilisée qu'elle ne l'est dans le domaine médical parce que l'on peut analyser à température ambiante des molécules lourdes sans les transformer par réactions chimiques en composés volatils. Ce travail expose la séparation de quatre 17-cétostéroïdes libres et des esters sulfates et glucuronides correspondants par CLHP par la technique du partage avec des phases inversées sur une colonne Micropak CH. Les différents paramètres sont étudiés pour améliorer la séparation. Les données de rétention permettent de comparer le caractère hydrophile des stéroïdes libres et conjugués.

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Journal of Chromatography, 118 (1976) 295–302

CHROM. 8853

CHROMATOGRAPHY OF HEMOGLOBINS ON CM-CELLULOSE WITH BIS-TRIS AND SODIUM CHLORIDE DEVELOPERS

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(Received October 28th, 1975)

SUMMARY

CM-Cellulose as an ion-exchange medium with Bis-tris as buffer and a gradient of sodium chloride provides a versatile system for the chromatography of hemoglobins. Changes in pH, Bis-tris concentration, and slope of the sodium chloride gradient provide means for markedly altering chromatographic behavior for special separations. Examples are given of the application of the method to normal samples and to those with hemoglobinopathies.

INTRODUCTION

Shortly after the first preparation of an ion-exchange cellulose by Sober and co-workers[1,2], CM-cellulose was applied to the chromatography of hemoglobin by Huisman *et al.*[3,4]. Later, as improved forms of CM-cellulose became available commercially, modifications have been described[5]. These procedures have separated the hemoglobins by means of a gradient of pH that was produced by phosphate buffers. In the development of microchromatographic methods for hemoglobins[6], buffers with Tris or Bis-tris and sodium chloride have been successfully applied. Indeed, with these buffers, human fetal hemoglobin (Hb-F) and adult hemoglobin (Hb-A) were well separated in contrast to their minimal separation in phosphate buffers with a pH gradient. Consequently, we have examined the behavior of human hemoglobins in this new system on more conventionally sized columns in order to ascertain its applicability to the analysis of complex mixtures.

* Contribution No. 5210.

MATERIALS AND METHODS

Blood samples

Blood from normal individuals and from those with various types of hemoglobinopathy were collected with EDTA as anticoagulant. Solutions for chromatography were prepared from blood by washing the cells three times with 0.9% NaCl, by hemolyzing with water equal to 1.5 times the packed cell volume plus 0.4 volume of carbon tetrachloride for 20 min at room temperature, by centrifuging twice to remove cellular debris, and finally by dialyzing the sample against a large volume of the appropriate developer overnight at 4°.

Solutions

Developer I is 0.03 *M* Bis-tris–HCl–0.03 *M* NaCl–0.01% KCN at pH 6.1 and contains 6.28 g Bis-tris [N,N-bis-(2-hydroxymethyl)-iminotris-(hydroxymethyl)-methane], 1.75 g NaCl, 0.1 g KCN, and HCl to pH 6.1 in 1 l.

Developer II is 0.03 *M* Bis-tris–HCl–0.12 *M* NaCl–0.01% KCN at pH 6.1 and is prepared by adding 5.25 g NaCl per liter to Developer I and adjusting the pH.

Developer III is 0.03 *M* Bis-tris–HCl–0.085 *M* NaCl–0.01% KCN at pH 6.1 and is prepared by adding 3.22 g NaCl per liter to Developer I and adjusting the pH.

Preparation of ion exchanger

A 50-g portion of CM-cellulose (CM-52, microgranular and pre-swollen; Whatman, Clifton, N.J., U.S.A.) was suspended in 300 ml of Developer I. After the ion exchanger had been settled twice in this buffer to remove fines, the pH of the stirred suspension was adjusted to pH 6.1, another settling was done, pH was checked, and finally the volumes of settled resin and supernatant fluid were adjusted to a ratio of 1:2. All chromatographic operations were done at room temperature.

Chromatographic procedure

A 20 × 1 cm column was poured from the slurry of equilibrated CM-52 and 50–100 ml of Developer I was passed through. After most of the liquid had been removed, the sample was carefully layered on the column and allowed to flow in. The tube above the column and the line through the pump to the gradient device were filled with Developer I. Development was accomplished with a linear gradient from a two-vessel system of which the mixer contained 650 ml of Developer I and the second vessel 650 ml of Developer II; this is the full gradient. Developer was passed through the column at 50 ml/h with a peristaltic pump. Fraction size was 5 ml. Absorbance was read at 415 nm for all fractions and at 280 nm in some instances. Conductance was determined on every tenth fraction. Because the conductance is a linear function of NaCl concentration, the NaCl concentration at any fraction of the chromatogram can be calculated easily from the measured conductance of the two solutions of a gradient.

If Hb-C is not present in the sample, the gradient may be decreased; 375 ml each of Developers I and III provide a shortened gradient of the same slope as the full grad ient.

Other procedures

DEAE-Sephadex chromatography[7,8], starch gel electrophoresis[9], and amino acid analysis by Beckman amino acid analyzer followed published procedures.

RESULTS AND DISCUSSION

Figs. 1–5 depict the separations that may be obtained from a variety of normal and abnormal hematological conditions. The identity of the components was established in one or more of four ways: starch gel electrophoresis, comparison with DEAE-Sephadex chromatography or with microchromatography, and/or amino acid analysis.

Cord bloods with common abnormal hemoglobins

The data in Fig. 1A derive from the cord blood of a newborn infant with sickle cell trait. The separation of Hb-F, Hb-A, and Hb-S is excellent in this system and Hb-F_1 separates from Hb-F_0 as it does in most chromatographic systems. The newborn child with Hb-C trait has the pattern in Fig. 1B. The conditions of chromatography were chosen to provide a relatively rapid movement of hemoglobins on the column. If an electrophoretically fast moving hemoglobin at alkaline pH were present, it would be virtually unadsorbed under these conditions. However, as will be discussed below, a pattern such as that in Fig. 1A can be translated along the volume axis by changing the NaCl gradient at constant pH and Bis-tris and KCN molarity and thus the rate of movement of more rapidly moving components can be retarded.

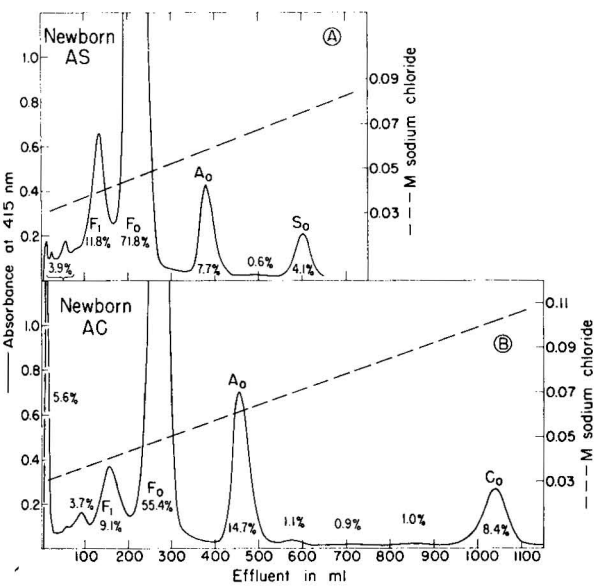

Fig. 1. Separation of hemoglobins in cord blood from an infant with sickle cell trait (A) and an infant with Hb-C trait (B). The shortened and full gradients respectively were used.

Adult samples with common abnormal hemoglobins

The hemoglobins of normal adults and of adults with combinations of hemoglobins A, S, and C yield chromatograms that are depicted in Figs. 2 and 3. In addition to Hb-A_2, a number of other minor components separate from the major Hb-A_0 of the normal individual (Fig. 2A). The very rapidly moving zones may contain some enzymes of the red cell[4] as well as the pyridoxal complex[10]. Traces of Hb-F_0 and minor components of Hb-A are not separable. Because no detailed study has been made of minor components and because they (with the exception of A_2 and F_1) have not been correlated with minor components as detailed by other chromatographic methods or by electrophoresis, they are labelled as A_x, A_y, etc. in the figures.

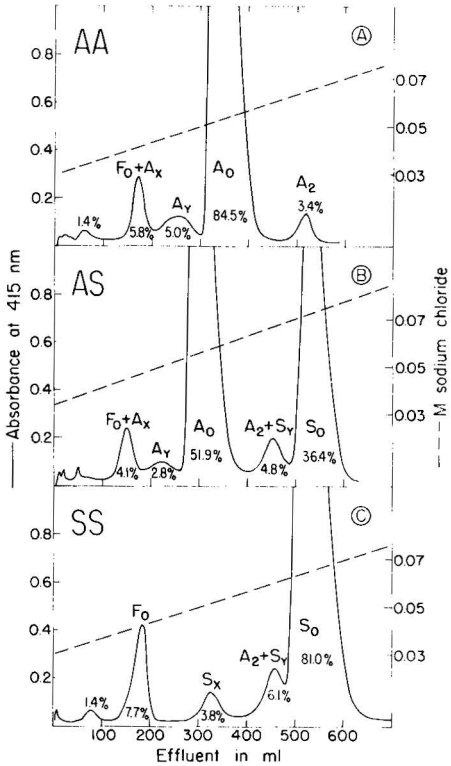

Fig. 2. Separation of hemoglobins of a normal adult (A) and of adults with sickle cell trait (B) and sickle cell anemia (C) by the shortened gradient.

When Hb-S is present, the separations in Figs. 2B (sickle cell trait) and 2C (sickle cell anemia) are obtained. A minor component(s) related to Hb-S (S_y) overlaps Hb-A_2 although in some chromatograms there may be partial separation (Fig. 4B). Hb-A_2, therefore, cannot be quantitatively determined in the presence of Hb-S by this method.

When Hb-C is present and the full gradient is used, Hb-C emerges, as shown in Figs. 1B, 3A, and 3B, before the end of the gradient. The separation of Hb-A_2 from

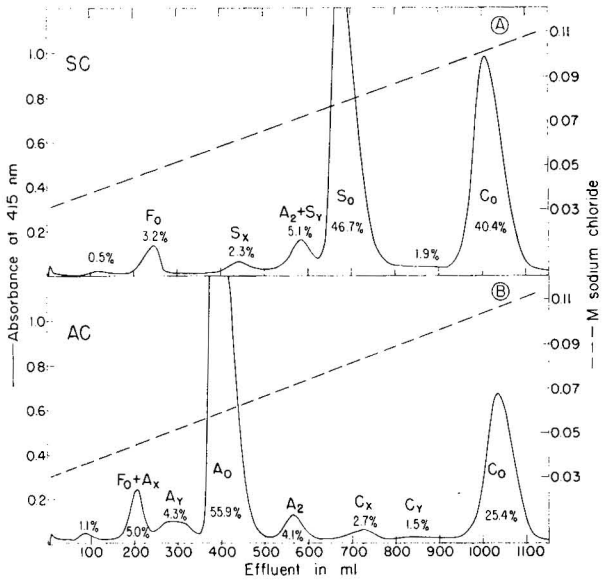

Fig. 3. Separation of hemoglobins of adults with SC disease (A) and Hb-C trait (B) by the full gradient

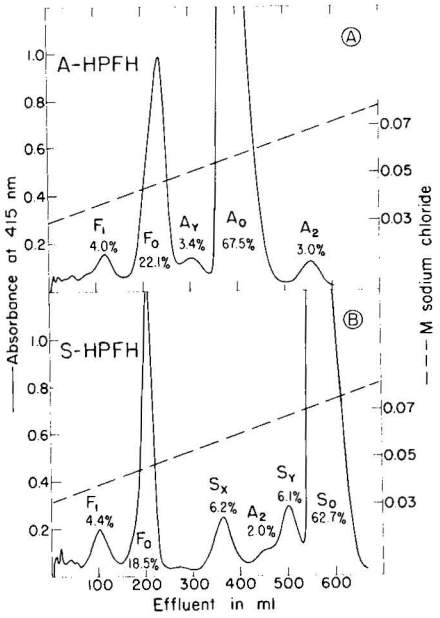

Fig. 4. Separation of hemoglobins in a heterozygote for the hereditary persistence of fetal hemoglobin (HPFH) (A) and in an HPFH individual with Hb-S (B) by the shortened gradient.

Hb-C is excellent and permits the quantitative determination of Hb-A_2 in the presence of Hb-C. Previous chromatographic procedures for this determination required about four days[5] in contrast to about a day by the present method.

Although the reproducibility of the procedure is good, slight changes in pH

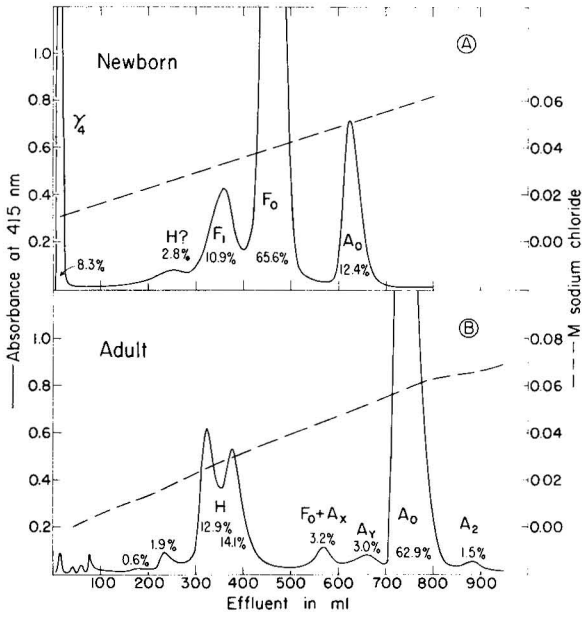

Fig. 5. Separation of hemoglobins in a newborn infant with Hb-Bart's (A) and in the father who has Hb-H disease (B) with a modified gradient (see text).

or NaCl concentration are responsible for the slightly different effluent volumes at which a given peak emerges. The NaCl molarity at the volume of emergence of the peak of any hemoglobin has been as follows: Hb-F_0, 0.039–0.050; Hb-A_0, 0.050–0.063; Hb-A_2, 0.060–0.074; Hb-S_0, 0.064–0.079; and Hb-C_0, 0.093–0.106. From these data as well as from a comparison of various chromatograms in Figs. 2 and 3, it is apparent that Hb-A_0 in an AS sample will be contaminated with a minor component of Hb-S (note the position of S_x in Fig. 2C and Fig. 3A as compared to Hb-A_0 in Fig. 2B). On electrophoresis on starch gel at pH 9, S_x does not behave like A_0 but is heterogeneous and moves like S_0 and F.

Other hemoglobinopathies and abnormal hemoglobins

The adult heterozygote for the hereditary persistence of fetal hemoglobin (HPFH) will have 15–30% Hb-F. The increased Hb-F of such a heterozygote is apparent in the chromatogram of Fig. 4A. When HPFH coexists with Hb-S, the chromatogram of Fig. 4B is obtained.

If Hb-S coexists with β-thalassemia, Hb-A is absent in the type termed S-β^0-thal and present in S-β^+-thal. The chromatogram of the former would have much the appearance of Fig. 4B except for a lower percentage of Hb-F. However, in S-β^+-thal, the S_x peak would contain Hb-A_0 and be present to the extent of 15–25%, and Hb-F would vary from case to case.

When other hemoglobins were chromatographed (not depicted), it was found that an Hb-D (possibly $D_{\text{Los Angeles}}$) moved more rapidly than Hb-S in a distinct peak although incompletely separated from Hb-S. Hb-Lepore (probably $\text{Lepore}_{\text{Washington}}$) has the mobility of Hb-A_2 as does Hb-E.

Figs. 5A and 5B present data from a Thailander in whom Hb-H(β_4) was detected by starch gel electrophoresis, and from his newborn child in whose hemoglobin Hb-Bart's (γ_4) was present. The mother had no electrophoretically abnormal hemoglobin. Because of the presence of these electrophoretically fast moving hemoglobins, the gradients were modified. For the chromatogram in Fig. 5A, the NaCl concentration in the two buffers was 0.01 *M* and 0.08 *M* in 0.03 *M* Bis-tris and 0.01 % KCN at pH 6.1, the total gradient was 1000 ml, and the column had been equilibrated with 0.01 *M* NaCl. For that in Fig. 5B, the column was equilibrated with 0.01 *M* Bis-tris–no NaCl–0.01 % KCN; 25 ml of this solvent was used for initial development with a subsequent 1000-ml gradient between no NaCl and 0.08 *M* NaCl in 0.03 *M* Bis-tris and 0.01 % KCN at pH 6.1. The movement of hemoglobins is retarded under these conditions but the NaCl molarity at which a given peak emerges is within the range that is observed with the other gradient. The virtually unabsorbed peak in the chromatogram of the newborn (Fig. 5A) is Hb-Bart's and there is little or no Hb-H. Hb-H formed two peaks (Fig. 5B) which were readily identified by amino acid analysis. Because of the lability of Hb-H, different methods of preparing hemolysates gave varying ratios of the two peaks of Hb-H in other experiments.

Technical considerations

Bis-tris has been chosen as a buffer for these procedures because its pK_a is 6.5 and therefore the buffer capacity at the pH of choice is good. A few chromatograms in this study used only 0.01 *M* Bis-tris with a linear gradient of 1300 ml total volume and NaCl molarities of 0.03 and 0.12 at pH 6.1 in the gradient vessels. In this system, reproducibility of point of emergence was somewhat variable probably because of poorer pH control at this concentration of Bis-tris. Consequently, 0.03 *M* Bis-tris has been used and the reproducibility has improved.

A slower flow-rate or a longer column does not improve separations. Most of the chromatograms in the figures used a flow-rate of 25 ml/h, but in more recent work, the flow-rate has been 50 ml/h. Consequently, the chromatogram is complete in about a day. In fact, the flow-rate may be increased to 75 ml/h without significant deterioration in the separations. Visual observations of the movement of the hemoglobins on the column suggest that most of the separation occurs in the upper 10 cm, and that each hemoglobin washes virtually unretarded through the lower half of the column. The columns have been re-equilibrated as many as five times and used again successfully.

The developing conditions with the linear gradient of Developers I and II provide a good and rapid separation of the normal hemoglobins and common variants. However, modification of the NaCl gradient or Bis-tris concentration provides a versatility that can be used with less common mixtures of hemoglobins. As an example, Fig. 5A may be compared with Fig. 2A. The slope of the gradient was identical but the start at 0.01 *M* NaCl instead of 0.03 *M* NaCl retarded Hb-A_0 about 275 ml of effluent. Similar retardation occurs if the gradient is started at 0.03 *M* NaCl but the Bis-tris is 0.01 *M* instead of 0.03 *M*. In summary, an increase in pH, NaCl or Bis-tris concentration, or in slope of the NaCl gradient speeds the movement of any hemoglobin and vice versa. With some experience, these variables may be adjusted to provide correct conditions for special separations.

If the Bis-tris–NaCl system is replaced by a gradient of 0.02–0.08 *M* phosphate

at pH 6.1 or a gradient of 0.0 *M*–0.1 *M* NaCl in 0.02 *M* phosphate at pH 6.1, the separations are similar but less satisfactory.

ACKNOWLEDGEMENTS

These studies were supported in part by grants N01-HB-3-3007 and HL-02558 from the National Institutes of Health, U.S. Public Health Service. Dr. Darleen Powars and Dr. Richard Barnes supplied most of the blood samples that were used in this study.

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Journal of Chromatography, 118 (1976) 303–312

CHROM. 8864

GEL PERMEATION CHROMATOGRAPHY

XII. COMPUTER-ASSISTED GEL PERMEATION CHROMATOGRAPHY AND LOW-ANGLE LASER LIGHT-SCATTERING PHOTOMETRY

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SUMMARY

An automated gel permeation chromatograph–low-angle laser light-scattering system capable of measuring the molecular-weight distribution of soluble polymers without the necessity of calibration is described. The automation system eliminates most of the tediousness associated with molecular-weight distribution measurement. The molecular-weight distributions and molecular-weight averages of a broad range of polystyrene standards were determined using this technique. The results obtained are shown to agree closely with literature data.

INTRODUCTION

In an earlier article[1] we described the use of a recently developed low-angle laser light-scattering (LALLS) photometer[2] coupled to an experimental gel permeation chromatograph[3] for measuring the molecular-weight distribution (MWD) of polymers without the necessity of calibration and band-broadening correction. It was demonstrated[1] that light-scattering photometers, when properly designed, could be used to monitor molecular weights of gel permeation chromatographic (GPC) effluents in a continuous fashion and in a real time mode. The raw data obtained consist of a dual trace [differential refractometer (DRI) and LALLS excess scattering] chromatogram from which the concentrations, the Rayleigh factors and the molecular weights of the GPC effluent fractions were calculated. Although the calculations involved in converting the raw data to molecular-weight averages and MWDs are simple, they are tedious. The manual digitization of the concentration and Rayleigh factor chromatograms is not only time consuming, it is also difficult to accomplish with high precision.

The continuous and real time characteristics of both the DRI and light-scattering intensity obtained from a directly coupled LALLS and GPC instrument makes this system ideally suitable for computer assisted data acquisition (direct digitization and storage of the LALLS and DRI analog signals) and reduction (reducing the data to molecular-weight distribution and averages). Consequently, some effort has been devoted to apply laboratory automation techniques to improve the performance of the gel permeation chromatograph–LALLS system.

Since the LALLS photometer does not need the high-precision flow-rate and temperature control required by the real time viscometer molecular-weight detector[3], the LALLS was coupled directly to a commercially available chromatograph (a Modified Waters Assoc. Model 200) rather than to the high-precision experimental gel permeation chromatograph[1]. As expected, the LALLS photometer performed equally well with the commercial gel permeation chromatograph as with the high-precision chromatograph. Narrow MWD polystyrenes (Pressure Chem., Pittsburgh, Pa., U.S.A.; Duke, Palo Alto, Calif., U.S.A.; and National Bureau of Standards, Washington, D.C., U.S.A.) covering a wide range of molecular weights were analyzed using the above system and the results obtained were compared with literature values.

INSTRUMENTATION

A commercially available chromatograph (Waters Assoc. Model 200 GPC) was modified to minimize the "hold up" volume of the mobile phase system and to relocate the DRI concentration detector so that outgasing could be minimized and coupling with the LALLS photometer facilitated. The modification consisted of the following:

(i) Removal of the original pumping system and replacing it with a Milton Roy "minipump" with a Laboratory Data Control Model 709 pulse dampener connected in parallel with the pump. Two sintered 5 μm metal filters (one between the solvent reservoir and the pump and the other between the pump and the sample injection valve) were used to insure removal of particulates from the mobile phase. A diagram of the mobile phase modification is illustrated in Fig. 1.

(ii) The DRI detector was lowered to a point just above the siphon collector. This prevented the formation of gas bubbles in the DRI cell and also shortened the 0.015-in.-I.D. PTFE tubing connector (a 1 m $\times$ 1/16 in. O.D. $\times$ 0.015 in. I.D. PTFE tubing was used to connect the fractionating column, LALLS and DRI detectors).

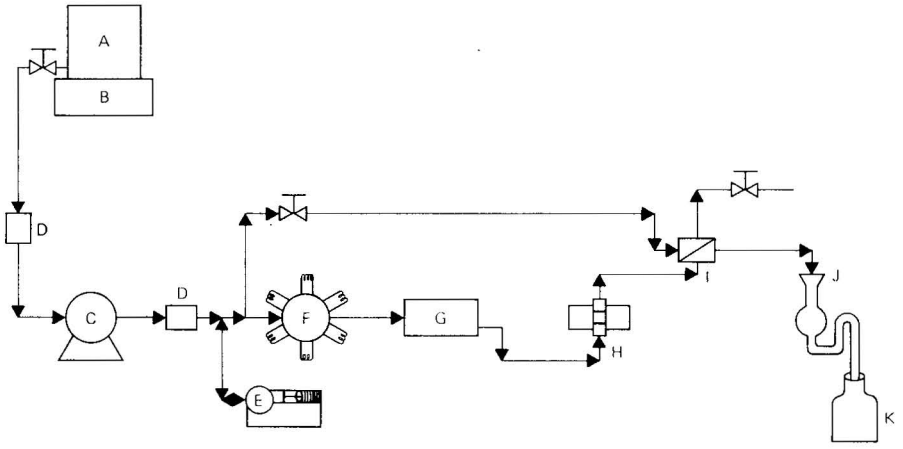

Fig. 1. Diagram of the modified GPC-200 mobile-phase lines. A = Solvent reservoir; B = magnetic stirrer; C = Milton Roy minipump; D = 5-μm sintered metal filter; E = LDC pulse dampener; F = Waters Assoc. automatic sample injection system; G = fractionating column; H = LALLS photometer; I = DRI; J = Siphon collector; K = waste bottle.

The above modification is not necessary for newer chromatographs with low holdup volume in the mobile phase lines and better located concentration detectors.

The LALLS photometer used in this work is essentially the same as the one described in the earlier article[1]. However, some modifications have been made on the design of the sample cell and its associated filter. The modification made was mainly done to improve the plumbing into and out of the sample cell and to reduce the mixing volume of the sample filter. The black PTFE light-scattering cell was replaced with a stainless-steel sample cell with a black PTFE insert. The PTFE insert not only served as a low reflecting surface but also served as an effective sealing gasket between the two fused silica sample cell windows. A sketch of this setup is shown in Fig. 2.

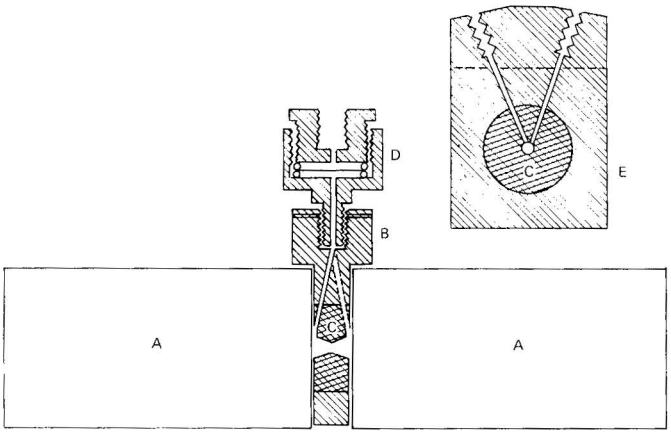

Fig. 2. Sketch of the improved cell design. A = Silica window; B = stainless-steel sample cell housing; C = black PTFE cell; D = modified millipore filter; E = stainless-steel cell housing.

The laboratory automation system hardware consisted of a signal amplifier for each of the analog signals (DRI and LALLS), a sensor-based computer (IBM System/7) for data acquisition, a host computer (IBM 360/195) for data reduction, a typewriter terminal (communication with the host computer) and a gas panel terminal (communication with the sensor-based computer). In our installation, the analog-to-digital converter is situated in the sensor-based computer, hence the analog signal had to be transmitted by a twisted pair of shielded cables over a distance of approximately 150 ft. To avoid an increase in the background electrical noise in the transmission lines, the low-level (0–100 mV) signals from both the DRI and LALLS detectors were amplified to 0–5 V at the chromatograph before being transmitted. A flow sheet of the data acquisition system is shown in Fig. 3.

The laboratory automation software consisted of a relatively simple data acquisition program which specifies to the sensor-based computer the following parameters:

(i) Data sampling frequency —This parameter refers to the time interval between sampling of the analog signal. This parameter can be varied over a very wide range, *i.e.*, 10^{-4}–60 sec.

(ii) The length of the experimental run —This parameter specifies the total number of hours or minutes required to complete the experiment.

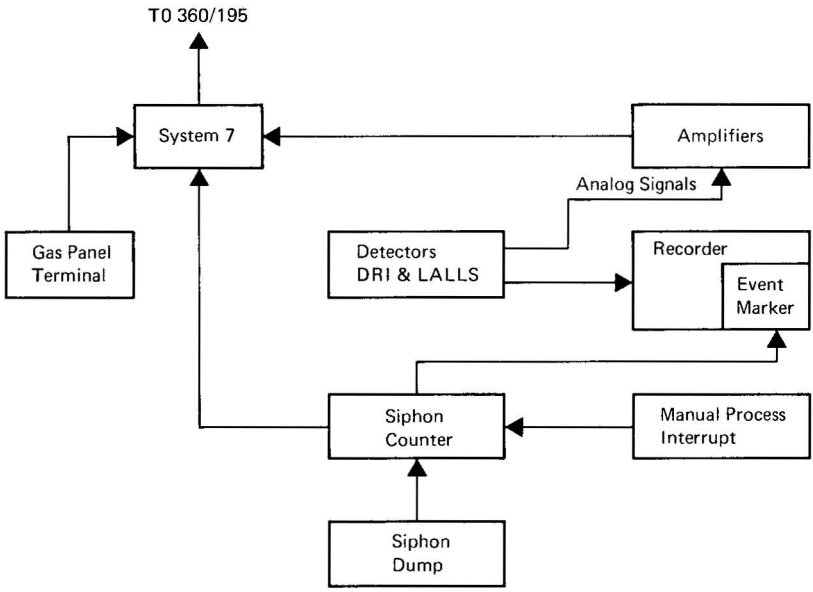

Fig. 3. Flow sheet diagram of the data acquisition system which shows the components of the laboratory automation hardware.

(iii) Data transmission and job submit option parameter—This parameter allows an option for complete automatic or manual data transmission from the sensor-based computer to the data processing (host) computer and data reduction at the termination of the analytical run. In the automatic mode, an unlimited number of samples (in our case we are limited to six samples, which is the maximum number our automatic sample injection system can hold per analytical run) could be processed without any intervention.

A flow chart of the data acquisition and reduction of the laboratory automation software is illustrated in Fig. 4. After the transmission of the data to the host computer, it is partitioned into six individual samples. Each sample chromatogram is reduced into MWD and molecular-weight averages via the following schematics:

(i) Both the DRI and LALLS signals are smoothed using a simplified least squares smoothing method[4].

(ii) The peak and baselines of both the DRI and LALLS chromatograms are located from the digitized chromatogram data array.

(iii) The excess scattering intensities and concentrations are computed from the chromatograms using the following equations:

$$R_{\theta_i} = K_1 Y_i \tag{1}$$

and

$$C_i = K_2 x_i / \Sigma x_i \tag{2}$$

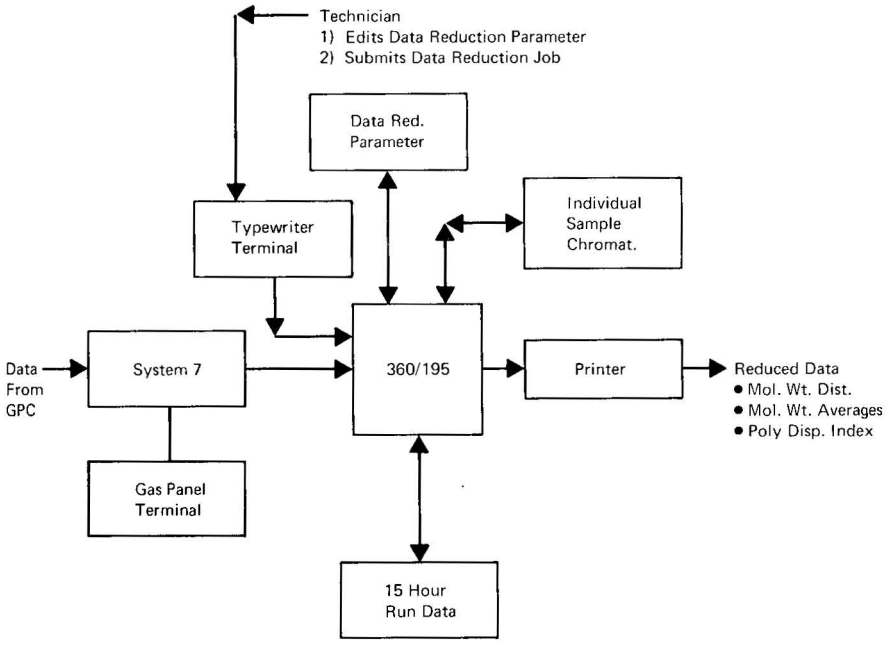

Fig. 4. Flow chart of the laboratory automation software which illustrates the different steps in reducing the raw data MWDs.

where Y_i and x_i are the digitized LALLS and DRI data and K_1 and K_2 are instrumental constants defined as follows

$$K_1 = F/P_0G \tag{3}$$

and

$$K_2 = WT/\Delta V \tag{4}$$

when F, P_0 and G are the attenuation factor, the transmitted light intensity, and the geometrical constant of the photometer, respectively[1], WT is the total sample weight injected, and ΔV is the volume increment of the effluent being considered.

(iv) The second virial coefficient, A_i, is obtained from the relationship

$$A_i = K_3 M_i^{-\alpha} \tag{5}$$

where K_3 and α are constants obtained empirically from a plot of A_i *versus* M_i. This is done by obtaining light-scattering data of various molecular weights and concentrations of the polymer in an off-line determination prior to the GPC–LALLS analysis.

(v) The molecular weight, M_i, is computed in an iterative mode using eqns. 5 and 6.

$$\frac{K_4 C_i}{R_{\theta_i}} = \frac{1}{M_i} + A_i M_i \tag{6}$$

where K_4 is the well-known polymer constant which depends on the viewing angle, the solvent refractive index and the refractive index increment.

(vi) The molecular-weight averages are calculated from the moments of the MWDs using Simpson's rule integration routines.

EXPERIMENTAL PROCEDURE

If high-accuracy MWD measurement is desired, the second virial coefficient, A_i, must be determined as a function of M_w. A_i can be determined in an off-line analysis (LALLS not coupled to the gel permeation chromatograph) by obtaining LALLS data at various concentrations of the polymer over a suitable range of molecular weights. If the polymer is not available over a range of molecular weights with relatively narrow MWD, A_i can be obtained by repeated runs in the gel permeation chromatograph–LALLS system at various total injected weights of the polymer. Fig. 5 shows a plot of the second virial coefficients with molecular weights. The K_3 and a obtained from polystyrene in tetrahydrofuran were $7.41 \cdot 10^{-3}$ and 0.187, respectively.

The refractive index increment ($\Delta N/\Delta C$) for polystyrene in tetrahydrofuran was measured using a Brice Phoenix differential refractometer calibrated with KCl solution. The $\Delta N/\Delta C$ obtained was 0.212 ml/g. The refractive index used for tetrahydrofuran was 1.405.

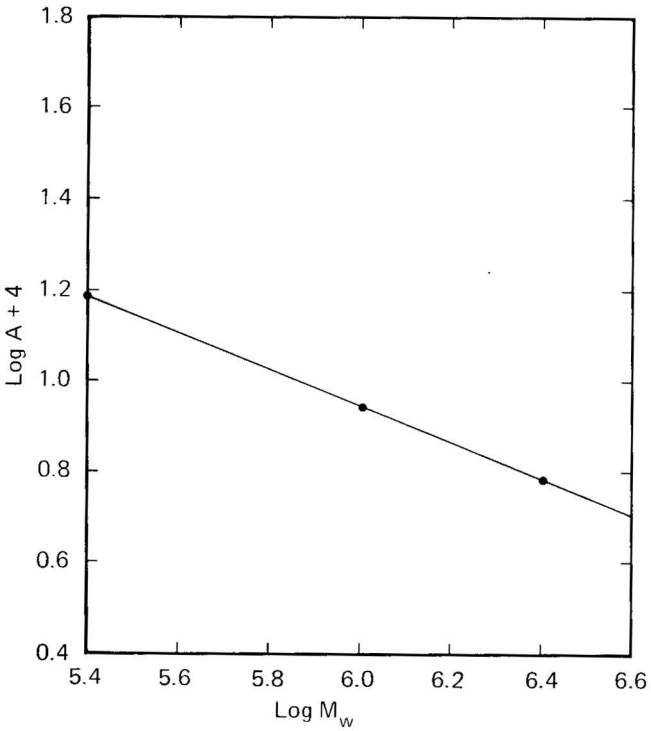

Fig. 5. Plot of the second virial coefficient (A) *versus* molecular weight (M_w) illustrating the strong dependence of A on M_w.

Sample solutions of various concentrations (the concentrations were adjusted according to molecular weights and the volume of the sample loops) were prepared. For example, for polystyrene of molecular weight $7.1 \cdot 10^6$ and a calibrated sample loop volume of 0.5202 ml, the concentration was $4.233 \cdot 10^{-4}$ g/ml or a total sample charge of 0.221 mg. For lower molecular weights, the concentrations and the sensitivity of the photometer were increased accordingly to give a good peak height of the LALLS chromatogram. The sensitivity of the DRI refractometer was likewise adjusted to provide a good signal-to-noise ratio.

After the correct range of LALLS and DRI detector sensitivities have been set, six samples can be analyzed in a completely automated mode. To start the analysis, the following sequence is followed:

(i) The calibrated sample loops of the automatic injector (Waters Assoc.) are loaded with six different sample solutions of precisely determined concentrations.

(ii) The data acquisition program is initialized through a gas panel terminal of the sensor-based computer.

(iii) The sample identifications and other parameters (K_1, K_4, K_3 and α) are entered into the data processing program (in the host computer) via a typewriter terminal.

(iv) Finally, the automatic injector is initialized and the samples are injected into the chromatograph at a programmed interval.

For six samples, at 1 ml/min flow-rate, the total time required to complete the analysis is about 15 h. In many cases, the length of time required to finish the analysis is not critical or of particular concern, for the analysis is usually conducted in the evening hours under conditions requiring no personnel attention or intervention. For example, if an analysis is started at 4:00 pm, the molecular-weight distribution and averages (in the form of digital printout and cumulative and distribution plots) of the six samples are usually ready the next morning.

DISCUSSION OF RESULTS

In common with other light-scattering techniques, one of the concerns in coupling the LALLS photometer to the gel permeation chromatograph is in making certain that particulate matter is filtered out from the effluent as completely as possible. Fortunately, LALLS has a very small scattering volume, *i.e.*, 0.02 μl. If particles were present in the sample cell in relatively low concentration, the probability that these particles reside in the scattering volume is not high and, furthermore, their residence times are normally short. Consequently, the particles appear as spikes in the LALLS chromatogram and not as a broad increase in the scattering of the solvent. Fig. 6 is an illustration of a case where the filter did not operate properly and a considerable amount of particulate matter (particle size in the order of less than 1 μm) from the column entered the LALLS sample cell. Despite the noisy signal one gets when particles are present in LALLS, the data obtained are usable, for the baseline of the spikes can still be reasonably interpreted as the scattering intensity without the particles. The same could not be said for light-scattering photometers with large scattering volumes where the scattering contribution of the particles could not be easily factored out. The presence of particles in a large scattering volume would not appear as individual spikes, but as the integral or the envelope of these spikes resulting in a

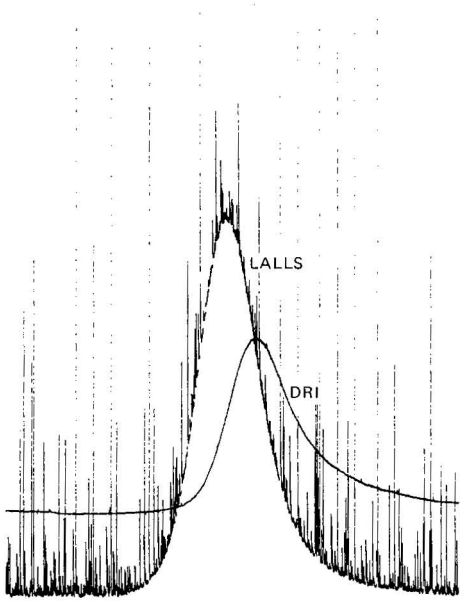

Fig. 6. Case where the filter did not operate properly and a considerable amount of particulate matter from the column entered the LALLS sample cell. Each spike represents a particle which passed through the scattering volume.

gross increase in scattering intensities in the same manner as an increase of polymer concentration or molecular weight in the solution.

The particles causing the spikes in Fig. 6 can be eliminated to a large extent by proper design of the filter cell and proper selection of the filtering medium. A dramatic result in the reduction of particles is illustrated in Fig. 7. It was found that by using a series of filtering media (two layers of a 0.2 μm FGL Millipore filter, topped with a 0.45-μm Flotronix membrane filter) the particles can be drastically reduced.

Table I shows the molecular weight averages of several narrow-MWD polystyrenes which are commonly used as calibration standard for GPC. Except for the Duke $7.1 \cdot 10^6$ sample, which is the highest-molecular-weight calibration standard we presently have, the weight average molecular weights (M_w) obtained by the normal gel permeation chromatograph (M_w calculated using a calibration curve but without

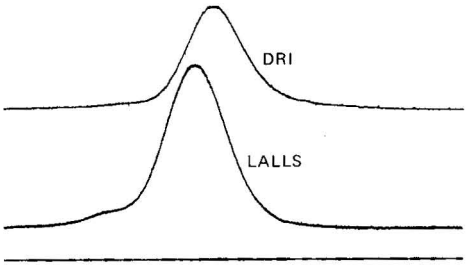

Fig. 7. LALLS chromatogram free of particles.

TABLE I

COMPARISON OF MOLECULAR-WEIGHT DISTRIBUTIONS OF POLYSTYRENE STANDARDS BY GPC AND GPC–LALLS METHODS

Sample	$M_w \times 10^{-5}$		$M_n \times 10^{-5}$		M_w/M_n	
	GPC	*GPC–LALLS*	*GPC*	*GPC–LALLS*	*GPC*	*GPC–LALLS*
Duke $7.1 \cdot 10^6$	69.0	73.0	54.3	70.6	1.28	1.03
PS-$1.8 \cdot 10^6$	17.4	16.0	7.94	15.4	2.19	1.04
PS-$670 \cdot 10^3$	6.10	6.05	5.01	5.64	1.22	1.07
PS-$411 \cdot 10^3$	4.46	4.30	3.80	4.17	1.17	1.03
PS-$179 \cdot 10^3$	1.81	1.76	1.62	1.67	1.12	1.06

band broadening correction) are all higher compared with the values obtained by the coupling of gel permeation chromatograph and LALLS photometer (direct calculation of molecular weight). On the other hand, the number average molecular weights (M_n) as obtained by GPC are in all cases lower than those obtained by GPC–LALLS. The net result is a higher M_w/M_n ratio for the GPC compared to that of the GPC–LALLS method. It is well known that for narrow-MWD polymers, the M_w/M_n calculated from GPC chromatograms and from a calibration curve without band broadening correction (reshaping of the chromatogram) give higher values than the actual. The question is: Does the M_w/M_n calculated directly from the concentration and LALLS chromatogram represents the actual M_w/M_n of the polymer. The answer to this question is, of course, negative. The M_w/M_n obtained by the GPC–LALLS method would be lower than the actual because the fractionating column does not have infinite resolution. That is, the GPC effluent is not composed of perfectly separated components but mixtures of different molecular weights, though their distribution may be narrow indeed. In fact, for a column with zero resolution (*i.e.*, columns packed with non-porous glass beads), a sample with a very broad distribution will have an M_w/M_n of unity via the GPC–LALLS method.

In this work, the fractionating column was composed of five columns with permeability limits extending from $6.5 \cdot 10^6$ to $5 \cdot 10^3$ Å (Waters Assoc. designation) with column efficiencies ranging from 800 to 1500 plates/ft. Hence, it was not surprising to have obtained M_w/M_n values close to the reported literature values of the polystyrene samples[5,6].

The odd result obtained for the Duke 7.1×10^6 polystyrene sample which gave a M_w by GPC lower than by the GPC–LALLS method could be explained by the uncertainty of the calibration curve for molecular weights higher than $7.2 \cdot 10^6$. The molecular weights of the fractions represented by retention volume earlier than the peak of the $7.1 \cdot 10^6$ sample were obtained by extrapolating the calibration curve beyond the retention volume of the highest molecular weight calibration standard. Since the calibration curve is logarithmic in molecular weight, a small error in the extrapolation can mean a large change in the value of the calculated M_w.

CONCLUDING REMARKS

It has been demonstrated that a light-scattering photometer coupled with a gel permeation chromatograph to measure light-scattering intensities of the chromato-

graph effluents in a continuous mode can be readily interfaced with a sensor based computer for on-line data acquisition and processing. In addition to the obvious advantages of laboratory automation, *i.e.*, elimination of the tedious and time-consuming manual data processing, high-precision data can be extracted from the chromatogram by signal-enhancement techniques. The presentation and formating of the processed data is also much improved with the computer-assisted chromatograph.

The effluent from a gel permeation chromatograph has been shown to be unsuitable for light-scattering measurement (particularly if the light-scattering photometer has a relatively large scattering volume) without careful filtration. However, it has also been demonstrated that, with proper filter-cell design and a good choice of filter media, particles from the effluent can be effectively removed.

The MWD measurement of NBS polystyrene and other narrow-MWD polystyrene standards showed results that agree well with published results. The heterogeneity index (M_w/M_n) obtained by the GPC–LALLS technique appears to be slightly but consistently lower than those reported for the polystyrene standards. This has been attributed to the less than perfect resolution of the column.

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Journal of Chromatography, 118 (1976) 313–317

CHROM. 8728

RESOLUTION OF OVERLAPPING GAS CHROMATOGRAPHIC PEAKS USING FAST FOURIER TRANSFORMATION*

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(First received June 13th, 1975; revised manuscript received September 2nd, 1975)

SUMMARY

A method is presented for the determination of the retention time and the area of overlapping gas chromatographic peaks. Fast Fourier transformation has been used for the resolution of the overlapping peaks, and the results are compared with the actual amounts of the components which are present.

INTRODUCTION

The application of minicomputers to the resolution of overlapping spectral bands requires the use of complicated algorithms. For example, in gas chromatography (GC), least-squares and other methods have been used to process the chromatograms[1–4], and the Fourier transformation has been used in the resolution of spectra[5].

The theoretical aspects of the application of the fast Fourier transformation (FFT) have been presented by Kirmse and Westerberg[6], who separated overlapping chromatographic peaks into the individual components. In order to increase the resolution of GC peaks with the help of FFT, the following procedure is used. A signal obtained from an analytical instrument (spectrometer, gas chromatograph, etc.) is treated by an analog-to-digital converter (ADC) and introduced into the memory of a computer. A fast Fourier transform is then made by the computer. A division by line-shape function and a multiplication by apodizing function are usually carried out simultaneously; this is necessary in order to reduce the truncation effect. It is possible to obtain a better resolution without using the Hamming window[6], but there is interference from side bands in the calculation of the chromatographic peaks (see Fig. 1). After these operations an inverse transform is carried out and the results of the analysis are obtained from the computer.

* Presented at the 5th Soviet–Italian Symposium on Chromatography, Tallinn, April 22–25, 1975.

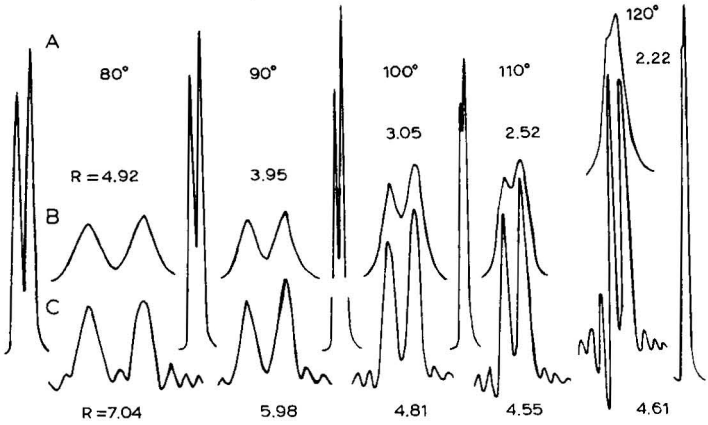

Fig. 1. Resolution of two overlapping peaks by fast Fourier transformation without the use of the Hamming window. A = Recorder output, B = ADC output and C = FFT deconvolution.

EXPERIMENTAL

A mixture of weighed amounts of *n*-, iso- and *sec.*-butanols was separated on a Perkin-Elmer Model 900 gas chromatograph. A signal from a hot-wire detector was converted into digital form by an ADC and then punched on to a tape at a frequency of four points per second. Computations were made with a Videoton-1010B computer (16 K, 32 bit) for which a program was written in Fortran. Different degrees of overlapping of the peaks were obtained by gradually increasing the oven temperature of the gas chromatograph. Measurements were made between 110 and 170°.

When applying FFT for increasing the resolution of peaks, it is necessary to know the half-width of the narrowest peak. Since the half-width of a peak is related approximately linearly to its retention time, the initial values of the half-widths of the overlapping peaks may be determined by constructing a calibration graph from the peaks (two at least) which are completely separated in the chromatogram. In practice, 1-pentanol and 1-hexanol were added to the mixture of alcohols under investigation. The peaks of the compounds which were added (Fig. 2, peaks 4 and 5) did not overlap with the peaks of the components of the mixture. The shape functions of the peaks should also be known when using this method, and the chromatographic peaks were assumed to obey a gaussian distribution.

RESULTS

The ADC output obtained from this experiment is presented in Fig. 3A. Whereas the mixture was almost entirely separated at 110°, it was impossible to separate the individual peaks of the mixture on increasing the temperature to 150°. The separated chromatograms obtained on the basis of the FFT output are presented in Fig. 3B, and the retention times of the overlapping peaks could be determined more precisely. In order to evaluate the accuracy of the FFT separation with reference to the measurements of the retention times, the relative retention times of the separated and overlapping peaks were compared. The results are shown in Fig. 4, in

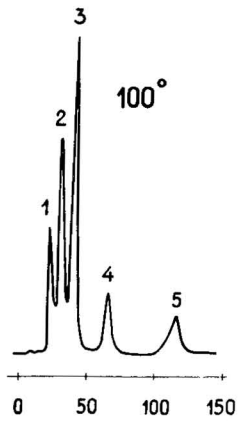

Fig. 2. Gas chromatograms of a mixture of butanols at 100°. Peaks: 1–3 = *sec.*-, iso- and *n*-butanol; 4 = 1-pentanol; and 5 = 1-hexanol.

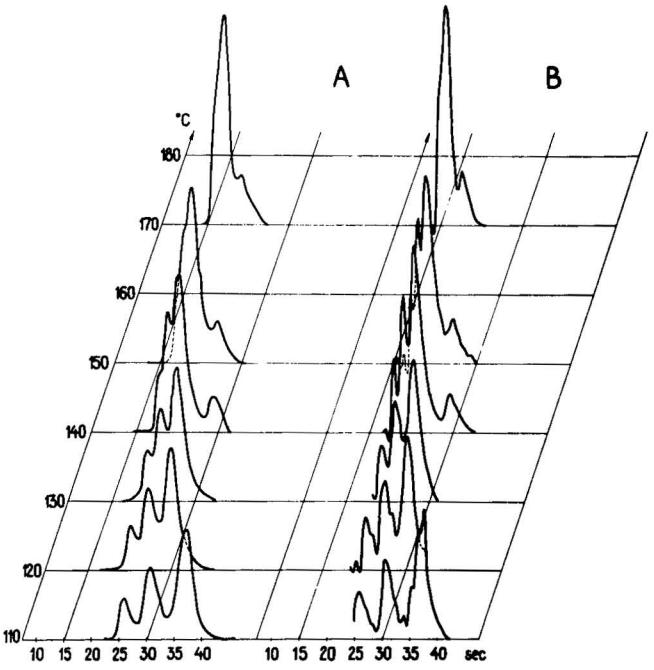

Fig. 3. Resolution of the overlapping peaks of three butanols. A = ADC output; B = FFT deconvolution.

co-ordinates of log α_i and $1/T$, where α_i is the retention time of a peak (i = 1–3) relative to that of peak 4 in Fig. 2. It is evident from the straight lines obtained that in this case by using FFT one can determine the retention times when the oven temperature is 150°, whereas direct measurement is possible only up to 130°. With FFT

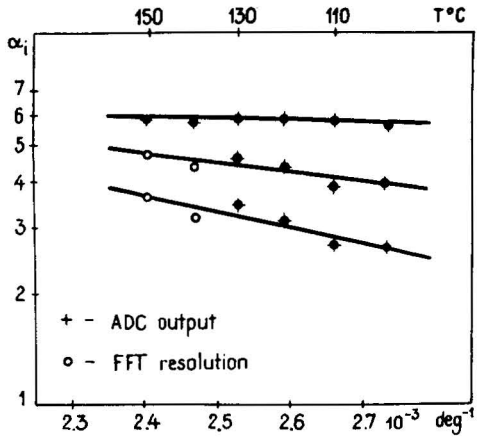

Fig. 4. Graphs of relative retention time against temperature.

it is also possible to determine the retention times with great precision even in a case of wide overlapping of the peaks.

The peak resolution, R, is expressed by

$$R = \frac{t_2 - t_1}{\sigma_2} \tag{1}$$

where t_1 and t_2 are the retention times of two peaks and σ_2 is the variance of the second peak. Hence it is possible to determine the extent of overlapping for which FFT separation is no longer effective. In the present case the value of R was 1.70. If FFT separation is not used, the value of R_2 for which deconvolution for determining the retention time is still possible is 2.7. The precision of the determination of areas of overlapping peaks by FFT is of great interest. A mixture of compounds of precise composition was prepared and the calculated ratio and actual ratio of the components were compared. Peaks which were not separated by FFT were calculated by digital integration. Methods which are available for determining areas are triangular, perpendicular-drop and digital integration, but the best results for FFT were obtained by using the peak amplitude and the retention time. The areas of the overlapping peaks were determined from the peak height and the variance, using the equation

$$S = \sqrt{2\pi} \cdot \sigma \cdot A \tag{2}$$

where A is the peak height, σ is the variance and S is the peak area. The variance, σ, was determined from the equation

$$\sigma = \sigma_1 \sqrt{1 - \left(\frac{\sigma_0}{\sigma_1}\right)^2} \tag{3}$$

where σ_0 is the initial value for FFT deconvolution and σ_1 is determined from the linear relation between the retention time and the variance of the peak. The results are shown in Fig. 5, where the ratios of the areas of the peaks are compared to the actual ratios

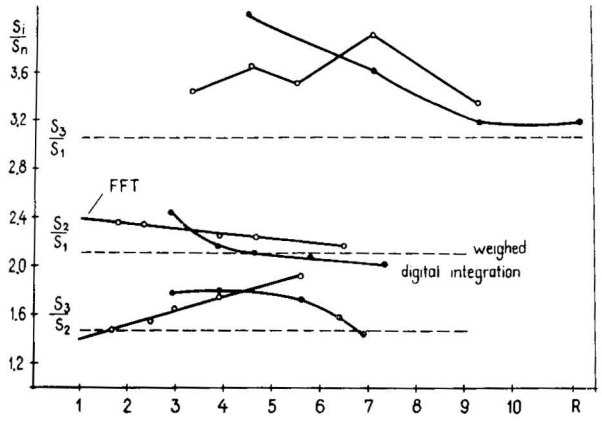

Fig. 5. Graphs of the ratio of the measured areas of overlapping peaks against the peak resolution.

in the cases of different extents of overlapping. It is seen that the results are not precise enough. Whereas the ratios of S_1 and S_2 approximate to the real values, those of S_2 and S_3 are different. This is apparently due to the influence of three overlapping peaks.

CONCLUSIONS

By using FFT in GC the retention times and the location of the individual components of overlapping peaks can be determined with greater precision. This technique may be considered as an alternative to direct separation. The mathematical separation of peaks permits information about the individual components to be obtained more quickly than from direct separation, where columns having different polarity, oven temperature and working conditions should be tested. On the other hand, mathematical data handling distorts the information and it is not quite clear how much information is lost in the processing of overlapping peaks. Thus peak deconvolution by FFT is a quick, but somewhat approximate, method, the application of which is justified only if great accuracy of data handling is not required for widely overlapping peaks.

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Journal of Chromatography, 118 (1976) 319–329

CHROM. 8906

GAS ADSORPTION STUDIES ON ION-EXCHANGED FORMS OF CRYSTALLINE ZIRCONIUM PHOSPHATE

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(Received October 23rd, 1975)

SUMMARY

Gas–solid chromatography was used to measure the heats of adsorption of eight aromatic and aliphatic adsorbates on crystalline α-zirconium phosphates. Four cation-exchanged phosphates were examined. Evidence was that both aromatic and aliphatic hydrocarbons were physically adsorbed in such a manner as to be screened from the cations in the cavities that are present in the zirconium phosphates. Surface areas are measured to enable entropies to be calculated. Entropy values are used to suggest modes of adsorption on to the crystalline surfaces.

INTRODUCTION

The properties of zirconium phosphates have been the subject of two comprehensive reviews[1,2]. The major portion of these reviews were devoted to descriptions of the variety of crystalline, "semi-crystalline" and gel phases which can be prepared. Very little is known of the structures of these materials but some of them have the same potential versatility, as porous media, as the zeolite minerals.

The literature contains several publications suggesting the use of zirconium phosphate as a catalyst for the dehydration of alcohols[3–6], the polymerization of ethylene oxide[7], and the isomerization of cyclohexane to methyl cyclopentene[6]. Other studies refer to the thermal decomposition of an ammonium zirconium phosphate[8] and the acid properties of a zirconium phosphate surface[9].

This study uses the technique of gas–solid chromatography to measure the heats of adsorption of aromatic and aliphatic hydrocarbons on crystalline α-zirconium phosphate (α-ZrP). The structure of this adsorbent in its hydrogen form is known and the position of other counter ions can be conjectured[1,2]. Here the hydrogen, lithium, sodium and potassium exchanged form of α-zirconium phosphates have been used.

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THEORY

Isotherms were constructed directly from elution peaks. The method of Cremer and Huber[10] was used in the manner suggested by Saint-Yrieix[11] taking into account the Bechtold correction[12]. A computer programme was written to provide (a) a least squares best fit to isotherm data; (b) the slope of appropriate isosteres to calculate the different heats of adsorption, ΔH; and (c) an interpolation to estimate the free energy of adsorption, ΔG, and the differential entropy of adsorption (ΔS) in the usual manner.

From these experimental values of ΔS, can be calculated ΔS^0 representing the difference in differential molar entropy between the three dimensional adsorbate in its standard state and adsorbed standard states. This can be done for the two models of localized and non-localized adsorption, described by de Boer and Kruyer[13], using the equation of Everett[14].

Thus for immobile (site) adsorption

$$-\Delta S_i^0 = -\Delta S - R \ln \theta/(1-\theta) \tag{1}$$

and for mobile adsorption, when the adsorbate is considered an ideal two-dimensional gas,

$$-\Delta S_m^0 = -\Delta S - R \ln A^0/A \tag{2}$$

where $\theta = \omega M/a\varrho_T t_p$, A^0 = standard molecular area, A = area per molecule, ω = moles of absorbate per gram of adsorbent, M = molecular weight of adsorbate, a = surface area of adsorbent, ϱ_T = density at temperature T, and t_p = thickness of adsorbate molecule[15].

The value $A^0 = 4.08\,T \times 10^{-12}$ m^2 was used throughout and A was determined from experimental values for a. Values of $-\Delta S_i^0$ and $-\Delta S_m^0$ were compared to ideal values for the two models. If the system behaved as the model of entropically ideal site adsorption, then $-\Delta S_i^0$ calculated from $-\Delta S$ with various amounts adsorbed should be independent of surface concentration of adsorbate and its value should represent the total loss of translational entropy.

So when the localised entropy of the adsorbed layer (${}_aS^0{}_{loc}$) is zero at $\theta = \frac{1}{2}$ we can expect

$$-\Delta S_i^0 = {}_gS^0{}_{tr} \tag{3}$$

where ${}_gS^0{}_{tr}$ is the translational entropy of the adsorbate.

If however the system was one of entropically ideal mobile adsorption $-\Delta S_m$ should still be independent of adsorbate concentration and should be given by

$$-\Delta S_m^0 = {}_gS^0{}_{tr} - {}_aS^0{}_{tr} \tag{4}$$

where ${}_aS^0{}_{tr}$ is the translational entropy of an ideal molar gas. ${}_gS^0{}_{tr}$ and ${}_aS^0{}_{tr}$ can be calculated[14]:

$$ {}_gS^0{}_{tr} = R \ln (M^{3/2} \cdot T^{5/2}) - 2.30 \tag{5} $$

and

$$ {}_aS^0{}_{tr} = 2/3\,{}_gS^0{}_{tr} + 1.52 \log T - 3.04 \tag{6} $$

(Note. Everett[16] has pointed out that

$$ {}_aS_{tr} = {}_a\bar{S}_{tr} + R \tag{7} $$

where ${}_a\bar{S}_{tr}$ = standard differential molar entropy of an ideal two-dimensional gas and this was taken into account.)

EXPERIMENTAL

Ion-exchanged samples were prepared from the hydrogen form of α-zirconium phosphate. Full details of their composition and characterization have already been published[17].

Crystalline zirconium phosphates were crushed and sieved. Aliquots of about 10 g of the 60–85 BSM fraction were used to pack gas chromatographic columns. Packing was carried out without undue agitation to avoid deaggregation of the 60–85 fraction. Packed columns were conditioned for 24 h at temperatures previously defined by thermogravimetric analyses[17]. This conditioning was repeated to constant weight.

Preliminary experiments showed that (a) a linear relationship existed between peak area and sample size and (b) the isotherms obtained were unaffected by changes in carrier gas flow-rate.

The chromatograph used was a Pye 104 Model 34 with a katharometer detector. The carrier gas was Mineral Helium (BOC, Lancs., Great Britain) dried by a molecular sieve 4A column. When various sample sizes were eluted from any column at identical conditions of gas flow-rate, temperature, etc., the elution peaks were calculated from any elution peak[18] since the diffuse edge of the peak was thus reproducible.

Surface areas were measured by an adaptation of the method of Kuge and Yoshikawa[19]. The method involves the "imposition" of a gas–liquid peak onto a gas–solid peak to give an overall peak shape as shown in Fig. 1. Interpretation of the concentration up to point X should correspond to the amount of adsorbate required to form a monolayer and by use of the molecular area of the area of the adsorbate the surface area can be obtained. The procedure was as follows.

Elution peaks of the type described were obtained and the number of moles/g adsorbent (n_X) corresponding to the point X (Fig. 1) calculated. Assuming the adsorbate to be adsorbed as a liquid film the volume of liquid (V_X) corresponding to n_X was calculated from information of the bulk density of the liquid at the temperature of the elution. This quantity (V_X) was divided by the probable thickness (tp) of the adsorbed layer and the surface area obtained.

Surface areas were also calculated by a BET nitrogen method[20] for comparison.

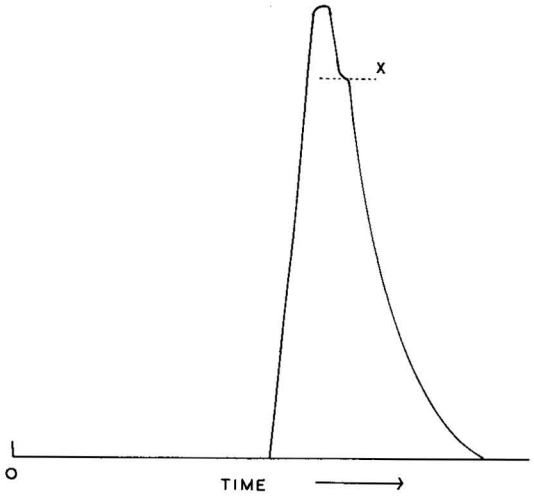

Fig. 1. Asymmetric gas–solid chromatography elution peak used for surface area calculations.

RESULTS

Surface area measurements are presented in Table I. A typical family of isotherms is shown for benzene adsorption on α-zirconium phosphate in its hydrogen form (Fig. 2). The example is extended to show ΔG and ΔH values (Fig. 3) as a function of surface coverage. Table II summarizes the data obtained for all the adsorbate–adsorbent systems studied. Figs. 4–10 show entropy data.

TABLE I

SURFACE AREA MEASUREMENTS

Adsorbent	*Adsorbate*	*Assumed orientation of adsorbate molec. on surface*	*Surface area by chromatographic method (m^2/g)*	*BET surface area (m^2/g)*
α-ZrPH	*n*-hexane	horizontal	10.8	32.5
	cyclohexane	horizontal	10.2	
	1-hexene	L-shaped C_4 thick	10.4	
	benzene	vertical	10.0	
α-ZrPLi	*n*-hexane	horizontal	23.8	42.2
	cyclohexane	horizontal	26.3	
	1-hexene	L-shaped C_3 thick	23.0	
α-ZrNa	*n*-hexane	horizontal	13.9	23.6
	cyclohexane	horizontal	14.1	
	benzene	vertical	14.3	
	1-hexene	L-shaped C_3 thick	13.3	
	cyclohexene	vertical	14.2–15.8	
α-ZrPK	benzene	vertical	10.4	20.1
	cyclohexane	horizontal	10.0	
	n-hexane	horizontal	10.2	

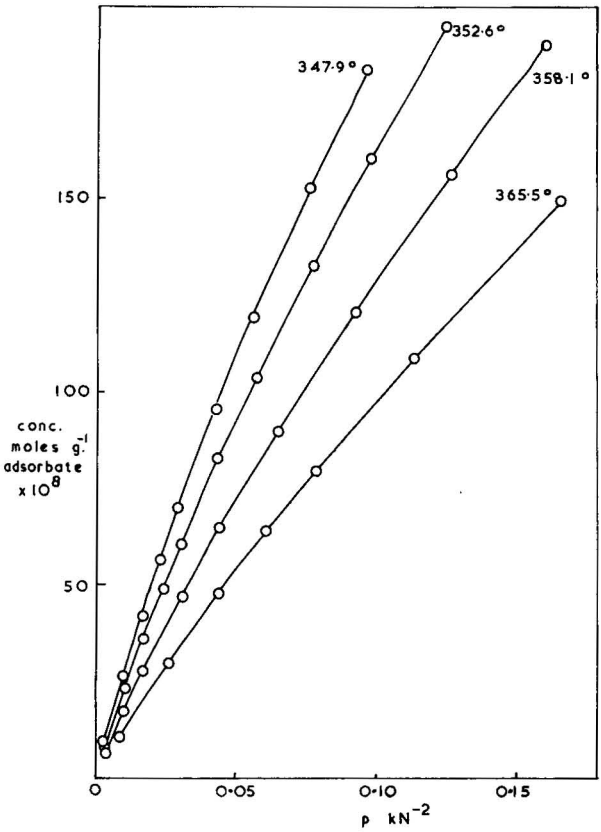

Fig. 2. Isotherms of benzene on α-ZrPH.

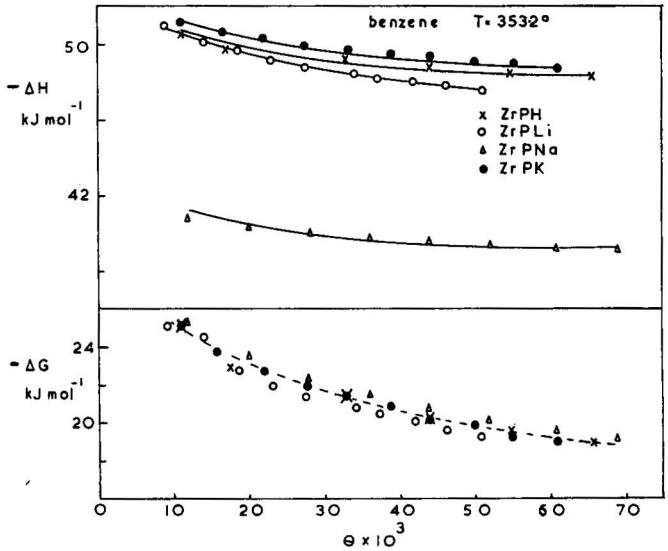

Fig. 3. Variation of ΔG and ΔH with surface coverage for benzene on ion-exchanged α-zirconium phosphates.

TABLE II

THERMODYNAMIC PARAMETERS OF ADSORPTION

Adsorbent	$-\Delta H$ *(kJ/mol) at* $\theta = 25 \cdot 10^{-3}$								
	Adsorbate								
	Benzene	*Toluene*	*Ethylbenzene*	*Cyclohexane*	*Cyclohexene*	*n'-Hexane*	*1-Hexene*	*n-Heptane*	*n-Octane*
α-ZrPH	49.4	59.0	62.8	35.2	45.6	37.7	48.1	43.6	46.9
α-ZrPLi	49.0	—	—	35.6	50.6	39.8	48.5	—	—
α-ZrPNa	40.2	—	—	36.4	39.4	36.4	43.9	—	—
α-ZrPK	50.2	—	—	36.8	41.9	43.1	47.7	—	—
$-\Delta G$ (kJ/mol) at $\theta = 25 \cdot 10^{-3}$	21.8*	23.4**	23.8**	14.2*	18.4*	16.6*	20.9*	17.2**	18.8**
Isostere temp. range	347–366	378–393	398–415	341–366	347–366	335–353	335–353	365–383	392–408

* Average value (± 1 kJ/mol) for all adsorbents.

** Measured on α-ZrPH.

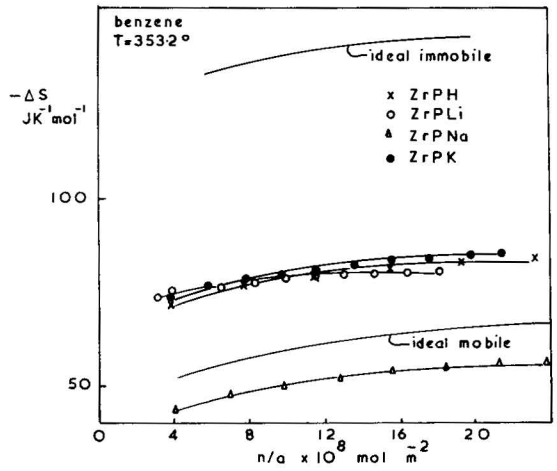

Fig. 4. Variation of $-\Delta S$ with n/a for benzene on α-ZrP.

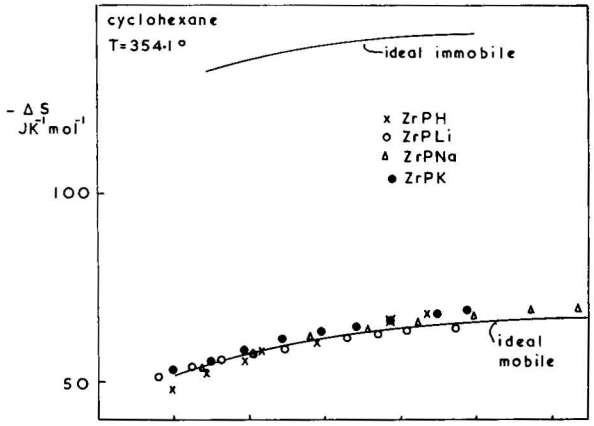

Fig. 5. Variation of $-\Delta S$ with n/a for cyclohexane on α-ZrP.

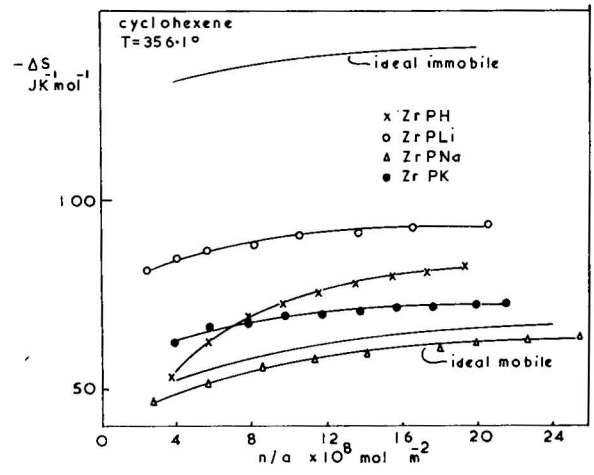

Fig. 6. Variation of $-\Delta S$ with n/a for cyclohexene on α-ZrP.

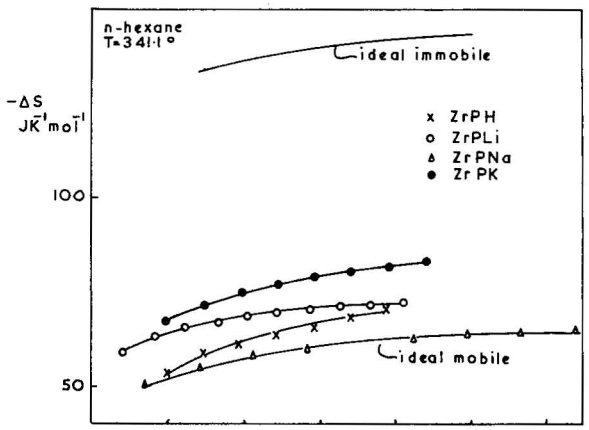

Fig. 7. Variation of $-\Delta S$ with n/a for n-hexane on α-ZrP.

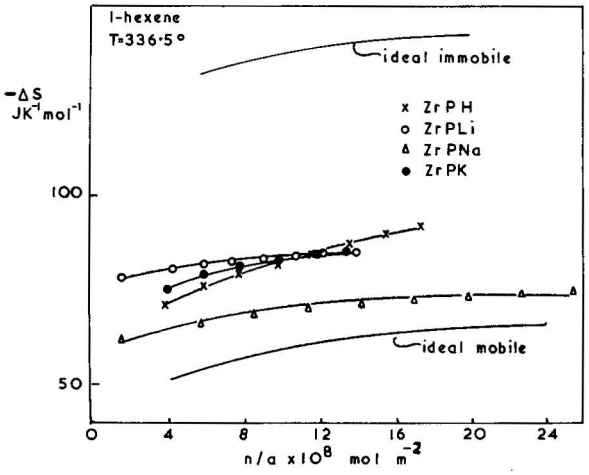

Fig. 8. Variation of $-\Delta S$ with n/a for 1-hexene on α-ZrP.

DISCUSSION

Surface area

Since surface area plays an important role in determining θ, A^0/A and hence the various entropy values, a pore volume was calculated as described by Gregg and Sing[21]. The value was more than one hundred times less than a theoretical pore volume calculated from the X-ray interlayer spacings[17]. This seems to imply that the adsorptions observed were not taking place in micropores.

At first sight, it seems that entropy calculations might be incorrect if a wrong surface orientation is chosen for calculations (*i.e. tp* will be wrong). It can be demonstrated that a maximum variation of only $\pm$ 4 J/°K arises between the cases of a benzene molecule (a) lying flat (tp = 340 pm) or (b) standing on "an edge" (tp = 680 pm).

Table I shows the orientations used in entropy calculations the choice of

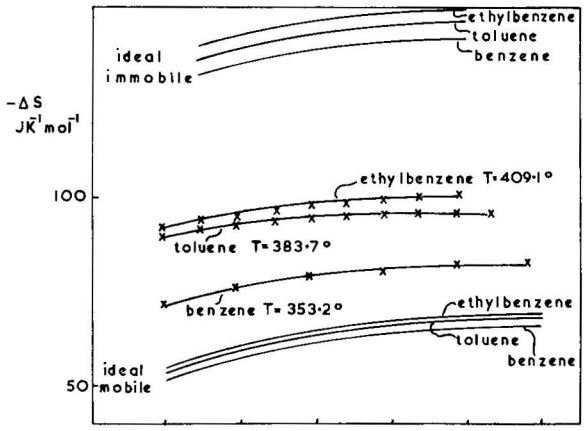

Fig. 9. Variation of $-\Delta S$ with n/a for benzene, toluene and ethylbenzene on α-ZrPH.

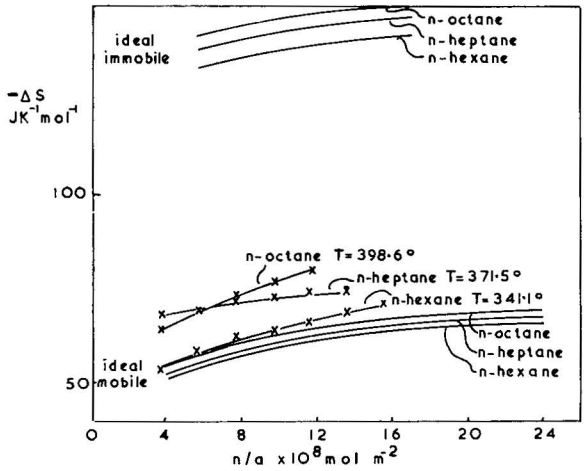

Fig. 10. Variation of $-\Delta S$ with n/a for *n*-octane, *n*-heptane, *n*-hexane on α-ZrPH.

orientation being that to give consistent results. It can be seen that the figures from the chromatographic method are at variance with those from the BET technique.

Heats of adsorption

Small changes in ΔH with surface coverage (*e.g.* Fig. 3) indicated a regular surface with little or no evidence of surface heterogeneity at the lower coverages studied. All the values measured were higher than the adsorbate latent heat of vaporization but not sufficient to suggest that anything other than physical adsorption was being observed. The adsorbates examined were from the classification of Kiselev[22], *viz.* (a) molecules capable only of non-specific action and (b) molecules capable of non-specific and specific interactions. Kiselev and Lopatkin[23] also suggest that the same specific and non-specific terminology be applied to adsorbents and, by their definition, the zirconium phosphates are all non-specific. Their expandable layer

structure and "zeolitic" water content might be expected to give specific interactions, but the critical cavity dimensions in the non-expanded forms of α-zirconium are about 260 pm[1,2], which, in a zeolite structure, will exclude all molecules except water and ammonia.

Entropies of adsorption

Lithium, sodium and potassium forms (see Figs. 4–8). All adsorbates (except 1-hexene) have close to complete mobility on the sodium zirconium phosphate, indeed cyclohexane and benzene are "super-mobile"[24], *i.e.* the adsorption is so weak that the entropy associated with the vibration replacing the lost degree of translational freedom is too strong to neglect.

In both the lithium and potassium forms entropy losses above the ideal state are observed and can be ascribed to lost degrees of rotational freedom. As apparently there are no direct ion–adsorbate interactions, these variations in entropy must arise from changes in surface potential energy.

Hydrogen form (Figs. 9 and 10). Here the entropy changes offer some indication of the form of restriction in degrees of freedom. Benzene, toluene and ethylbenzene are increasingly restricted. This is Kemball's anchoring effect[24], that substituent groups have on an aromatic nucleus. Again, in the series *n*-hexane, *n*-heptane, *n*-octane, there is an increase of entropy equivalent to about 6 $J \cdot °K^{-1} \cdot mol^{-1}$ per carbon atom increase in the hydrocarbon chain. Kemball[25] calculates that an increase from C_6 to C_7 should provide an additional entropy of about 20 $J \cdot °K^{-1} \cdot mol^{-1}$, so, for these adsorbates on α-hydrogen zirconium phosphate, no new restrictions to bond rotation occur as chain length increases. Increases in moments of inertia for overall rotation must be the main contributors to the entropy losses.

Generally the presence of a double bond causes an increase in restriction in comparison to the adsorption of the unsaturated homologue. This generally is true for all the adsorbents studied (*e.g.* compare Figs. 4 and 5).

Free energies of adsorption

The ΔG values for a particular adsorbate are nearly independent of adsorbent. This supports the earlier suggestion that non-specific adsorption occurs and that the alkali metal ions are screened from the adsorbate molecules.

CONCLUSION

Adsorption on α-zirconium phosphates is at the surface and not within zeolite-like cavities within the structures. It seems that only oxygen-containing molecules have strong interactions with zirconium phosphate surfaces. This concurs with their potential catalytic usage[3–7]. Attempts to separate alcohols by gas chromatography have not been possible owing to these strong interactions[26].

ACKNOWLEDGEMENTS

One of us (D.L.) wishes to thank the University of Salford for the award of a studentship. We are also grateful to Dr. R. P. W. Scott for helpful discussions.

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CHROM. 8902

QUANTITATIVE DETERMINATION OF NALTREXONE AND BETA-NALTREXOL IN HUMAN PLASMA USING ELECTRON CAPTURE DETECTION

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(Received October 28th, 1975)

SUMMARY

A gas–liquid chromatographic method is described for the determination of naltrexone and beta-naltrexol in human plasma following derivatization with pentafluoropropionic anhydride using electron capture detection. The lower sensitivity of the method for absolute standards is 5–10 pg. Following an acute 100-mg dose to a subject, peak levels of naltrexone of 15 ng/ml at 2 h and of beta-naltrexol 84 ng/ml at 4 h were observed. The levels of both compounds decreased by 24 h after the dose: naltrexone to 2.9 ng/ml and beta-naltrexol to 25 ng/ml. Following chronic administration for two weeks of 100 mg per day the peak levels of naltrexone and beta-naltrexol increased to 26.9 and 131 ng/ml at 2 h, respectively, but by 24 h both compounds were at levels similar to those following a single dose. Thus no accumulation of either drug or metabolite in the plasma was seen following chronic naltrexone administration.

INTRODUCTION

Naltrexone was synthesized by Blumberg *et al.*[1] in 1965. It is a potent orally effective narcotic antagonist with a reasonably long time course of action and thus is considered useful in the out-patient treatment of opiate addicts following detoxification from either methadone or heroin[2,3]. The urinary excretion of naltrexone and its major metabolite beta-naltrexol[4,5] in man has been reported recently[6,7]. However, owing to the low concentrations in human plasma, quantitative determinations of naltrexone and beta-naltrexol have not been accomplished. In this report we describe a method for the quantitative determination of naltrexone and beta-naltrexol in the plasma of a subject taking an acute dose and chronic therapeutic doses of naltrexone.

MATERIALS AND METHODS

Chemicals and reagents

Beta-naltrexol · HCl (N-cyclopropylmethyl-7,8-dihydro-14-hydroxynormor-

phine) was a gift of Dr. R. Willette of NIDA (Bethesda, Md., U.S.A.), and naltrexone·HCl (N-cyclopropylmethyl-7,8-dihydro-14-hydroxynormorphinone) and naloxone·HCl, (N-allyl-7,8-dihydro-14-hydroxynormorphinone) were provided gratis by Endo Labs. (Garden City, N.Y., U.S.A.). The solvents used were glass-distilled, GC grade purchased from Burdick and Jackson (Muskegon, Mich., U.S.A.). All aqueous solutions, buffers and dilute acids, were prepared using double glass-distilled water. Pentafluoropropionic anhydride (PFPA) and glassware siliconizing fluid of Dri-Film® SC-87 were purchased from Pierce (Rockford, Ill., U.S.A.).

Stock solutions

Standard solutions of naltrexone (25 ng/ml), beta-naltrexol (200 ng/ml) and internal standard naloxone (250 ng/ml) were prepared in double glass-distilled water.

Calibration curves and quantitation

Naltrexone and beta-naltrexol standard curves were prepared by adding 1.25, 2.5, 12.5 and 25 ng of naltrexone, 5, 10, 20 and 40 ng of beta-naltrexol, 6.25 ng of naloxone to each of 1.0 or 2.0 ml of blank plasma. The samples for both standard curves were extracted according to the method described below, derivatized by PFPA and chromatographed. The peak height or area ratios were plotted against the respective concentration of standards and the slope determined. The unknown sample peak height or area ratio was divided by the slope to determine the concentrations of naltrexone and beta-naltrexol in the unknown samples.

Extraction procedure

All glassware was siliconized with a 5% (v/v) solution of Dri-Film in toluene. The following substances were added to 20-ml round-bottom centrifuge tubes: Antifoam A (A.H. Thomas, Philadelphia, Pa., U.S.A.) 0.5 to 2 ml of plasma and 25 μl of aqueous internal standard containing 6.25 ng of naloxone. The pH was adjusted to 8.5 with 1.0 ml of saturated sodium bicarbonate and the aqueous phase was shaken with 12 ml of chloroform. After 10-min shaking and 5-min centrifugation the chloroform layer was transferred into clean centrifuge tubes containing 5 ml of 0.5 *N* HCl. After back extraction into acid the chloroform layer was discarded and 4.5 ml of the HCl phase was transferred into clean centrifuge tubes and neutralized with 2.5 *N* NaOH. The pH was adjusted to 8.5 with saturated sodium bicarbonate and the aqueous phase extracted with 7 or 8 ml of chloroform. After shaking and centrifugation the chloroform was transferred to a clean 15-ml test tube and evaporated to dryness on a rotary flash evaporator (Evapo-o-mix, Buchler). To the dry residue 100 μl of PFPA were added, the tube was tightly stoppered (00 size hollow nalgene stoppers; A.H. Thomas) and placed in a heating block at 110° for 40 min. After the reaction the samples were stored overnight at −16° and usually analyzed the following day. The anhydride was evaporated at room temperature under a stream of nitrogen. The dried sample was taken up in 50–100 μl of ethyl acetate and 1–2-μl aliquots were injected into the gas chromatograph. Fig. 1 shows a chromatogram for two standards of beta-naltrexol and naltrexone extracted by the method described above.

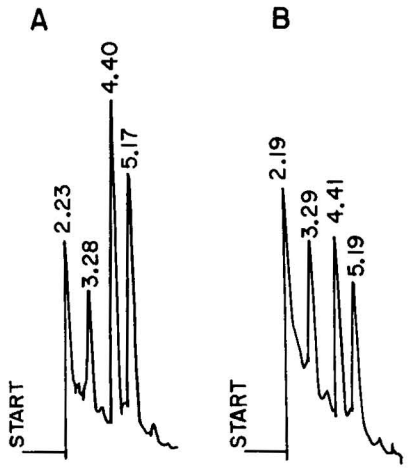

Fig. 1. Chromatogram A represents 6.25, 40, and 25 ng of naloxone, beta-naltrexol and naltrexone, respectively, extracted from 1 ml of plasma. Chromatogram B represents half the above concentration of naltrexone and beta-naltrexol while using the same amount of internal standard, naloxone. The retention times for naloxone, beta-naltrexol, and naltrexone are 3.28, 4.40, and 5.17 ± 0.02 min, respectively. The signal is automatically suppressed over the first 2.2 min by time programming. The first apparent peak at 2.20 ± 0.03 min is the time the signal is attenuated to scale.

Gas–liquid chromatographic conditions

Analysis was performed under isothermal conditions on a Hewlett-Packard Model 5830A gas chromatograph, equipped with a ^{63}Ni linear electron capture detector operating at 1×10^9 mA standing current. The column was 6 ft. × 2 mm I.D. packed with 3% OV-22 on Supelcoport, 80–100 mesh. The carrier gas was a mixture of 10% methane in argon at a flow-rate of 35 ml/min. The injection port temperature was 250°, the detector temperature 300° and the column oven temperature 215°.

Subject

The subject in this study was a paid volunteer from the New York State Office of Drug Abuse Services. He was informed of the study protocol and signed an informed written consent prior to the study. Blood (12–15 ml) was drawn 1, 2, 4, 8, 12 and 24 h after drug administration. The blood was centrifuged and the plasma separated from the cells and stored at −17° until analysis.

RESULTS AND DISCUSSION

A solvent extraction method and derivatization process was developed for the electron capture analysis of naltrexone and beta-naltrexol in human plasma. The recovery of naltrexone and beta-naltrexol from plasma within the concentration range of the standards was 77 ± 2% and 54 ± 2%, respectively. When derivatization was carried out under milder conditions (70° for 25 min), in the presence of solvent (benzene–PFPA, 4:1), pentafluoro-derivatives different from those shown in Fig. 1

were obtained for naloxone, beta-naltrexol, and naltrexone. Their retention times were 4.45, 3.24 and 7.27 min, respectively. Absolute standard curves and standards extracted from plasma and derivatized either neat or in benzene provided linear relationships between the concentration and either peak height or area ratios of naltrexone, beta-naltrexol, and the internal standard naloxone.

The detector response for beta-naltrexol was greater than that for naltrexone when compared with the derivatization moieties obtained under neat conditions. Since in the clinical plasma samples naltrexone levels were extremely low, the neat derivatization condition was used. Both naltrexone and beta-naltrexol are stable at −16° for at least one week in excess PFPA either before or after reaction. However, once the derivatizing agent is removed and the derivatives reconstituted in solvent, it is necessary to inject them onto the gas chromatograph as soon as possible since the derivatives rapidly decompose in solvent. The derivatives were found to be equally soluble in either ethyl acetate or acetonitrile but less soluble in non-polar solvents such as benzene.

In order to make a simultaneous determination of naltrexone and beta-naltrexol in all plasma samples over a 24-h period, the choice of sample aliquot becomes critical. This occurs because in some samples the concentration of beta-naltrexol is as much as 15 times higher than naltrexone. For plasma samples taken after 12 h, aliquots of 0.6–2.0 ml were found adequate, whereas for plasma samples taken earlier than 12 h 0.2–0.4 ml aliquots were used.

Plasma levels of naltrexone and beta-naltrexol

To ascertain the practical application of the method developed, plasma levels of naltrexone and beta-naltrexol were determined in a subject following single and multiple doses of naltrexone. Serial blood samples were taken after an acute dose of 100 mg naltrexone at 1, 2, 4, 8, 12 and 24 h after the dose. Following the 24-h sample, the same subject received 100 mg naltrexone daily for 14 days and then blood samples were taken following the last 100-mg dose to evaluate the plasma levels of naltrexone and beta-naltrexol following chronic naltrexone treatment. Fig. 2 shows that in the acute study naltrexone reached a peak at 2 h of 15 ng/ml and by 24 h the level declined to 2.9 ng/ml. The rapid rise of beta-naltrexol in plasma following a single dose indicates a rapid rate of biotransformation of naltrexone to beta-naltrexol. A peak level of 84 ng/ml of beta-naltrexol was found at 4 h and the levels decrease to 25 ng/ml by 24 h after the dose.

Following chronic treatment the peak concentration at 2 h of both naltrexone and beta-naltrexol were higher in the plasma, 26.9 ng/ml and 131.0 ng/ml, respectively. However, by 24 h after the dose the levels of these compounds were approximately at the same concentration as in the acute study. If this pattern of naltrexone disposition is confirmed in other subjects and is representative of the population, no substantial accumulation of naltrexone or beta-naltrexol would be expected following chronic naltrexone treatment.

In conclusion, the method described in this report provides sufficient sensitivity for the quantitative determination of naltrexone and beta-naltrexol in human plasma following single and multiple therapeutic doses. As compared to the bis(trimethylsilyl)trifluoroacetamide derivatives of naltrexone and beta-naltrexol measured by flame ionization detection, the PFPA derivatives using electron capture detection

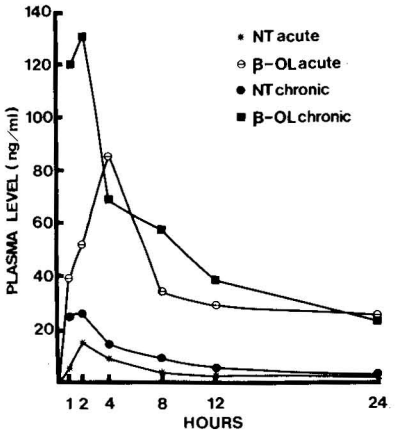

Fig. 2. Plasma levels of naltrexone (NT) and beta-naltrexol (β-OL) in a subject following the administration of an acute 100-mg oral dose of naltrexone (NT and β-OL acute) and following chronic doses of 100 mg naltrexone for 14 days (NT and β-OL chronic).

increases the sensitivity of the method of absolute standards approximately a thousand-fold and for plasma extracted materials approximately a hundred-fold.

ACKNOWLEDGEMENTS

These studies were supported in part by NIDA Contract No. ADM-45-74-133. The authors wish to express their gratitude to Drs. R. Resnick and J. Volavka (New York Medical College) for the clinical plasma samples.

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Journal of Chromatography, 118 (1976) 337–343

CHROM. 8898

THE APPLICATION OF THIN-LAYER GEL FILTRATION CHROMATOGRAPHY IN THE RAPID ASSESSMENT OF PROTEIN–PROTEIN COUPLING REACTIONS

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(Received November 17th, 1975)

SUMMARY

The use of enzymes as markers when covalently coupled to various antigens or antibodies has wide application in medical science. A variety of bifunctional reagents has been used to produce the conjugates, and there have been some attempts to investigate the conditions of reaction necessary to obtain good coupling, whilst preserving the biological function of the molecules.

The present study describes the influence of coupling conditions on the coupling of a α-amylase and albumin by a range of commercially available bifunctional coupling reagents, and the application of thin-layer gel filtration chromatography for the rapid qualitative/semi-quantitative assessment of coupling reactions.

INTRODUCTION

Protein–protein conjugates have been used as reagents in a variety of analytical techniques including immunoelectrophoresis, immunodiffusion, haemagglutination and enzyme immunoassay[1–4]. Enzyme- or ferritin-labelled antibodies have also found use as immunohistochemical markers. Such conjugates have been prepared using a range of coupling agents, including dialdehydes, diisocyanates, carbodiimides, bismaleimides, nitrogen mustards, *s*-triazines, bisdiazobenzidines and fluoronitrobenzenes. These studies, however, have not always demonstrated that optimum conditions were employed for the coupling reaction, and the relative merits of each coupling agent in different systems have not been fully explored.

The criteria for the assessment of coupling agents are related to the efficiency of coupling, and the subsequent maintenance of the integrity of biological activity of the conjugated proteins. Impairment of biological function will reduce the sensitivity of analytical procedures utilising these coupled proteins. Various techniques have been employed for assessing the results of protein–protein coupling reactions, and purifying the conjugated species. These have included ultracentrifugation, electro-

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phoresis, precipitin studies, chemical analysis and gel filtration in columns. These methods do not readily lend themselves to the rapid assessment of the large numbers of conjugation reactions required for deciding optimum coupling conditions. We present here an account of the use of thin-layer gel filtration (TLG) for rapid qualitative and semi-quantitative assessment of conjugation in model protein–protein coupling reactions between human albumin and α-amylase.

EXPERIMENTAL

Materials

Human albumin and α-amylase (Grade II bacterial) were purchased from Sigma (London) (London, Great Britain) and used without further purification. Amylase substrate was prepared essentially by the method of Rinderknecht *et al.*[5]. Remazol Brilliant Blue R salt (Calbiochem, Los Angeles, Calif., U.S.A.) was covalently linked to corn starch (Sigma) and the insoluble product was obtained in quantitative yield as a waxy solid (RBB starch).

3,6-Bis(acetoxymercurimethyl)dioxane was prepared from mercury(II) acetate and allyl alcohol[6]. 4,4′-Diaminobibenzyl, m.p. 129–131° (ref. 7: m.p. 132°) was obtained by reduction of the corresponding 4,4′-dinitro compound available via nitration of bibenzyl[7].

Methods

Sephadex® G-200 Superfine (Pharmacia, Uppsala, Sweden) (21.0 g) was equilibrated for three days at room temperature in 500 ml of phosphate buffer saline pH 7.2–7.4 (phosphate 0.02 *M*, NaCl 0.5 *M*), prior to use. The gel was spread 0.6 mm thick on 20 × 40 cm glass plates. Following overnight equilibration in a Pharmacia TLG apparatus, the plates were placed flat, and 20-μl samples (8–10 per plate) were applied using an Eppendorf automatic pipette. The plate was inclined at an angle of 10° and developed for 8–10 h. A replication technique was then used to detect the chromatographed substances, by soaking up the excess buffer with a sheet of filter paper (Whatman 3MM, 180 g/m^2).

The protein stain used was Coomassie Brilliant Blue R250, 0.25% in methanol–glacial acetic acid (90:10, v/v). Paper replicates were stained for 15 min and excess stain was removed by washing three times with water, then twice with methanol–glacial acetic acid–water (50:10:50, v/v), and finally twice more with water.

α-Amylase activity was localised by a modification of the method described by Rosalki[8] for use with cellulose acetate electrophoresis strips. Agarose (1.5%) was dissolved in phosphate buffer saline pH 7.2 (phosphate 0.015 *M*, NaCl 0.15 *M*). Agarose gel (20 ml) was melted in a boiling water bath and RBB starch (1 g) was added. The mixture was then poured on a 20 × 20 cm glass plate, giving a gel of 0.5 mm thickness. The wet filter-paper replica was placed face-down on top of the gel and left in the open at room temperature for up to one hour. The areas of amylase activity showed up as blue spots on the paper replica.

Immunoelectrophoresis was performed essentially by the method of Scheidegger[9]. Agarose gel (1%) was made up in barbitone buffer pH 8.6 (ionic strength 0.025) containing polyethylene glycol 6000 (4%) and sodium azide (0.02%). The melted gel was poured 0.8 mm thick on to 10 × 10 cm glass plates, and 1-μl samples

were pipetted into each well. Electrophoresis, in barbitone buffer pH 8.6 (ionic strength 0.05), was performed at constant voltage of 2–3 V/cm for 55 min. Double diffusion, using 50 μl of antiserum in each trough, was allowed to proceed at room temperature for 20–24 h. The gels were then press-dried[10] to remove soluble proteins and stained as for the TLG replicates.

Typical coupling reaction

Solutions of human albumin (500 μl, 20 mg/ml) and α-amylase (700 μl, 20 mg/ml) in phosphate buffer (0.1 *M*, pH 7.8) were treated with 40 μl of a solution of hexamethylene diisocyanate (100 mg/ml in acetone), and the mixture was agitated gently at 0–4° for 24 h. The reaction mixture was then dialysed against 2 l of ammonium carbonate solution (0.1 *M*) at 0–4° for 16 h, and finally centrifuged at 4000 *g* for 15 min. The supernatant was then subjected to TLG, and filter paper replicates were stained for protein and enzyme.

RESULTS AND DISCUSSION

Mixtures of human albumin and α-amylase have been reacted under varying conditions of protein concentration, (2, 20 g/l), pH (2–10.8), and reagent concentration (10^{-4}–10^{-2} *M*) with a range of commercially available bifunctional protein–protein coupling reagents (Table I). α-Amylase was chosen as the enzyme for this model study by virtue of its stability and low cost, both highly desirable features of an enzyme label.

The general approach to assessing each of the bifunctional, protein–protein coupling reagents is outlined in the scheme[11] below.

Reagent	→	Coupling conditions	→	Qualitative/semi-quantative assessment	→	Characterisation of coupled products
		Temperature, pH, reagent and protein concentrations		Thin-layer gel filtration chromatography		Immunoelectrophoresis

Reaction mixtures were subjected to TLG and a paper replicate of the chromatogram stained for either protein or α-amylase activity. In these studies TLG has proved an extremely valuable analytical tool for the simultaneous and rapid assessment of large numbers of reaction mixtures. Inspection of protein stained replicates quickly reveals the extent of protein coupling and any polymerisation of the reactant proteins. A typical protein stained replicate is shown in Fig. 1.

A comparison of results obtained by TLG and conventional gel filtration on a column of Sephadex G-200, for a mixture of albumin and α-amylase which had been treated with hexamethylene diisocyanate at 38° for 2 h, is shown in Fig. 2. The enzyme stained replica (b) indicated that the high-molecular-weight products have very little amylase activity. Initial attempts to visualise amylase activity after replication utilised the starch–iodine reaction. However, the reaction also detects the presence of albumin[12], and the use of an insolubilised dyed starch, previously reported for the localisation of α-amylase activity on cellulose acetate electrophoresis strips[8]. was adopted instead.

TABLE I

PROTEIN–PROTEIN COUPLING REAGENTS

Suppliers: a, Aldrich, Milwaukee, Wisc., U.S.A.; b, BDH, Poole, Great Britain: c, Cambrian Chemicals, Croydon, Great Britain; d, Eastman Organic Chemicals, Rochester, N.Y., U.S.A.; e, kindly supplied by I.C.I. Organics Division, Manchester, Great Britain; f, Sigma (London), London, Great Britain; g, Pierce & Warriner, Chester, Great Britain.

Type	*Product*	*Supplier*
Dialdehydes	Malondialdehyde	a
	Glutaraldehyde	b
	Terephthal-dicarboxaldehyde	a
Diisocyanates	Hexamethylene diisocyanate	a
	p-Phenylene diisocyanate	c
	m-Phenylene diisocyanate	c
	Toluene-2,4-diisocyanate	b
	3,3′-Dimethoxy-4,4′-biphenyl diisocyanate	d
	4,4′-Diphenylmethane diisocyanate	e
	4,4′-Dicyclohexylmethane diisocyanate	e
s-Triazines	Cyanuric chloride	a
	Procion yellow MX-4R	e
Bismaleimides	N,N′-*o*-Phenylene dimaleimide	a
	N,N′-*p*-Phenylene dimaleimide	a
Carbodiimides	1-Ethyl-3-(dimethylaminopropyl)-carbodiimide	f
Nitrogen mustards	1,1-Bis(2-chloroethyl)-4,4′-bipiperidine	a
Isoxazoles	N-Ethyl-5-phenyl isoxazolium-3′-sulphonate	f
Sulphonyl chlorides	*m*-Benzene disulphonyl chloride	a
Mercurials	3,6-Bis(acetoxymercurimethyl)dioxane	
Bisdiazonium salts	*o*-Dianisidine	f
	p,p′-Diaminodiphenyl ethane	
Fluoronitrobenzenes	1,5-Difluoro-2,4-dinitrobenzene	a
	Bis(4-fluoro-3-nitrophenyl)sulphone	f
Imidoesters	Dimethyl suberimidate	g
	Dimethyl adipimidate	g

High-molecular-weight products of coupling reactions, selected on the basis of the TLG analysis, were characterised by immuno electrophoresis. Samples were run in duplicate and stained for protein and α-amylase activity, in order to detect conjugates of α-amylase and albumin. These analyses revealed that the extent of coupling of α-amylase to albumin was very low since little enzyme activity could be demonstrated for products showing immunological activity with anti-albumin. This finding was in agreement with the results from TLG which suggested that with many of the coupling reagents, especially those which effected coupling via amino groups (diisocyanates) or thiol groups (mercurials), albumin reacted preferentially to form albumin polymers.

In general protein–protein coupling was insignificant at low protein concentrations (2 g/l) as compared with corresponding reactions at protein concentrations of 20 g/l. The effect of reagent concentration and pH on a typical series of coupling reactions is illustrated by results obtained with several diisocyanates (Table II).

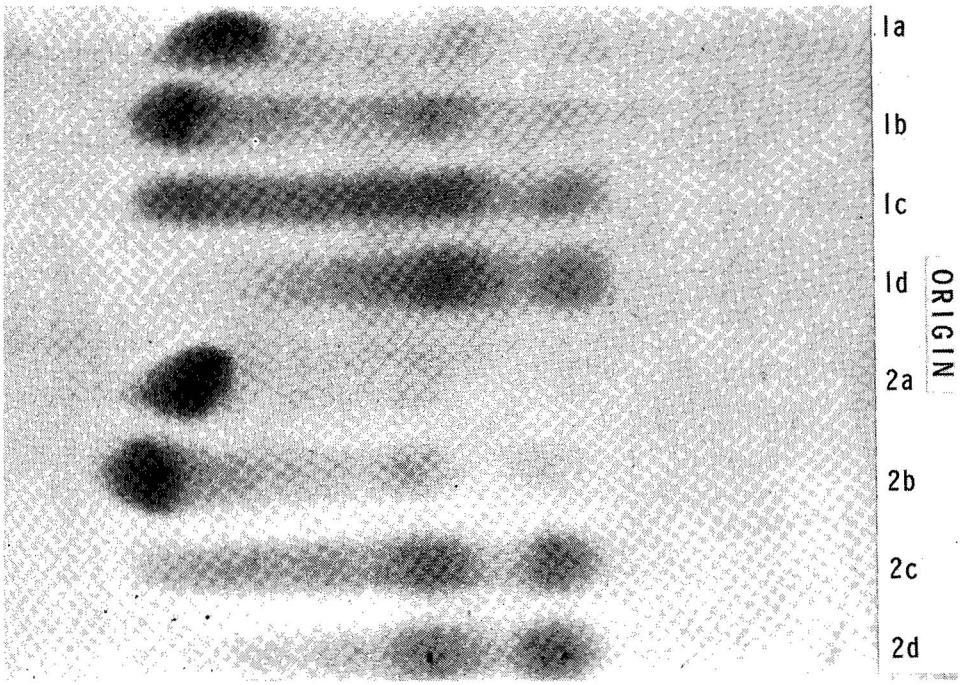

Fig. 1. Replicate of thin-layer gel filtration chromatogram of mixtures of α-amylase (A) and albumin (B) following treatment with glutaraldehyde. Protein concentration 20 g/l; pH: 1, 7.4; 2, 7.0; concentration of glutaraldehyde: a, 3.2×10^{-2} *M*; b, 1.6×10^{-2} *M*; c, 8.0×10^{-3} *M*; d, 4.0×10^{-3} *M*.

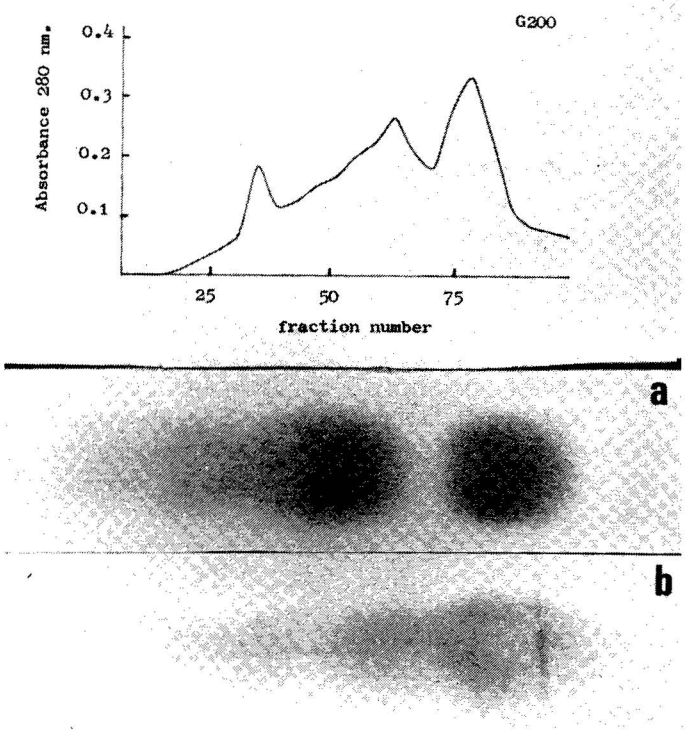

Fig. 2. Comparison of TLG and gel-filtration on a column. Top: reaction of albumin and α-amylase with hexamethylene diisocyanate at 38° for 2 h. Bottom: paper replicates stained for (a) protein and (b) α-amylase.

TABLE II

SUMMARY OF RESULTS FOR THE COUPLING OF α-AMYLASE AND ALBUMIN BY THE BIFUNCTIONAL COUPLING REAGENTS, *m*- AND *p*-PHENYLENE DIISOCYANATE, TOLUENE-2,4-DIISOCYANATE, AND HEXAMETHYLENE DIISOCYANATE

c Denotes the formation of high molecular weight products. Protein concentration, 20 g/l. Reaction mixtures contained equimolar proportions of α-amylase and albumin.

Coupling reagent	*Concentration (M)*	*pH*				
		6.6	*7.0*	*7.4*	*7.8*	*8.2*
p-Phenylene diisocyanate	5×10^{-3}				c	c
	1×10^{-2}			c	c	c
	2×10^{-2}	c	c	c	c	c
	4×10^{-2}	c	c	c	c	c
m-Phenylene diisocyanate	5×10^{-3}					c
	1×10^{-2}				c	c
	2×10^{-2}	c	c	c	c	c
	4×10^{-2}	c	c	c	c	c
Toluene-2,4-diisocyanate	5×10^{-3}				c	c
	1×10^{-2}	c	c	c	c	c
	2×10^{-2}	c	c	c	c	c
	4×10^{-2}	c	c	c	c	c
Hexamethylene diisocyanate	5×10^{-3}				c	c
	1×10^{-2}			c	c	c
	2×10^{-2}			c	c	c
	4×10^{-2}			c	c	c

ncreasing reagent concentration led to increased formation of high-molecular-weight products, whilst the combination of high pH and high reagent concentration produced insoluble products. The trend towards increased reaction at high pH can be understood from the mechanism by which diisocyanates couple proteins. Diisocyanates effect coupling principally via reaction with free amino groups on proteins. At low pH values such groups are protonated and therefore unreactive towards an isocyanate.

In the model system α-amylase–albumin the most reactive coupling reagents were found to be glutaraldehyde, cyanuric chloride, 1-ethyl-3-(dimethylaminopropyl)-carbodiimide (ECDI), toluene-2,4-diisocyanate (TDIC), and N-ethyl-5-phenyl isoxazolium-3′-sulphonate (Woodwards Reagent K). In contrast to the other coupling reagents listed in Table I, these five coupling reagents produced good yields of soluble high-molecular-weight products.

The low yield of conjugates which was observed with many of the bifunctional coupling reagents may be attributed first to the poor solubility of the reagents in aqueous media, although this was overcome in part by using the reagents dissolved in a water-miscible solvent, *e.g.* ethanol or dioxane, and secondly to the relatively unreactive nature of α-amylase as compared with albumin.

CONCLUSIONS

Design of successful coupling experiments requires an appreciation of (a) the specificity of the coupling reagent, and (b) the relative reactivity of the two proteins

to be coupled. The latter is related to the nature and number of functional groups on the protein available for reaction with the coupling reagent. TLG offers a method whereby the influence on a protein–protein coupling reaction of coupling conditions, type of coupling agent, and reactivity of the two proteins, may be rapidly assessed, thus guiding the selection of an appropriate coupling reagent and conditions for a particular pair of proteins.

For the system α-amylase–albumin the most reactive coupling reagents were found to be glutaraldehyde, cyanuric chloride, ECDI, TDIC, and Woodwards Reagent K.

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Journal of Chromatography, 118 (1976) 345–356

CHROM. 8908

GAS–LIQUID CHROMATOGRAPHY OF SEVERAL FAMILIES OF ISOMERIC 1,2,3-TRISUBSTITUTED CYCLOHEXANOLS AND CYCLOPENTANOLS AND THEIR ACETATES*

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(Received October 6th, 1975)

SUMMARY

The gas chromatographic retention times for several families of isomeric 1,2,3-trisubstituted cyclohexanols and cyclopentanols and their acetates have been determined on two columns (DEGS and QF-1) which differ in polarity and hydrogen bonding characteristics. The effects of acetylation, configuration, ring size, and column properties on the retention times of the 1,2,3-trisubstituted alcohols are examined.

INTRODUCTION

The relationship between chromatographic retention behaviour and molecular structure has been a widely discussed topic. The retention index system of Kováts[1] has been accepted as a general method of compound identification and has been recently investigated by Schomburg and Dielmann[2]. Ashton *et al.*[3] have found for extensive sets of isomeric dihalocycloalkanes that the order of elution of the isomers is the same (except for the fluoro compounds) and that individual isomers can be quickly identified from their retention times. Intramolecular hydrogen bonding has been shown to have a marked effect on the retention times of aminocyclohexanols[4] and nitrophenols[5].

In our studies on the formation and scission of 3-substituted cyclohexene and cyclopentene oxides[6–14], gas–liquid chromatography was used extensively and effectively both for preparative separations of stereoisomers and for the analysis of product mixtures. However, no systematic comparative examination was made of the gas chromatographic behaviour of the wide variety of stereochemically closely related 1,2,3-trisubstituted cyclopentanols and cyclohexanols resulting from the oxide scission

* Issued as DREO Report No. 735.
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reactions and the ensuing structural elucidation studies. In the work now to be described, the gas chromatographic retention times of these compounds were measured under identical conditions on two columns of widely differing polarities (DEGS and QF-1). The resultant data are discussed from the standpoint of the effect of functionality, location, and steric orientation of substituents on retention times and the possible utility of the latter for tentative structural assignments.

EXPERIMENTAL

An F & M Scientific Research Chromatograph, Model 5750 (Hewlett-Packard), equipped with a thermal conductivity detector (bridge current 150 mA) and a Model 3370 B electronic integrator (Hewlett-Packard), which provided a read-out of retention times, were used. Two columns (10 ft. × 1/8 in. O.D. stainless steel, DMCS treated) were employed. The solid support in both cases was Chromosorb G AW DMCS, 80–100 mesh, and the stationary phase was 4% DEGS in one column and 4% QF-1 in the other. Instrumental conditions were standardized as follows; temperatures: column 170°, injector 240°, detector 300°; carrier gas: helium, flow-rate 25 ml/min.

The compounds (10 mg) were dissolved in methanol (50 μl) and 1 μl of cyclohexanol was added as an internal chromatographic standard. Aliquots (1 μl) of the resultant solutions were injected into the chromatograph. A specific compound was selected with respect to each column (DL-1,3-di-O-methyl-(1,3/2)-1,2,3-cyclohexanetriol for the DEGS column; DL-2-O-acetyl-1,3-di-O-methyl-(1,3/2)-1,2,3-cyclohexanetriol for the QF-1 column) and repeated injections were made at random intervals to monitor the reproducibility of the method. The dead volumes for each column were determined by injecting 2-μl aliquots of air into each of the columns under the standard conditions of operation specified above.

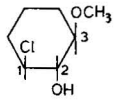
RESULTS AND DISCUSSION

The retention times reported in this paper have been adjusted for the appropriate dead volumes (*i.e.*, 0.72 min for the DEGS column and 0.74 min for the QF-1 column). Injections (15) of DL-1,3-di-O-methyl-(1,3/2)-1,2,3-cyclohexanetriol at random intervals on the DEGS column gave an average retention time of 4.92 $\pm$ 0.04 min. Similarly, twelve injections of DL-2-O-acetyl-1,3-di-O-methyl-(1,3/2)-1,2,3-cyclohexanetriol on the QF-1 column gave an average retention time of 7.51 $\pm$ 0.05 min. The retention time for cyclohexanol (internal standard) was 0.71 $\pm$ 0.02 min on DEGS and 0.49 $\pm$ 0.02 min on QF-1. Thus, retention times relative to cyclohexanol may be calculated for all the compounds by subtracting from the values shown in Tables I–IV the appropriate value for cyclohexanol.

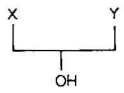
It is well known that hydroxyl groups in substituted cyclopentanols and cyclohexanols can form intramolecular hydrogen bonds with other functional groups (such as alcohols, ethers, halogens, etc.) when certain configurational conditions are met[15–20]. For example, both *cis*- and *trans*-1,2-cyclohexanediols (diequatorial *trans*-1,2) form intramolecular hydrogen bonds, and the phenomenon also occurs with *cis*- but not with *trans*-1,2-cyclopentanediols[15–17]. Also, *cis*- (diaxial *cis*-1,3 only) but not *trans*-1,3-cyclohexanediols[15] and, similarly, *cis*- but not *trans*-1,3-cyclopentane-

diols[21–23] form intramolecular hydrogen bonds. Consequently, it might be anticipated that families of compounds, which show a systematic positional and steric relationship between a hydroxyl group and another functional group capable of participating in intramolecular hydrogen bond formation, would show a corresponding systematic behaviour in chromatographic retention times. This behaviour should be especially true with polar columns which effect their separations mainly by intermolecular hydrogen bonding effects. The availability of a considerable number of 1,2,3-trisubstituted cyclohexanols and cyclopentanols of well defined geometry from our oxide ring opening studies[6–14] provided a ready means to examine the validity of this hypothesis.

The results of the investigation are given in Tables I–IV. Retention times are in minutes and are corrected for the instrument dead volume. The symbols C5 and C6 at the headings of the columns in the Tables indicate cyclopentane and cyclohexane compounds respectively. Horizontal rows (each of which represents compounds with the same configuration) have been designated by the capital letters A–E. Columns (each of which represents a family of isomers) have been designated by the small letters a–h. The numbers beneath the retention times are those assigned to individual compounds for identification purposes and increase consecutively from top to bottom of the columns. The symbols i, ii, ..., v indicate the elution order within a particular family of isomers. For example, a compound such as 9 would be listed in the Tables as having configuration A and shown in Table I, column c as C6, X = Cl, Y = OCH_3. It should also be noted in the Tables that X can be on position 1 or 2 while Y is always on position 3.



Compound 9



Configuration A

Retention times of the substituted cyclohexanols and cyclopentanols on DEGS (Table I)

Table I lists the retention times of substituted cyclohexanols and cyclopentanols on the DEGS column. The order of elution of isomers in the columns a–h is identical with one exception (in column f the order of elution of bromodiols 24 and 25 is the reverse of that found in the other columns). Therefore, the compound elution order on this polar column could be used to make tentative configurational assignments within similar types of isomeric families. As expected, the disubstituted compounds (configuration E) elute first since these have lower molecular weights and since there is one functional group less to interact with the column. The trisubstituted alcohols elute in the following configurational order, C < D < A < B.

It is well known that hydrogen bonding of alcohols to the column is a major factor in determining their retention times[4,5]. The infrared (IR) studies of Kuhn[15,16] have shown that in cyclopentane- and cyclohexane-diols hydrogen bonding strength has the following order *cis*-1,3(C6) > *cis*-1,2(C5) > *cis*-1,2(C6) > *trans*,1,2(C6). Darby *et al.*[21] have reported that a strong intramolecular hydrogen bond exists in *cis*-cyclopentane-1,3-diol. The frequency shift, and hence the strength of the hydrogen bond, for the bonded hydroxyl group (63 cm^{-1} to lower wave number compared with

TABLE I

RETENTION TIMES OF SUBSTITUTED CYCLOHEXANOLS AND CYCLOPENTANOLS ON DEGS

See text for explanation of symbols.

Configuration	*a; C6* $X = OCH_3$ $Y = OCH_3$	*b; C5* $X = OCH_3$ $Y = OCH_3$	*c; C6* $X = Cl$ $Y = OCH_3$	*d; C5* $X = Cl$ $Y = OCH_3$	*e; C6* $X = Br$ $Y = OCH_3$	*f; C6* $X = Br$ $Y = OH$	*g; C6* $X = OH$ $Y = OCH_3$	*h; C5* $X = OH$ $Y = OCH_3$
A) 	4.92 1,iv	5.45 5,iv	9.07 9,iv	7.77 14,iv	13.26 19,iv	33.52 24,v	13.14 28	23.69 31
(B) 	5.45 2,v	—	9.59 10,v	10.83 15,v	14.02 20,v	32.64 25,iv	—	—
(C) 	3.03 3,ii	2.15 6,ii	4.74 11,ii	2.98 16,ii	7.45 21,ii	22.82 26,ii	12.38 29	11.85 32
	—	3.85 7,iii	7.08 12,iii	5.06 17,iii	9.93 22,iii	32.60 27,iii	—	—
	1.56 4,i	1.66 8,i	2.50 13,i	2.64 18,i	3.84 23,i	3.84 23,i	8.17 30	8.96 33

the free hydroxyl) was about equal to that found by Kuhn[15,16] for *cis*-cyclopentane-1,2-diol (61 cm^{-1}). Sable *et al.*[22,23] have also reported the occurrence of intramolecular *cis*-1,3 hydrogen bonding in cyclopentane alcohols. No hydrogen bonding was detected by Kuhn[15,16] in the *trans*-1,3(C6) and the *trans*-1,2(C5) diols. In an IR study on hydrogen bonding in halohydrins, Nickon[18] found that hydrogen bonds for the *cis*-1,2 and *cis*-1,3 arrangements in the cyclohexane series are stronger than for the corresponding *trans* compounds. He also found that the bonding strengths between hydroxyl groups and halogens decrease in the order I > Br > Cl.

Although, as will be shown later, hydrogen bonding is important in explaining the retention times of the substituted cyclohexanols and cyclopentanols, other factors must be taken into account when considering absolute retention values. For example, even though the alcohols in rows C and D (which can form *cis*-1,2 and *cis*-1,3 hydrogen bonds) elute prior to the alcohols in rows A and B, where no *cis* hydrogen bonding is possible (diols in columns f, g and h excepted), on acetylation, the acetates in rows C and D generally still elute prior to those in rows A and B (Table II). Nor can the retention times be explained entirely in terms of configuration since the alcohols in row C with the functional groups arranged (3,2/1) are eluted second and the alcohols in row B which also have the groups arranged (3,2/1) are eluted last. The above situation differs from that found in the dihalocyclobutane, -cyclopentane, -cyclohexane and -cycloheptane isomers[3] where the order of elution of the isomers is the same for each set of dihalocycloalkanes (except for the fluoro compounds) (1,1- < *trans*-1,2- < *trans*-1,3- < *trans*-1,4- < *cis*-1,3- < *cis*-1,4- < *cis*-1,2-). That is, in these dihalo compounds, retention times vary regularly with configuration.

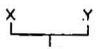
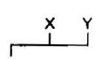
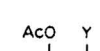
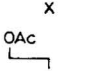
Hydrogen bonding effects become apparent when differences between retention times of alcohols of the same configurations in the C5 and C6 series are examined and during comparison of the retention times of the alcohols with those of their respective acetates. In row C (Table I), the cyclopentanols have smaller retention times than the corresponding cyclohexanols. This behaviour could be due to a lower molecular weight and to a stronger *cis*-1,2 hydrogen bond in the cyclopentane series[15,16]. Intramolecular hydrogen bonding would reduce the retention time by reducing the intermolecular hydrogen bonding to the column (DEGS forms strong intermolecular hydrogen bonds with alcohols as illustrated by the McReynolds constants[24]). In rows A, B, D, and E, except for compounds 9 and 14, 12 and 17, the cyclopentanols have longer retention times than the corresponding cyclohexanols. This behaviour is considered to be due to the fact that *trans*-1,2 intramolecular hydrogen bonding can take place in cyclohexanols but not in cyclopentanols[15,16].

When comparing the retention times of alcohols with the same configuration, the dimethoxy alcohols (columns a and b) have the shortest retention times, followed by the 3-methoxychlorohydrins (columns c and d; the retention times of compounds 16 and 3 are identical within experimental error), the 3-methoxybromohydrins (column e; the retention times of compounds 28 and 19 are identical within experimental error), the 3-methoxydiols (columns g and h), and then the bromodiols (column f). In agreement with the McReynolds constants[24], the importance of intermolecular hydrogen bonding on this column is shown here by the long retention times of the diols when compared with those of the other compounds of similar molecular weight. Thus, the retention times of the diols in columns g and h are much greater than those of the corresponding dimethoxy alcohols in columns a and b. Similarly,

TABLE II

RETENTION TIMES OF SUBSTITUTED CYCLOHEXANOL AND CYCLOPENTANOL ACETATES ON DEGS

See text for explanation of symbols. The values in parentheses indicate percentage change in retention time of the acetates relative to the corresponding alcohols (Table I).

Configuration	*a; C6* $X = OCH_3$ $Y = OCH_3$	*b; C5* $X = OCH_3$ $Y = OCH_3$	*c; C6* $X = Cl$ $Y = OCH_3$	*d; C5* $X = Cl$ $Y = OCH_3$	*e; C6* $X = Br$ $Y = OCH_3$	*f; C6* $X = Br$ $Y = OAc$	*g; C6* $X = OAc$ $Y = OCH_3$	*h; C5* $X = OAc$ $Y = OCH_3$
(A) 	9.16 (+86) 34,v	3.43 (−37) 38,iv	12.58 (+39) 42,v	4.58 (−41) 47,iv	17.76 (+34) 52,v	38.08 (+14) 57,v	19.27 (+47) 61	9.90 (−58) 64
(B) 	4.90 (−10) 35,iv	—	9.56 (0) 43,iv	6.66 (−39) 48,v	13.34 (−5) 53,iii,iv	25.95 (−20) 58,iii	—	—
(C) 	3.86 (+27) 36,ii	2.84 (+32) 39,ii	5.58 (+18) 44,ii	3.48 (+16) 49,ii	8.13 (+9) 54,ii	25.48 (+12) 59,ii	13.36 (+8) 62	10.14 (−14) 65
(D) 	—	2.90 (−25) 40,iii	9.33 (+32) 45,iii	3.90 (−23) 50,iii	13.35 (+34) 55,iii,iv	36.25 (+11) 60,iv	—	—
(E) 	1.91 (+22) 37,i	1.06 (−36) 41,i	2.99 (+20) 46,i	1.62 (−39) 51,i	4.62 (+20) 56,i	4.62 (+20) 56,i	5.95 (−27) 63	4.10 (−54) 66

the retention times of the bromodiols in column f are much longer than those of the corresponding methoxybromohydrins in column e. The above arguments become even more convincing when the retention times of the acetates are compared with those of the alcohols.

Retention times of the substituted cyclohexanol and cyclopentanol acetates on DEGS (Table II)

In contrast to the situation found in Table I, the retention times of the cyclopentane acetates on DEGS (Table II) are substantially shorter in every instance than those of the corresponding cyclohexane acetates (compare columns a and b, c and d, g and h). The values in parentheses beneath the retention times in Table II indicate the percentage change in retention time of the acetate relative to that of the corresponding alcohol on the same column. For the most part, the change in retention time on acetylation can be explained in terms of hydrogen bonding. In *trans*-1,2-disubstituted cyclohexanols (row E) intramolecular hydrogen bonding is possible[15,16]. On acetylation, the opportunity for hydrogen bonding no longer exists and one would expect an increase in retention time (also a small increase in retention time would probably be expected because of the increase in molecular weight). Thus, the disubstituted cyclohexane acetates 37, 46, and 56 exhibit increases in retention times of 20 to 22%. The negative change in retention time (−27%) for the cyclohexane diacetate 63 can be explained by the fact that in the diol one hydroxyl can bond intramolecularly while the other cannot.

In *trans*-1,2-disubstituted cyclopentanols intramolecular hydrogen bonds do not form and hence these alcohols form strong intermolecular bonds with the column. On acetylation, the formation of intermolecular hydrogen bonds with the column is no longer possible and thus the percentage change in retention times for the acetates 41, 51, and 66 relative to their alcohols are −36, −39, and −54%, respectively. The same reasoning explains the percentage changes in retention times found for the trisubstituted acetates in row A. That is, the presence of *trans*-1,2 intramolecular hydrogen bonds leads to positive changes in retention times for the trisubstituted cyclohexanols on acetylation (compounds 34, 42, 52, 57, and 61) while the absence of *trans*-1,2 intramolecular hydrogen bonds in the cyclopentanols leads to negative changes in retention times on acetylation (compounds 38, 47, and 64). These interpretations are substantiated by the negative change (−26%) in retention time exhibited on this column by *trans*-3-methoxycyclohexanol on acetylation (retention times of alcohol and acetate 3.04 and 2.25 min, respectively). Since no internal hydrogen bonding can take place in this alcohol, the change in retention time is negative.

Acetylation of the trisubstituted alcohols in row C (*cis*-1,2 hydrogen bonding can occur in both cyclohexanols and cyclopentanols), as expected, produces a positive percentage change in every case except for the diol 32, in which one hydroxyl can bond internally while the other cannot. In row D, the percentage change for the cyclohexanols is positive, as would be predicted, since both *trans*-1,2 and *cis*-1,3 hydrogen bonding are possible in the corresponding alcohols (the retention time data does not distinguish between the two). The negative percentage change for the cyclopentane acetates 40 and 50 is unexpected since a *cis*-1,3 hydrogen bond should be possible in the corresponding alcohols 7 and 17. Thus, it appears that a *cis*-1,3 intramolecular bond is not formed in the alcohols 7 and 17 (or at best a weak bond is

TABLE III

RETENTION TIMES OF SUBSTITUTED CYCLOHEXANOLS AND CYCLOPENTANOLS ON QF-1

See text for explanation of symbols.

Configuration	*a; C6* $X = OCH_3$ $Y = OCH_3$	*b; C5* $X = OCH_3$ $Y = OCH_3$	*c; C6* $X = Cl$ $Y = OCH_3$	*d; C5* $X = Cl$ $Y = OCH_3$	*e; C6* $X = Br$ $Y = OCH_3$	*f; C6* $X = Br$ $Y = OH$	*g; C6* $X = OH$ $Y = OCH_3$	*h; C5* $X = OH$ $Y = OCH_3$
(A) 	2.34 1,v	1.46 5,iv	3.43 9,v	1.76 14,iv	4.53 19,v	4.04 24,v	3.05 28	2.49 31
(B) 	2.27 2,iv	—	2.79 10,iv	2.14 15,v	3.55 20,iii,iv	3.89 25,iv	—	—
(C) 	1.66 3,ii	1.21 6,ii	2.19 11,ii	1.61 16,iii	3.17 21,ii	1.65 26,ii	3.01 29	2.15 32
(D) 	—	1.31 7,iii	2.75 12,iii	1.56 17,ii	3.55 22,iii,iv	3.80 27,iii	—	—
(E) 	1.04 4,i	0.64 8,i	1.23 13,i	0.78 18,i	1.59 23,i	1.59 23,i	1.66 30	1.16 33

formed). This conclusion is reinforced by the highly negative percentage change in retention time observed in proceeding from the diol 31 (in which *cis*-1,3 hydrogen bonding is a possibility) to the diacetate 64.

In row B, the negative value for the percentage change in retention time for compound 48 is as expected since no internal hydrogen bonds can form in the corresponding alcohol 15. In the bromodiol 25, which corresponds to the diacetate 58, one hydroxyl can bond internally while one cannot, leading to a negative value. For the cyclohexane series, the slightly negative or unchanged retention times for the acetates 35, 43, and 53 corresponding to the alcohols 2, 10, and 20 are the only examples, except for the diol cases discussed above, in which the values for the percentage changes in retention times are not positive. This behaviour is probably due to the fact that a sizeable proportion of the alcohols 2, 10, and 20 can exist in the conformation in which the hydroxyl group occupies the axial position. In this conformation, no intramolecular hydrogen bond can form.

When families of isomeric acetates are compared in the cyclohexane series, those with the same orientation elute in an order which approximates the increase in molecular weight. For example, in row A (Table II), the molecular weights of the cyclohexane acetates increase in the order $34 < 42 < 61 < 52 < 57$ whereas the retention times increase in the order $34 < 42 < 52 < 61 < 57$. For any row in Table II, the retention times of the cyclopentane acetates increase as the molecular weight increases. In contrast to the excellent separations of the alcohols (Table I), the retention times of acetates in isomeric families are often almost identical (*e.g.*, compounds 39 and 40, 43 and 45, 53 and 55, 58 and 59). Therefore, the elution order on DEGS in families of isomeric acetates of this type would be unsuitable for tentative structural assignments.

Retention times of the substituted cyclohexanols and cyclopentanols on QF-1 (Table III)

As anticipated, the retention times of the alcohols on this less polar column (Table III) are substantially shorter than on DEGS (Table I). The decreasing importance of hydrogen bonding on QF-1 is also shown by the fact that without exception the retention times of the cyclopentanols in Table III are shorter than those of the cyclohexanols. In addition, the retention times of the diols are often smaller than those found for the comparable chlorohydrins or bromohydrins (*e.g.*, $28 < 9$ and 19; $29 < 21$; $24 < 19$; $26 < 21$).

The increase in molecular weight is clearly becoming more important in determining the magnitude of the retention times than are hydrogen bonding effects. The elution order within a given isomeric series of alcohols on QF-1 more closely resembles that found for the acetates on DEGS than that found for the alcohols on DEGS.

Retention times of the substituted cyclohexanol and cyclopentanol acetates on QF-1 (Table IV)

From Table IV, it is evident that some difunctional acetates on QF-1 actually have longer retention times than the trifunctional acetates in the same series (*e.g.*, $37 > 35$ and 36; $46 > 43$; $56 > 53$). It is noteworthy that this phenomenon occurs only in the cyclohexane series and involves only trifunctional acetates in rows B and C which have two of the groups in a *cis*-1,2 relationship. The acetates in rows A and D, in which the three groups have an all *trans* relationship (all the groups can

TABLE IV

RETENTION TIMES OF SUBSTITUTED CYCLOHEXANOL AND CYCLOPENTANOL ACETATES ON QF-1

See text for explanation of symbols. The values in parentheses indicate percentage change in retention time of the acetates relative to the corresponding alcohols (Table III).

Configuration	*a; C6* $X = OCH_3$ $Y = OCH_3$	*b; C5* $X = OCH_3$ $Y = OCH_3$	*c; C6* $X = Cl$ $Y = OCH_3$	*d; C5* $X = Cl$ $Y = OCH_3$	*e; C6* $X = Br$ $Y = OCH_3$	*f; C6* $X = Br$ $Y = OAc$	*g; C6* $X = OAc$ $Y = OCH_3$	*h; C5* $X = OAc$ $Y = OCH_3$
(A) 	7.51 (+220) 34,v	2.71 (+86) 38,iv	8.86 (+158) 42,v	3.75 (+113) 47,iv	12.29 (+171) 52,v	22.54 (+458) 57,iii	16.73 (+448) 61	6.62 (+166) 64
(B) 	2.12 (−7) 35,ii	—	2.54 (−9) 43,i	4.97 (+134) 48,v	3.47 (−2) 53,i	17.62 (+354) 58,ii	—	—
(C) 	1.56 (−6) 36,i	2.23 (+84) 39,ii	5.07 (+132) 44,iii	2.90 (+80) 49,ii	6.25 (+97) 54,iii	23.50 (+1,324) 59,iv,v	13.53 (+350) 62	7.98 (+272) 65
(D) 	—	2.40 (+83) 40,iii	7.08 (+157) 45,iv	3.17 (+103) 50,iii	9.39 (+165) 55,iv	23.47 (+518) 60,iv,v	—	—
(E) 	2.37 (+128) 37,iii	1.30 (+103) 41,i	3.35 (+172) 46,ii	1.90 (+144) 51,i	4.35 (+174) 56,ii	4.35 (+174) 56,i	7.57 (+356) 63	4.52 (+290) 66

be equatorial), have substantially longer retention times than the difunctional acetates in row E. This unusual relationship in the cyclohexane series undoubtedly owes its origin to the known enhanced accessibility of the equatorial *vs.* axial substituents[25–27]. In the difunctional compounds, the two groups can both be equatorial, whereas in rows B and C the compounds exist as a mixture of two conformations in which either one or two of the functional groups may be axial. Thus, it would appear that a large proportion of the trifunctional acetates, which have shorter retention times than their difunctional counterparts, exist as the conformer with two axial groups. This observation is supported by the 220 MHz NMR coupling constants observed for these trifunctional acetates[28].

Recent conformational studies on cyclopentane compounds by Lambert *et al.*[29] (and refs. cited therein), suggest that there would be much less difference in the accessibility of cyclopentane functional groups to the column as configurations change than is the case in the cyclohexane series. This statement is supported by two observations from Table IV. First, the retention times of all trifunctional cyclopentane acetates in columns b, d, and h are substantially greater than those of the corresponding difunctional cyclopentane acetates (unlike the situation found in the cyclohexane series). Second, the differences in retention times within isomeric families of trifunctional acetates are much smaller in the cyclopentane series than in the corresponding cyclohexane series. For example, the retention times of the trifunctional cyclopentane acetates in column b, range from 2.23 to 2.71 min while for the corresponding cyclohexane acetates (column a), the retention times have a much larger range (1.56 to 7.51 min). Similarly, in column d, the range is from 2.90 to 4.97 min while in the corresponding cyclohexane acetates (column c) the range is much larger (2.54 to 8.86 min).

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Journal of Chromatography, 118 (1976) 357–362

CHROM. 8875

GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE MEASUREMENT OF METHYL UREA IN BLOOD AND URINE

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(Received October 13th, 1975)

SUMMARY

A technique for the measurement of methyl urea in biological fluids is described based upon gas–liquid chromatography of its trifluoroacetyl derivative. The method requires 10 ml of either blood or urine and is capable of measuring methyl urea at concentrations of less than 5 mg/l.

INTRODUCTION

In recent years a number of reports have appeared in the literature which have been concerned with the presence of methyl urea in body fluids. These communications have dealt with its investigation as a diuretic[1], a poison[2], a normal end product of metabolism[3], and as a metabolite of the antitumour agent hydroxyurea[4]. Unfortunately all these studies have been limited by the lack of a sensitive and specific assay for methyl urea. While its identification has been possible by use of thin-layer chromatography and high-voltage electrophoresis, followed by staining with *p*-dimethylaminobenzaldehyde[5], such a procedure is not readily applied to precise quantitation.

Only one serious attempt has been made to measure accurately concentrations of methyl urea in blood[6]. This technique used ^{14}C-labelled methyl urea as a tracer for the unlabelled compound and changes in blood concentrations were calculated from changes in total radioactivity. Results obtained therefore depended upon the assumption that the label remained within the parent molecule, a supposition which is by no means necessarily true.

It is the purpose of this article to report an assay system for methyl urea in body fluids suitable for the study of its biochemistry and pharmacology.

MATERIALS AND METHODS

Silica gel M.F.C. was obtained from Hopkin and Williams (Chadwell Heath, Essex, Great Britain) and trifluoroacetic anhydride (TFAA) from BDH (Poole,

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Dorset, Great Britain). Nonanoic acid methyl ester and urease were supplied by Sigma (Kingston-upon-Thames, Surrey, Great Britain).

A solution of urease was made up by grinding tablets (Type III, 80 units per tablet) in a volume of water sufficient to give a final enzyme concentration of 80 units/ml. The theoretical rate of hydrolysis of urea by 1 ml of this solution was 34 mg/min at 30° and pH 7.0.

The gas–liquid chromatographic procedure described by Evans[7] has been applied in which separation is achieved on a 7 ft. × 3/16-in.-I.D. column of 10% polyethylene glycol adipate on Diatomite CAW, 100–120 mesh (J.J.'s Chromatography, Kings Lynn, Norfolk, Great Britain). The column was housed in a Pye Model 104 gas chromatograph (Pye Unicam, Cambridge, Great Britain), incorporating a flame ionisation detector connected to a 1-mV recorder (Leeds and Northrup, North Wales, Pa., U.S.A.) with a chart speed of 10 in./h.

Chromatography was carried out isothermally at 130° using nitrogen as the carrier gas at a flow-rate of 40 ml/min.

Procedure

Measure 10-ml volumes of blood or urine into 30-ml glass tubes and add to each 1 ml of molar phosphate buffer, pH 7.0. To tubes which contain urine add 1 ml of urease solution and to those which contain blood 0.2 ml of urease. After careful mixing incubate all tubes at 37° for 1 h. At the end of this time wash the contents of the tubes into beakers with approximately 60 ml of absolute ethanol. Bring those beakers containing urine rapidly to the boil on an electrical hot plate and then filter the hot solutions through Whatman No. 1 filter paper into 150-ml flasks. Beakers containing blood should not be treated in this way but well mixed at room temperature before also being filtered into flasks. Thoroughly wash the filter papers with more ethanol and add the washings to the filtrate.

Place the flasks onto a rotary film evaporator (Type 319, from James Jobling, Stone, Staffordshire, Great Britain, has the advantage of being able to handle four flasks at once) and reduce the volumes of the alcoholic extracts to approximately 5 ml. Wash these solutions into further 30-ml glass tubes with more alcohol and reduce them to dryness at 60° under a stream of nitrogen.

Treat each deposit with 1 ml of methanol to dissolve any soluble residue and follow with 9 ml of chloroform. Centrifuge the tubes at 1200 *g* for 5 min and gently pour the resulting supernatants onto 10-cm columns of silica gel suspended in 10% methanol in chloroform and contained in 300-mm × 10-mm-I.D. glass columns each fitted with a stopcock and sintered glass disc. Repeat the extractions and apply the resulting solutions also to the columns. Run the 20 ml of extract into the columns and discard the eluate. Now elute the methyl urea by use of 80 ml of 15% methanol in chloroform, in each case collecting the eluate into a tube immersed in water maintained at approximately 70° such that the solvent rapidly evaporates as it leaves the column.

Remove any traces of solvent which remain by use of a stream of nitrogen and treat each residue with 5–10 drops of TFAA for 2–3 min, making sure that all traces of deposit come into contact with the acetylating reagent. Blow off excess TFAA with nitrogen and dissolve the solid which remains in 1.0 ml of ethyl acetate containing 0.015% v/v nonanoic acid methyl ester as internal standard. The resulting solution is ready for injection into the chromatograph.

Prepare standards for injection by taking to dryness under nitrogen 0–2 ml volumes of a 1 g/l solution of methyl urea in methanol. Treat with TFAA and dissolve in ethyl acetate containing internal standard as for the test solutions.

RESULTS

A standard curve for methyl urea is shown in Fig. 1. It has been obtained by plotting peak height, expressed as the ratio to that of the internal standard, against concentration for solutions corresponding to blood and urine concentrations of between 0 and 200 mg/l. It can be seen that the best straight line through the points does not go precisely through the origin. This divergence, though small, has been a consistent finding and its cause has not been conclusively established. It is, however, most likely to be due to loss of methyl urea during gas chromatography, the amount of loss being constant for each injection and unrelated to concentration.

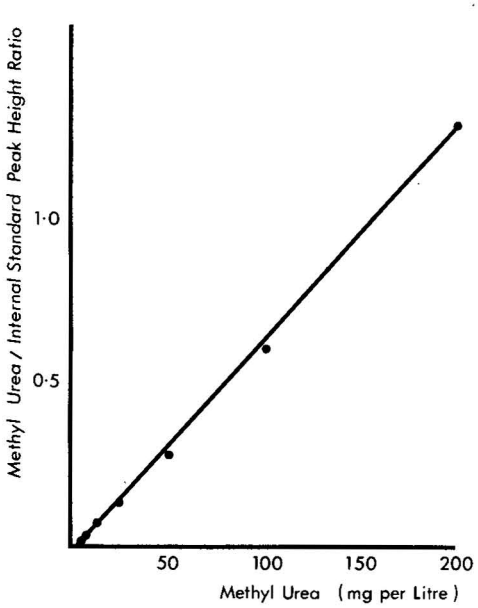

Fig. 1. Standard curve for methyl urea.

Because of this phenomenon, best results for the concentration of methyl urea in test solutions require reference to a standard curve. Less precise results can be obtained by use of the equation:

$$\text{Methyl urea concentration in blood or urine (mg/l)} = \frac{\text{Peak height ratio in test}}{\text{Peak height ratio in standard}} \times \frac{\text{Value of standard (mg/l)}}{10}$$

Investigations into the reproducibility of the chromatographic stage of the

analysis revealed a coefficient of variation of 3.1% ($N = 12$), while recoveries of methyl urea added to blood and urine at a concentration of 100 mg/l averaged for blood 96%, range 92–102% ($N = 12$), and for urine 94.5%, range 89.5–105% ($N = 12$).

Application of this method to specimens of blood and urine from twenty normal persons has in no instance revealed the presence of methyl urea at concentrations above the minimum detectable, approximately 4 mg/l under these conditions. Chromatograms typical of those obtained on normal specimens are shown in Figs. 2 and 3 with, for comparison, one obtained following the injection of 4 μl of ethyl acetate solution containing 4 μg of the methyl urea derivative (Fig. 4).

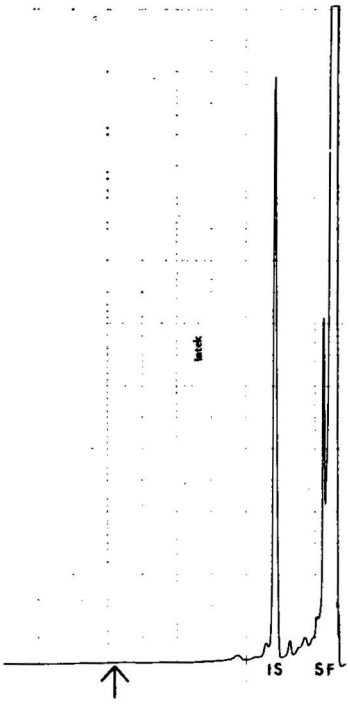

Fig. 2. Chromatogram obtained for a specimen of normal blood. SF = Solvent front; IS = internal standard. The arrow indicates the position expected to be occupied by methyl urea.

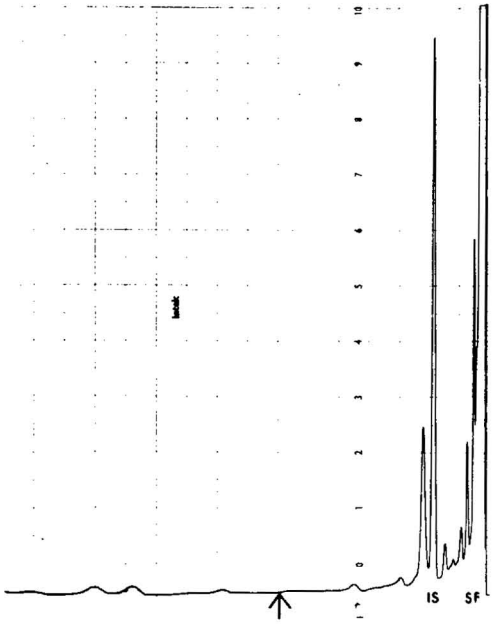

Fig. 3. Chromatogram obtained for a specimen of normal urine. SF = solvent front; IS = internal standard. The arrow indicates the position expected to be occupied by methyl urea.

DISCUSSION

Reiser[8] in 1964 was the first to apply gas–liquid chromatography to the separation of substituted ureas including methyl urea. His method did not involve the conversion of the ureas to more volatile derivatives with the result that in order to avoid serious loss of the materials which he was investigating chromatographic analyses had to be performed extremely rapidly using high carrier gas flow-rates, high temperatures and short columns. As a consequence his separations were frequently

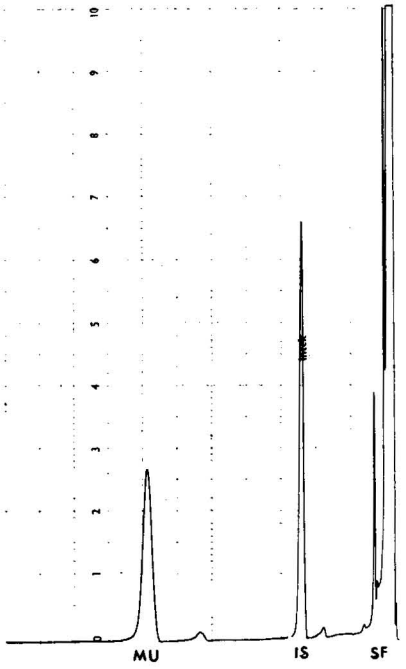

Fig. 4. Chromatogram obtained following injection of 4 μl of a standard solution containing 4 μg of methyl urea as its trifluoroacetate. SF = solvent front; IS = internal standard; MU = methyl urea.

poor, the method was insensitive and was unsuitable for quantitative measurements. The application of the trifluoroacetylation technique to methyl urea as described here has overcome many of these difficulties.

Using this technique twelve specimens can be processed ready for chromatography during the course of a normal working day. Since at the same time it would not be difficult to carry out chromatography on a further twelve specimens, the effective rate of analysis is twelve specimens per day despite the complete analysis taking nearly two days.

Methyl urea is itself extremely stable and in unpreserved blood or urine can be kept at 4° for at least two months without any signs of loss. Similarly, once having been extracted, it is stable indefinitely. Consequently, specimens can be stored for long periods of time if access to a chromatograph is limited. Once converted to its trifluoroacetate more care is required in the handling of methyl urea, since its susceptibility to breakdown in the presence of water is considerable. Stored in a desiccator away from contact with water it can be kept for many weeks, but under less stringent conditions of storage decay will begin rapidly.

Data quoted here have been based upon solution of methyl urea, following trifluoroacetylation, in 1 ml of internal standard solution. While the use of such a volume is necessary for concentrated and highly pigmented urines, smaller volumes can be used for blood and more dilute urines with a corresponding increase in sensitivity.

ACKNOWLEDGEMENTS

Grateful thanks are due to Dr. J. B. Holton for the provision of laboratory facilities.

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Journal of Chromatography, 118 (1976) 363–370

CHROM. 8805

THE DETERMINATION OF PHANQUONE IN BIOLOGICAL MATERIAL BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A gas–liquid chromatographic method for the quantitative determination of phanquone is described, based on the formation of a dimethoxime prior to its extraction from biological material. The sensitivity of the procedure is about 15 ng/ml in biological fluid.

INTRODUCTION

4,7-Phenanthroline-5,6-dione (phanquone) (I), the active principle of Entobex, marketed by Ciba-Geigy, Basle, Switzerland, cannot be extracted from biological material with the usual organic solvents. By treating the aqueous biological sample with methoxyamine, phanquone can be converted into the 5,6-dimethoxime (II), which is a stable, extractable compound that can be isolated by base specific extraction and subjected to quantitative gas–liquid chromatography (GLC).

In the organism, phanquone is partly reduced to 4,7-phenanthroline-5,6-dione (V), which shows the same pharmacological activity as the parent compound. As the hydroquinone, under standard reaction conditions used for the derivatization, yields the same derivative as phanquone, the levels determined in biological samples represent the sum of unchanged phanquone and its hydroquinone.

In order to correct for losses during derivatization, purification procedures and quantitative gas chromatography, known amounts of 10-methyl-4,7-phenanthroline-5,6-dione (C 14 574) (III) are added to the biological sample as an internal standard.

MATERIALS AND METHODS

Reagents

All the chemicals used were of analytical-reagent grade and were tested for purity by carrying out blank runs.

Extraction procedure and derivative formation

The procedure is carried out according to the following scheme:

To 1–3 ml of plasma or urine:

Add 0.5 ml of internal standard (*ca.* 600 ng/ml in 0.1 *N* HCl), 1 ml of buffer, pH 3 (67.8 g of citric acid, 25.9 g of NaOH, 17.4 g of KCl in 1000 ml of water) and 0.5 ml of 20% (w/v) solution of methoxyamine hydrochloride. Heat the stoppered tubes in a water-bath at 70° for 2 h.

↓

Add 1.5 ml of 1 *N* NaOH, 3000 ml of buffer, pH 10 (24.7 g of H_3BO_3, 29.8 g of KCl, 14.1 g of NaOH in 1000 ml of water) and 6 ml of toluene, shake for 15 min on a mechanical shaker at 200 strokes/min.

↓

Centrifuge for 5 minutes at 3 rpm (rotor radius = 7 cm). Transfer the organic phase into a clean tube and shake with 5 ml of 1 *N* H_2SO_4 for 15 min at 200 strokes/min.

↓

Aspirate and discard the organic phase. To the aqueous phase add 0.8 ml of 6 *N* NaOH, 1 ml of buffer, pH 10, and 5 ml of toluene. Shake for 15 min at 200 strokes/min.

↓

Transfer the organic phase into a clean tube and evaporate to dryness under a stream of nitrogen at 50°.

↓

Add 0.2–0.8 ml of toluene to the dry residue and inject aliquots of 2–5 μl into the gas chromatograph.

Gas chromatography

The instruments used were a Pye Unicam Model 74, Series 104, gas chromatograph with a pulsed (150 μsec) electron capture detector (^{63}Ni, 10 mCi), an Infotronics Model CRS 208 integrator and a W+W Model 1100 recorder. The columns were 5 ft. long, 2 mm I.D. (Pyrex glass) packed with 3% JXR on Chromosorb G, AW and DMCS, 80–100 mesh.

The temperatures used were: column oven, 210°; detector, 300°; injector, 220°. The carrier gas was nitrogen at a flow-rate of 30 ml/min. Under these conditions, the retention times of the derivatives of phanquone and the internal standard are about 10 and 13.7 min, respectively. The columns were conditioned for 24 h at 280° with a flow-rate of 15 ml/min.

It should be noted that amounts above 5 ng were not injected as the response ceases to be linear owing to saturation of the detector.

Calibration graph

A calibration graph for the analysis of phanquone in human plasma was prepared by adding phanquone and internal standard to fresh human plasma and following the above procedure. The internal standard (III) (288 ng per sample) and phanquone were each added as 0.5 ml of a solution in 0.1 *N* hydrochloric acid. The amounts added ranged from 66 to 500 ng of phanquone per 3-ml sample. The lowest concentration thus determined on this graph was 22 ng/ml. The peak area ratios were plotted against amounts (nanograms per sample) as shown in Fig. 1. Three separate determinations were made for each concentration. The deviation of individual values from the average did not exceed 10%.

The precision was tested by analyzing ten prepared plasma samples with concentrations unknown to the analyst. For each concentration, three independent analyses were carried out (Table I). Simultaneously, control analyses were performed with concentrations of 80, 300 and 500 ng per sample and the peak area ratios from

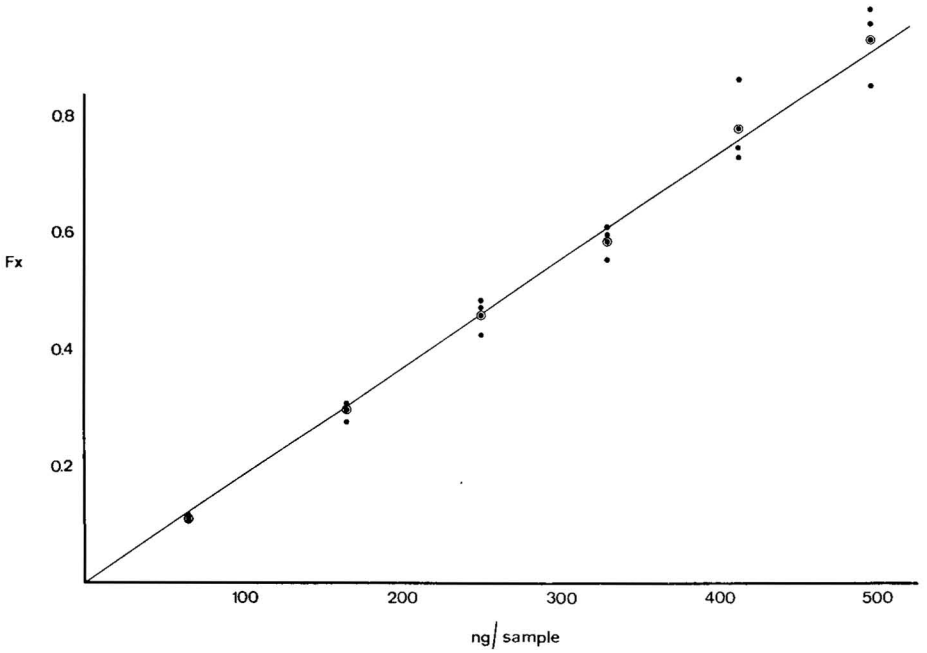


Fig. 1. Calibration graph for human plasma. Sample volume: 3 ml. Internal standard: 288 ng of C 14 574 per sample. Final residue, after analytical procedure, dissolved in 200 μl of toluene (1–5 μl injected). *Fx* = peak area of phanquone-dimethoxime/peak area of C 14 574-dimethoxime.

these test analyses were compared with the original calibration graph so as to ensure that the analytical system was stable.

TABLE I

ANALYSES OF SERUM SAMPLES WITH PHANQUONE CONCENTRATIONS UNKNOWN TO THE ANALYST

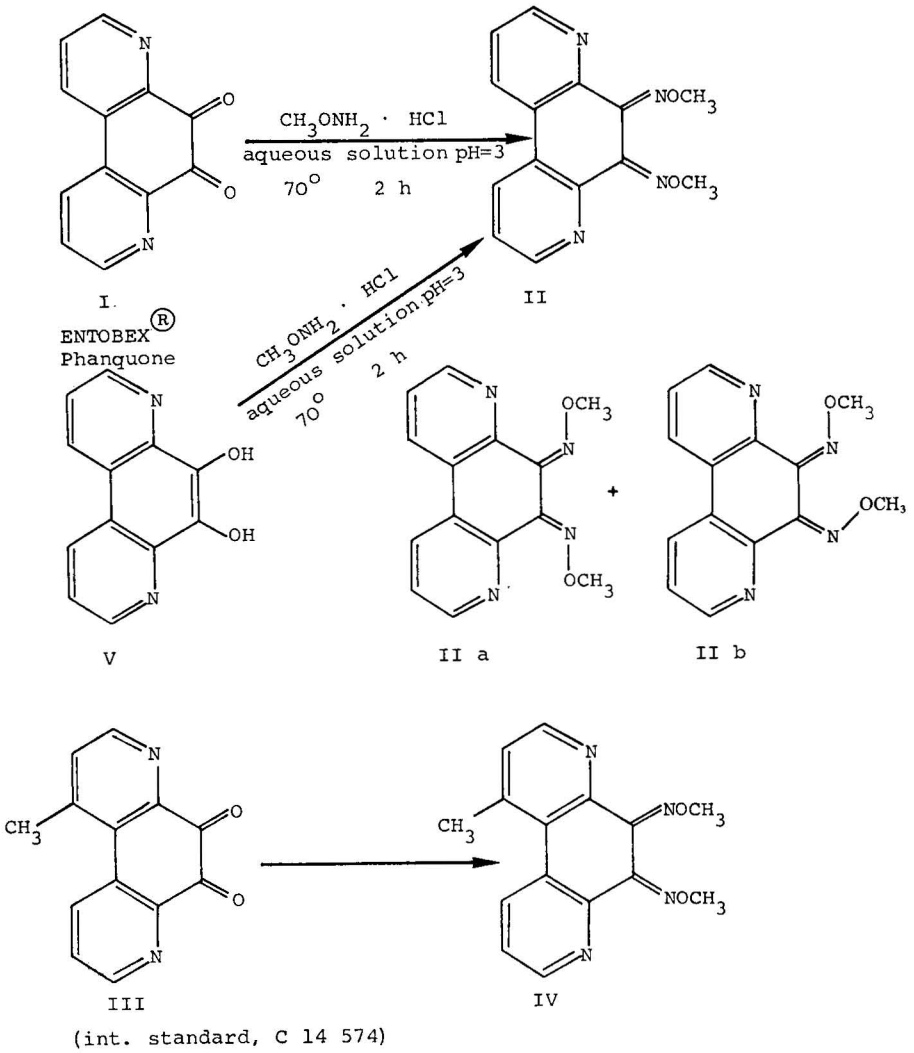
Each sample was analyzed in triplicate.

Sample No.	*Phanquone concentration present (ng/ml)*	*Phanquone concentration found (ng/ml)*		*Deviation of mean found value from concentration present (%)*
		Mean	*S.E.*	
1	0	0	0	0.0
2	135	122	2.0	− 9.6
3	33	33.6	0.7	+ 1.8
4	67.6	60	2.9	−11.0
5	16.5	18	0.6	+ 9.0
6	268	240	9.9	−10.0
7	0	0	0	0.0
8	122	142	7.2	+16.0
9	243	244	6.7	+ 0.4
10	76	62	5.0	−18.0

RESULTS AND DISCUSSION

Physical and chemical properties of phanquone dimethoxime

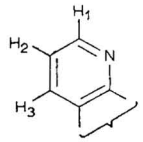
In order to ascertain the structures of the dimethoxime derivatives, 4,7-phenanthroline-5,6-dione (I) and 10-methyl-4,7-phenanthroline-5,6-dione (III) (Scheme 1) were subjected to derivatization in milligram amounts. The GLC of the products showed single peaks and the mass spectra were in accordance with structures II and IV. The dimethoxime (II) can be separated by thin-layer chromatography into two isomers (IIa and IIb), which can be identified by their NMR spectra. In the symmetrical isomer IIa, the two OCH_3 groups (and the protons) are equivalent. Isomer IIb shows no symmetry. Therefore, the two OCH_3 groups give rise to two different signals.



Scheme 1. Formation of the dimethoximes of phanquone and derivatives suitable for gas–liquid chromatography.

TABLE II

NMR DATA FOR THE TWO ISOMERS OF THE DIMETHOXIME (II)



Group	δ (ppm)			J (Hz)
	IIa	IIb		IIa
OCH_3	4.26	4.2	4.23	–
H_1 H'_1	8.71	7.75	7.73	$J_{(1,2)} = 4.5$
H_2 H'_2	7.43	7.42	7.42	$J_{(1,3)} = 1.5$
H_3 H'_3	8.15	8.15	8.14	$J_{(2,3)} = 7.5$

4,7-Phenanthroline-5,6-diol (V), which is known to be formed from phanquone, also leads to the 5,6-dimethoxime (II) when subjected to the reaction with methoxyamine (Scheme 1). Phanquone dimethoxime can be extracted from aqueous solution with several solvents (toluene, chloroform, ethyl acetate). Although extraction yields with chloroform and ethyl acetate are higher, toluene was chosen as the solvent as it extracts fewer impurities from biological material that interfere with the GLC determinations.

The pH dependence of the partition of phanquone dimethoxime between toluene and aqueous buffer solutions and plasma is shown in Fig. 2. Purification of extracts from biological material was effected by re-extraction into acid (0.1 *N* sulphuric acid) and back-extraction into toluene at pH 10.

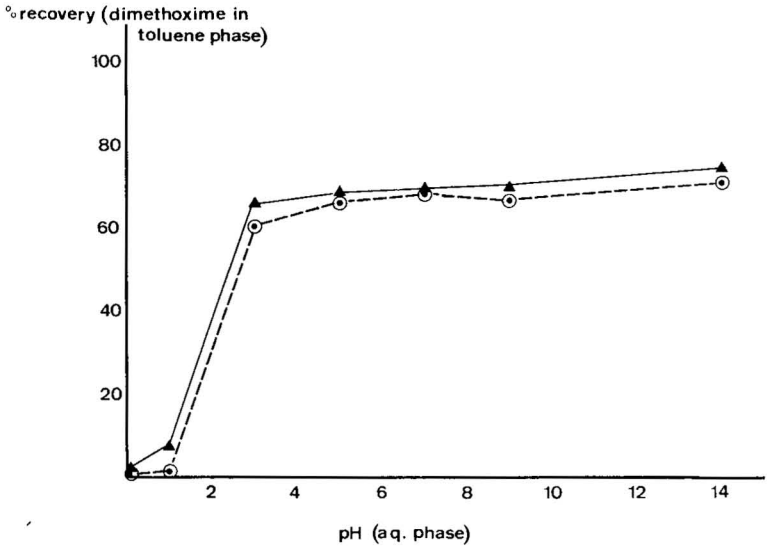


Fig. 2. pH dependence of the extractability of phanquone dimethoxime from water (○) and from plasma (▲). Conditions: 1 μg of ^{14}C-labelled phanquone dimethoxime, 6 ml of buffer solution (pH 0–14) and 10 ml of toluene.

Optimization of derivatization

^{14}C-labelled phanquone was used for all experiments carried out to optimize the reaction conditions. Reaction mixtures were extracted with chloroform at pH 6–7 and aliquots of the organic layer were measured for total radioactivity on a liquid scintillation counter. The percentage of dimethoxime in the reaction product was determined by thin-layer chromatography of the reaction mixture extracts on silica gel with chloroform–acetone (9:1) as eluent, followed by radiometry of the plates. Treatment of phanquone with methoxyamine hydrochloride in aqueous solution

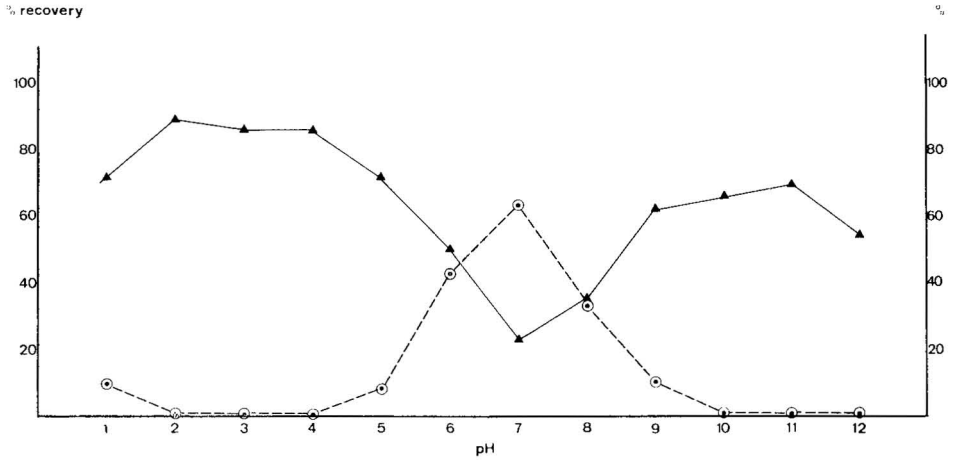


Fig. 3. pH dependence of the formation of mono- and dimethoxime derivatives. Conditions: 1 μg of phanquone in 1 ml of 0.02 *N* HCl, 100 ml of methoxyamine hydrochloride and 1 ml of buffer solution (pH 1–12); 2 h at 70°. ▲, Formation of dimethoxime; ⊙, formation of monomethoxime.

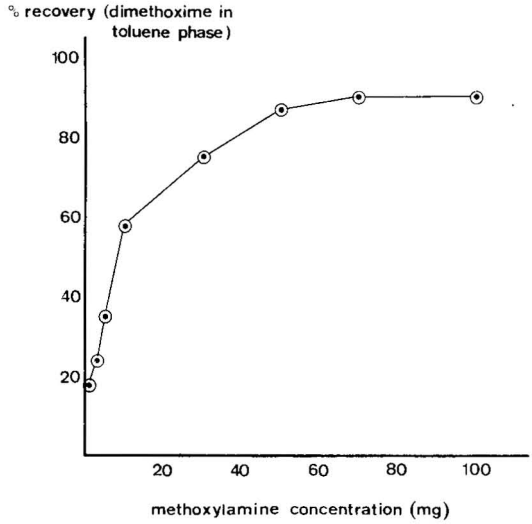


Fig. 4. Dependence of formation of phanquone dimethoxime on methoxyamine hydrochloride concentration. Conditions: 1 μg of phanquone in 1 ml of 0.02 *N* HCl, 1–100 mg of methoxyamine hydrochloride and 1 ml of buffer (pH 3.0); 2 h at 70°.

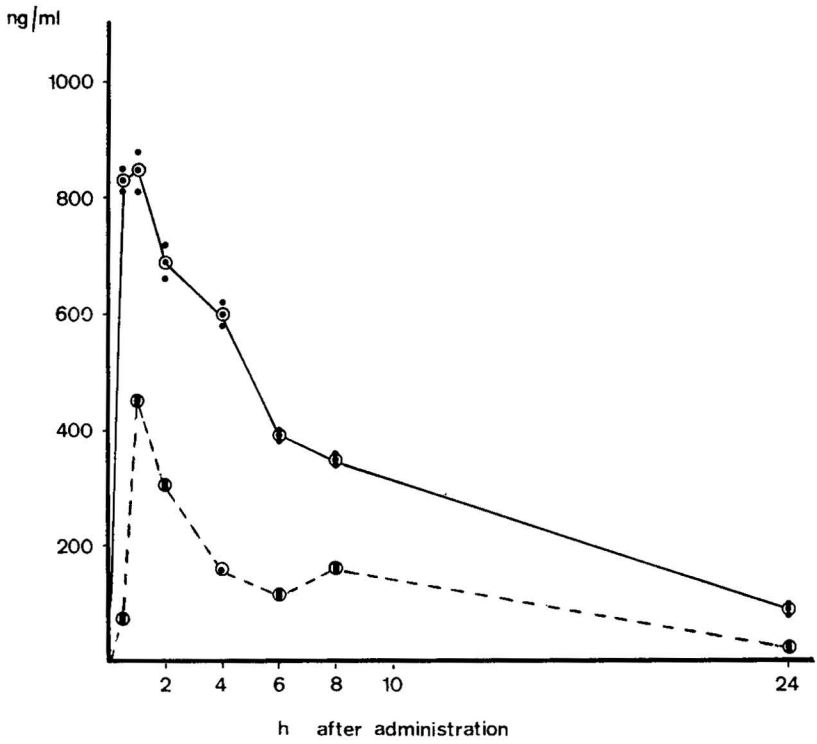


Fig. 5. Concentrations of unchanged phanquone in the plasma of two volunteers after oral doses of 50 mg (broken line) and 100 mg (solid line) of Entobex. Each sample was analyzed in triplicate.

leads to mixtures of the phanquone monomethoxime and dimethoxime, only the dimethoxime being suitable for the GLC determination.

The ratio of monomethoxime to dimethoxime in aqueous reaction mixtures is extremely pH dependent (Fig. 3). At pH 2–4, 85% of the reaction product consists of dimethoxime, whereas at pH 7 the monomethoxime is the major component (65%).

The reagent methoxyamine hydrochloride must be present in a very large excess (70–100 mg per 2 ml of sample, *i.e.*, 70,000–100,000 fold by weight) in order to obtain the dimethoxime in good yields (Fig. 4). Maximum yields are obtained after 2 h at 70°. Using standard optimal conditions (100 mg of methoxyamine hydrochloride in 1 ml of water, 1 ml of buffer solution (pH 3), 1 ml of phanquone solution, 2 h at 70°) for 100 ng of phanquone per sample, the yields of dimethoxime are approximately 80% from aqueous solutions, approximately 70% from plasma and 60% from blood.

Application of the method to the analysis of human plasma and urine

Plasma samples from two healthy human volunteers (A, 49 years, 73 kg; B, 35 years, 65 kg) were analyzed following the ingestion of A, 50 mg and B, 100 mg of phanquone (as commercial Entobex tablets).

Blood samples were collected at 0, 1/2, 1, 2, 4, 6, 8 and 24 h after ingestion of the dose. Immediately after collection, the blood samples were mixed with heparin

and centrifuged. The plasma samples were frozen until analysis. The samples were then processed as described above and chromatographed (Fig. 5).

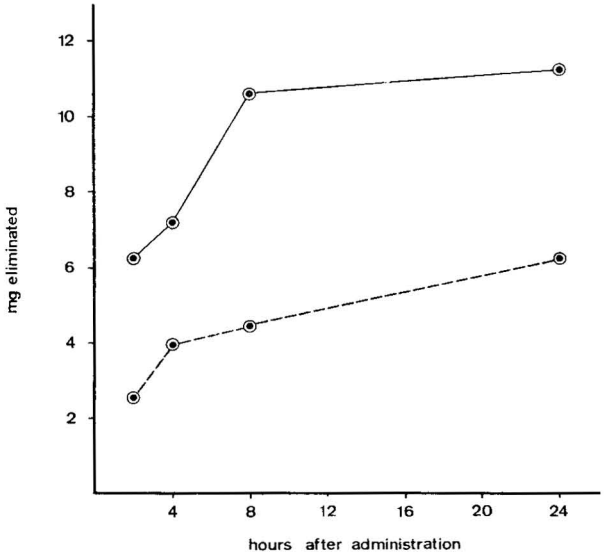


Fig. 6. Total amount of unchanged phanquone excreted in urine at 0, 2, 4, 8 and 24 h after administration of 50 mg (broken line) and 100 mg (solid line).

Urine samples were collected during the intervals 0–2, 2–4, 4–8 and 8–24 h after administration of the oral dose. The urine samples were also kept frozen until required for analysis. The total amount of unchanged phanquone excreted between 0 and 24 h was 12.5% of the dose after the 50-mg dose and 11.2% of the dose after the 100-mg dose (Fig. 6).

ACKNOWLEDGEMENT

We thank Dr. H. Sauter (Central Function Research, Ciba-Geigy) for providing and evaluating NMR Spectra.

Journal of Chromatography, 118 (1976) 371–386

CHROM. 8910

DETERMINATION OF 7-IODO-1,3-DIHYDRO-1-METHYL-5-(2′-FLUOROPHENYL)-2H-1,4-BENZODIAZEPIN-2-ONE (Ro 7-9957) AND ITS MAJOR BIOTRANSFORMATION PRODUCTS IN BLOOD AND URINE BY ELECTRON CAPTURE–GAS–LIQUID CHROMATOGRAPHY

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(Received November 3rd, 1975)

SUMMARY

A sensitive and specific electron capture–gas chromatographic assay was developed for the determination of 7-iodo-1,3-dihydro-1-methyl-5-(2′-fluorophenyl)-2H-1,4-benzodiazepin-2-one (I) and its major metabolites in blood and urine. The overall recovery of I and its N-desmethyl metabolite (II) from blood is apparently quantitative. The recovery of the major urinary metabolite, the N-desmethyl-3-hydroxy analog (IV), and the minor metabolites, the N-desmethyl analog (II) and the N-methyl-3-hydroxy analog (III) added to urine as authentic reference standards ranged from 80 to 85%. The sensitivity limits of detection are of the order of 2–3 ng of I and 4–5 ng of II per ml of blood or urine. The method was applied to the determination of blood levels and the urinary excretion pattern in a dog following oral and intravenous administration of a 1-mg/kg dose (total 13 mg), and in man following the intravenous administration of single 5- and 10-mg doses. The N-desmethyl metabolite II was more predominant in dog blood than was the orally or intravenously administered I, but II was barely measurable in human blood.

INTRODUCTION

The 1,4-benzodiazepine class of compounds is clinically important because of their extensive use as tranquilizers, hypnotics and muscle relaxants[1].

The compound 7-iodo-1,3-dihydro-1-methyl-5-(2′-fluorophenyl)-2H-1,4-benzodiazepin-2-one (I) is a new member of this series[2]. It differs from diazepam in having an iodo group instead of a chloro group in position 7 and a fluoro group in position 2′ of the 5-phenyl ring (Fig. 1). The compound is of clinical interest as an anticonvulsant agent in epilepsy[3].

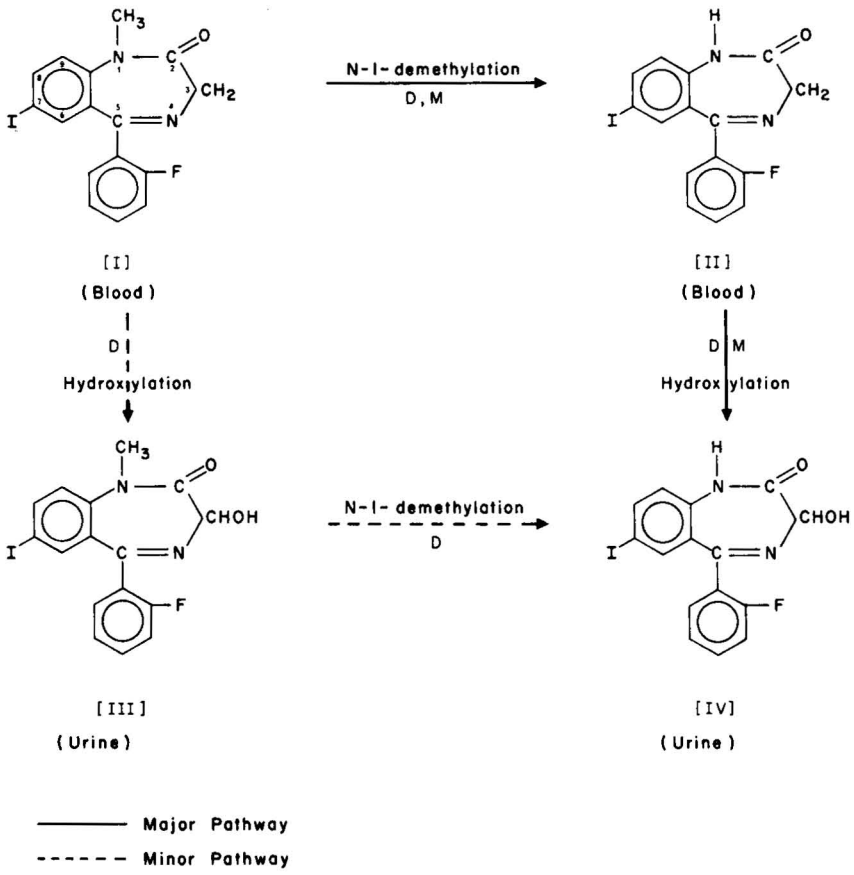

Fig. 1. Postulated pathways of the biotransformation of I in the dog (D) and in man (M) based on those reported for diazepam in these species[4,9].

Since I is analogous to diazepam its biotransformation in the dog and in man was expected to be similar to that of diazepam[4]. Authentic reference compounds of the expected metabolites were synthesized (Table I) and used to identify and quantitate the major metabolites of I in the dog and in man. It was found as expected that this compound I was rapidly and extensively metabolized producing measurable amounts of the N-desmethyl metabolite (II) in the blood and the N-desmethyl-3-hydroxy analog (IV) in the urine (Fig. 1).

The response of I and II to electron capture–gas–liquid chromatography (GLC–ECD) was sufficiently sensitive for quantitation in the nanogram range. The GLC assay reported here is a modification of that developed for medazepam and diazepam[5], and also employs the liquid phase OV-17 to resolve the intact drug I from its major metabolites in blood and urine. Diazepam is used as the reference standard and the high sensitivity of the ^{63}Ni electron capture detector (ECD) is used in the pulsed d.c. operational mode for the determination of I and its metabolites as the intact benzodiazepin-2-ones.

TABLE I

NAMES AND PHYSICAL PROPERTIES OF THE COMPOUNDS REFERRED TO IN FIGS. 1 AND 5

The 7-iodo-1,4-benzodiazepin-2-ones were synthesized by G. F. Field. The benzophenones were prepared from their respective benzodiazepin-2-ones by acid hydrolysis in 6 *N* HCl[2]. The 9-acridanones and quinazolinones were synthesized by R. I. Fryer using published procedures[10,11].

Type	*Compound*	*Chemical Name*	*Mol. wt.*	*M.p. (°C)*
1,4-Benzodiazepin-2-ones	I	7-Iodo-1,3-dihydro-1-methyl-5-(2′-fluorophenyl)-2H-1,4-benzodiazepin-2-one	395.15	107–110
	II	7-Iodo-1,3-dihydro-5-(2′-fluorophenyl)-2H-1,4-benzodiazepin-2-one	380.16	222–224
	III	7-Iodo-1,3-dihydro-3-hydroxy-1-methyl-5-(2′-fluorophenyl)-2H-1,4-benzodiazepin-2-one	410.19	188–190
	IV	7-Iodo-1,3-dihydro-3-hydroxy-5-(2′-fluorophenyl)-2H-1,4-benzodiazepin-2-one	396.16	184–187
Benzophenones	V	2-Methylamino-5-iodo-2′-fluorobenzophenone	355.16	97–100
	VI	2-Amino-5-iodo-2′-fluorobenzophenone	341.12	102–105
9-Acridanones	VII	2-Iodo-10-methyl-9-acridanone	335.15	194–196
	VIII	2-Iodo-9-acridanone	321.11	>350
Quinazolinones	IX	6-Iodo-4-(2-fluorophenyl)-1-methyl-2(1H)-quinazolinone	380.16	242–246
	X	4-(2-Fluorophenyl)-6-iodoquinazolin-2(1H)-one	366.14	>350

The method was applied to the determination of blood levels and the urinary excretion of I and its metabolites in a dog given intravenous and oral doses of 1 mg/kg, and in man following the intravenous administration of single 5- and 10-mg doses.

EXPERIMENTAL

Analysis for I and its major metabolites in blood

Parameters for GLC analysis

Column. The column packing was a pre-tested preparation containing 3% OV-17 on 60–80 mesh Gas-Chrom Q (Applied Science Labs., State Park, Pa., U.S.A.) packed in a U-shaped 4 ft. × 4 mm I.D. borosilicate glass column. The column was conditioned at 325° for 4 h with no flow of carrier gas, followed by 12 h at 275° with

carrier flowing at a rate of 40 ml/min. The useful life span of such a column was about 4–5 months of continuous use.

Instrumental parameters. A Micro-Tek Model MT-220 gas chromatograph, (Tracor, Austin, Tex., U.S.A.) equipped with a ^{63}Ni ECD containing a 15-mC ^{63}Ni β-ionization source was used. Argon–methane (9:1), oil-pumped and dry (Matheson Gas Products, East Rutherford, N.J., U.S.A.), was used as the carrier gas; the column head pressure was adjusted to 40 p.s.i.g. and the flow-rate to 120 ml/min with the detector purge gas adjusted to 20 ml/min. The temperature settings were as follows: oven 250°, injection port 280°, detector 325°. The conditions of column head pressure, flow-rate and oven temperature must be adjusted so as to obtain a retention time of 4.5 min for diazepam. Under these conditions the retention times of I and the N-desmethyl metabolite II are 8.0 and 11.4 min, respectively. A typical chromatogram is shown in Fig. 2. The solid-state electrometer (Micro-Tek, No. 8169) input was set at 10^2 and the output attenuation was 32 giving a response of 3.2×10^{-9} A for full-scale deflection (f.s.d.), the chart speed was 30 in./h, and the time constant on the 1.0-mV recorder (Model No. 194, Honeywell, Fort Washington, Pa., U.S.A.) was 1 sec (f.s.d.). The response of the ^{63}Ni ECD (operated in the pulsed d.c. mode) to I, II and the reference standard diazepam showed maximum sensitivity at 50 V d.c.

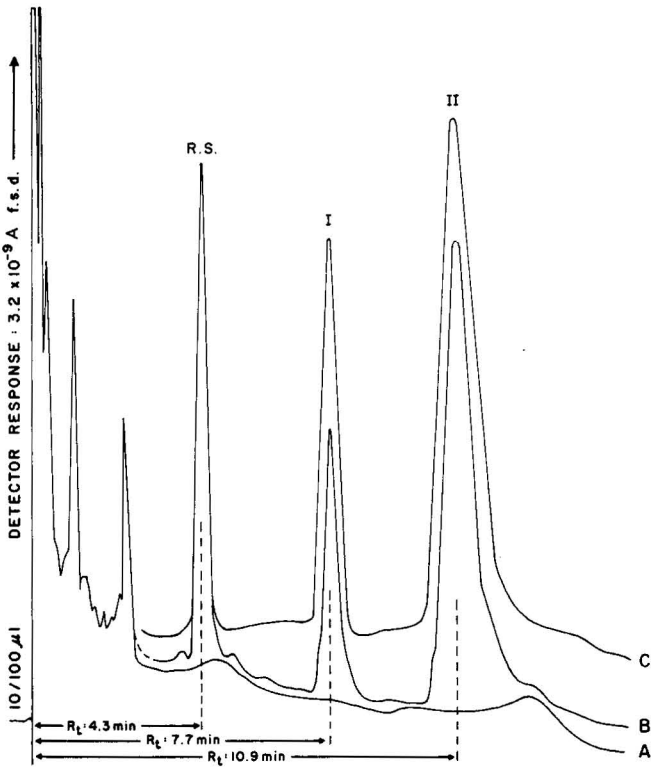

Fig. 2. Gas chromatograms of benzene–methylene chloride (9:1) extracts of A, control dog blood; B, control dog blood containing added authentic standards; C, dog blood post intravenous administration of I. R_t = retention time; R.S. = reference standard (diazepam).

with a 270-μsec pulse rate and a 4-μsec pulse width. Under these conditions 1.0 ng of I and 3 ng of diazepam give nearly full-scale deflection on the 1.0-mV recorder. The minimum detectable amount of I is 1–2 ng/ml of blood.

Assay in blood

Preparation of standard solutions. The respective benzodiazepin-2-ones that are required as analytical standards are listed in Table I. Weigh out 10.00 mg each of the free base of I and its N-desmethyl metabolite II into separate 10-ml volumetric flasks. Dissolve I in 1.0 ml of absolute methanol followed by 1 ml of absolute ethanol and make up to volume with acetone–*n*-hexane (1:4). Dissolve II in 2 ml of acetone and make up to volume with *n*-hexane. Prepare a stock solution of diazepam (10.00 mg) as described for I. These stock solutions A contain 1 mg/ml. Prepare working standard solutions B in acetone–*n*-hexane (1:4) containing a mixture of the authentic standards I and II and the reference standard (diazepam) in the following concentrations in separate 10-ml volumetric flasks as indicated in Table II.

TABLE II

CONCENTRATIONS OF STANDARD SOLUTIONS FOR ASSAY IN BLOOD

Figures in parentheses represent concentrations (ng per 10 μl) injected for GLC analysis.

Standard solution	*Concentration (ng/ml)*		
	I	*II*	*Diazepam*
B_1	25 (0.25)	50 (0.5)	300 (3.0)
B_2	50 (0.50)	100 (1.0)	300 (3.0)
B_3	75 (0.75)	150 (1.5)	300 (3.0)
B_4	100 (1.0)	200 (2.0)	300 (3.0)

Aliquots of solutions B_1 to B_4 (10 μl) are injected into the chromatograph to establish the ECD response to the compounds and the parameters for GLC analysis. Additionally, 100-μl aliquots of solutions B_1 to B_4 are added to blood as the internal standards to monitor the relative recovery with respect to the reference standard (diazepam).

The recovered internal standards are used to prepare a calibration curve (Fig. 3) for the quantitation of the concentrations of I and II in biological specimens. A new calibration curve has to be made with each set of unknowns.

Reagents. All reagents must be of analytical reagent grade (> 99% purity) and all inorganic reagents are made up in distilled de-ionized water. These include 1 *M* H_3BO_3–Na_2CO_3–KCl buffer (pH 9.0) prepared as previously described[5] and normal saline (0.9% NaCl in water). The organic solvents used are benzene and methylene chloride (nanograde) (Mallinckrodt, St. Louis, Mo., U.S.A.). Acetone is pesticide grade and *n*-hexane is 99 mol% pure "H-301" (Fisher, Pittsburgh, Pa., U.S.A.). A mixture of benzene–methylene chloride (9:1) is used as the solvent for

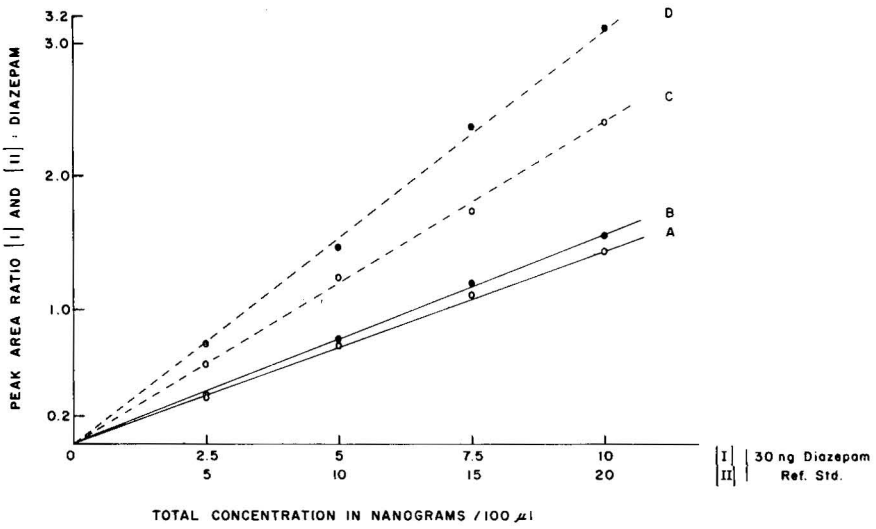

Fig. 3. ECD calibration curves of I, and its major blood metabolite II. A and B, external standard and internal standard curve of I, respectively. C and D, external and internal standard curve of II, respectively.

extraction, and a mixture of acetone–*n*-hexane (1:4) is used as the solvent for GLC–ECD analysis.

Procedure. Into a 15-ml conical centrifuge tube containing 30 ng of diazepam (added as a reference standard) add 1.0 ml of whole blood, 1 ml of normal saline solution (0.9% NaCl in water), 3 ml of borate buffer (pH 9.0) and mix well on a Vortex action mixer for 30 sec. Then add 8 ml of benzene–methylene chloride (9:1) for extraction. Along with the samples process a specimen of control blood (taken preferable from the subject prior to medication) and four 1-ml specimens of control blood to which 100 µl of solutions B_1, B_2, B_3 or B_4 are added to comprise a set of internal standards. Seal the tubes with PTFE stoppers and extract by shaking for 10 min on a reciprocating shaker (Eberbach, Ann Arbor, Mich., U.S.A.) at about 80–100 strokes per min. Centrifuge the samples for 10 min at 8–10° in a refrigerated centrifuge (Model PR-J with a No. 253 rotor; Damon/IEC Division, Needham, Mass., U.S.A.) at 2600 rpm (1500 *g*). Transfer a 7-ml aliquot of the supernatant into a fresh 15-ml centrifuge tube and evaporate to dryness at 60° in the water-bath of a N-EVAP Model No. N-07 evaporator (Organomation Assoc., Worchester, Mass., U.S.A.) under a stream of pure dry nitrogen. Vacuum dry the residue (over Drierite) in a vacuum desiccator for 10 min to remove all traces of moisture, dissolve the residue in 100 µl of acetone–*n*-hexane (1:4) and inject a suitable aliquot (5–10 µl) for EC–GLC analysis. The above method is limited to the analysis of 1 ml of whole blood or less. If greater sensitivity is required, then up to 4 ml of blood can be extracted with diethyl ether using a procedure which requires extensive clean up, details of which have been published[6].

The peaks due to the intact drug I, its metabolite II and the reference standard are identified by their respective retention times (Fig. 2), and their respective peak

areas are determined either by measuring peak height (cm) × width at half-height (cm) using the slope baseline technique or by electronic digital integration.

Calculations. The peak area ratios of I and II to diazepam in the aliquots of the unknowns injected are used to interpolate the concentrations directly from the respective internal standard curves (Fig. 3, curves B and D). The sensitivity of the GLC–ECD assay is of the order of 2–3 ng of I and 4–5 ng of II per ml of blood.

If further dilution of the unknown sample (*i.e.*, $< 100\ \mu l$) is necessary because of high levels of I and II, the reference standard may be diluted to such an extent that the peak area ratio technique becomes impractical. In this event direct calibration has to be employed whereby calibration curves of peak area of the recovered internal standards of I and II *vs.* concentration are plotted and used for the quantitation of the unknowns. Furthermore, the amounts of I and II per aliquot of the unknown sample injected have to be corrected for the dilution of the total sample.

Assay in urine

Preparation of standard solutions. Prepare a working solution C containing a mixture of 3.0 μg II, 25 μg III and 10 μg IV in 10 ml of acetone–*n*-hexane (1:4) by combining the appropriate aliquots of their stock solutions (A). A 10-μl volume of solution C contains 3.0 ng II, 25 ng III and 10 ng IV. Inject suitable aliquots of solution C into the gas chromatograph to establish the ECD calibration curves for each compound, and 100-μl aliquots are added to urine as internal standards for the quantitation of these metabolites.

Procedure. The sample preparation and extraction procedure used is similar to that described for diazepam[5]. Into a 50-ml erlenmeyer flask add 5.0 ml urine, 5 ml phosphate buffer (pH 5.3) and 0.1 ml of Glusulase (1 % of total volume). Along with the samples run a 5-ml specimen of control urine (taken preferably from the subject prior to medication) and duplicate 5-ml specimens of control urine containing 100 μl of C equivalent to 100 ng of IV, 30 ng of II and 250 ng of III added as internal standards. The samples are then incubated and processed exactly as described for diazepam[5] with one exception. The 6.0 *N* HCl extract must be neutralized carefully in an ice-bath by the dropwise addition of 6.0 *N* NaOH to prevent excessive heat of neutralization which could partially hydrolyze the compounds to their benzophenones. The sample is titrated to pH 9.0 using a pH meter. This is necessary to effect optimal extraction of the N-desmethyl metabolites II and IV.

Quantitation of the urinary metabolites. The presence of several metabolites in the urine results in a complex chromatogram (Fig. 4). Therefore, the direct calibration technique has to be used for quantitation. The analysis of a 5–10-μl aliquot usually results in a peak for IV which is off-scale thus necessitating further dilution prior to re-injection. The peaks for the N-desmethyl analog II and the N-methyl-3-hydroxy analog III, however, are on-scale and can be directly quantitated. The amount of each urinary component is calculated directly by comparison of the peak areas to that of the respective internal standards[5].

RESULTS AND DISCUSSION

Compounds I and II were quantitatively extracted from blood buffered to pH 7.0 and 9.0. Extraction of blood buffered to pH 9 with borate buffer gave "cleaner"

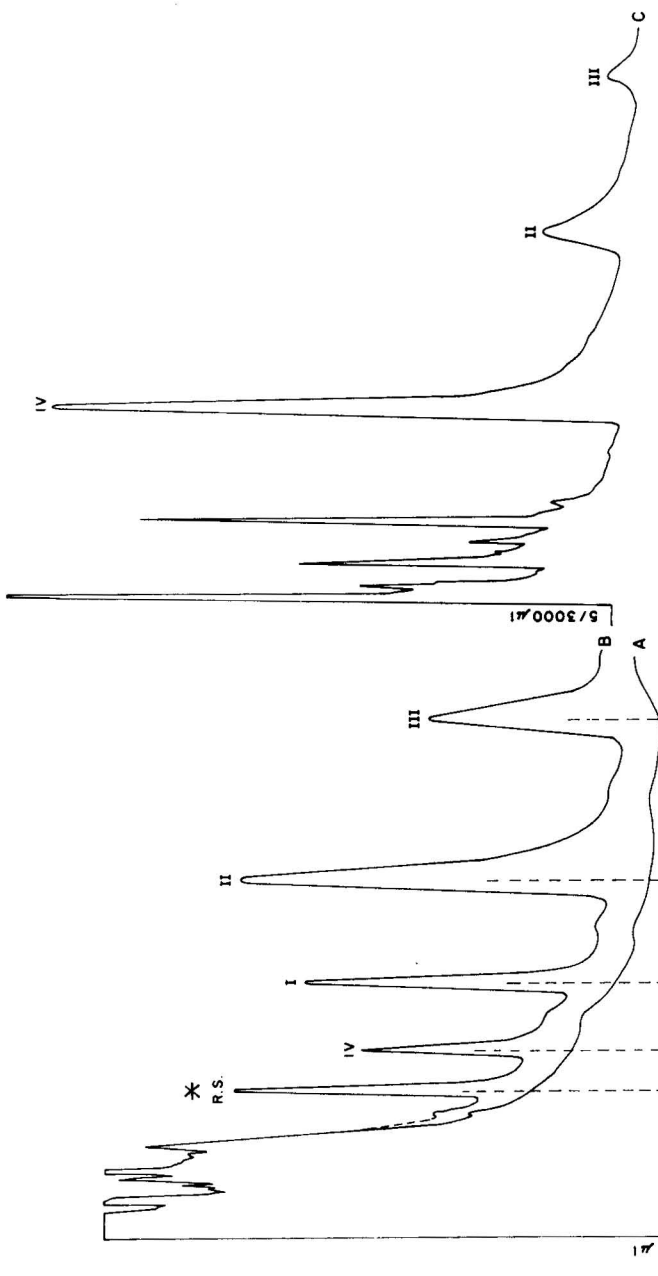

Fig. 4. Gas chromatograms of diethyl ether extracts of A, control dog urine; B, control dog urine containing added authentic standards; C, dog urine (0–24 h) post medication (intravenous or oral). R_t = retention time. * R.S. = reference standard (diazepam).

control chromatograms by GLC analysis and was preferred. When authentic standards of I and II in acetone–*n*-hexane (1:4) were analyzed by GLC, the two components were well resolved, the peak due to I was very symmetrical, whereas that due to II showed tailing. The chromatograms obtained from the addition of both compounds to either control blood or to 6 *N* HCl in the absence of blood were significantly different. By the former procedure, the peak due to I was eluted at the same retention time as before, but its sensitivity to the EC detector was enhanced. The peak due to II not only showed greater sensitivity to the ECD, but it was also eluted as a sharper, Gaussian-shaped peak with a retention time which was shorter by about 1 min than that of authentic II. By the latter procedure, the same observations were noted but to a lesser extent. These phenomena indicated the formation of either a chemical derivative or an adsorption complex, especially in the presence of lipids extracted from blood. Consequently, the recovery of I and II from blood when determined against an external standard curve of the pure authentic compounds I and II is greater than 100%; mean, 106% ± 5.0 for I and 112% ± 5.0 for II (Fig. 3, curves A and C). It is possible that the extracted lipids either interact with exposed active sites on the column and reduce surface adsorption effects through hydrogen bonding or form an adsorption complex with the amide bond $-\overset{H}{\underset{|}{N_1}}-\overset{O}{\underset{\|}{C_2}}-$ of the N-desmethyl metabolite II, thus minimizing its adsorption on the column. The use of internal standards of I and II added in varying concentrations to blood and taken through the entire procedure is essential for obtaining valid quantitation of I and II in unknown specimens.

The known chemical reactions and derivatives of I and II are shown in Fig. 5. Both compounds undergo hydrolysis in strong acid at 100° to their respective benzophenones which can be cyclized in dimethylformamide (DMF) in K_2CO_3 at 100° to the 9-acridanone derivatives[7]. Furthermore, the N-desalkyl-1,4-benzodiazepin-2-ones undergo thermolytic rearrangement during GLC analysis to form quinazolines[8] which could be responsible for the anomalous behavior of II. Authentic reference standards of the quinazolinones IX and X were synthesized and used as analytical standards to test this hypothesis. It was found that neither the respective benzophenones, the 9-acridanones, nor the quinazolinones were the derivatives formed during GLC analysis. Consequently, the chemical nature of the derivatives and/or complexes formed has yet to be elucidated.

The determination of the actual percentage recovery is not necessary for purposes of quantitation on a routine basis, because the internal standards taken through the entire assay give valid analytical data since they automatically compensate for these anomalies and for the recovery of both components in the unknowns. The added reference standard diazepam (whose recovery is > 95%) serves as the index of any procedural variation incurred in sample processing.

Application of the method in biological specimens

Studies in the dog. The blood levels and the urinary excretion of I and its major metabolites were determined in a dog following the administration of a single 1-mg/kg dose (total 13 mg) by intravenous (I.V.) and oral routes. The blood level curves are shown in Fig. 6. Following I.V. administration the intact drug I was measurable for

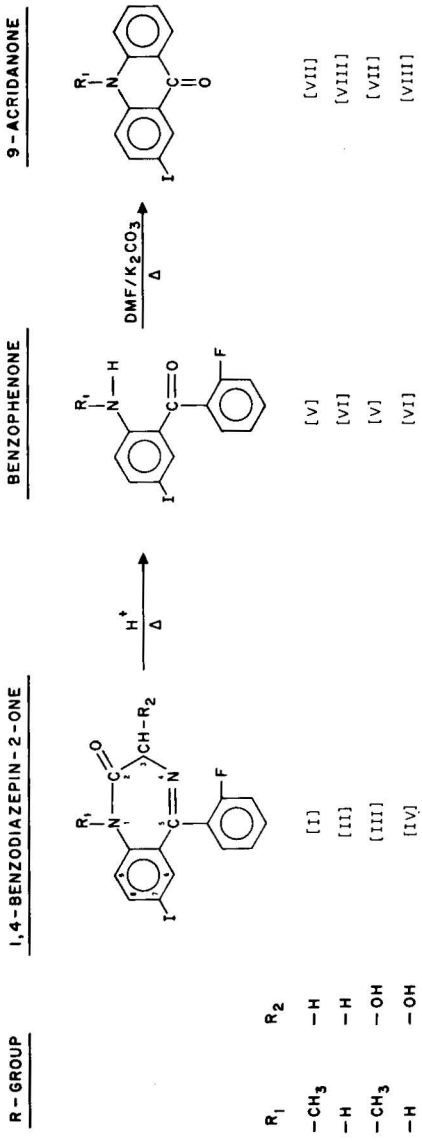

Fig. 5. Reactions of I and its major metabolites.

up to 24 h. The concentration of I declined very rapidly from a peak level of 1.3 μg/ml at 1 min to threshold levels at 12 and 24 h. The N-desmethyl metabolite II was seen almost immediately after the I.V. administration of I; its levels reached a peak (0.40 μg/ml) at about 10 min and declined progressively with time with a half-life of about 6 h.

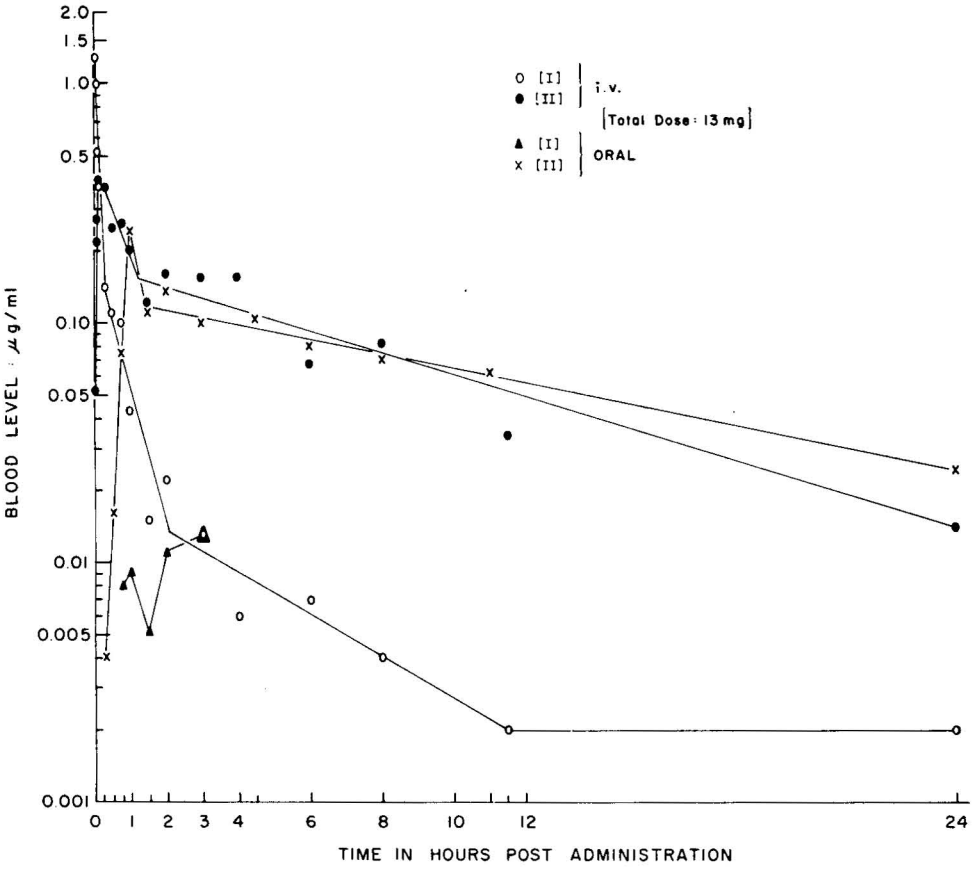

Fig. 6. Blood level fall-off curves of I and its N-desmethyl metabolite II following a single 1 mg/kg dose by oral and intravenous (i.v.) routes in the dog.

Following oral administration, the intact drug was seen in very low amounts from 45 min to 3 h post dosing whereas II was again the major blood component. It was measurable at 15 min, reached a peak at 1 h (0.25 μg/ml) and declined progressively with time, with a half-life of about 10 h. The blood levels of II resulting from the i.v. or oral administration of I were very similar indicating both rapid absorption of I and rapid biotransformation to II.

The urinary excretion data from specimens collected over a 48-h period in the same animal are shown in Table III. The major metabolite was the N-desmethyl-3-hydroxy analog IV which accounted for 3.83% of the dose following i.v. and 4.11%

TABLE III

URINARY EXCRETION DATA ON I AND ITS METABOLITES IN A DOG

N.M. = Not measurable: <0.002 μg I, <0.004 μg II, <0.050 μg III, <0.020 μg IV, per ml of urine.

Dose	*Route*	*Excretion period (h)*	*Excreted (μg)*			
			I	*II*	*III*	*IV*
1 mg/kg (13 mg)	Intravenous	0–24	N.M.	2.8	8.3	385
		24–48	N.M.	1.7	N.M.	113
		Total	...	4.5	8.3	498
		% of dose excreted	—	0.04	0.06	3.83
1 mg/kg (13 mg)	Oral	0–24	N.M.	3.4	11.1	408
		24–48	N.M.	N.M.	N.M.	126
		Total	—	3.4	11.1	534
		% of dose excreted	—	0.03	0.08	4.11

of the dose following oral administration. No measurable amounts of the intact drug I were seen in any of the urine specimens. The N-desmethyl analog II and the N-methyl-3-hydroxy analog III each accounted for less than 0.1% following i.v. and oral administration. The low amounts of the metabolites recovered suggest either extensive tissue distribution and/or an alternate route of elimination.

Characterization of the blood and urinary metabolites in the dog by thin-layer chromatography. Two-dimensional thin-layer chromatography (TLC) was used to confirm the identity of I and its metabolites seen in the dog. The acetone–*n*-hexane solutions (remaining after GLC analysis) of the diethyl ether extracts of blood and urine following oral dosing were pooled, concentrated by evaporation and transferred onto Merck F_{254} pre-coated silica gel G/F chromatoplates. The plates were developed in vapor saturated chambers (lined with Whatman No. 1 paper) in two dimensions using chloroform–heptane–ethanol (5:5:1) in the first dimension and heptane–ethyl acetate–ammonia (50:50:1) in the second dimension. Authentic standards of I, II, III and IV were run as markers along the margins.

The chromatogram of the pooled blood specimen representing 26 ml showed the presence of only the intact drug I and its N-desmethyl analog II as ultraviolet (UV) absorbing spots when viewed under a short-wave UV lamp (254 nm), thus confirming the components seen by GLC–ECD.

The chromatogram of the pooled urine specimen following i.v. and oral administration representing 10 ml each was run in one dimension in chloroform–heptane–ethanol (5:5:1) and showed the presence of trace amounts of the N-desmethyl metabolite II, and the N-methyl-3-hydroxy metabolite III. The major component was the N-desmethyl-3-hydroxy analog IV, again confirming the GLC findings.

Studies in man. Four healthy adult volunteers, three males and one female ranging in age from 21 to 51 years with no previous history of benzodiazepine therapy were selected. Two subjects (V. J. and S. S.) were administered a single 5 mg i.v. dose of I while the other two (K. K. and S. K.) were administered a single 10 mg i.v. dose of I. Blood specimens were drawn prior to administration (0-h control),

TABLE IV

BLOOD LEVELS (μg/ml) OF I AND ITS N-DESMETHYL METABOLITE II IN 4 PATIENTS FOLLOWING INTRAVENOUS ADMINISTRATION OF SINGLE 5- AND 10-mg DOSES

Doses = patient V.J., 5 mg = 0.05 mg/kg; S.S., 5 mg = 0.082 mg/kg; K.K., 10 mg = 0.116 mg/kg; S.K., 10 mg = 0.120 mg/kg. Ages: V.J. (female), 51 year; S.S (male), 21 year; K.K. (male), 21 year; S.K. (male), 23 year. N.M. = Not measurable: <0.001 μg/ml for I and <0.002 μg/ml for II. N.S.T. = No sample taken.

Time	*V.J.*		*S.S.*		*K.K.*		*S.K.*	
	I	*II*	*I*	*II*	*I*	*II*	*I*	*II*
0 h	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
5 min	0.59	N.M.	0.240	N.M.	1.54	0.002	2.56	0.004
10 min	0.30	N.M.	0.170	N.M.	0.51	0.003	0.47	0.004
15 min	0.18	N.M.	0.150	0.002	0.36	0.003	0.41	0.004
20 min	0.10	N.M.	0.130	0.002	0.35	0.004	0.23	0.005
25 min	0.09	N.M.	0.120	0.002	0.26	0.003	0.19	0.005
30 min	0.06	N.M.	0.100	0.002	0.24	0.003	0.18	0.005
45 min	0.05	N.M.	0.089	0.002	0.20	0.004	0.17	0.005
1 h	0.05	N.M.	0.066	0.002	0.20	0.004	0.16	0.005
1.5 h	0.04	N.M.	0.062	0.002	0.14	0.004	0.15	0.006
2 h	0.03	N.M.	0.054	0.002	0.13	0.004	0.13	0.006
3 h	0.02	N.M.	0.058	0.003	0.11	0.004	0.11	0.006
4 h	N.S.T.	N.S.T.	N.S.T.	N.S.T.	N.S.T.	N.S.T.	0.13	0.007
6 h	N.S.T.	N.S.T.	N.S.T.	N.S.T.	N.S.T.	N.S.T.	0.18	0.006
24 h	0.04	0.004	0.086	0.012	0.13	0.015	0.09	0.017

and at the specific time points as shown in Table IV. A control urine specimen and a 0–24 post dosing specimen were also collected from each subject.

The blood level data on the four subjects are given in Table IV and plotted graphically in Figs. 7 and 8, respectively. Measurable blood levels of the intact drug were seen in all four subjects at the first sampling time point of 5 min after administration and were measurable throughout the 24-h experimental period. The blood level curves of I showed either a biphasic or triphasic fall-off pattern with a secondary peak seen at 6–24 h. The initial phase was seen in the 0–30 min period with apparent half-lives ranging from 5 to 18 min, while the second phase seen in the 0.5–3-h period in the four subjects indicated apparent half-lives ranging from 1.5 to 3.5 h during this time period. Additional blood samples were drawn at 3 and 6 h from the fourth subject (S. K.) and an increase in the blood levels of I was seen at these times. Therefore, in the other three subjects the blood levels of I may also have increased between 3 and 24 h. This rise may reflect recirculation from tissue storage depots. In sharp contrast to the dog, in man the N-desmethyl metabolite II was seen in trace amounts in the blood ranging from 2 to 7 ng/ml in three out of the four subjects over virtually the entire experimental period. It is interesting to note that the levels of this metabolite at the 24-h point were higher than at any other point in time.

The 0–24 h urinary excretion of I and its major metabolites in these four subjects are given in Table V in terms of total amount (μg) excreted, comprising the "free" or directly extractable fraction represented by I and II and the "bound" or glucuronide-sulfate conjugated fraction represented by the 3-hydroxy analogs III and IV. The latter metabolites are extractable only after enzymatic deconjugation with

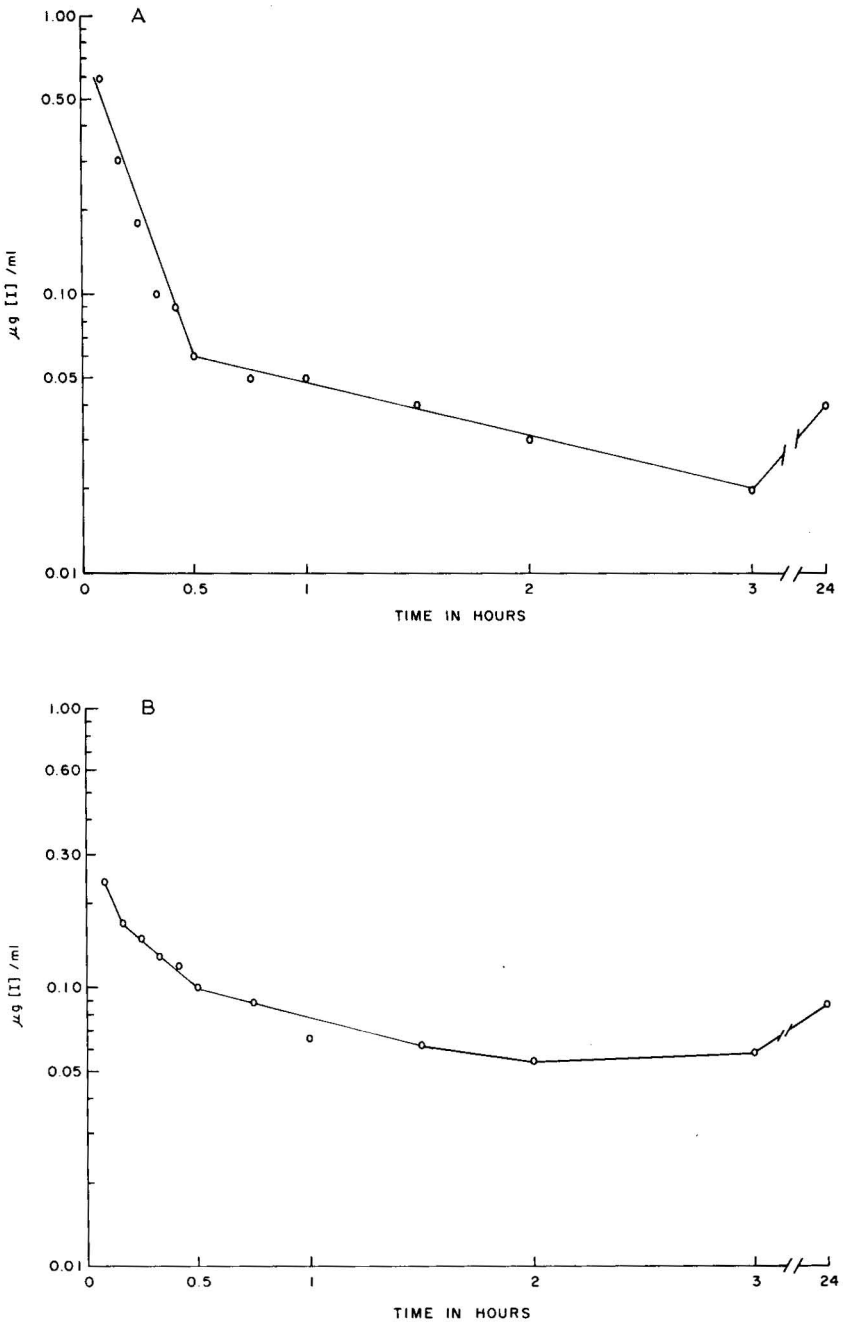

Fig. 7. Blood level fall-off curves of I in (A) patient V.J. and (B) patient S.S. following the intravenous administration of a single 5-mg dose.

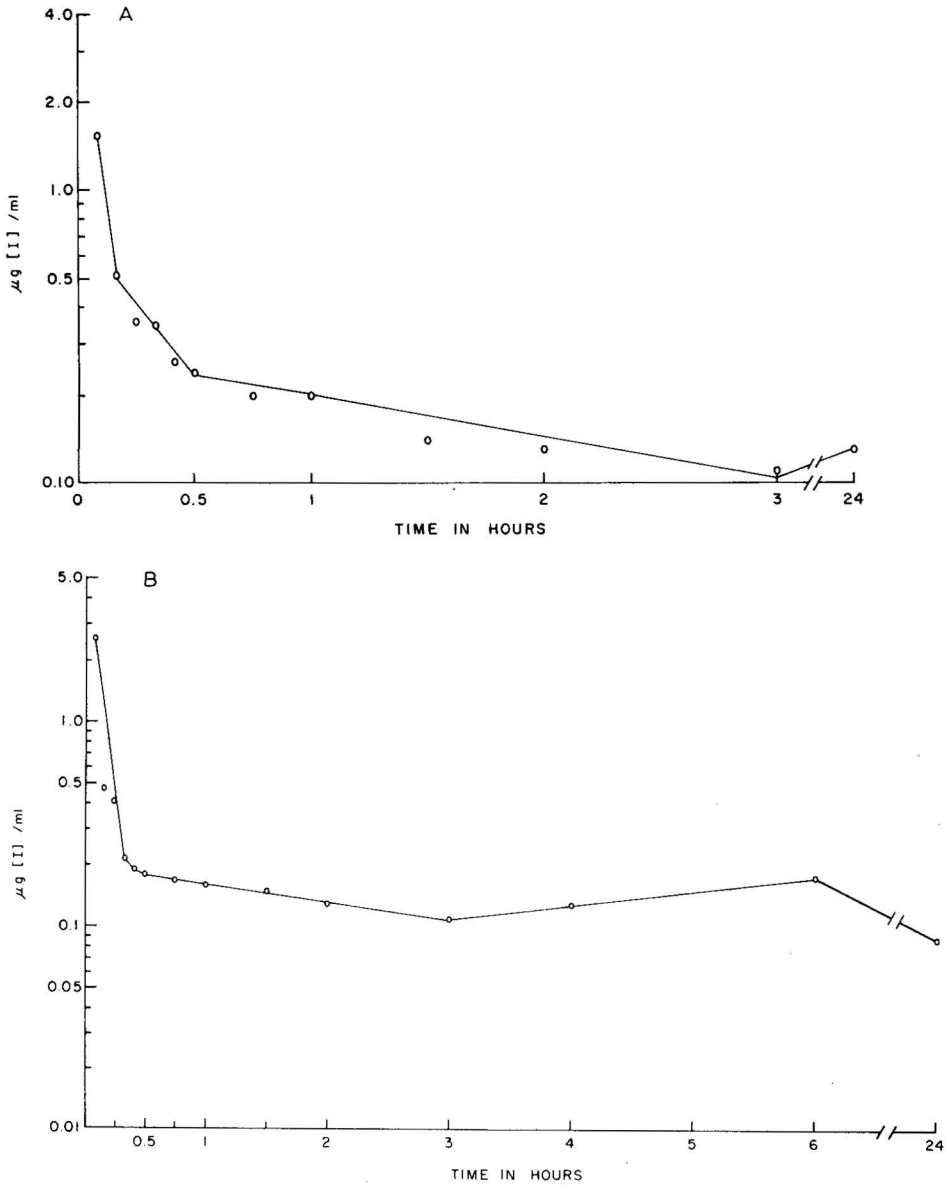

Fig. 8. Blood level fall-off curves of 1 in (A) patient K.K. and (B) patient S.K. following the intravenous administration of a single 10-mg dose.

Glusulase®. The total percent of the administered dose recovered was quite low and ranged from 0.18 to 1.94% in the four subjects. No measurable amounts of the intact drug or the N-methyl-3-hydroxy analog III were seen in any subject. The major metabolites recovered were the N-desmethyl analog II and the N-desmethyl-3-hydroxy analog IV present in an approximately 1:2 ratio respectively in three out of four subjects and in a 1:1 ratio in the fourth subject.

TABLE V

URINARY EXCRETION DATA ON I AND ITS METABOLITES IN 4 PATIENTS FOLLOWING THE INTRAVENOUS ADMINISTRATION OF SINGLE 5- AND 10-mg DOSES

Collection period: 0–24 h (total volume voided). N.M. = Not measurable: <0.001 μg/ml for I and <0.050 μg/ml for III.

Patient	*Dose*	*Total excreted (μg)*				
		*I**	*II**	*III***	*IV***	*% of dose recovered*
V.J.	5 mg (765 ml)	N.M.	4.59	N.M.	4.59	0.18
S.S.	5 mg (1230 ml)	N.M.	30.75	N.M.	66.42	1.94
K.K.	10 mg (1495 ml)	N.M.	28.41	N.M.	73.26	1.02
S.K.	10 mg (864 ml)	N.M.	29.38	N.M.	58.75	0.88

* "Free" or directly extractable compounds.

** "Bound" or conjugated compounds.

The data indicate that the biotransformation pathway of I in man and in the dog is analogous to that of diazepam in these two species[4,9]. The compound appears to undergo N-demethylation to II more rapidly in the dog than in man based on the blood levels of II in both species. Hydroxylation in the C-3 position of the benzodiazepine ring results in the oxazepam-like analog IV which is excreted in the urine of both species as a conjugate. Hydroxylation in the C-3 position to produce III without either prior or subsequent N-demethylation appears to be negligible in man and only a minor route in the dog.

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Journal of Chromatography, 118 (1976) 387–393

CHROM. 8801

A SPECIFIC GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF MICROSOMAL STYRENE MONOOXYGENASE AND STYRENE EPOXIDE HYDRATASE ACTIVITIES

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(Received October 3rd, 1975)

SUMMARY

A gas chromatographic (GC) method for the determination of the metabolite resulting from the activities of microsomal styrene monooxygenase (epoxide synthetase) and epoxide hydratase using styrene or styrene epoxide as substrates has been developed. The determination of the activities of both enzymes is based on the GC determination of phenylethylene glycol after its esterification with *n*-butylboronic acid. Kinetic parameters for both enzymes are given.

INTRODUCTION

The biotransformation of a variety of chemicals through the epoxide–diol pathway[1,2] seems to be important in the formation of active intermediates that may cause damage to cellular components[3,4]. The enzymes involved in the formation (monooxygenase, E.C. 1.14.1.1) and hydration of epoxides (epoxide hydratase, E.C. 4.2.1.63) play a crucial role in this pathway. The comprehension of their behaviour to different stimuli *in vivo* and *in vitro* and the study of their distribution in various tissues could be helpful in establishing if there is a correlation between the accumulation of epoxide and the onset of toxic effects.

While the method for determination of the activities of monooxygenase and hydratase separately (with different substrates) has been reported by several workers[5–7], no methods that allow the simultaneous determination of activities of these two enzymes utilizing homogeneous substrates seem to be available.

This paper presents a simple gas chromatographic (GC) method for the assay of the metabolite resulting from the activities of microsomal monooxygenase and epoxide hydratase when styrene and styrene oxide are utilized as substrates.

EXPERIMENTAL

Chemicals

Styrene, styrene oxide and phenylethylene glycol were purchased from Merck

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(Darmstadt, G.F.R.); *n*-butylboronic acid from Pierce (Rockford, Ill., U.S.A.); nicotinamide-adenine dinucleotide phosphate ($NADP^+$), glucose-6-phosphate and glucose-6-phosphate dehydrogenase from Boehringer (Mannheim, G.F.R.); and *m*-dinitrobenzene from EGA-Chemie (Steinheim/Albuch, G.F.R.). All other reagents were obtained from Carlo Erba (Milan, Italy).

Microsomal preparation

The microsomes were prepared using Charles River CD male (150–180 g) rats according to the method of Kato and Takayanaghi[8]. Only a slight modification to the composition of the solution used for the homogenization was made. This solution consisted of Tris–hydrochloric acid buffer (0.05 *M*, pH 7.4) that was 0.15 *M* in potassium chloride and 5 m*M* in magnesium chloride.

The microsomal pellets were re-suspended in the same buffer to a final protein concentration of 10–20 mg/ml. The protein concentration was determinated by the method of Lowry *et al.*[9] using bovine serum albumin as a standard.

Assay of styrene monooxygenase

The incubation mixture in a final volume of 5 ml contained the NADPH-generating system (2.5 μmoles of $NADP^+$, 50 μmoles of glucose-6-phosphate and 2 units of glucose-6-phosphate dehydrogenase), the Tris–hydrochloric acid buffer and about 10 mg of microsomal protein. After pre-incubation at 37° for 10 min, the reaction was started by the addition of 100 μl of an acetone solution of styrene to obtain a final substrate concentration of 5 m*M*. The reaction was carried out with shaking for 5 min and stopped by the addition of 1 ml of 0.6 *N* sulphuric acid. The samples were then agitated for 12 h at room temperature and subsequently extracted twice with 5 ml of ethyl acetate after being made alkaline with 2 ml of 0.6 *N* sodium hydroxide solution.

The acidification of the styrene epoxide induces more than 95% transformation into the diol. The combined extracts were dried under vacuum at room temperature and the phenylethylene glycol content in the residue was determined as described below. The recovery of phenylethylene glycol under these conditions was about 90%.

The enzymatic activity was expressed in nanomoles of glycol formed per minute of incubation per milligram of microsomal protein.

Assay of epoxide hydratase

The activity of this enzyme was determined as described previously for styrene monooxygenase with the following exceptions: (a) 5 m*M* styrene oxide was used instead of styrene as substrate, (b) the NADPH-generating system was omitted and (c) the reaction was stopped by adding 1 ml of 0.6 *N* sodium hydroxide solution and samples were immediately extracted twice with ethyl acetate.

The spontaneous hydration of styrene oxide that occurred under the conditions used was less than 5% with respect to the enzymatic hydration and it was subtracted in the calculation of the enzymatic activity.

Preparation of samples for GC analysis

The residue, after evaporation of ethyl acetate, was dissolved in 100 μl (monooxygenase assay) or 400 μl (hydratase assay) of an acetone solution of *m*-dinitro-

benzene (0.5 μg/μl) used as an internal standard. To each 100 μl of above solution, 2.5 μl of *n*-butylboronic acid solution (100 mg/ml in dimethylformamide) were added and 1 μl of the sample was injected into the gas chromatograph.

Apparatus and conditions

A Carlo Erba Model GI gas chromatograph equipped with a flame ionization detector was used. The GC column was a 2.5-m glass tube (I.D. 2 mm; O.D. 4 mm) packed with 3% OV-17 and Gas-Chrom Q (100–120 mesh). The operating conditions were: column temperature, 170°; oven temperature, 240°; nitrogen (carrier gas) flow-rate, 30 ml/min; and chart speed, 1 cm/min.

Mass spectrometry

In order to confirm the formation of the *n*-butylboronate of phenylethylene glycol[10], a mass spectrometric analysis was carried out on an LKB 9000 instrument under the following conditions: ion source temperature, 290°; ionization energy, 70 eV; and trap current, 60 μA. The sample was introduced either by a direct inlet system (DIS) or by GC under the conditions described above but with helium as the carrier gas at a flow-rate of 30 ml/min.

RESULTS

The gas chromatograms of the *n*-butylboronic ester of phenylethylene glycol and the internal standard *m*-dinitrobenzene are shown in Fig. 1.

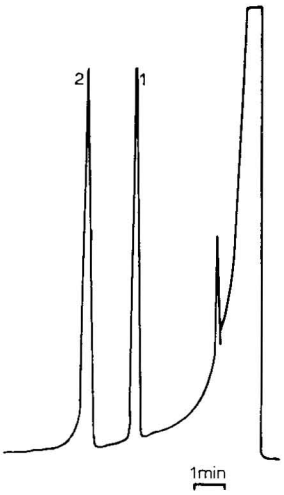

Fig. 1. Gas chromatographic separation of the ester of phenylethylene glycol with *n*-butylboronic acid (1) and *m*-dinitrobenzene (2) under the conditions described in the text.

The identification of the *n*-butylboronate of phenylethylene glycol was checked by means of mass spectrometry (MS) and GC–MS. The mass spectra of the glycol and of its ester with butylboronic acid are shown in Fig. 2.

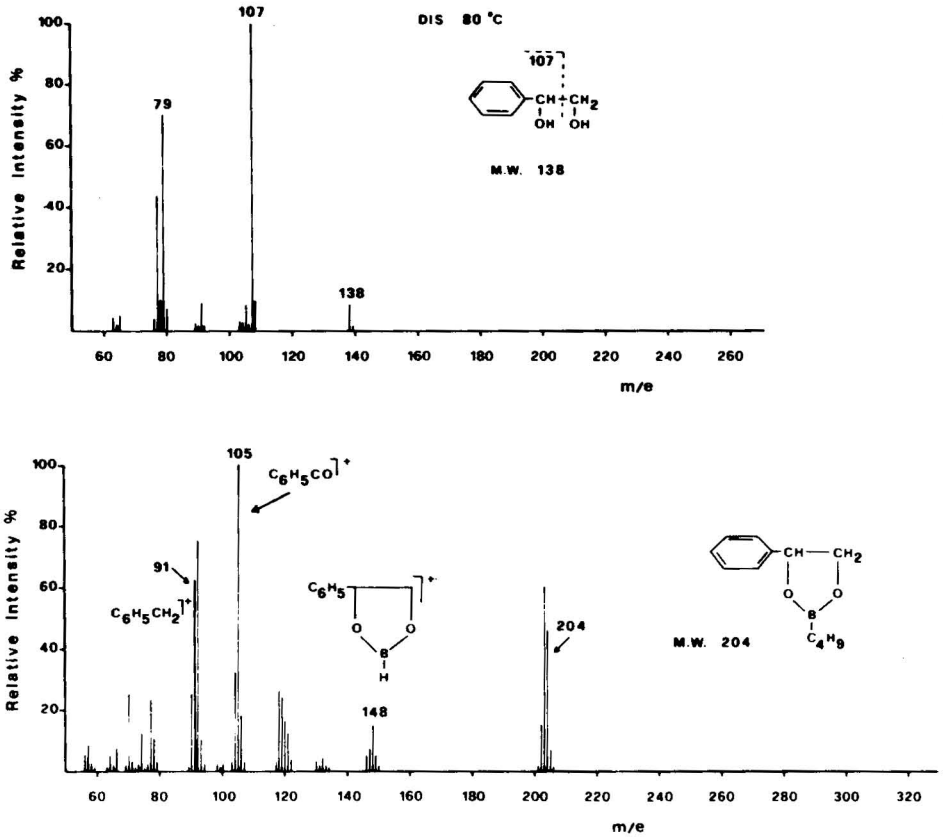

Fig. 2. Mass spectra of phenylethylene glycol (above) and butylboronic derivative of phenylethylene glycol (below).

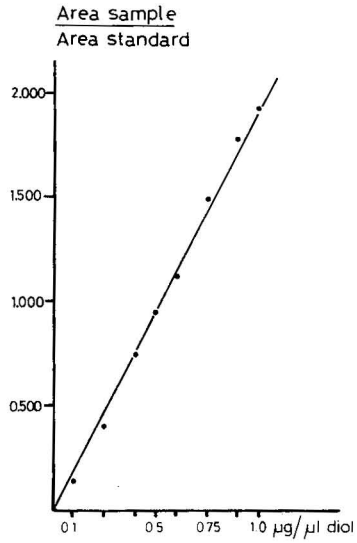

Fig. 3. Calibration graph for phenylethylene glycol after esterification with *n*-butylboronic acid. Internal standard: *m*-dinitrobenzene (0.125 µg/µl).

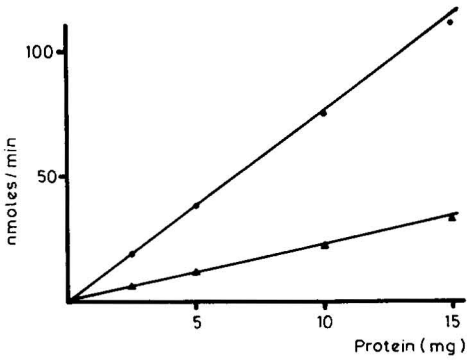

Fig. 4. Effect of microsomal protein concentration on the activity of hydratase (●) and monooxygenase (▲).

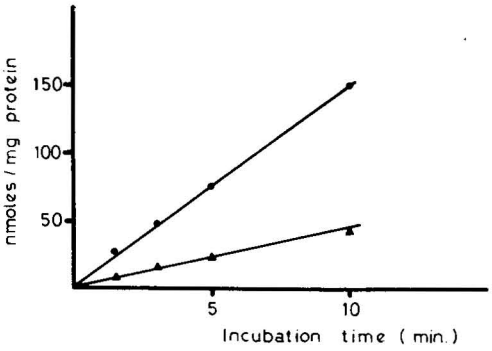

Fig. 5. Effect of incubation time on the amount of product formed from hydratase (●) and monooxygenase (▲).

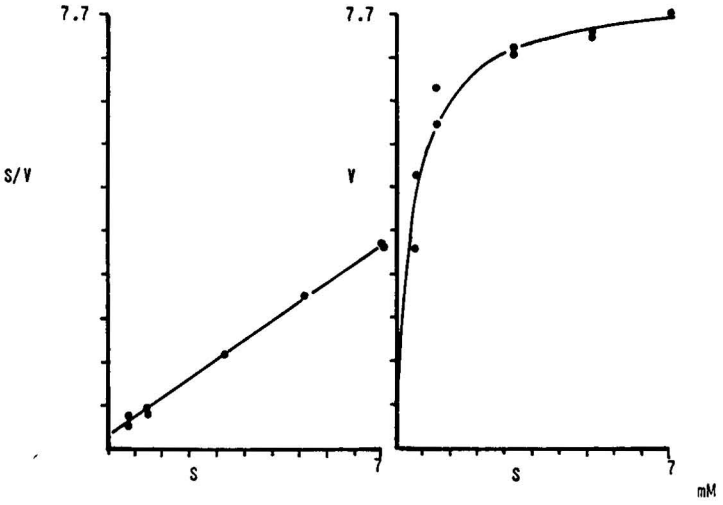

Fig. 6. Activity of monooxygenase in the presence of different concentrations of styrene. Left, Woolf plot; right, Michaelis–Menten plot.

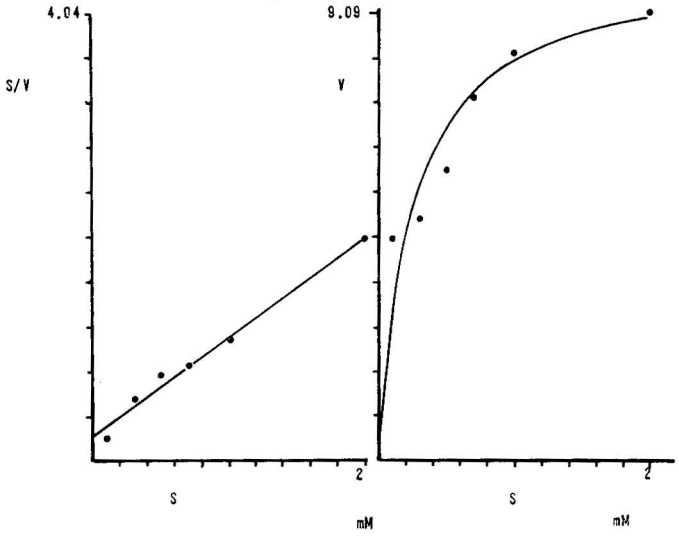

Fig. 7. Activity of epoxide hydratase in the presence of different concentrations of styrene epoxide. Left, Woolf plot; right, Michaelis–Menten plot.

The calibration graph for phenylethylene glycol is shown in Fig. 3. The graph is linear from 0.1 to 1 $\mu g/\mu l$.

The results in Figs. 4 and 5 indicate that the amounts of glycol formed during the enzymatic reaction catalyzed by monooxygenase or hydratase are linear up to an incubation time of 10 min and in the range of microsomal protein from 2.5 to 15 mg. In all of the subsequent experiments, therefore, an incubation time of 5 min and an amount of microsomal protein of 10 mg were used.

Fig. 6 shows the Woolf and Michaelis–Menten plots for the monooxygenase activity.

The saturation of the enzyme by the substrate (styrene) is observed at a concentration of 3 mM. The apparent K_m and $V_{max.}$ values are 0.43 mM and 4.77 nmoles/min per milligram of protein, respectively.

Fig. 7 shows the Woolf and Michaelis–Menten plots for epoxide hydratase using styrene epoxide as substrate. The apparent K_m and $V_{max.}$ values are 0.25 mM and 11.17 nmoles/min per milligram of protein, respectively.

DISCUSSION

The determination of epoxide hydratase involves the use of radioactive styrene epoxide[7], while the monooxygenase (epoxide-forming enzyme) activity is determined by using a variety of substrates[5–7]. This paper describes a simple GC method for the determination of both enzymatic activities by employing the homogeneous non-radioactive substrates styrene epoxide and styrene.

In the presence of monooxygenase obtained from rat liver, styrene is transformed into an epoxide but a consistent fraction of this epoxide is metabolized to form the diol, phenylethylene glycol. As the diol can be formed only by the epoxide

pathway[11,12], it is possible to determine the total epoxide formed by measuring the diol formed both enzymatically and chemically after acidification of the epoxide that is not hydrated during the incubation. The chemical hydration of the epoxide accomplished by addition of sulphuric acid is necessary in order to avoid under-estimation of the amount of epoxide formed.

In the presence of epoxide hydratase, the diol is derived quantitatively only from the disappearance of the epoxide. Therefore, by using the two substrates, but by measuring only one final metabolite, namely phenylethylene glycol, it is possible to determine the activities of two enzymatic systems separately. The determination of phenylethylene glycol is simple and quantitative because its butylboronic derivative is suitable for GC analysis.

The kinetic data obtained in these studies indicate that the affinity of styrene epoxide for the hydratase is about twice as great as that of styrene for the epoxide-forming enzyme. Not only the affinity constant is different but also the amount of the hydratase is greater than the epoxide-forming system, as shown by the $V_{max.}$ values. Therefore, the formation of the epoxide seems to be the rate-limiting step in the epoxide–diol pathway.

The method described may be of use in studies on the inhibition or the activation of these two enzymatic activities, which are of significance for the accumulation of toxic metabolites.

ACKNOWLEDGEMENT

This work was supported by a grant from the Gustavus and Louise Pfeiffer Research Foundation, New York, U.S.A.

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Journal of Chromatography, 118 (1976) 395–402

CHROM. 8936

MASS FRAGMENTOGRAPHIC QUANTITATION OF GLUTAMIC ACID AND γ-AMINOBUTYRIC ACID IN CEREBELLAR NUCLEI AND SYMPATHETIC GANGLIA OF RATS

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(Received October 2nd, 1975)

SUMMARY

A method for the simultaneous quantitation of glutamic acid and γ-aminobutyric acid (GABA) in tissue by mass fragmentography has been developed. The amino and carboxylic groups of the two amino acids were in a convenient one-step reaction derivatized with pentafluoropropionic anhydride and hexafluoroisopropanol. Deuterium-labeled glutamic acid and GABA and a homologue of GABA have been used as internal standards. The usefulness of the technique has been demonstrated by measurements in parts of rat cerebellum and in the superior cervical ganglion.

INTRODUCTION

The role of γ-aminobutyric acid (GABA) as an inhibitory transmitter is now fairly well established[1,2]. The findings of a deficiency of GABA[3] and of glutamic acid decarboxylase (the enzyme that decarboxylates glutamic acid to GABA)[4,5] in the brains of patients with Huntington's chorea, have stimulated the research concerning the function of this compound.

Several methods for the analysis of GABA including enzymatic[6–8] and gas chromatographic[9] techniques have been described. In this paper we report on the simultaneous determination of GABA and its precursor glutamic acid by the sensitive and specific method of mass fragmentography. We will show that this method can be used to determine the two amino acids in small punches (about 50 μg protein) of discrete rat brain nuclei. The combination of microdissection and the analytical technique of mass fragmentography has previously proved useful for studies on other neurotransmitters at the synaptic level[10,11]. In an abstract, Cattabeni *et al.*[12] recently reported a mass fragmentographic analysis of GABA using the trimethylsilyl derivative.

EXPERIMENTAL

Reagents and reference compounds

The following compounds were commercially available: pentafluoropropionic

anhydride (PFPA, distilled before use) and 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) from Pierce (Rockford, Ill., U.S.A.); L-glutamic acid and 5-amino-*n*-valeric acid (AVA) hydrochloride from K & K Labs. (Plainview, N.Y., U.S.A.); GABA from Calbiochem (Los Angeles, Calif., U.S.A.); L-[2,3,3,4,4-$^2H_5$]glutamic acid (glutamic acid-d_5) from Merck, Sharp and Dohme (Montreal, Canada); deuterium chloride (20% in deuterium oxide; 100.0 atom %D) and deuterium oxide (99.7 atom %D) from Aldrich (Milwaukee, Wisc., U.S.A.).

[4,4-$^2H_2$]Glutamic acid (glutamic acid-d_2) and γ-amino[2,2-$^2H_2$]butyric acid (GABA-d_2) were synthesized by heating glutamic acid and GABA (200 mg of each), respectively, at 130° in sealed tubes with 1 ml of 8% deuterium chloride in deuterium oxide. The solutions were reacted for three periods, each of 12 days. After each period the solvent was evaporated by a stream of nitrogen gas and replaced by new deuterium chloride solution. After the last heating period the crystals obtained were dissolved in protium water to exchange active deuterium atoms by protium. When the water was evaporated white crystals of glutamic acid-d_2 and GABA-d_2 were obtained.

Tissue preparation

Male Sprague-Dawley rats (about 150 g) were killed by exposing their heads for 2.2–2.5 sec to a focused high-intensity microwave beam as described by Guidotti *et al.*[13]. The superior cervical ganglia and the brains were dissected out and immediately frozen in dry ice. In the experiments where the concentration of glutamic acid and GABA were determined in regions of cerebellum, this part of the brain was sliced in a cryostat at −4°. From these frozen slices the deep nuclei *(n. fastigii, interpositus* and *lateralis)* and the cortex (molecular, Purkinje cell and granular layers) of cerebellum were punched out using a hollow steel tube (I.D. 0.8 mm) as described for other brain nuclei[10].

Procedure

The tissue was homogenized in glass homogenizer tubes (Kontes, Vineland, N.J., U.S.A.) in 80% aqueous ethanol (which has been shown to efficiently extract GABA[14]) containing the internal standards for the quantitations. Whole cerebella were homogenized in 500 μl of a solution containing 380 and 750 nmoles/ml of glutamic acid-d_5 and AVA, respectively [or, in some experiments (see Table I), 380 and 300 nmoles/ml of glutamic acid-d_2 and GABA-d_2, respectively]. Cerebellar deep nuclei and cortex were homogenized in 100 and 200 μl, respectively, of a solution containing 42 and 84 nmoles/ml of the two internal standards. Superior cervical ganglia (pooled from 2 or 3 rats) were homogenized in 150 μl of a solution of 3.3 nmoles AVA per ml.

After homogenization the tubes were centrifuged at 12,000 *g* for 5 min at −2°. The supernatant (only 50 μl from the whole cerebellum) was transferred to glass vials and evaporated to dryness by a stream of nitrogen. Fifty microlitres of HFIP and 100 μl PFPA were added, the vials were sealed, heated for 1 h at 60° and then stored at 4°. Just before the mass fragmentographic analysis, the reaction mixture was evaporated to dryness. The residue was dissolved in 10–100 μl of ethyl acetate and 1–3 μl were injected into the gas chromatograph–mass spectrometer.

When small samples of tissue were analyzed, protein was determined in the

TABLE I

MASS FRAGMENTOGRAPHIC QUANTITATION OF GLUTAMIC ACID AND GABA IN WHOLE RAT CEREBELLUM USING DIFFERENT INTERNAL STANDARDS

Concentrations are expressed in μmoles per g wet weight (mean ± standard error of the mean). Number of determinations are in parentheses.

Internal standard	*Glutamic acid*	*GABA*
Glutamic acid-d_2	11.3 ± 0.3 (5)	
Glutamic acid-d_5	12.0 ± 0.2 (5)	
GABA-d_2		1.46 ± 0.04 (5)
AVA		1.52 ± 0.04 (15)

pellet of the homogenate according to Lowry *et al.*[15]. The amount of glutamic acid and GABA was then expressed per mg of protein.

Gas chromatography–mass spectrometry

An LKB Model 9000 gas chromatograph–mass spectrometer with a multiple ion detector (LKB-Produkter, Bromma, Sweden) was used. The separations were made on a 2.5 m × 3 mm I.D. silanized glass column packed with 3% OV-17 on Gas-Chrom Q, 100–120 mesh (Applied Science Labs., State College, Pa., U.S.A.), maintained at a temperature of 115°. The temperature of the flash heater was 200° and the ion source was kept at 270°. The flow-rate of the helium carrier gas was 25 ml/min. The ionizing potential and trap current were 80 eV and 60 μA, respectively. When mass spectra of reference compounds were recorded, the column temperature was 95°.

RESULTS AND DISCUSSION

Watson *et al.*[16] have reported a procedure to derivatize simultaneously carboxylic groups with a fluorinated alcohol and phenolic groups with a fluorinated anhydride. With this in mind we have in a convenient way derivatized glutamic acid and GABA. The carboxylic groups were esterified with HFIP and the amino groups acylated with PFPA. Recently, a gas chromatographic method with electron capture detection for the determination of GABA was reported[9]. Pearson and Sharman used the same type of reagents (HFIP and trifluoroacetic anhydride) to derivatize GABA.

The mass spectra and the proposed fragmentation patterns of the PFP–HFIP derivatives of glutamic acid, GABA and the internal standards are shown in Figs. 1 and 2. The base peak at *m/e* 202 was used to monitor glutamic acid by mass fragmentography. The corresponding peaks (*m/e* 204 and 206) were used for the detection of the internal standards, di- and pentadeuterium-labeled glutamic acid. Either of these internal standards may be used for the quantitation of glutamic acid. The dideuterium-labeled species can be synthesized in each laboratory at a low cost compared to the commercially available glutamic acid-d_5. One of our goals with this methodology is to study the incorporation of stable isotopes from, *e.g.*, [^{13}C]glucose into glutamic acid and GABA *in vivo*. The formed [^{13}C]glutamic acid may interfere with the peak at *m/e* 204 if glutamic acid-d_2 is used and we have therefore chosen

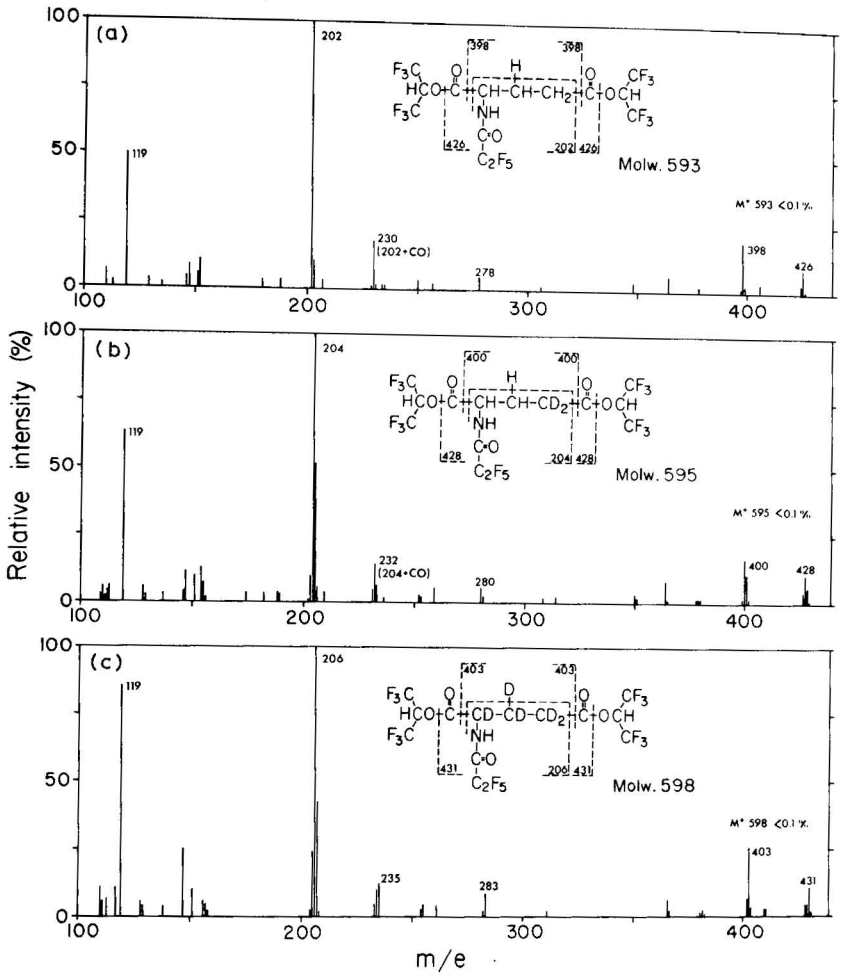

Fig. 1. Mass spectra and proposed fragmentation pattern of the HFIP–PFP derivatives of glutamic acid (a), glutamic acid-d_2 (b) and glutamic acid-d_5 (c).

glutamic acid-d_5 as an internal standard. The derivatives of GABA, GABA-d_2 and AVA have the common base peak at *m/e* 176 (Fig. 2). Thus, this fragment cannot be monitored when GABA-d_2 is used as an internal standard. As GABA and AVA have different retention times in the gas chromatographic system, the base peak can be used to record both GABA and AVA independently.

Fig. 3 shows mass fragmentograms obtained from an analysis of glutamic acid and GABA in deep cerebellar nuclei using glutamic acid-d_5 and AVA as internal standards, respectively. The retention times for the different compounds were for glutamic acid and glutamic acid-d_5 92 sec (peaks 1), for GABA 133 sec (peaks 2), and for AVA 232 sec (peaks 3).

Standard curves for the quantitation of glutamic acid and GABA were prepared by analyzing a series of standard solutions of these two compounds by the

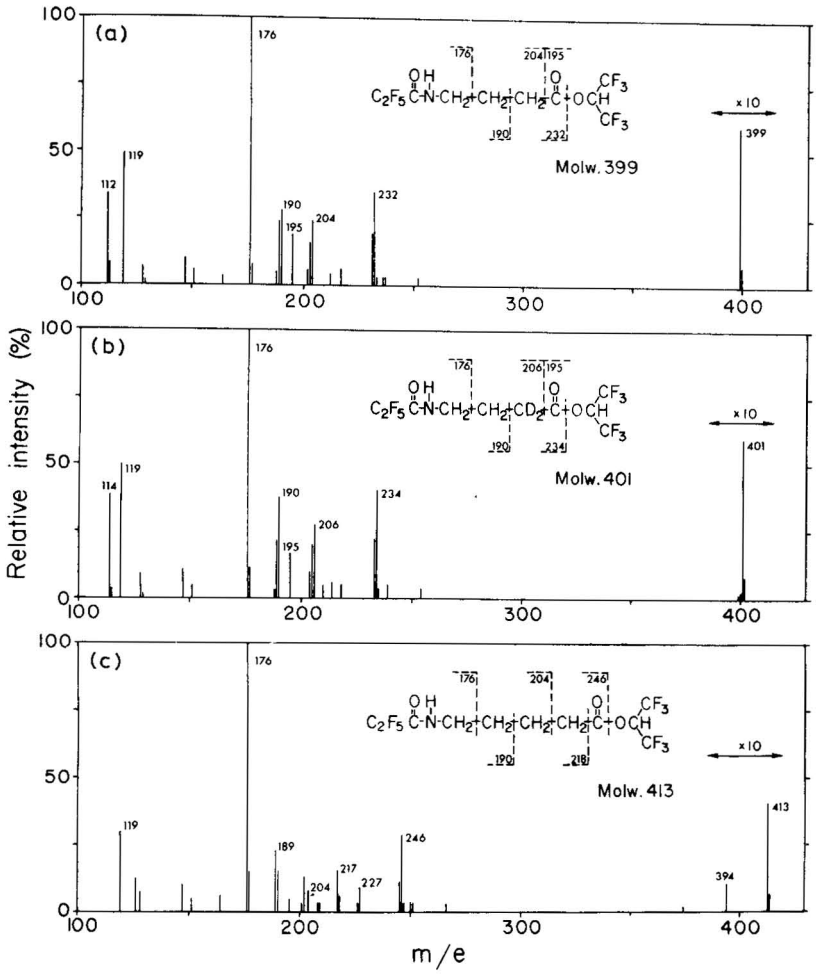

Fig. 2. Mass spectra and proposed fragmentation pattern of the HFIP–PFP derivatives of GABA (a), GABA-d_2 (b) and AVA (c). The intensity of the ions in the region of m/e 400 is magnified 10 times.

procedure described in Experimental. The peak height ratio between glutamic acid or GABA and the internal standard was plotted against the known concentration of the actual compound. Table I shows the results from the determination of glutamic acid and GABA in whole rat cerebellum. When the two different deuterium-labeled glutamic acids were used as internal standards, similar concentrations of glutamic acid were obtained. The concentrations of GABA determined with either GABA-d_2 or AVA as internal standard were almost identical (1.46 ± 0.04 and 1.52 ± 0.04 μmoles/g wet weight, respectively). This shows that AVA can be used as an internal standard for quantitation of GABA. The concentrations of glutamic acid and GABA in the whole rat cerebellum obtained here are similar to those reported by some other investigators[14,17].

As GABA is an important transmitter of cerebellar Purkinje, Golgi, basket

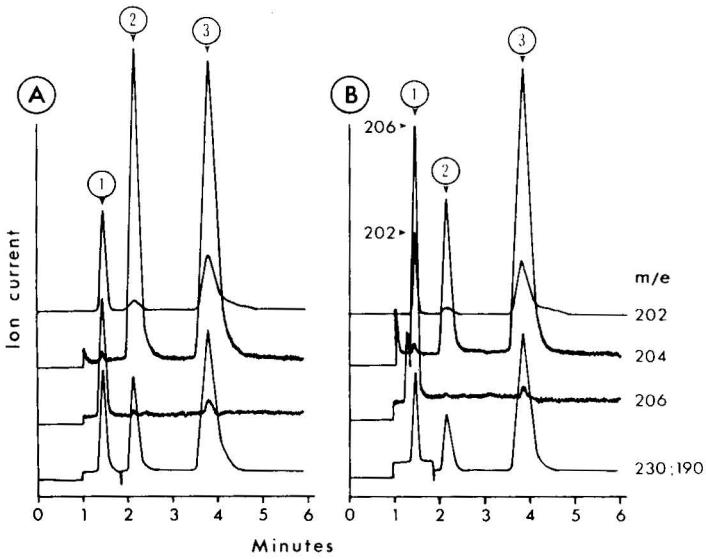

Fig. 3. Mass fragmentograms obtained from HFIP–PFP derivatives of (A), reference glutamic acid and glutamic acid-d_5 (peaks 1), GABA (peaks 2) and AVA (peaks 3); and (B) material obtained from rat cerebellar deep nuclei homogenized in an 80% aqueous ethanol solution containing glutamic acid-d_5 and AVA as internal standards. The mass spectrometer was in this case set to detect *m/e* 202 (glutamic acid), 204 (GABA and AVA) and 206 (glutamic acid-d_5). The fourth channel was initially used to detect a second fragment of glutamic acid (*m/e* 230) and 110 sec after injection this channel was changed to detect *m/e* 190, a fragment of both GABA and AVA. The relative sensitivities on the four channels used to detect *m/e* 202, 204, 206 and 230 (190) were 1:10:10:3.3.

and stellate cells we have started our investigations on the regulation of GABA-ergic mechanisms in this part of the central nervous system. The concentration of GABA was higher in deep cerebellar nuclei than in the cortex ($P < 0.01$; Table II). This finding is in line with a three-fold higher activity of glutamic acid decarboxylase in *n. interpositus* than in the cerebellar cortex[19]. The cerebellar nuclei contain nerve terminals of the Purkinje cells, which have their cell bodies in the cortex. Interestingly, we found higher levels of glutamic acid in the cortex than in the deep cerebellar nuclei

TABLE II

MASS FRAGMENTOGRAPHY OF GLUTAMIC ACID AND GABA IN CEREBELLAR REGIONS OF RAT

Values are mean ± S.E.M. Numbers of determinations are in parentheses.

Sample	*Glutamic acid*	*GABA*
Quantitation (μmoles per mg protein)		
Deep nuclei	84 ± 7 (5)	24.2 ± 1.6 (5)
Cortex	120 ± 6 (5)	14.2 ± 1.5 (5)
*Identification**		
Standards	0.321 ± 0.003 (8)	1.07 ± 0.02 (7)
Deep nuclei	0.316 ± 0.003 (5)	1.08 ± 0.02 (5)
Cortex	0.310 ± 0.003 (5)	1.05 ± 0.01 (5)

* Glutamic acid: *m/e* 230/202; GABA: *m/e* 204/190.

TABLE III

MASS FRAGMENTOGRAPHY OF GABA IN SUPERIOR CERVICAL SYMPATHETIC GANGLIA OF RAT

Quantitation: 309 ± 22 pmoles per mg protein (12 determinations). Values are mean ± S.E.M. Number of determinations are in parentheses.

Sample	*m/e 190/176*	*m/e 204/176*
Standards	0.285 ± 0.003 (14)	0.167 ± 0.002 (15)
Ganglia	0.287 ± 0.004 (11)	0.165 ± 0.003 (12)

($P < 0.01$; Table II). Glutamic acid and GABA were each identified by the ratio of two characteristic fragments (Table II and Fig. 3).

We believe that in studies of GABA the use of microwave to sacrifice the rats is mandatory. When rats are killed with a high-intensity microwave beam, the glutamic acid decarboxylase is promptly inactivated; thus, the GABA content is stabilized to a level close to that existing *in vivo*. The "punching" of nuclei from brain slices allows studies of the regulation of GABA with a direct reference to the synaptic organization of the tissue under study. By mass fragmentography both GABA and its precursor glutamic acid can be measured simultaneously with high sensitivity and specificity. Moreover, by labeling the glutamic acid pool with stable isotopes (*e.g.*, infusion of [^{13}C]glucose) the turnover rate of these two amino acids may be determined in brain nuclei.

The presence of endogenous GABA in the rat superior cervical ganglion has previously not been shown. Nagata *et al.*[20] reported that the GABA concentration in this ganglion was less than 1 nmole/mg protein; this was the lower limit of sensitivity of their method. Using the mass fragmentographic technique described in the present report, GABA was identified by the ratio between three fragments (*m/e* 176, 190 and 204; see Table III) at the gas chromatographic retention time of GABA. The concentration of GABA was 309 ± 22 pmoles/mg protein, which is only a few percent of the level found in rat brain. As the superior cervical ganglion contains fairly high concentrations of glutamic acid[20], this compound was not further investigated.

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Journal of Chromatography, 118 (1976) 403–408

CHROM. 8793

HIGH-SPEED LIQUID CHROMATOGRAPHIC DETERMINATION OF PHENYLPYRUVIC ACID

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(First received August 8th, 1975; revised manuscript received October 10th, 1975)

SUMMARY

A high-speed liquid chromatographic method has been developed for the determination of urinary phenylpyruvic acid. This acid is converted by treatment with naphthalene-2,3-diamine into 3-benzyl-2-hydroxybenzoquinoxaline, which is extracted into carbon tetrachloride for separation. 2-Mercaptoethanol is a useful stabilizer, and 2-chlorothioxanthone is a suitable internal standard. The method is specific, and the results are not affected by the presence of such 2-oxo-acids as pyruvic acid, 2-oxobutyric acid, 2-oxoglutaric acid, and 4-hydroxyphenylpyruvic acid.

INTRODUCTION

Phenylketonuria is an inherited disease that may cause mental deficiency if not properly treated. The disease is characterized primarily by a metabolic error that prevents phenylalanine in the body from being converted into tyrosine. As the amount of phenylalanine increases, the amino acid is de-aminated to phenylpyruvic acid (PPA), the blood concentration and urinary excretion of which become excessive. The determination of PPA offers a useful technique for differentiating between phenylketonuria and hyperphenylalaninaemia.

Hitherto, PPA has usually been determined by colorimetric methods depending on a reaction with ferric chloride[1,2] or 2,4-dinitrophenylhydrazine[3]; these methods, however, lack specificity. Although the enol–borate complex method[4–6] is more sensitive and selective, it is affected by the presence of 4-hydroxyphenylpyruvic acid, which is usually formed at increased levels in tyrosinaemia.

This paper describes the development of a high-speed liquid chromatographic determination of PPA; the method is based on the formation of 3-benzyl-2-hydroxybenzoquinoxaline (BHQ) by a reaction between PPA and naphthalene-2,3-diamine.

EXPERIMENTAL

The apparatus used in this work was a Shimadzu–Du Pont liquid chromatograph 840 equipped with a UV absorption detector (254 nm). The separation was

carried out on a 1-m column of Zipax Permaphase ETH (Shimadzu Seisakusho, Kyoto, Japan).

PPA, naphthalene-2,3-diamine and 2-chlorothioxanthone were obtained from Tokyo Organic Chemicals (Tokyo, Japan). The other reagents and organic solvents used were of reagent grade.

Preparation and purification of naphthalene-2,3-diamine

Naphthalene-2,3-diamine was stable as its sulphate, which was prepared by adding 50% aqueous sulphuric acid to an ethanol solution of the diamine; the product was recrystallized from 2% aqueous sulphuric acid.

Preparation of BHQ

BHQ was prepared by interaction of PPA (500 mg) and naphthalene-2,3-diamine (450 mg) in methanol (20 ml) and 2 *N* aqueous hydrochloric acid (20 ml) at about 40° for 1 h. The product was filtered off, washed with water, dried and recrystallized from ethyl acetate; its m.p. was 270 ± 1°. Analysis: (calculated for $C_{19}H_{14}ON_2$) C, 79.79%, H, 4.93%, N, 9.80%; found C, 79.77%, H, 4.92%, N, 9.68%.

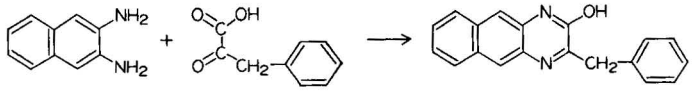
Procedure

To 100 μl of an aqueous sample solution were added 5 ml of 2.5 *N* aqueous hydrochloric acid containing 3 mg of naphthalene-2,3-diamine sulphate, 10 μl of 2-mercaptoethanol and 3 μg of 2-chlorothioxanthone in 100 μl of methanol. The mixture was warmed in a water-bath at about 80° for 2 h, then cooled, and the BHQ formed was extracted into 10 ml of carbon tetrachloride. The organic phase was washed with 10 ml of 3 *N* aqueous hydrochloric acid, the carbon tetrachloride was evaporated in a rotary evaporator, the residue was dissolved in a few drops of NN-dimethylformamide, and 10 μl of this solution were subjected to high-speed liquid chromatography under the conditions shown in Fig. 1.

RESULTS AND DISCUSSION

Hinsberg[7] reported originally that 2-oxo-acids react with *o*-phenylenediamine to form quinoxaline derivatives in aqueous acidic media; this reaction has been applied to the fluorimetric[8] or gas chromatographic determination[9] of 2-oxo-acids. In our work, such a reaction is used to prepare a derivative of PPA for high-speed liquid chromatographic separation.

As the result of preliminary experiments on a few types of aromatic *o*-diamines, naphthalene-2,3-diamine was chosen as a suitable reagent.



This derivatization of PPA is considered to result in a large increase in its affinity for non-polar solvents. Various polar compounds occur in biological samples, and, accordingly, a combination of this derivatization with reversed-phase chromatography offered a convenient method for the determination of PPA in such samples.

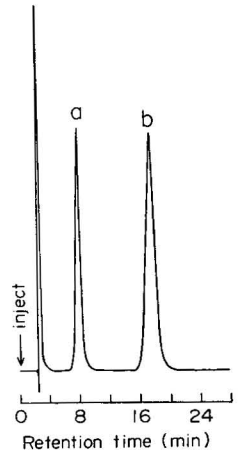


Fig. 1. Liquid chromatogram of a standard mixture of BHQ (peak a) and 2-chlorothioxanthone (internal standard; peak b). Operating conditions: column, 1 m of Permaphase ETH; mobile phase, water–acetonitrile (18:7); column temperature, 40°; flow-rate, 0.5 ml/min; detector, UV photometer.

For this purpose, a 1-m column of Zipax Permaphase ETH was used. Fig. 1 shows a typical chromatogram obtained from a standard mixture of BHQ and the internal standard (2-chlorothioxanthone). A good separation is attained in only 20 min, even without a gradient-elution technique.

As a result of an investigation into the effect of hydrochloric acid concentration in the reaction mixture, 2.5 *N* was selected as a suitable concentration. Fig. 2a shows a chromatogram obtained by allowing PPA to react with naphthalene-2,3-diamine in 2.5 *N* hydrochloric acid. A peak possibly attributable to a by-product can be seen ahead of the peak for BHQ. The ratio of these two peaks varied with slight changes in the reaction conditions, and the results were significantly affected. Spikner and Towne[8] reported that sulphuric acid was the preferred medium for a similar reaction, because

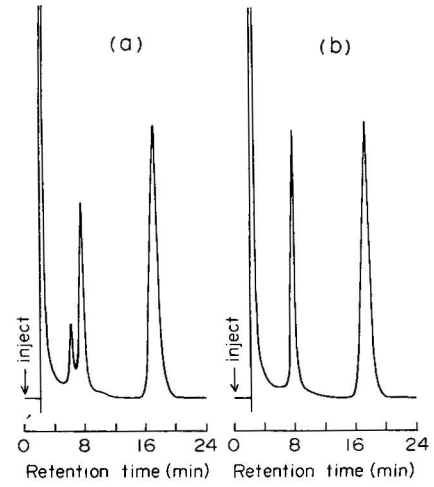
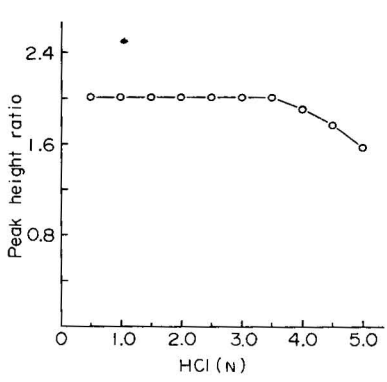


Fig. 2. Effect of 2-mercaptoethanol on the reaction.

aromatic *o*-diamines and the quinoxaline products were stabilized against oxidation in this medium. We attempted to carry out the reaction under similar conditions, but did not obtain good results; however during this work, it was fortunately discovered that formation of the by-product was depressed by addition of 2-mercaptoethanol. As shown in Fig. 2b, the addition of 10 μl of 2-mercaptoethanol to the reaction mixture suppressed formation of the by-product almost completely. The other reaction conditions, *e.g.*, temperature, time and concentration of naphthalene-2,3-diamine were also investigated.

Next, the extractability of BHQ was studied. This compound could be easily extracted from the aqueous layer with such varied organic solvents as carbon tetrachloride, dichloromethane, diethyl ether, chloroform and ethyl acetate; carbon tetrachloride was chosen as being the most suitable, as it separated clearly from the aqueous phase as a lower layer, which was convenient for the separation procedure. Fig. 3 shows the relationship between the extractability of BHQ into carbon tetrachloride and the hydrochloric acid concentration in the aqueous layer; BHQ was completely extractable at hydrochloric acid concentrations below 3 *N*. After the BHQ had been extracted into carbon tetrachloride, the extract was washed with 3 *N* hydrochloric acid in order to remove as much as possible of the excess of naphthalene-2,3-diamine. Under these extraction conditions, the internal standard 2-chlorothioxanthone was also almost completely extracted.



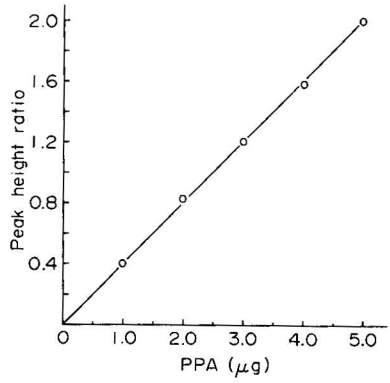


Fig. 3. Relationship between extractability of BHQ into carbon tetrachloride and hydrochloric acid concentration in the aqueous layer.

Fig. 4. Calibration graph for determination of PPA.

Fig. 4 illustrates the calibration graph obtained by the recommended procedure. The relative peak-height ratios of BHQ to internal standard were plotted against the amount of PPA in the solution; the relationship was rectilinear, at least in the concentration range shown.

In this method, the results were not affected by the presence of such 2-oxo-acids as pyruvic, 2-oxobutyric, 2-oxoglutaric and 4-hydroxyphenylpyruvic acids.

In recovery tests, the method was applied to normal human urine to which had been added 3.00 μg of PPA per 100 μl; the reproducibility was determined by carrying out eleven identical analyses, with the results shown in Table I. The chromatogram obtained in the recovery experiments is shown in Fig. 5; no interfering

TABLE I
RECOVERY OF PPA FROM URINE (100 μl)

Amount added (μg)	*Amount found* (μg)	*Recovery* (%)
3.00	2.96	98.7
3.00	3.07	102.3
3.00	3.09	103.0
3.00	2.96	98.7
3.00	3.06	102.0
3.00	3.04	101.3
3.00	3.00	100.0
3.00	3.10	103.3
3.00	3.10	103.3
3.00	3 04	101.3
3.00	2.96	98.7
		($\sigma_{rel.}$ = 1.85%)

peak was observed. Normal urine was used directly in these experiments, but the urine from a phenylketonuric patient should first be appropriately diluted with water, owing to the large amount of PPA in such urine[6].

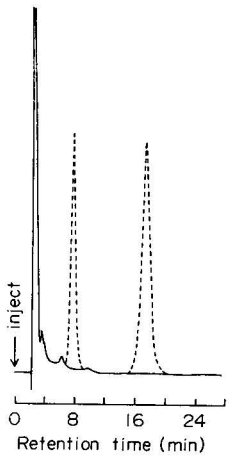


Fig. 5. Liquid chromatogram obtained in recovery experiments from urine: ———, urine blank.

CONCLUSIONS

A method for the high-speed liquid chromatographic determination of PPA in urine has been developed. It is more sensitive, selective and reproducible than are previously reported methods, such as those involving use of 2,4-dinitrophenylhydrazine, ferric chloride or the enol–borate complex.

We are currently studying the application of this method to serum.

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Journal of Chromatography, 118 (1976) 409–410

CHROM. 8880

Note

Gas chromatographic determination of dicyclohexylurea in the active esters of amino acids

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(Received September 30th, 1975)

The reaction between the carboxyl group of amino acids or peptides and halo- or nitrophenols to form the so called active esters is one of the most important reactions of modern peptide chemistry. As the condensing reagent of this reaction is dicyclohexylcarbodiimide, an equivalent quantity of dicyclohexylurea (DCU) is also formed. Because of its unfavourable crystallization properties the removal of the latter from active esters is not an easy task, therefore the quantitative determination of DCU, as the contaminant of active esters, in amounts down to 0.1% is one of the important tasks of peptide analysis.

As DCU is titrimetrically and spectrophotometrically inactive and its –CO–NH– grouping which may serve as the basis for colorimetric measurements can be found in peptides and protected amino acids as well, we decided to use gas chromatography to solve the above problem.

No data have been found in the literature regarding the gas chromatography of DCU. Reiser[1] described the gas–liquid chromatographic separation of some alkyl substituted urea derivatives on glass beads covered with 0.5% Carbowax 20M but no groups larger than butyl were investigated. Recently Evans[2] chromatographed some N,N′-disubstituted urea derivatives as the trifluoroacetates on Diatomite C covered with 10% PEGA.

As the derivatization of small amounts of DCU in the presence of large quantities of amino acids or peptides cannot be carried out DCU was chromatographed without derivatization.

After having failed to obtain suitable chromatograms on various stationary phases it has been found that this non-volatile material (m.p. 227°) can only be chromatographed on non-polar methylsilicone phases; JXR on Chromosorb W HP has been selected. Using this column at 190° DCU gives a symmetrical peak (retention time 3.0 min) sufficiently separated from the solvent peak and that of docosane (5.1 min) used as the internal standard.

A linear relationship has been found between the ratio of the peak areas and the amount of DCU within the concentration range 0.1–2.5 μg DCU/μl. The relative molar response of DCU (relative to docosane) is 0.22. As about 50 μg of the active ester is injected on to the column this means that 0.2–5% DCU contamination can smoothly be determined with the described method. This was confirmed by the evaluation of serial tests on model mixtures. A relative standard deviation of $\pm$ 1.8%

was found when a model mixture containing 2.0% DCU in *tert.*-butoxycarbonyl glycine pentachlorophenyl ester was investigated.

The materials investigated included pentachlorophenyl, pentafluorpohenyl and *p*-nitrophenyl esters of amino acids such as glutamine, asparagine, histidine, glycine, alanine, phenylalanine, nitroarginine as well as some peptides with benzyloxycarbonyl or *tert.*-butoxycarbonyl protecting groups at the amino terminals. It should be noted that these materials are not chromatographed under the conditions described and no thermal decomposition leading to products interfering with the gas chromatographic determination has been found either. This naturally means that relatively large quantities of these materials are accumulated at the injector zone of the column. It is advisable therefore to replace the column load in this zone by a fresh one after about 10 injections.

EXPERIMENTAL

A Hewlett-Packard 7620 gas chromatograph with a flame ionization detector was used in this study. The chromatographic conditions were as follows: Column, glass tube, 4 ft. × 2 mm I.D., packed with 3% JXR on Chromosorb W HP, 90–100 mesh; Oven temperature, 190°; Vaporizer zone and detector temperature, 260°; Carrier gas (nitrogen) flow-rate, 35 ml/min.

Procedure

A 0.05-g amount of the material to be investigated is dissolved in 1 ml of ethanol–chloroform (1:1) containing 0.4 mg of docosane and 1 μl of this solution is injected on to the column. The DCU content is calculated from the ratio of the peak areas by means of a calibration graph.

ACKNOWLEDGEMENTS

The authors thank Mrs. Zs. Falka for her technical assistance.

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Journal of Chromatography, 118 (1976) 411–413

CHROM. 8881

Note

Analysis of steroids

XXVI*. Transformation of 3-methoxy-2,5(10)-diene steroids during their gas chromatographic analysis

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A great number of steroids undergo decomposition or transformation when chromatographed at elevated temperatures (200–250°) which is usually necessary for the evaporation of these less volatile materials. The best known examples are the cleavage of the 17-side chain of corticosteroids[1,2], formation of D-homo derivatives from 17-hydroxy-20-keto pregnane derivatives[3], dehydration of 4-en-3-hydroxy steroids[4], formation of cyclo derivatives from 3-sulphonyloxy-5-ene steroids[5], the fission of the alkoximes of ketosteroids to nitriles[6] and the decomposition of cholesterol heptafluorobutyrate to the corresponding 3,5-diene[10]. A common feature of these and some other reactions of this type is that they are either not unidirectional and quantitative under chromatographic conditions or it is very difficult to use them as the basis of reliable analytical procedures.

The present paper aims to extend the number of the above reactions by describing our observations on the re-arrangement of 3-methoxy-2,5(10)-dienes to 3-methoxy-3,5-dienes which under suitable conditions have been found to meet the requirements of quantitative analytical methods.

EXPERIMENTAL

A Hewlett-Packard 7620 gas chromatograph was used equipped with a flame ionization detector (FID).

Methanolic solutions of the samples were injected directly onto the column (6 ft. × 4 mm I.D., packed with 80–100 mesh Gas-Chrom Q coated with 1% QF-1, 3% OV-210, 3% SE-30, 3% JXR or, 3% OV-225). Temperatures: column oven 230°, vaporizer zone and detector 250°. The flow-rate of the carrier gas (nitrogen) was 45 ml/min.

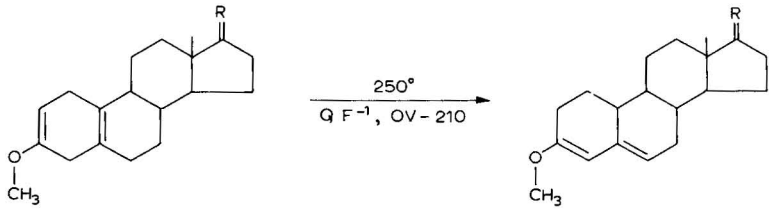
The same instrument was used for trapping the reaction products. The FID was replaced by a water-cooled U-tube and all solid materials eluted from the column after the repeated injections of the methanolic solution were collected.

* Part XXV: *Boll. Chim. Farm.*, 114 (1975) 98.

The ultraviolet (UV) and infrared (IR) spectra of the trapped materials were recorded by means of Pye-Unicam SP-1800 and Perkin-Elmer 257 instruments, respectively.

RESULTS AND DISCUSSION

During our analytical studies aimed at developing a quantitative method for the simultaneous determination of 3-methoxy-2,5(10)-oestradien-17β-ol (I) and 3-methoxy-2,5(10)-oestradien-17-one (II) it was found that their separation was excellent when the ketone-selective silicone QF-1 was used as the stationary phase (retention time for I 8.0 min and for II 9.8 min). The symmetry and reproducibility of the peaks enabled us to determine 1% of I in the presence of II.



I R = β-OH, H
II R = O

These good results seemed to be surprising to us as 3-alkoxy-2,5(10)-dienes are well known to be unstable, aromatization and formation of Δ^4-3-ketones being the main reaction routes of their transformation. Taking this into consideration it seemed to be unlikely that I and II were chromatographed intact. To clear up this problem the materials eluted from the column were trapped and subjected to spectroscopic investigation. The UV spectra of the materials obtained (white needles) in methanol show an intense maximum at 242 nm which is not affected by treatment with sodium borohydride[7–9]. This indicates the presence of the conjugated 3-methoxy-3,5-diene grouping (the parent non-conjugated 3-methoxy-2,5(10)-diene system is spectrophotometrically inactive). Further evidence supporting this structure was furnished by the IR spectra: the $\nu_{C=C}$ vibrations appearing with medium intensity at 1627 and 1651 cm^{-1} are also characteristic of the 3-methoxy-3,5-diene system.

The trapped materials were dissolved in methanol and re-chromatographed on the same column. The excellent agreement of the retention times and the acceptable agreement of the peak areas indicated that more than 85% of I and II were instantaneously transformed to the corresponding 3,5-dienes (see reaction equation) so that neither side reactions nor remarkable losses need be considered during the chromatographic process.

We repeated these investigations with all stationary phases listed under Experimental. It is interesting to note that similar results were only obtained using OV-210. All of the other columns resulted in chromatograms of poor quality and the spectra of the trapped materials showed that they were mixtures of various deriv-

atives. As both QF-1 and OV-210 contain fluoroalkyl groups it seems to be likely that the reason for the unidirectional re-arrangement in the case of these stationary phases is the directing effect of this grouping on the transformation of 3-methoxy-2,5(10)-dienes.

ACKNOWLEDGMENT

The authors are indebted to Mrs. Zs. Falka for valuable technical assistance.

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Journal of Chromatography, 118 (1976) 414–417

CHROM. 8928

Note

High-performance liquid chromatography of sterigmatocystin and other metabolites of *Aspergillus versicolor*

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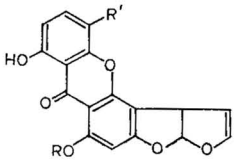
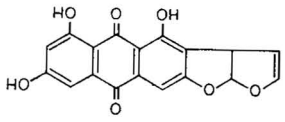
(First received October 14th, 1975; revised manuscript received November 24th, 1975)

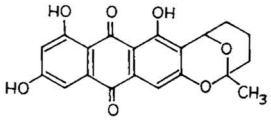
The fungus *Aspergillus versicolor* produces a large number of metabolites including the known carcinogen sterigmatocystin (I)[1] and the related compounds 5-methoxysterigmatocystin (II)[2] and demethylsterigmatocystin (III)[3]. In addition it produces the anthraquinone pigments versicolorin A (IV)[4], versicolorin C (V)[4], averufin (VI)[5] and avermutin (VII)[6]. These latter materials are of potential concern because of the similarity of the furofuran ring system of versicolorin A to that of the known carcinogens sterigmatocystin and aflatoxins B_1 and G_1 (ref. 7), and because certain dimeric hydroxyanthraquinones such as luteoskyrin and rugulosin are known toxins[8]. It is thus of interest to develop a method for the detection of these metabolites in cultures of *A. versicolor*.



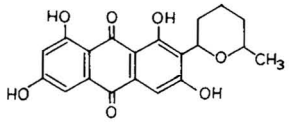
I: R = CH_3, R′ = H
II: R = CH_3, R′ = OCH_3
III: R = H, R′ = H



IV
V: dihydro- IV



VI



VII

Previous methods for the analytical detection of sterigmatocysin by thin-layer chromatography (TLC) techniques have been published[9,10], but little is known about the separation of the anthraquinone pigments. Previous methods for their separation have relied heavily on column chromatography on silica gel[4] or cellulose[11] and on TLC[12]. In our hands, however, the column techniques yielded incomplete separation and even TLC was unable to resolve averufin and avermutin; these compounds have been separated in the past by multiple development TLC[13]. In view of these problems with classical techniques, we turned to high-performance liquid chromatography (HPLC) in an attempt to develop a satisfactory separation. A recent paper describes the separation of some naturally occurring anthraquinones by HPLC and summarizes previous literature on their separation by other methods[14].

EXPERIMENTAL

Liquid chromatographic separations were performed on the apparatus previously described[15]. The pre-packed μPorasil® column, 4 mm I.D. × 30 cm, was obtained from Waters Assoc. (Milford, Mass., U.S.A.), and the prepacked Partisil-10 PAC® column, 4.6 mm I.D. × 25 cm, was obtained from Whatman (Bridewell, N.J., U.S.A.). Both columns were packed with particles of 10-μm mean particle size, and were used as received without pretreatment except for equilibration with the solvent systems used. Slight changes in the capacity ratios for the compounds studied were noted as the columns became older; the values reported are for columns that had been in use for approximately 200 h actual running time. The pressure was isobaric, and the sample injector and columns were at room temperature. Solvents (chloroform, hexane, ethyl acetate) were Burdick and Jackson "distilled in glass" grade or A.C.S. reagent grade (acetic acid–*n*-propanol) supplied by J. T. Baker (Phillipsburgh, N.J., U.S.A.). Solvent compositions are given in Table I; the solvent flow-rate was 2.0 ml/min in all cases. Sample injection was of a 10-μl aliquot of a qualitative standard stock solution.

TABLE I

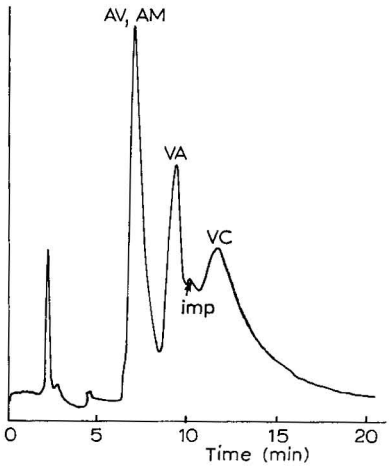
RETENTION VOLUMES AND CAPACITY FACTORS OF *A. VERSICOLOR* METABOLITES

System A: μPorasil; hexane–*n*-propanol–acetic acid (99.3:0.7:0.1). System B: μPorasil; hexane–ethyl acetate–acetic acid (83:17:1). System C: Partisil-10 PAC; hexane–chloroform–acetic acid (65:35:1).

Metabolite	*System A*		*System B*		*System C*	
	Retention vol. (ml)	*Capacity factor*	*Retention vol. (ml)*	*Capacity factor*	*Retention vol. (ml)*	*Capacity factor*
Sterigmatocystin	18.6	3.1	14.5	2.2	10.2	1.7
Demethylsterigmatocystin	—	—	—	—	5.6	0.5
5-Methoxysterigmatocystin	—	—	—	—	13.8	2.6
Averufin	14.0	2.1	11.5	1.6	44.6	10.7
Avermutin	14.0	2.1	11.5	1.6	50.0	12.2
Versicolorin A	18.8	3.2	14.5	2.2	70.6	17.5
Versicolorin C	23.5	4.2	17.2	2.8	84.8	21.3

RESULTS AND DISCUSSION

Of several solvent mixture–packing combinations investigated, three systems proved effective at separating the mixtures of compounds tested. These systems are summarized in Table I, and Figs. 1 and 2 show typical separations for two of the systems. The use of small amounts of acetic acid in the solvent was found to be helpful in suppressing tailing of the anthraquinone peaks, presumably because it suppresses the ionization of these strongly acidic quinones.



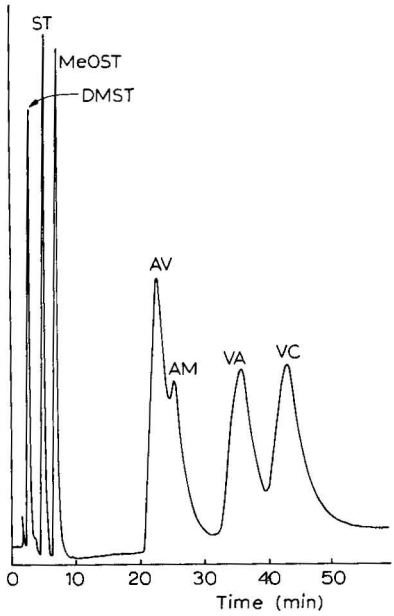


Fig. 1. HPLC separation of averufin (AV), avermutin (AM), versicolorin A (VA), versicolorin C (VC) on μPorasil. Solvent system, A (see Table I); flow-rate, 2.0 ml/min. imp. = impurity.

Fig. 2. HPLC separation of sterigmatocystin (ST), demethylsterigmatocystin (DMST), 5-methoxysterogmatocystin (MeOST), avermutin (AM), averufin (AV), versicolorin A (VA), and versicolorin C (VC) on Partisil-10 PAC. Solvent system, C (see Table I); flow-rate, 2.0 ml/min.

It is particularly noteworthy that the Partisil-PAC column is capable of separating averufin (VI) from avermutin (VII), in addition to separating the three sterigmatocystins. The silica gel microparticle column (μPorasil) gave good separations of averufin and the versicolorins, but did not separate averufin from avermutin. In addition, the sterigmatocystin peak overlapped the versicolorin A peak on this column. The bonded phase cyano-type packing thus seems to be packing material of choice for the separation of materials such as hydroxylated anthraquinones and xanthones; this conclusion has been confirmed by a recent study[16].

ACKNOWLEDGEMENTS

This work was supported by the U.S. Department of Health, Education, and

Welfare, Food and Drug Administration, on Contract 223-74-2146. Appreciation is expressed to Miss Sue Ellen Jolly for technical assistance. We thank Dr. J. S. E. Holker for an authentic sample of avermutin and information on its separation from averufin by TLC.

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Journal of Chromatography, 118 (1976) 418–424

CHROM. 8901

Note

The analysis of impurities in ethchlorvynol by gas chromatography–mass spectrometry

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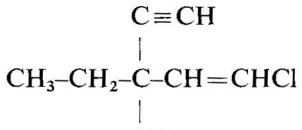
(Received October 27th, 1975)

Ethchlorvynol (1-chloro-3-ethyl-1-penten-4-yn-3-ol; I) is a widely used synthetic sedative. The reports of ethchlorvynol abuse resulting in intoxication, addiction, or death have been well documented[1–3]. In our work it was noticed that a pure sample of ethchlorvynol (Placidyl[1]) gave many peaks by gas–liquid chromatography. This led us to believe that many impurities may be present in the commercial product as well as in the sample supplied by Abbott.



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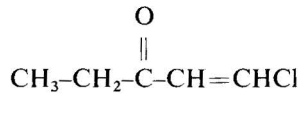


II

Ethchlorvynol exerts its action on the central nervous system and the extent of depression can be altered by these impurities. For this reason it is imperative that the impurities be identified. Various methods[1,4–8] have been described for determining ethchlorvynol in biological specimens. Washburn and co-workers[9,10] reported ethchlorvynol ketone (1-chloro-1-penten-3-one) (II) in 0.1% concentration as an impurity in the pharmaceutical preparation.

The purpose of this paper is to identify other impurities by means of gas chromatography–mass spectrometry (GC–MS).

EXPERIMENTAL

Several 500-mg capsules of Placidyl (Abbott, Chicago, Ill., U.S.A., Lot Nos. 854-1499 and 43-320-AF) and a 5-g sample of ethchlorvynol were obtained. The samples were analyzed directly. The mass spectra were taken on a Finnigan Model 3200 gas chromatograph–mass spectrometer which was coupled to a Finnigan Model 6100 data system. The samples were chromatographed through a 5 ft. × 2 mm glass column, packed with 3% OV-17 on Gas Chrom Q, 80–100 mesh. A column temperature of 90° and a helium flow-rate of 20 ml/min were used. The electron impact (EI) mass spectra were obtained at 70 eV with the ion source set at 180°.

For the chemical-ionization (CI) mass spectra methane was used as the reactant gas with a source pressure of 1 torr. The emission was 1 MAMP power voltage was 2 V, and the source temperature was 120°.

RESULTS AND DISCUSSION

The gas chromatograms and gas chromatograms–mass spectra were similar for both the capsule obtained and the sample supplied by Abbott. The major compound I as well as small amounts of 1-chloro-3-methyl-1-penten-4-yn-3-ol (III), 1-chloro-3-

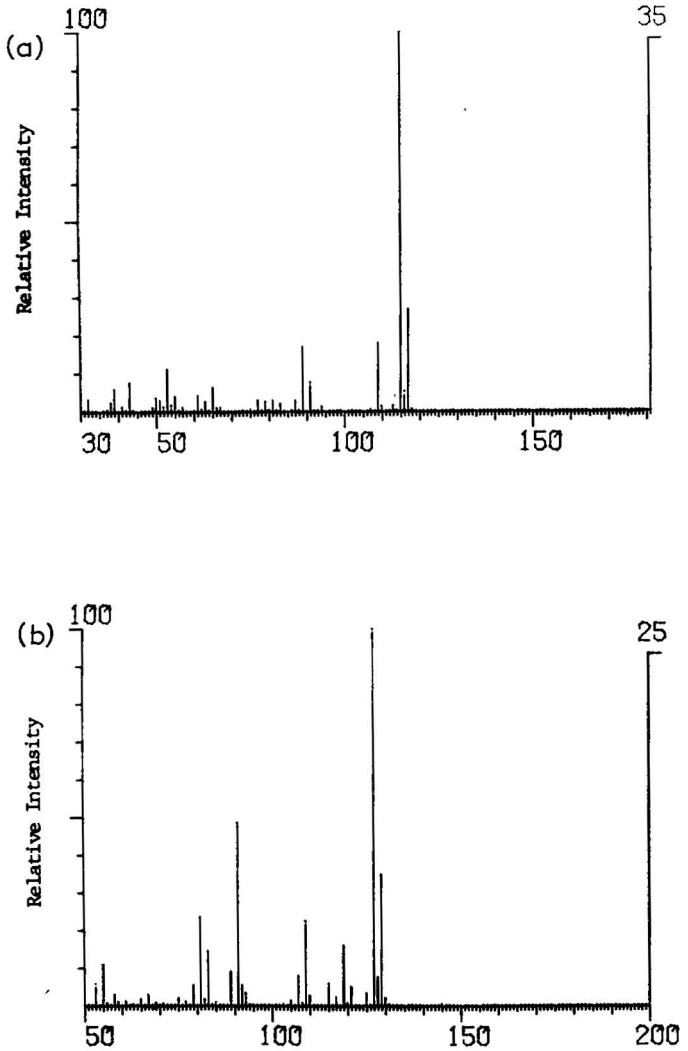
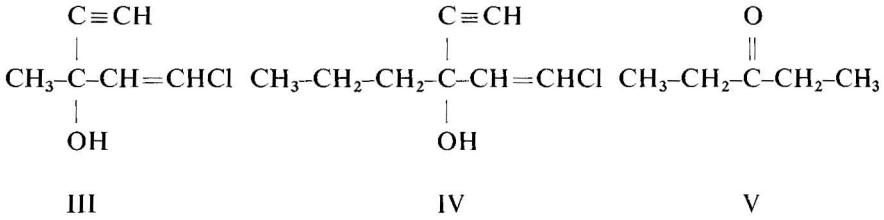
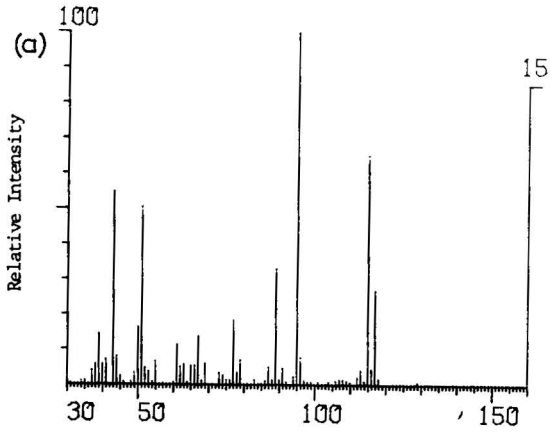


Fig. 1. Mass spectra of ethchlorvynol (I) by (a) EI and (b) CI.

propyl-1-penten-4-yn-3-ol (IV), and 3-pentanone (V) were found in both the capsule and vial. In addition the capsule contained a small amount of either 1,1- or 1,2-dichloro-3-ethyl-1-penten-4-yn-3-ol (VI).





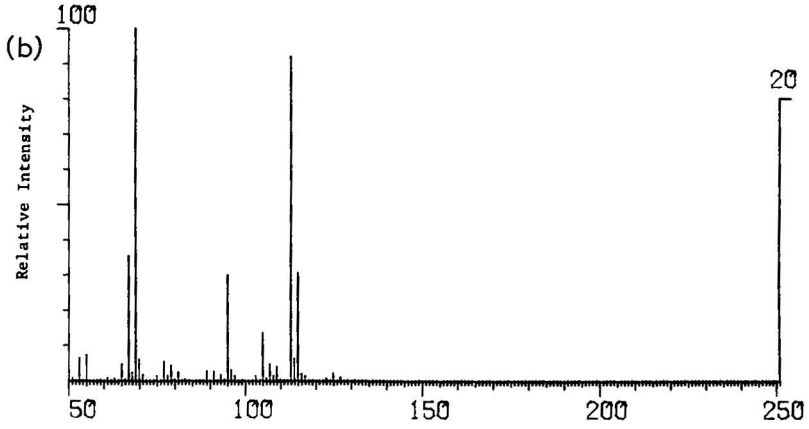
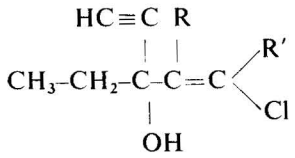


Fig. 2. Mass spectra of III by (a) EI and (b) CI.



VI R = H, R′ = Cl or R = Cl, R′ = H

The mass spectra of I, III, IV, V, and VI are shown in Figs. 1–5. The analysis of the various alcohols present as impurities is summarized in Table I. The EI mass spectrum of ethchlorvynol agrees very closely with that reported by Fales *et al.*[11]. The alcohols did not show prominent parent ions by EI. With the exception of III, the major route of decomposition was the loss of the alkyl portion of the molecule. The methyl derivative III showed loss of chlorine from the parent ion as the major route,

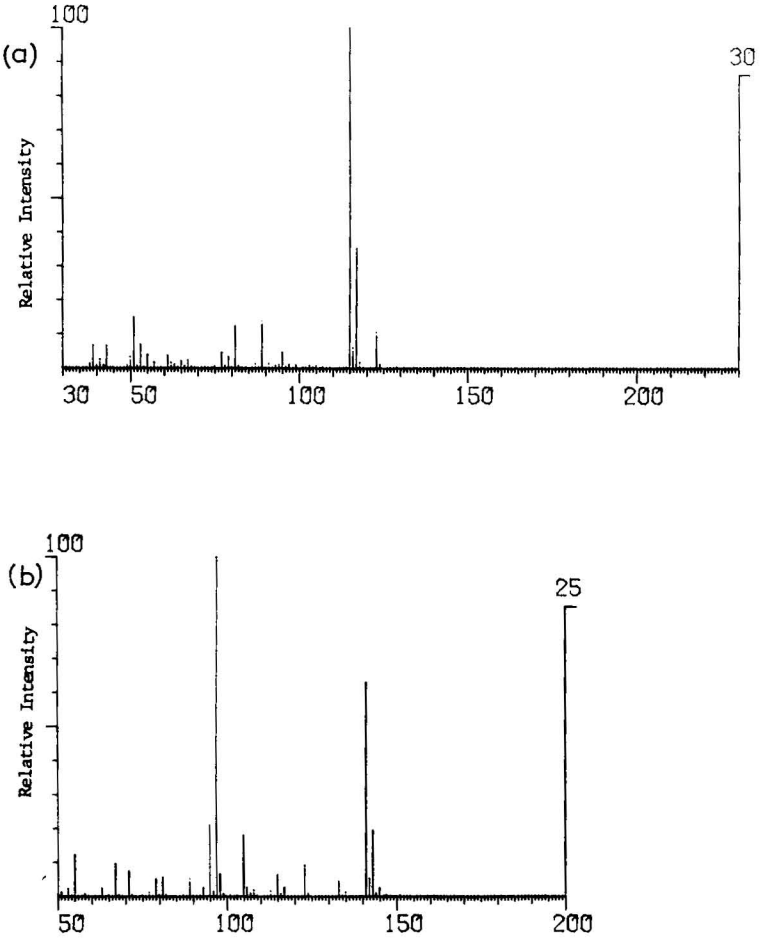


Fig. 3. Mass spectra of isomers of IV by (a) EI and (b) CI.

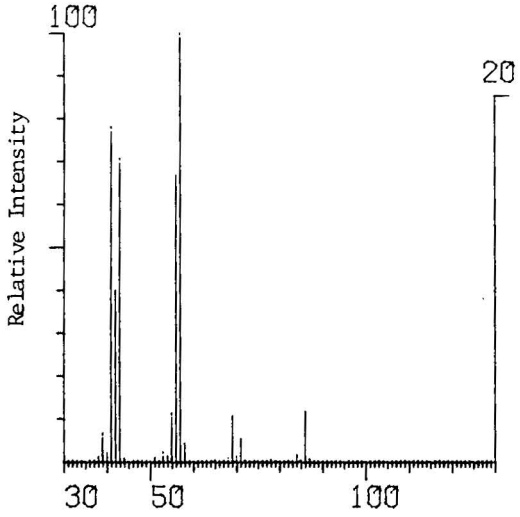


Fig. 4. Mass spectrum of V by EI.

TABLE I

FRAGMENTATION PATTERNS OF THE VARIOUS ALCOHOLS IN ETHCHLORVYNOL PREPARATION

Compound	*EI*				*CI*	
	Fragment cleaved from parent	*Peak (m/e)*	*Intensity relative to base peak*	*M or M+1 − fragment*	*Peak (m/e)*	*Intensity relative to base peak*
I	alkyl	115	100	(M+1) − water	127	100
	alkyl	117	27	(M+1) − water	129	35
	chlorine	109	18	M − chlorine − water	91	48
III	alkyl	115	65	(M+1) − water	113	92
	alkyl	117	27	(M+1) − water	115	30
	chlorine	95	100	(M+1) − chlorine	95	30
				M − alkenyl chlorine	69	100
IV	alkyl	115	100	(M+1) − water	141	64
	alkyl	117	37	(M+1) − water	143	22
	chlorine	123	12	M − water − chlorine	105	19
				M − alkenyl chlorine	97	100
VI	alkyl	149	100	(M+1) − water	161	88
	alkyl	151	77	(M+1) − water	163	54
	alkyl	153	05	(M+1) − water	165	11
	chlorine	143	42	(M+1) − alkenyl chlorine − hydrogen	117	100
	chlorine	145	13	(M+1) − alkenyl chlorine ± hydrogen	119	79
				(M+1) M alkenyl chlorine + hydrogen	121	15

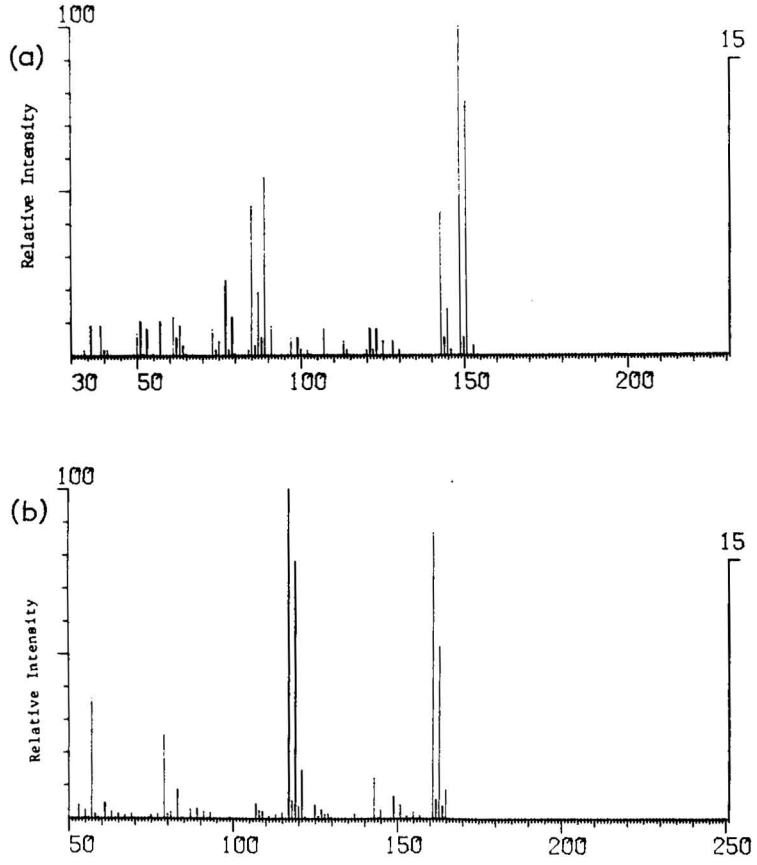


Fig. 5. Mass spectra of VI by (a) EI and (b) CI.

which was the secondary route for the other alcohols. Since the GC trace showed two peaks whose mass spectra were very similar, it is believed that these represent the two geometric isomers of compound IV. The order of elution from the gas chromatograph of the sample (Abbott) was V, III, I, and isomers of IV and VI. The CI spectra of III and IV exhibited loss of alkenyl chloride radical as the primary route. Although none of the alcohols possessed a M + 1 ion, the loss of water from the M + 1 ion was the secondary route with the exception of I, which exhibited dehydration as its major route. The dichloro derivative VI of ethchlorvynol exhibited large peaks at 161, 163 and 165 which were due to the loss of water from the protonated molecular ion; however the base peak was at *m/e* 117. Quite possibly the 117, 119 and 121 peaks were due to two different ions, each containing a single chlorine atom (^{35}Cl) with masses of 117 and 119. This is conceivable by the rearrangement of a chlorine atom to the C_2H_5–C(OH)(C≡CH)– moiety accompanied by the gain (*m/e* 119) or loss (*m/e* 117) of a hydrogen atom. Other routes include loss of chlorine and loss of water and chlorine from the parent ion. 3-Pentanone was detected and compared to the EI spectrum of a known

sample. A parent peak, *m/e* 86 was present but the major peak was represented by loss of ethyl radical (*m/e* 57). The secondary route was loss of ethane (*m/e* 56) followed by loss of methyl (*m/e* 41).

The GC results showed that the amount of ethchlorvynol in capsules varied from 94.040% to 99.784%; the sample from the bottle consisted of 99.48% ethchlorvynol.

Although the concentration of the impurities was small compared with that of ethchlorvynol, it is essential from the toxicological point of view that these impurities be identified. This report showed that a number of tertiary alcohols similar in structure to ethchlorvynol are the major impurities. Work is in progress in our laboratory to study the acute and chronic toxicity of these compounds.

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Journal of Chromatography, 118 (1976) 425–428

CHROM. 8950

Note

The gas–liquid chromatographic separation of selected catecholamines on polyamide A103

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(Received October 31st, 1975)

The use of polyamide A103 (Poly A103) phase for gas–liquid chromatographic (GLC) analysis of standard catecholamine solutions has been reported using mixed trifluoroacetyl–trimethylsilyl (TFA–TMS) derivatives[1]. In the present study this method of analysis has been applied to human urine for identification of conjugated dopamine (DA), tyramine (TYR), normetanephrine (NM) and metanephrine (MN). An additional method for catecholamine analysis employing trimethylsilyl (TMS) derivatives of NM, MN, DA, TYR, and norepinephrine (NE) on Poly A103 is presented.

EXPERIMENTAL

Preparation of TMS derivatives of DA, TYR, MN, NM, NE

Aliquots (100–500 μmoles) of each catecholamine solution, prepared in 0.001 *N* HCl, were dried in a Virtis Bio-Dryer. After flushing the vials with nitrogen, 50 μl of BSA [N,O-bis(trimethylsilyl)acetamide] (Applied Science Lab., State College, Pa., U.S.A.) was injected through a PTFE tape cover, screw caps were put in place, and the vials were heated at 76° for 3–4 h. Aliquots of the TMS derivative solutions were injected directly on the gas chromatograph.

Preparation of TFA–TMS derivatives of DA, TYR, NM, MN, NE

Solutions of TYR (14 μmoles), DA (12 μmoles), MN (109 μmoles), NM (109 μmoles), NE (102 μmoles) were prepared in 0.001 *N* HCl and brought to dryness under vacuum (Virtis Bio-Dryer). Mixed TFA–TMS derivatives were prepared by a modification of the method of Cancalon and Klingman[1]. Each standard catecholamine solution was dissolved in 50 μl methylene chloride and 50 μl of trifluoroacetic anhydride (Eastman, Rochester, N.Y., U.S.A.) was added. The reaction time was 7 min at 140°. After cooling and drying, 50 μl of pyridine and 20 μl of BSTFA [bis (trimethylsilyl)trifluoroacetamide] (Supelco, Bellefonte, Pa., U.S.A.) were added. The vials were heated at 105° for 25 min, cooled in ice and reduced in volume in the Bio-Dryer. Acetone was added to bring the final volume of solution to 7–8 μl and the entire solution was injected on the gas chromatograph.

Preparation of urinary NM, MN, DA and TYR derivatives

Conjugated NM, MN, DA and TYR were isolated from human urine using sequential Dowex 50-X4 (H^+) columns. An aliquot of urine was applied to the first column, the water effluent was collected, hydrolyzed at pH 1.0, and applied to a second column. MN, NM, and DA were eluted with 1 *N* and TYR with 2 *N* HCl. The eluates were lyophilized and derivatives prepared as described above except that 50 μl of pyridine was the solvent for the BSA reaction.

Gas–liquid chromatography

All GLC analyses were performed on glass columns (4 ft. × 4 mm I.D.) containing 3% Poly A103 on 100–120 mesh Gas-Chrom Q (Applied Science Lab.). The TMS derivatives were separated isothermally at 160°, 170° and 180° as well as by temperature programming from 150° to 200° at a rate of 4°/min. The mixed (TFA–TMS) derivatives were separated isothermally at 180° and 200° in addition to the temperature program of Cancalon and Klingman[1], 75–250° at 4°/min. The following conditions were employed for these studies: injection port, 230°; flame ionization detector, 250°; carrier gas (helium) flow-rate, 60 ml/min.

RESULTS AND DISCUSSION

Table I presents the chromatographic separation of the TMS derivatives prepared by reaction with BSA. No attempt was made to prepare quantitatively these derivatives and in fact, other derivatives may be seen in low concentration[2]. The complete separation of MN and NM was not achieved by either the isothermal or the temperature program conditions tested. Isothermally the overall separation of the catecholamines was best accomplished at 170° (Fig. 1), without improvement at either 160° or 180°. The temperature programmed chromatogram resembled that obtained isothermally at 170°. Low concentrations of MN and NM on both systems gave a double peak with NM preceding MN.

TABLE I

GAS CHROMATOGRAPHIC SEPARATION OF THE TMS DERIVATIVES OF CATECHOLAMINES ON POLY A103

Derivative	*Relative retention time, isothermal at 180°*	*Temperature program (4°/min)*
NE	0.45	168°
NM	0.51	170°
MN	0.54	170°
TYR	0.68	180°
DA	1.00	190°

The separation of urinary NE, MN, NM and DA by Dowex-50 column chromatography has been reported[3]. In the present study TYR was eluted with 2 *N* HCl. The BSA-prepared TMS derivatives of the appropriate fractions of TYR, MN and NM, and DA have been successfully chromatographed by temperature programming (150–200°) on Poly A103.

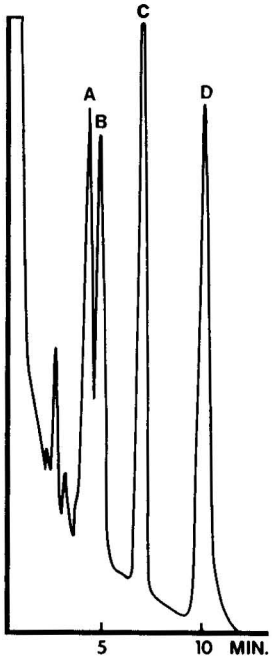

Fig. 1. Gas–liquid chromatogram on Poly A103 of the BSA prepared TMS derivatives of (A) norepinephrine, (B) metanephrine and normetanephrine, (C) tyramine, and (D) dopamine. Isothermal conditions were employed at 170°.

The mixed TFA–TMS derivatives of DA, TYR and NE have been reported by Cancalon and Klingman[1]. In the current study the positions of MN and NM at 190 and 202°, respectively, (Table II) are added to the temperature programmed chromatogram. In addition, the isothermal (180°) separation of the mixed TFA–TMS derivatives of MN, TYR, NM and DA on Poly A103 is here reported. Difficulty was encountered with the formation of the mixed derivative of NE. Two peaks were found on the temperature programmed chromatogram at 170° and 190°; the previously reported[1] NE derivative should elute at 212°. The NM derivative also gave a second peak at 170°. In both cases the two peaks were also detected by the isothermal GLC system. The mixed derivative of urinary DA was readily chromatographed by temperature programming (Fig. 2). By comparison with the standard catecholamine

TABLE II

GAS CHROMATOGRAPHIC SEPARATION OF THE TFA–TMS DERIVATIVES OF CATECHOLAMINES ON POLY A103

Derivative	*Relative retention time, isothermal at 180°*	*Temperature program (4°/min)*
MN	0.43	190°
TYR	0.65	200°
NM	0.71	202°
DA	1.00	210°

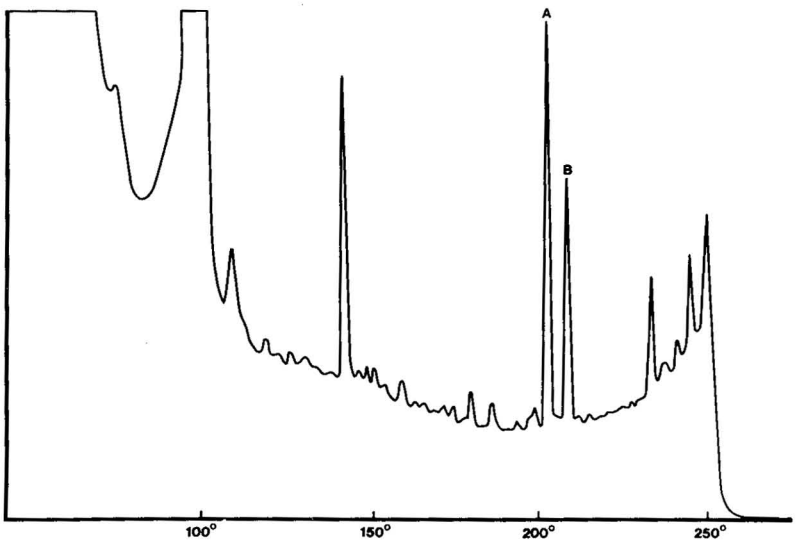

Fig. 2. Gas–liquid chromatogram on Poly A103 of an eluate from a Dowex-50 column of human urine. Dopamine (B) is separated from an unknown component (A) by use of temperature programming of the mixed TFA–TMS derivative.

elution temperatures (Table II) the peak preceding DA is neither MN nor NM, thereby demonstrating the complete separation of MN and NM from DA on the Dowex column. The mixed derivative of urinary TYR was similarly chromatographed with virtually no additional components seen.

It is concluded that Poly A103 is a useful phase for the GLC analysis of NE, NM and MN, TYR and DA utilizing either the TMS or TFA–TMS derivatives. This system has been applied to the study of urinary catecholamines.

ACKNOWLEDGEMENTS

This work was supported by contract N00014-68-A-0516, Office of Naval Research, Project Themis, and by a grant from Wallace Laboratories, Cranbury, N.J.

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Journal of Chromatography, 118 (1976) 429–432

CHROM. 8937

Note

Hochdruck-Flüssigkeits-chromatographische Analyse der Katecholamine Dopamin und Noradrenalin als Fluorescaminderivate

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(Eingegangen am 6. Oktober 1975; geänderte Fassung eingegangen am 26. November 1975)

Die Katecholamine Dopamin und Noradrenalin lassen sich mit dem Reagenz Fluorescamin zu fluoreszierenden Verbindungen umsetzen, deren Trennung durch Dünnschicht-Chromatographie (DC) von Imai *et al.*[1] und Hochdruck-Flüssigkeits-Chromatographie (HPLC) ebenfalls von Imai[2] beschrieben wurde. Bei der HPLC-Methode ist die geringste mit einem Fluorimeter bestimmbare Menge an Amin mit etwa 10 ng (= 60 pMol) angegeben, als Trennmaterial diente ein spezielles Gel.

Von den Reaktionsbedingungen sind bisher nur die Einflüsse des pH-Wertes und verschiedener Puffer systematisch untersucht worden[2]. Das Ziel dieser Arbeit war, die DC-Methode auf die HPLC zu übertragen und die einzelnen Reaktionsparameter im Hinblick auf eine grösstmögliche Empfindlichkeit der Methode zu optimieren.

EXPERIMENTELLES

Die folgenden Geräte wurden verwendet: Varian Aerograph 4000 Flüssigkeits-Chromatograph, DuPont 836 Fluorescence Detector, 8-μl Quarzküvette (Excitation Filter 325–385 nm, Emission Filter 451 nm); 25-cm Stahlsäule (I.D. 2 mm), gefüllt mit LiChrosorb Si 60-10 (10 μm, Fertigsäule der Fa. Varian (Darmstadt, B.R.D.) "Micropak SI-10"); Aminco-Bowman Spectrophotofluorometer.

Chemikalien: alle organischen Lösungsmittel z.A. (E. Merck, Darmstadt, B.R.D.), Fluram (= Fluorescamin; Serva, Heidelberg, B.R.D.). Eisessig (100%), Natriumchlorid, Dinatriumhydrogenphosphat, Tris(hydroxymethyl)aminomethan, alle z.A. (Merck); Dopamin- und Noradrenalin-Hydrochlorid (= 3-hydroxytyramine hydrochloride und DL-arterenol hydrochloride, B grade-Calbiochem, Luzern, Schweiz).

Lösungen: 0.1 *M* Phosphatpuffer (pH 8.0); 0.05 *M* Trispuffer (pH 8.0); 0.1 *N* Salzsäure; Fluorescaminlösung, 0.2 mg/ml Aceton.

Methodik

Für die DC-Trennung der Katecholamine als Fluorescaminderivate setzten Imai *et al.*[1] als Fliessmittel ein Gemisch aus Benzol, Dioxan und Essigsäure (90:25:5)

* Geschäftsführender Direktor: Prof. Dr. Dr. J. Rutenfranz.

ein. Ausgehend von dieser Zusammensetzung wurde mit einer Kieselgel-Säule (10 μm-Teilchen) die HPLC-Trennung optimiert. Das Ziel war, eine gute Auftrennung mit einer grösstmöglichen Empfindlichkeit (d.h. Peakhöhe) zu kombinieren. Für das Produkt aus der Auftrennung R und der Peakhöhe h sollte ein Maximum in Abhängigkeit von der Zusammensetzung der mobilen Phase ermittelt werden.

Bei den Untersuchungen zur Optimierung der Umsetzung mit Fluorescamin wurden folgende Einflüsse berücksichtigt: Reaktionstemperatur, Gehalt an Aceton im Reaktionsgemisch, Fluorescaminüberschuss, Extrahierbarkeit mit organischen Lösungsmitteln, Stabilität der Fluorophore.

Analysenvorschrift

0.2 ml Probelösung (bis zu 1 μg Amin) werden mit 0.3 ml 0.1 M Phosphatpuffer (pH 8.0), 0.3 ml Fluorescaminlösung (0.2 mg/ml Aceton) und 0.2 ml bidest. Wasser gemischt. Nach der Sättigung mit Natriumchlorid erfolgt die Extraktion mit 0.5 ml Essigsäureäthylester (Schütteldauer eine Minute). Jeweils 50 μl der 0.8 ml umfassenden organischen Phase (Aceton wird quantitativ mitextrahiert) werden zur HPLC auf die Säule gegeben. HPLC-Bedingungen: siehe Fig. 2.

ERGEBNISSE UND DISKUSSION

Hochdruck-Flüssigkeits-Chromatographie

Eine Erhöhung des Anteils an Essigsäure im Elutionsgemisch Benzol–Dioxan–Essigsäure verschlechtert die Auftrennung von Noradrenalin und Dopamin als Fluorescaminderivate, jedoch sind 2% Essigsäure erforderlich, um symmetrische Peakformen zu erhalten. In Abhängigkeit von der Zusammensetzung der mobilen Phase und der Reaktionsbedingungen treten nämlich bei Noradrenalin zwei Peaks unterschiedlicher Grösse auf, für deren Auftrennung dieser Anteil an Essigsäure erforderlich ist.

Die Erhöhung des Dioxangehaltes verringert die Auftrennung der Hauptkomponenten von Noradrenalin und Dopamin, erhöht jedoch die Empfindlichkeit. Für das Produkt aus der Auftrennung R und der Peakhöhe h ergibt sich in Abhängigkeit vom Dioxangehalt bei 22% ein Maximum (Fig. 1).

Bei der DC an Kieselgel[1] und der HPLC an den Hitachi 3011 und 3010-OH Gelen[2] wurde für Noradrenalin von Imai *et al.* jeweils nur ein Reaktionsprodukt erhalten. Eine Erklärung für das Auftreten eines zweiten Peaks kann bisher nicht gegeben werden.

Reaktionsbedingungen

Die Reaktionsbedingungen lassen sich so optimieren, dass der zweite Peak für Noradrenalin im Verhältnis zum Hauptpeak möglichst klein bleibt und so die Möglichkeiten der quantitativen Analyse kaum beeinträchtigt (Fig. 2).

Der Acetongehalt in der Reaktionslösung muss 30–40% betragen, Fluorescamin mindestens in 40-fachem molaren Überschuss vorliegen (Fig. 3a und b).

Die Reaktionstemperatur wurde von Imai *et al.* in der ersten Arbeit[1] mit 4° angegeben. Umsetzungen bei 4, 15, 25 und 40° haben keine signifikanten Unterschiede in den Peakhöhen ergeben.

Der Einfluss des pH-Wertes und die Unterschiede zwischen Phosphat- und

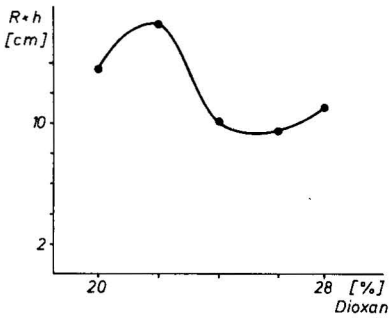

Fig. 1. Optimierung des Elutionsgemisches: Abhängigkeit des Produktes aus Auftrennung (R) und Peakhöhe (h) vom Dioxangehalt.

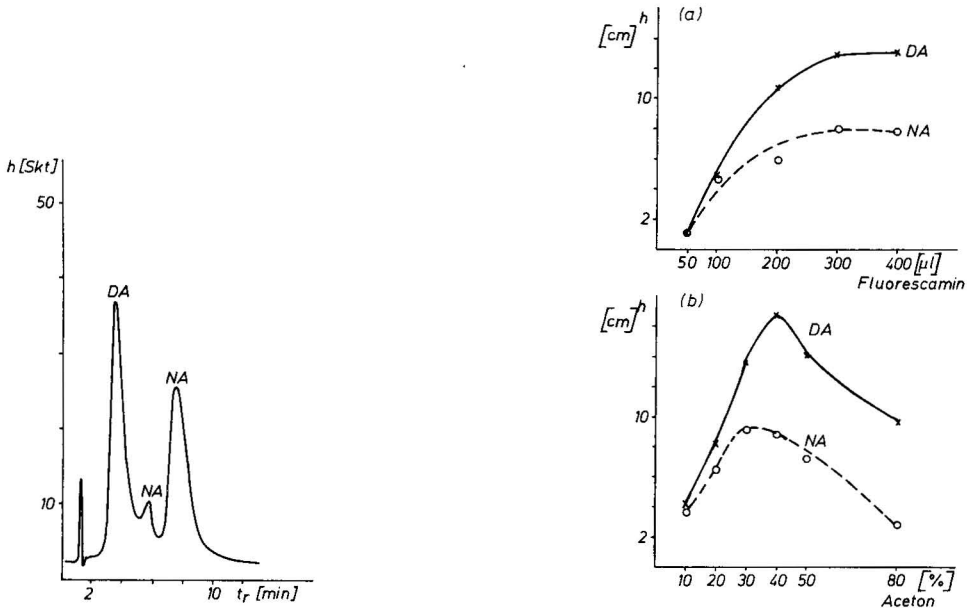

Fig. 2. Säule, Fertigsäule Micropak SI-10, 25 cm × 2 mm I.D., gefüllt mit Kieselgel LiChrosorb SI 60-10, (10 µm); Druck, 50 bar; Temperatur, 22°; Mobile Phase, Benzol–Dioxan–Essigsäure (76:22:2); Durchfluss, 0.50 ml/min; Probenmenge, 50 µl (1 µg/ml Amin); Detektor, DuPont Fluorescence Detector 836, 8 µl Quarzküvette, (Exitation Filter 325–385 nm, Emission 451 nm).

Fig. 3. (a) Abhängigkeit der Umsetzung (= Peakhöhe) von der Fluorescaminmenge, (b) Abhängigkeit der Umsetzung vom Acetongehalt des Reaktionsgemisches (je 1 µg Amin umgesetzt).

Boratpuffer wurden bereits von Imai[2] festgestellt. Aufgrund der komplexierenden Wirkung der Borationen werden nur geringe Fluoreszenzintensitäten gemessen. Verwendet man einen Trispuffer (pH 8.0), so tritt in der wässrigen Phase eine mit der im Phosphatpuffer vergleichbare Fluoreszenz auf. Jedoch lassen sich aus dem Trispuffer die Fluoreszenzprodukte mit Essigsäureäthylester nicht extrahieren, was vermutlich auf eine Wechselwirkung zwischen den Fluorophoren und den Pufferionen oder -molekülen zurückzuführen ist.

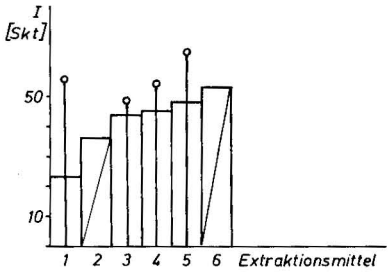

Fig. 4. Extrahierbarkeit der Fluorescaminderivate (am Beispiel des Noradrenalins). Rechteck, Messung in der Küvette; ○, Peakhöhe der HPLC-Analyse; Diagonale, kein Peak in der HPLC-Analyse; Extraktionsmittel: 1, Methyläthylketon; 2, *n*-Butanol; 3, Methyl-*n*-propylketon; 4, Diisopropyläther; 5, Essigsäureäthylester; 6, Isobutanol.

Für die HPLC-Analyse empfiehlt es sich wegen des organischen Elutionsgemisches, die wässrig–acetonische Lösung nicht direkt einzuspritzen, sondern die fluoreszierenden Verbindungen vorher mit einem organischen Lösungsmittel zu extrahieren, wodurch ausserdem eine Anreicherung möglich ist.

Die Messungen zur Extrahierbarkeit der Fluorophore wurden zuerst in Küvetten mit einem Spectrophotofluorometer am Beispiel des Noradrenalins durchgeführt (Anregung: 390 nm, Fluoreszenz: 490 nm). In Isobutanol und Essigsäureäthylester erhält man die höchsten Fluoreszenzintensitäten (Fig. 4), wobei die wässrige Phase mit Natriumchlorid gesättigt werden muss. Werden diese Extraktionslösungen jedoch auf die Säule gegeben, so finden sich in Iso- und *n*-Butanol keine Noradrenalin- bzw. Dopamin-Fluorescamin-Produkte. Dagegegen ist der Blindpeak in diesen Lösungen um das 2–3-fache gegenüber der Blindlösung höher. Eine Auftrennung dieser Peaks an Kieselgel war nicht möglich. Wie die Fig. 4 zeigt, erzielt man für die HPLC-Analyse mit Essigsäureäthylester die besten Ergebnisse.

Bei der Extraktion mit Methyläthylketon wird in der Küvette ein relativ hoher Blindwert gemessen. Die Differenz zwischen Probenmesswert und Blindwert ist im Vergleich zu den Peakhöhen der HPLC-Analyse gering (Fig. 4). Möglicherweise spielen hierbei Quencheffekte oder Verunreinigungen im Lösungsmittel eine Rolle. Die Fluoreszenzintensitäten der Fluorescaminprodukte sind in Essigsäureäthylester und auch in der wässrigen Phase etwa 20 min annähernd stabil, nach zwei Stunden haben sie um etwa ein Drittel abgenommen. Die Bestimmungsgrenze liegt bei etwa 200–500 pg je Einspritzung und Amin.

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Journal of Chromatography, 118 (1976) 433–436

CHROM. 8899

Note

Separation of inorganic isomers by thin-layer chromatography

III. Square planar geometric isomers

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(Received November 11th, 1975)

In the first publication in this series[1], we described the separation by thin-layer chromatography (TLC) on silica gel of a number of square planar non-electrolytic geometric isomers of platinum(II) that we had separated previously by column chromatography[2] along with the separation of two pairs of non-electrolytic binuclear platinum(II) isomers for which column chromatography had proven unsuccessful. In our second paper[3], we extended our TLC separations to include a number of different types of octahedral geometric isomers of various metals. In the present paper we report the TLC separation of a number of non-electrolytic as well as electrolytic square planar geometric isomers of other metals in addition to platinum, including the three isomers of $[Pt(NH_3)(py)(Br)(NO_2)]$. Inasmuch as we demonstrated in our first article[1] that separations can be carried out quantitatively and with significant amounts of material (*ca.* 200 mg of total mixture), all the separations reported here are strictly qualitative. In addition to using mixtures prepared from pure isomers, we also applied the method to materials that should theoretically exist in more than one isomeric form.

EXPERIMENTAL

Isomer samples were kindly provided by the persons listed alphabetically under Acknowledgements (designated by initials in Table I). All eluents were C.P. or reagent grade. Generous samples of the adsorbents used, SilicAR® TLC-7F and TLC-7G, were provided by Mallinckrodt (St. Louis, Mo., U.S.A.). Microscope slides (75 × 25 mm) were used for all separations except for sample 8 (Table I) which required 200 × 50 mm plates to achieve separation. Plates were developed by the ascending technique, and iodine vapor was used for visualization. The thiosemicarbazide complexes (samples 5–7) could also be detected by spraying the developed plates with $FeCl_3$ solution. Further details are given in previous articles[1–3].

RESULTS AND DISCUSSION

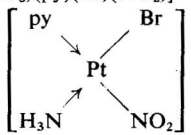
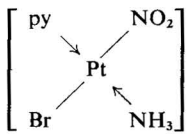
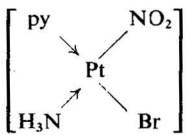
The results obtained are summarized in Table I. R_F values were reproducible to ± 0.03. Although many developing solvents and mixtures were evaluated, only the

TABLE I

THIN-LAYER CHROMATOGRAPHY OF TETRACOORDINATE GEOMETRIC ISOMERS

Abbreviations of sources: see Acknowledgements.

No.	*Isomer*	*Source*	*Developing solvent*	R_F		ΔR_F	*Type of separation*
				cis	*trans*		
Type MA_2BC							
1	$[Ir(Ph_3P)_2(CO)Cl]$* one form (lemon yellow)	B.W.M.[4]	Benzene	0.00	0.61	0.61	Complete
Type $M(AB)_2$							
2	$[Pt(glycinate)_2]$ *trans* (white); *cis* (white)	F.J.[5]	Methanol–dichloromethane (1:1)	0.61	0.85	0.24	Partial (smearing of spots)
3	$[Cu(tyrosinate)_2]$ *trans* (L) (dark blue); *cis* (DL) (blue-violet)	F.J.[6]	Methanol–water (1:1)	0.4–1.0 (considerable tailing)	0.63	?	Partial
4	$[Ni\{(CH_3)_2N\cdot CH_2C(=NOH)\text{-}C(CH_3)=NO\}_2]$*,** *cis–trans* mixture; *trans* (orange); *cis* (red)	E.U.[7]	*n*-Hexane or dichloromethane	—	—	—	None
5	$[Ni(thiosemicarbazide)_2]^{2+}$ *cis*- and *trans*-SO_4; *cis*- and *trans*-$(NO_3)_2$; *trans*-Cl_2	R.A.H.[8]	(a) Acetone–water (1:1)	0.82; $(NO_3)_2$ (pink-red)	0.62; $SO_4\cdot 3\,H_2O$ (brown)	0.20	Complete
			(b) Methanol–water (1:1)	0.00; SO_4 (red-brown)	0.15; Cl_2 (greenish brown)	0.15	Complete
			(c) water	0.74; SO_4 (red-brown)	0.00; $SO_4\cdot 3\,H_2O$ (brown)	0.74	Partial (tailing)
			(d) Dissolved in C_7H_8, developed in water	Variable; $(NO_3)_2$ (pink-red)	Variable; Cl_2 (greenish brown)	—	Partial (partition chromatography)

6	$[Pt(thiosemicarbazide)_2]^{2+}$ *trans*-Cl_2 (light yellow); *cis*-SO_4 (light orange)	R.A.H.[8]	Water	0.60	0.50	0.10	Partial
7	$[Pd(thiosemicarbazide)_2]^+$ *cis*-$(NO_3)_2$; *cis*-SO_4; *trans*-Cl_2	R.A.H.[8]	(a) Acetone–water (1:1)	0.00; $(NO_3)_2$ (yellow-orange)	0.36; Cl_2 (yellow)	0.36	Partial (considerable tailing)
			(b) Various solvents	SO_4; decomposes (yellow)	Cl_2; decomposes (yellow)	–	None
Type MABCD							
8	$[Pt(NH_3)(py)(Br)(NO_2)]$	A.D.G.[9]	Dissolved in dichloromethane; developed in acetone–chloroform (1:3)		(a) 0.00 (b) 0.32 (c) 1.00		Complete
	(a) 	(very pale yellow)					
	(b) 	(yellow)					
	(c) 	(pale yellow)					

* Previously separated by column chromatography[10].

** These compounds have since been found to be coordination polymers rather than *cis–trans* isomers[12].

most successful combinations, *i.e.* those resulting in maximum differences between R_F values and minimum tailing, are shown. The following samples, listed by type and number, were successfully separated: MA_2BC:1; $M(AB)_2$:2 and 3 (partial), 5(a) and (b), 5(c) and (d) (partial), 6 and 7(a) (partial); and MABCD:8. The remaining samples (4, 7(b), 9, and 10) could not be separated. We have previously separated samples 1 and 4 by column chromatography on Al_2O_3. With several exceptions (samples 3, 5(a), 5(c), and 6), R_F values for the *trans* isomer are greater than those for the *cis* isomer.

CONCLUSIONS

The advantages of TLC in the separation of isomers has been discussed in previous papers[1,3]. In column chromatography, with only a few exceptions, the *trans* isomer is more mobile than the *cis* isomer and is therefore eluted first[11]. In TLC, on the other hand, while we have likewise generally found the *trans* isomer to be more mobile, *i.e.* R_F *trans* $> R_F$ *cis*, exceptions to this general rule appear to be more numerous and may even depend upon the developing solvent (sample 5). Therefore, while TLC behavior may have some value in proof of configuration, it should be used with more caution than column chromatography and should always be supplemented by other data.

ACKNOWLEDGEMENTS

We gratefully acknowledge the donors of the Petroleum Research Fund, administered by the American Chemical Society (Grant 1152-B), the National Science Foundation (Undergraduate Research Participation Program Grants GY 2607 and GY 9916), and the California State University, Fresno Research Committee for support of this research. We also wish to thank Gary L. Anderson and Kenneth Berryhill for experimental assistance and the following persons, listed in alphabetical order, for kindly providing experimental samples of isomers: Anna D. Gel'man, Roland A. Haines, František Jursík, Bernard W. Malerbi, and E. Uhlig.

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Journal of Chromatography, 118 (1976) 437–438

CHROM. 8890

Book Review

Extraction chromatography, (Journal of Chromatography Library, Vol. 2), edited by T. Braun and G. Ghersini, Elsevier, Amsterdam, London, New York, and Akadémiai Kiadó, Budapest, 1975, XVII + 566 pp., price Dfl. 130.00, US$ 54.25, ISBN 0 444 99878 0.

One of the areas of rapid and steady development in chromatography has been that of extraction chromatography. This discipline was actually initiated about 16 years ago, when S. Siekierski and J. W. Winchester independently reported the separation of inorganic substances on columns involving liquid–liquid extractants used in spent nuclear fuel. Although developed initially in highly specialized nuclear laboratories, this technique has received marked general attention recently, finding more and more successful applications in many analytical problems.

Despite its increased significance in separation science, in the past it has at best been treated as review articles or as chapters in books concerned with other subjects. We are indeed fortunate that Prof. T. Braun (L. Eötvös University, Budapest) and Prof. G. Ghersini, (CISE, Milan) have now filled this gap. The book contains chapters by 20 authors from Europe, U.S.S.R. and Japan, each responsible for a chapter in which a particular aspect of extraction chromatography is presented and thoroughly discussed.

The first chapter commences with a general account by S. Siekierski of the theoretical aspects of extraction chromatography. Topics included are (i) the role of the support in the thermodynamic activities of the extractants and extracted complexes, (ii) the extraction mechanism and (iii) dynamic factors that affect the plate height. In the next chapter, I. Akaza describes the relationship between extraction chromatography and liquid–liquid extraction in a concise manner. Chapter 3, by P. Markl and E. R. Schmid, deals with the detailed experimental techniques of the method, informative even for beginners, giving good illustrative examples. The next two chapters deal with the most essential components of the technique, *i.e.*, the organic stationary phase (by G. Ghersini) and its supporting materials (by G. S. Katykhin), without presenting merely a catalogue. In the former chapter, performance characteristics of the stationary phases and their effect on the overall behaviour of chromatographic columns are thoroughly discussed. The many different extractants used in column chromatography and distribution data on these solvents (Chapter 4) and full information on the currently available support materials (Chapter 5) are presented and discussed, both chapters serving as good guide for selecting and developing chromatographic systems. In Chapter 6, I. Stronski writes about the extraction chromatographic methods that are now available for the separation of metals and non-metals. Particular reference is made to the problems involved in radiochemical separations of mother–daughter pairs and of separations of closely related elements in particular groups of the Periodic Table.

The next few chapters deal with fields where extraction chromatography has

contributed to a significant extent in solving particular problems in analytical chemistry and radiochemistry. Chapter 7, by W. Müller, deals with the liquid–liquid extraction and extraction chromatography of actinides, with many examples of actinide separations. Chapter 8, by S. Siekierski and I. Fidelis, covers the fundamentals of lanthanide extraction, including a full discussion of the double–double effect and its significance in lanthanide and actinide separations. A survey of lanthanide separations is also given. The subject of Chapter 9 is the separation and burn-up analysis of fission products by M. Bonnevie and K. Joon. In Chapter 10, C. Testa gives many analytical procedures for radiotoxicological determinations in which extraction chromatography has been successfully incorporated. Pre-concentration of trace amounts of metals is the subject of Chapter 12, by I. P. Alimarin and T. A. Bolshova.

Independent chapters are provided on the use of chelating agents as the stationary phase (Chapter 11, by F. Šebasta) and the use of cellular plastics (foam materials) as the support (Chapter 13, by T. Braun and A. B. Farag.) Foam materials have been investigated extensively by Braun and Farag during the last few years, either unloaded or as a means of immobilizing hydrophobic organic reagents and extractants for the collection and separation of inorganic or organic species from aqueous solution [see also *Talanta*, 22 (1975) 699].

Extraction chromatography is not confined to the column technique, but is also developing as forms of paper and thin-layer chromatography. This is the subject of Chapter 14, by G. Ghersini and E. Cerrai. Detailed experimental techniques are given and the relationships between R_F and D and/or retention volumes are critically reviewed. In addition, extensive literature data on R_F values are given. The final chapter, by E. Eschrich and W. Drent, gives a comprehensive bibliography covering the literature, including both column and laminar systems, up to the end of 1972, some references from 1973 also being included. Some 629 papers are listed with full titles. This is followed by a table that surveys experimentally important data on column extraction chromatographic investigations.

This book will no doubt serve both as an excellent standard text and as the authoritative handbook of extraction chromatography, and indeed will serve as the best reference source in many laboratories, particularly analytical and radiochemical laboratories.

The binding and cover are excellent, but unfortunately there are many misprints. Much of the text is poorly printed, and the type styles are often incorrect. Technical terms and notations are not always consistent throughout the book; *e.g.*, both distribution coefficient and distribution ratio are used for the same subject, and sometimes the technical terms are inadequate (*e.g.*, nitric complex, p. 284). Fundamental mathematical treatments often overlap, and the reader interested in practical techniques should note carefully that, *e.g.*, loading procedures are considered in several places throughout the text. In spite of these shortcomings, the book is a worthwhile contribution and will be invaluable to the analytical chemist and radiochemist, as well as to students, physicists and research workers in biology and medicine.

Chiba (Japan) ROKURO KURODA

Journal of Chromatography, 118 (1976) 439–440

CHROM. 8845

Book Review

Advances in chromatography, Vol. 12, edited by J. C. Giddings, E. Grushka, R. A. Keller and J. Cazes, Marcel Dekker, New York, 1975, XV + 278 pp., price US$ 22.75.

The twelfth volume in the series *Advances in Chromatography* continues to provide the chromatographer with stimulating reading. The book contains seven contributions covering a wide range of subjects and each will be considered in turn, but in general the contributions maintain the same high standard that has been found in previous volumes of this series, and in this, the Editors are to be congratulated.

Chapter 1 by Phyllis R. Brown provides a survey of the use of "Liquid chromatography in pharmacology and toxicology". Basically this review provides a list of the various applications in the field mentioned. but with little emphasis on the problems posed by liquid chromatography. It should prove quite useful to workers in the field in providing guide-lines for obtaining separations. However, one is left somewhat in doubt by the meaning of Fig. 1, the two parts of which bear little if any correspondence.

Chapter 2 by Leon Segal on the "Chromatographic separation and molecular weight distribution of cellulose and its derivatives", provides an excellent review on the problems involved in the analysis of cellulose. Two techniques are discussed, precipitation chromatography and gel permeation chromatography and the latter is shown to be the preferred technique for this problem. This is a well documented review with 68 references.

Chapter 3 by G. J. Fallick on "Practical methods of high speed liquid chromatography" gives a very useful summary of the technique together with a comprehensive account of how to tackle unknown samples. The review is highly biased to one company's products, no reference is made to the fact that other commercial equipment is capable of undertaking the work proposed. Nevertheless, a very useful review, and recommended to beginners in the field.

Chapter 4 by V. R. Maynard and E. Grushka on the "Measurement of diffusion coefficients by gas-chromatography broadening techniques" provides a comprehensive critical review on the title subject, and physical chemists would be advised to consider the findings. However, it is somewhat difficult to understand what is meant by the third proposition on page 108. What has been written has no meaning. The Van Deemter equation (eqn. 11, p. 109) is incorrect, the second term should be $B/\bar{u}$. A useful table of diffusion coefficients is provided in the Appendix.

Chapter 5 by J. Sherma on the "Gas-chromatographic analysis of polychlorinated biphenyls and other non-pesticide organic pollutants" will provide the interested reader with a comprehensive survey of the methods currently in use for the sampling, clean-up, by various techniques, of halogenated materials classed as pollutants of the ecosphere. Well documented with 173 references.

Chapter 6 by D. H. Smith on "High performance electrometer systems for gas

chromatography" is outside the competence of the reviewer to comment on. However, the affiliation of the author puts little doubt upon the authenticity of the data provided, and an electronics expert will undoubtedly appreciate these writings.

Chapter 7 by A. Nonaka on "Steam carrier gas–solid chromatography" provides information on this little used technique in gas chromatography and demonstrates how the use of steam as the carrier gas can be invaluable for the analysis of highly polar materials such as amines and acids. Apparatus and general requirements for the use of steam as a carrier gas are discussed in detail together with the effect of this mobile phase on the flame ionization detector. There can be little doubt that this technique has a potential that requires further exploration.

The reviewer found this book stimulating to read, and although some of the subject matter is highly specialised and not of wide interest, a large amount of useful information can be gleaned. The book is well produced with clear type and adequate figures.

Brighton (Great Britain) C. F. SIMPSON

Journal of Chromatography, 118 (1976) 441–442

CHROM. 8893

Book Review

Thin-layer chromatography abstracts 1971–1973, by Ronald M. Scott and Münime Lundeen, Ann Arbor Sci. Publ., Ann Arbor, Mich., 1974, 589 pp., price £ 10.40.

The third volume of the series of abstracts on thin-layer chromatography (TLC) includes 1337 entries covering the period from 1971 to the first half of 1973, with some papers from 1969 and 1970. The entries are divided into 22 chapters according to the groups of compounds involved; a further chapter is devoted to the theory and techniques of TLC. Abstracts in individual chapters are assembled randomly, without any classification. The book is supplied with an author index in which, however, only the first author of each paper is listed (although this fact is not mentioned anywhere).

Before formulating any judgement about this publication, it is necessary to consider the question of what the reader is expected to gain from such bibliographic and abstracts literature. The main aim is obviously to save time for the worker who has to search and follow the literature in order to solve a particular problem. It is therefore necessary that a bibliography should provide information rapidly and should be reliable in covering a defined time period exhaustively. When a bibliography deals with a certain type of a technique, it is necessary to compile it in such a way that certain types of compounds or even a particular compound can easily be found. How are these demands met in this book by Scott and Lundeen? It is possible to say that it fulfils the first demand, *i.e.*, it provides information rapidly. Also, the content of abstracts can be considered to be satisfactory as it pinpoints the most important experimental data. Unfortunately, the meeting of the other demands is considerably less satisfactory. This bibliography is far from exhaustive and it is not possible to rely upon it and to omit following the original literature. On average, only about 25% of published papers are quoted but with some groups of compounds this proportion is as low as 10%. Although it is dealt with in the TLC bibliography, in the chapter on theory and techniques, only 27 papers published in 1971–73 are quoted, whereas during this period at least 250 papers were published on this topic. Similarly, in the chapter devoted to antibiotics, only 13 papers are quoted but a brief search of the literature offered over 100 papers that deal with TLC of antibiotics.

Because the book is not supplied with a subject index, the division into 23 chapters is not detailed enough. Thus, if one is searching for a particular compound, one has to look through the whole chapter (which in the case of lipids, for example, contains 100 pages).

Too rapid editorial work, of course, leads to errors. In this book, it is possible to find errors in titles, in abbreviations of journals, in authors' names, and in references in the author index. The listing of some papers in individual chapters can be questioned. The reader will probably miss chapters devoted to such important groups of compounds as vitamins, steroid glycosides, nitro compounds and proteins. The

selection of journals that have been abstracted is limited. Of 27 references in the chapter on theory and techniques, 26 come from the *Journal of Chromatography*. In the chapter on pesticides, 104 of the 109 papers quoted come from two journals. As an example, there is not a single paper quoted in this particular chapter from the *Journal of the Association of Official Analytical Chemists*, which contains a vast number of papers in this area. No books or reviews on TLC are quoted.

Although the authors do not claim that this is an exhaustive bibliography on TLC, the reader would expect to find at least the most important papers on a particular group of compounds, especially as far as the technique itself is concerned. However, this is not the case here. It is believed that there are other bibliographies available in both book and journal form that offer much more information than this book.

Journal of Chromatography, 118 (1976) 443

CHROM. 8891

Book Review

Rodd's chemistry of carbon compounds, Supplement to the 2nd ed., Vol. I, Parts A and B, edited by M. F. Ansell, Elsevier, Amsterdam, 1975, XVI + 268 pp., price Dfl. 110.000.

The second edition of Volume I, Parts A and B, of *Rodd's Chemistry of Carbon Compounds* was published in 1964 and 1965, *i.e.*, more than 10 years ago. Even now, however, all of the volumes of this work, which became popular among organic chemists as the major reference book in organic chemistry, have not been published. In order to bridge this time gap between individual volumes, each volume is repeatedly extended by supplements, which are divided into chapters corresponding to those in the parent volume. The aim of these supplements is to provide the most recent information in the appropriate field. The task of the editor in this work is difficult, as his success depends on the efficiency of contributors supplying their chapters in time, which obviously does not always happen. The only way of solving this problem is to add such a chapter to the next volume of this supplement. This is exactly what happened in the case of Chapter 2 (Unsaturated acyclic hydrocarbons) of the present volume. It should be appreciated that the way in which this has been done will probably not disturb the reader.

This supplement consists of seven chapters. It deals with aliphatic compounds in the following order: saturated hydrocarbons, unsaturated hydrocarbons (bibliography only), their derivatives such as halogens, monohydric alcohols (and their ethers and esters), sulphur analogues and nitrogen derivatives (nitro and nitroso compounds, amines, quaternary ammonium compounds, etc.). The last and largest chapter is devoted to aliphatic organometallic and organometalloidal compounds. The examples in individual chapters are illustrative and sometimes sufficiently recent (references from 1973). On the other hand, from the literature references in individual chapters it is obvious that the time interval over which the individual chapters were supplied to the editor was rather lengthy. Individual chapters are presented in a very condensed and concise form, which is easy to understand. With a few exceptions (see pp. 103 and 114), the editor has succeeded in eliminating duplications in the reactions covered in individual chapters. Perhaps the chapter on fluorinated hydrocarbons should have been completed by a brief note on the anomalous course of Yarovenko's reaction, and in the chapter devoted to hydroperoxides a brief note on the new aspects on the mechanism of olefin oxidations by singlet oxygen would have been useful.

Prague (Czechoslovakia) J. KOPECKÝ

Journal of Chromatography, 118 (1976) 444–445

CHROM. 8894

Book Review

Immobilized biochemicals and affinity chromatography, (Advances in Experimental Medicine and Biology, Vol. 42; Proceedings of the Symposium on Affinity Chromatography and Immobilized Biochemicals, Charleston, S.C., November 7–9, 1973), edited by R. B. Dunlap, Plenum, New York, 1974, XI + 377 pp., price US$ 22.50.

Affinity chromatography and immobilized biochemicals represent two new biochemical frontiers that have emerged in recent years through the ingenious development and application of solid-phase biochemical techniques. The rapid advance in this field is reflected in the numerous symposia that have taken place and in the reviews that have been published in journals and monographs. The book reviewed here contains most of the papers presented at the Symposium on Affinity Chromatography and Immobilized Biochemicals held in November, 1973.

In the first part, "Affinity Chromatography", contributions are included that report numerous examples of the successful application of this method to the isolation of enzymes, specific peptides and proteins on various supports, and also make the reader aware of certain disadvantages of the method. An example is the leakage of the coupled ligand into the solvent (March *et al.*, Wilchek) or the non-specific binding of proteins by supports with hydrophobic spacers, which give rise to the so-called hydrophobic chromatography (Hofstee). A valuable report for many readers will certainly be the chapter "Quantitative Parameters in Affinity Chromatography", which deals with the theory of affinity chromatography (Nishikawa *et al.*). Papers are also devoted to radioimmunoassay and covalent affinity chromatography and affinity chromatography using group-specific adsorbents. Specific elution and the structural requirements of ligands are also discussed.

Although a number of the examples quoted are included in the book *Affinity Chromatography** by C. R. Lowe and P. D. G. Dean (Wiley-Interscience, London, 1974, 272 pp.), the practical aim of the book reviewed here makes it a suitable supplement to the former book. In contrast, *Methods in Enzymology, Vol. 34, Affinity Techniques*, edited by W. B. Jakoby and M. Wilchek (Academic Press, New York, 1974, 810 pp.), offers much more information from both the practical and theoretical points of view.

The second part of the book, "Immobilized Biochemicals", describes the preparation, characterization and application of enzymes immobilized on glass, stainless steel, metal oxide supports, agarose, polyacrylamide gels, collagen, cellulose and Sephadex. Chapters on the use of membrane-bound enzymes and water-encapsulated enzymes in enzyme reactors are also included. The use of immobilized enzymes both as helpful heterogeneous catalysts and as tools in studies on the three-dimensional structure of proteins is discussed. There are interesting chapters on the

* Review: *J. Chromatogr.*, 118 (1976) 282.

use of immobilized asparaginase in the treatment of leukaemia and of immobilized cofactors and enzymes in the analysis of blood and tissues.

Books dealing with related problems are *Insolubilized Enzymes*, edited by M. Salmona, C. Saronio and S. Garattini (Raven Press, New York, 1974, 226 pp.), *Immobilized Enzymes for Industrial Reactors*, edited by R. A. Messing (Academic Press, New York, 1975, 232 pp.) and *Methods in Enzymology, Vol. 44, Immobilized Enzymes*, edited by K. Mosbach (Academic Press, New York, in press).

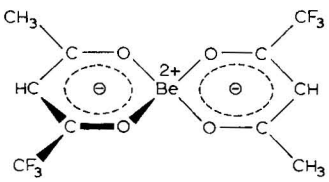
The book reviewed here contains useful material covering most of the important aspects of affinity chromatography and immobilized biochemicals and the reader will find in it a great deal of theoretical and practical information. The book contains a useful subject index.

Prague (Czechoslovakia) JAROSLAVA TURKOVÁ

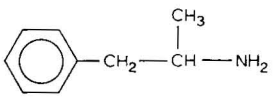
Author Index

Erratum

J. Chromatogr., 113 (1975) 303–356
Page 318, the formula should read



Page 325, the formula for amphetamine should read



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journal of
chromatography news section

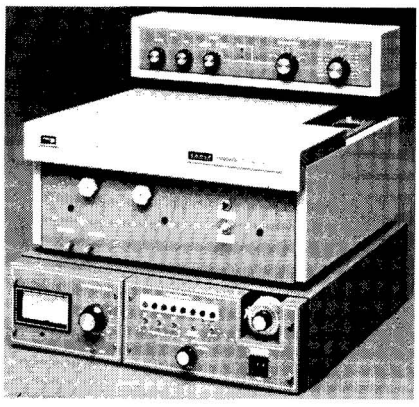
APPARATUS

N-808

SERIES-S ANALYTICAL GAS CHROMATOGRAPHS FROM CARLE

A 6-page technical bulletin No. 0105 is available describing the Series-S version of Carle Instruments' analytical gas chromatograph line. Series-S analytical gas chromatographs feature built-in automatic valving and multi-column systems pre-planned for specific applications.

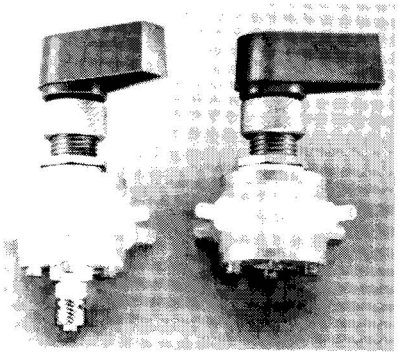
The fifteen different applications for which Series-S configurations are currently available are described in an easy-to-read table. Summarized are analyses as natural gas, ammonia production gases, respiratory gas, refinery gas streams, impurities in ethylene, propylene or other polymer feedstocks, impurities in chlorine, transformer oil headspace gas, hydrogen product gas.



N-821

HAMILTON INERT VALVE WITH TEFLON BODY

A miniature inert valve with an all-Teflon body has been introduced by Hamilton. The Model 2X valve (which replaces the former model with metal body) provides a reliable flow system control for laboratory handling of ultrapure or highly corrosive fluids, or for use in other manufacturers' equipment. It is available in 2-, 3-, 4- and 5-way configurations, and is leak-proof up to 100 p.s.i.



A spring-loaded rotor plug on the valve maintains constant pressure for even distribution of liquids or gases through the terminal holes.

N-819

HEWLETT-PACKARD JOURNAL

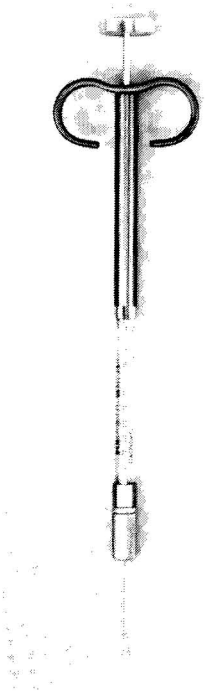
The October 1975 issue of the Hewlett-Packard Journal (Vol. 27, No. 2) features among others an article on flow control in high-pressure liquid chromatography.

N-810

HAMILTON HIGH-PRESSURE SYRINGES

Hamilton is introducing three precision syringes designed specifically for high-pressure chromatography.

The HP 1805 50-μl syringe introduced last year has proven so popular the company has added 10-μl, 25-μl, and 100-μl syringes to the line. These syringes have been pressure-tested to operate at working pressures up to 400 atm. (6000 p.s.i.).



They are equipped with adjustable friction locks to avoid plunger slippage, and with internal blow-out stops to prevent ejection of the plunger against several thousand pounds of inlet pressure.

Specially designed finger grips, a large plunger button, guides to prevent plungers from bending, and Teflon tips on the plungers minimize high-pressure injection problems and make the syringe easy to operate with one hand.

Additional features include a jam nut which allows adjustment of the needle to zero on the glass barrel; a replaceable glass barrel assembly; a metal holder; and removeable, interchangeable parts.

N-823

TECHNICON ION-EXCHANGE CHROMATOGRAPHY SYSTEM

The Technicon NC-2P single-column ion-exchange system consists of modular components, interconnected to form an integrated, automatic analyzer for separation, detection and quantitative analysis of amino acids in plant, fish or animal tissue. The system can be used to separate and analyze the free acidic, neutral, and basic amino acids and related compounds found in physiologic fluids and tissue extracts in 7.5 h. Similarly, hydrolysates of proteins or peptides can be separated and analyzed in less than 2 h. In both cases, results are consistent within ±3% for concentrations of individual components from 0.01–0.10 μmoles.

The NC-2P system may also be used for column effluent monitoring by colorimetric or fluorimetric analyses other than the reaction with ninhydrin, as commonly used for amino acids, for example, analysis of column-separated carbohydrates and/or proteinaceous compounds by the Folin–Ciocalteau (or Lowry modification) reaction.

The modules of the NC-2P system can easily be converted to a Technicon Autoanalyzer II continuous-flow analytical system by the addition of a few components, providing capability for performing a wide range of wet chemical analyses.

Also available is a conversion kit, which enables users of original Technicon NC-1 systems to upgrade their instrumentation to achieve the advanced capabilities of the NC-2P system.

N-814

TLC DATA REDUCTION DEVICES FROM KONTES

This line of data reduction devices includes calculating and digital integrators, baseline correctors, digital printers, recorders, and switching junctions designed especially for the thin-layer chromatographer.

CHEMICALS

N-800

COLUMNS WITH REPRODUCIBLE RETENTION TIMES FOR PROCESS GAS CHROMATOGRAPHS

Analabs announces a series of columns with reproducible retention times guaranteed to within 15 sec. Columns with reproducible retention times offer a solution to the problem of column change-over to a new column when a breakdown occurs. They are also useful in finger-printing formulations and other quality-control work where retention times are critical.

N-806

ANALABS ENVIRONMENTAL GC ANALYSIS SUPPLIES

Analabs announces a catalog, "what's new in gas chromatography", which features supplies for environmental analysis. Listed are vinyl chloride, PAH's PCB's PCT's and other standards together with many newly developed laboratory accessories and supplies for environmental researchers.

N-817

LIPID HANDBOOK

A new 146-page handbook of lipids, carbohydrates and amino acids has been released by Supelco. This third edition includes chemical structures, physical and chemical properties of fatty acids, esters, glycerides, phospholipids, glycolipids, tocopherols, steroids, bile acids, carbohydrates and amino acids. Also included is a section on reagents useful for preparing derivatives of these compounds.

For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

PROCEDURES

N-791

BIO-RADIATIONS NO. 19

Bio-Radiations No. 19, Bio-Rad's technical periodical, includes five major articles plus a column of short notes on helpful laboratory procedures. The major articles include: Cyclic nucleotide purification and attendant separation of cAMP and cGMP; Electrophoretic destaining without immersion; Accurate liquid dispensing with reproducibility of better than 0.2%; Econo-Columns™, low-cost chromatographic columns that are available in lengths up to 120 cm; Bio-Rad's Gel Tube Eliminator removes glass tubes from tube gels without damaging the gel.

N-792

WATERS ASSOC. BROCHURE ON MOLECULAR WEIGHT DISTRIBUTION

A brochure from Waters Assoc., called "A New Way To Tell Good From Bad" describes the application of a fundamental polymer parameter (the molecular-weight distribution) to quality and process control, competitive product analysis, research and development, and product stability determinations. Examples are given along with a brief discussion of why the molecular-weight distribution can be of great value to the producers and formulaters of plastics and polymers.

N-815

KONTES TLC NEWSLETTER

Kontes is publishing a quarterly newsletter on quantitative thin-layer chromatography (TLC). The newsletter will feature abstracts of papers published recently stressing the practical features of *in situ* quantitative TLC methods. The abstracts will cover compounds, adsorbent, solvent systems, detection methods, quantitation method, sensitivity, linearity and sample work-up.

NEW BOOKS

Chromatography – A laboratory handbook of chromatographic and electrophoretic methods, edited by E. Heftmann, Van Nostrand Reinhold, New York, Cincinnati, Toronto, London, Melbourne, 3rd (revised) ed., 1975, xxxi + 969 pp., price £30.90, ISBN 0 442 23280 2.

Annual reports and analytical spectroscopy, Vol. 4 (reviewing 1974), edited by C. Woodward, Chemical Society, London, 1975, xii + 268 pp., price £12.00, US$30.00, ISBN 0 85990 245 4.

Amino acids, peptides and proteins, Vol. 7, edited by R.C. Sheppard, Chemical Society, London, 1976, 450 pp., price £21.00, US$57.75, ISBN 0 85186 064 8.

Inorganic chemistry of the main-group elements, Vol. 3, edited by C.C. Addison, Chemical Society, London, 1976, 550 pp., price £29.00, US$79.75, ISBN 0 85186 772 3.

Inorganic chemistry of the transition elements, Vol. 4, edited by B.F.G. Johnson, Chemical Society, London, 1976, 562 pp., price £27.00, US$74.25, ISBN 0 85186 530 5.

Die chemische Industrie und ihre Helfer 1975–1976, Edition Selka, Industrieschau-Verlagsgesellschaft mbH, Darmstadt, 1976, 610 pp., price DM 35.50.

Gas and liquid chromatography abstracts – Cumulative indexes 1969–1973, edited by C.E.H. Knapman, Applied Science Publ., London, 1976, v + 381 pp., price £16.00, ISBN 0 85334 643 7.

MEETINGS

FACSS – 3rd ANNUAL MEETING

The Third Annual Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies will be held in Philadelphia, Pa. on November 15–19, 1976.

The meeting is co-sponsored by the XIX Colloquium Spectroscopicum Internationale and the Sixth International Conference on Atomic Spectroscopy.

In addition to Award Symposia and Plenary Lectures, symposia on specific sub-topics will include: spectroscopy, general (emission, absorption, fluorescence); molecular spectroscopy (IR, UV, visible, Raman); magnetic resonance; mass spectroscopy; electron and X-ray spectroscopy, X-ray diffraction; chemical analysis for elements; microchemical analysis; microscopy (optical, electron, microprobe); electrochemical analysis; chromatography. Specific symposia are also being considered on: trends in teaching analytical chemistry; computers in analytical chemistry; pattern recognition; forensic chemistry; food chemistry; air and/or waste monitoring; process analysis; instrument calibration and standards (methods and materials). A large laboratory equipment exhibition and technical workshops will be coordinated with the scientific program.

Those wishing to submit papers should send 3 copies of a 250–300-word abstract in English for review to: Dr. Edward C. Dunlop, Program Chairman, FACSS 1976, DuPont Experimental Station, Wilmington, Del. 19898, U.S.A., to arrive before May 1, 1976. Upon acceptance of a paper, a long abstract of 750–1000 words will be required for publication and distribution at the meeting.

For further details, general information and registration, contact: Dr. Edward G. Brame, Jr., General Chairman, FACSS 1976, DuPont Experimental Station, Wilmington, Del. 19898, U.S.A.

INTERNATIONAL MICROSYMPOSIUM ON CHROMATOGRAPHY AND RELATED TECHNIQUES

The Institute of Analytical Chemistry of the Czechoslovak Academy of Sciences in Brno is organising an International Microsymposium on Chromatography and related techniques. This symposium will be held in Brno, Czechoslovakia, on September 27–30, 1976, on the occasion of the 20th anniversary of the Institute.

The symposium should propose ideas about the state and the trends of development of the chromatographic methods in the course of seven plenary lectures followed by discussion.

The registration fee is US$40.00. Further information is available from the Institute of Analytical Chemistry of the Czechoslovak Academy of Sciences, Leninova 82, 662 28 Brno, Czechoslovakia.

CALENDAR OF FORTHCOMING MEETINGS

April 21–23, 1976
Vienna, Austria

6th Annual Symposium on Recent Advances in the Analytical Chemistry of Pollutants

Contact:
H. Malissa, Technische Hochschule Wien, Getreidemarkt 9, A-1060 Vienna, Austria; or R.W. Frei, P.O. Box 182, CH-4013 Basel, Switzerland

May 17–19, 1976
Wilmington, Del., U.S.A.

2nd International Symposium on Column Liquid Chromatography

Contact:
Dr. Paul F. Levy, Instrument Products Division, Du Pont Company, Wilmington, Del. 19898, U.S.A. (Further details published in Vol. 118, No. 1)

May 24–26, 1976
Montreux, Switzerland

Spektrometertagung

Contact:
Mr. H. Bräm, P.O. Box 140, CH-5401 Baden, Switzerland

June 8–10, 1976
Cambridge, Mass., U.S.A.

10th Miles International Symposium on Impact of Recombinant Molecules on Science and Society

Contact:
Paul C. Oakley, Miles Laboratories, Stoke Court, Stoke Poges, Bucks SL2 4LY (Great Britain)

June 17–18, 1976
Ghent, Belgium

International Symposium on Quantitative Mass Spectrometry in Life Sciences

Contact:
Prof. Dr. A. de Leenheer, Symposium Chairman, Laboratories of Medical Biochemistry and Clinical Analysis, 135 De Pintelaan, B-9000 Ghent, Belgium (Further details published in Vol. 118, No. 2)

June 21–23, 1976
Milan, Italy

International Symposium on Mass Spectrometry in Drug Metabolism

Contact:
Dr. A. Frigerio, Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy. (Further details published in Vol. 117, No. 1)

July 5–9, 1976
Birmingham, Great Britain

11th International Symposium on Chromatography

Contact:
The Executive Secretary, Chromatography Discussion Group, Trent Polytechnic, Nottingham, Great Britain. (Further details published in Vol. 109, No.1)

July 5–9, 1976
Munich and Ettal, G.F.R.

3rd International Meeting on Boron Chemistry

Contact:
Dr. W. Fritsche, Gesellschaft Deutscher Chemiker, D-6000 Frankfurt/Main 90, P.O. Box 900440, G.F.R.

July 6–9, 1976
Leeds, Great Britain

5th International Conference on Non-Aqueous Solutions

Contact:
Dr. J.B. Gill, Department of Inorganic and Structural Chemistry, The University of Leeds, Leeds LS2 9JT, Great Britain

July 12–16, 1976
Hamburg, G.F.R.

7th International Symposium on Organic Sulphur Chemistry

Contact:
Dr. W. Fritsche, Gesellschaft Deutscher Chemiker, D-6000 Frankfurt/Main 90, P.O. Box 900440, G.F.R.

July 25–31, 1976
Hamburg, G.F.R.

Tenth International Congress on Biochemistry

Contact:
Dr. W. Fritsche, Gesellschaft Deutscher Chemiker, P.O. Box 900440, D-6000 Frankfurt/Main 90, G.F.R.

August 2–4, 1976
Hamburg, G.F.R.

International Symposium on Isoelectric Focusing and Isotachophoresis

Contact:
Dr. B.J. Radola, Technical University Munich, D-8050 Freising-Weihenstephan, G.F.R.

August 27–29, 1976
Ghent, Belgium

European Symposium of the International Association of Forensic Toxilogists

Contact:
Professor A. Heyndrickx, Department of Toxicology, State University of Ghent, Hospitaalstraat 13, 9000 Ghent, Belgium

September 7–9, 1976
Amsterdam, The Netherlands

3rd International Symposium on Analytical Pyrolysis

Contact:
Miss Ria Priester, FOM-Institute for Atomic and Molecular Physics, Kruislaan 407, Amsterdam 1006, The Netherlands. (Further details published in Vol. 109, No. 2)

September 6–10, 1976
Hamburg, G.F.R.

17th International Conference on Coordination Chemistry

Contact:
Dr. W. Fritsche, Gesellschaft Deutscher Chemiker, D-6000 Frankfurt/Main 90, P.O. Box 900440, G.F.R.

September 12–18, 1976
Munich, G.F.R.

10th Congress of the International Academy of Legal Medicine and Social Medicine

Contact:
Congress Bureau, Deutsches Reisbüro GmbH, Direktion, D-6000 Frankfurt/M 1, Eschersheimer Landstr. 25/27, Department 442

September 27–30, 1976
Szeged, Hungary

1st Danube Symposium on Chromatography

Contact:
Hungarian Chemical Socity, P.O. Box 240, H-1368 Budapest, Hungary. (Further details published in Vol 117, No. 1)

September 27–30, 1976
Brno, Czechoslovakia

International Microsymposium on Chromatography and Related Techniques

Contact:
Institute of Analytical Chemistry of the Czechoslovak Academy of Sciences, Leninova 82, 662 28 Brno, Czechoslovakia (Further details published in Vol. 118, No. 3)

November 1–4, 1976
Houston, Texas, U.S.A.

Chromatography '76. 11th International Symposium on Advances in Chromatography

Contact:
Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, Texas 77004, U.S.A. (Further details published in Vol. 117, No. 1)

November 15–19, 1976
Philadelphia, Pa., U.S.A.

FACSS – 3rd Annual Meeting

Contact:
Dr. Edward G. Brame, Jr., General Chairman, FACSS 1976, DuPont Experimental Station, Wilmington, Del. 19898, U.S.A. (Further details published in Vol. 118, No. 3)

March 28–April 1, 1977
Dortmund, G.F.R.

6th European Symposium on Fluorine Chemistry

Contact:
Dr. D. Naumann, Department of Inorganic Chemistry, University of Dortmund, P.O. Box 500500, D-4600 Dortmund 50, G.F.R.

May 22–27, 1977
Davos, Switzerland

International Symposium on Microchemical Techniques 1977

Contact:
Dr. W. Merz, BASF A.G. Untersuchungslaboratorium, WHU, D-6700 Ludwigshafen, G.F.R. (Further details published in Vol. 117, No. 1)

Advances in Chromatography 1975

Proceedings of the Tenth International Symposium, held in Munich, November 3-6, 1975

Special volume: Reprinted from the Journal of Chromatography, Vol. 112
edited by **A. ZLATKIS.**

associate editors: E. BAYER, L.S. ETTRE and I. HALÁSZ.

1975. 752 pages. US $74.95/Dfl. 180.00. ISBN 0-444-41382-0.

This volume represents the proceedings of the Tenth International Symposium on the Advances in Chromatography. All aspects of chromatography are covered by the 61 papers presented by scientists from 15 different countries. The increased importance of combined techniques such as GC-MS is evidenced by more than a dozen papers describing various advanced systems and their application in biochemical-clinical and environmental analysis. Following the renaissance of liquid column chromatography, a similar upsurge is appearing in thin-layer chromatography. The papers on further advances in selective detectors, high-performance open tubular colomns and special techniques such as head-space analysis demonstrate the still-continuing development of gas chromatography.

Advances in Chromatography 1974

Proceedings of the Ninth International Symposium held in Houston, Texas, November 4-7, 1974.
Special volume: Reprinted from the Journal of Chromatography, Vol. 99.

edited by **A. ZLATKIS,** and **L.S. ETTRE.**

1974. 789 pages. US $66.75/Dfl. 160.00. ISBN 0-444-41267-0

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
P.O. Box 211, Amsterdam, The Netherlands

Distributed in the U.S.A. and Canada by:
AMERICAN ELSEVIER PUBLISHING COMPANY
52 Vanderbilt Ave., New York, N.Y. 10017, U.S.A.

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

PUBLICATION SCHEDULE FOR 1976

Journal of Chromatography (incorporating *Chromatographic Reviews*)

MONTH	D 1975	J	F	M	A	M	J	J	A	S	O	N	D
JOURNAL	115/1 115/2	116/1 116/2	117/1 117/2	118/1 118/2	118/3 119	120/1 120/2	121/1 121/2	122 123/1	123/2	124/1 124/2	125/1 125/2	125/3 126	128/1 128/2
REVIEWS*					127/1				127/2			127/3	

* Volume 127 will consist of *Chromatographic Reviews*. The issues comprising this volume will not be published consecutively, but will appear at various times in the course of the year.

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

Types of Contributions. (a) Original research work not previously published in a generally accessible language in other periodicals (Full-length papers). (b) Review articles. (c) Short communications and Notes. (d) Book reviews; News; Announcements. (e) Bibliography of Paper Chromatography, Thin-Layer Chromatography, Column Chromatography, Gas Chromatography and Electrophoretic Techniques. (f) Chromatographic Data.

Submission of Papers. Three copies of manuscripts in English, French or German should be sent to: Editorial office of the Journal of Chromatography, P.O. Box 681, Amsterdam, The Netherlands. For *Review articles*, an outline of the proposed article should first be forwarded to the Editorial office for preliminary discussion prior to preparation.

Manuscripts. The manuscript should be typed with double spacing on pages of uniform size and should be accompanied by a separate title page. The name and the complete address of the author to whom proofs are to be sent should be given on this page. Authors of papers in French or German are requested to supply an English translation of the title. A short running title of not more than 50 letters (including spaces between the words) is also required for Full-length papers and Review articles. All illustrations, photographs, tables, etc., should be on separate sheets.

Heading. The title of the paper should be concise and informative. The title should be followed by the authors' full names, academic or professional affiliations, and addresses.

Summary. Full-length papers and Review articles should have a summary of 50–100 words. 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 will be 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. Particular attention should be paid to the size of the lettering to ensure that it does not become unreadable after reduction. Sharp, glossy photographs are required to obtain good halftones. Each illustration should have a legend, all the *legends* being typed together on a *separate sheet*. Coloured illustrations are reproduced at the author's expense.

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 using superscript numerals. In the reference list, periodicals[1], books[2], and multi-author books[3] 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 R. D. Marshall and A. Neuberger, in A. Gottschalk (Editor), *Glycoproteins*, Vol. 5, Part A, Elsevier, Amsterdam, 2nd ed., 1972, Ch. 3, p. 251.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*.

Proofs. Two sets 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.

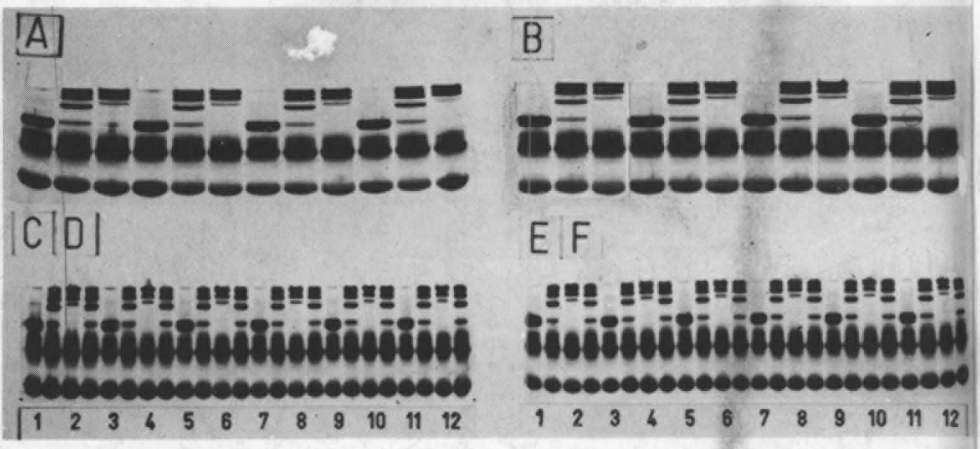
News. News releases of new products and developments, and information leaflets of meetings should be addressed to: The Editor of the News Section, Journal of Chromatography, Elsevier Scientific Publishing Company, P.O. Box 330, Amsterdam, The Netherlands.

Subscription orders. Subscription orders should be sent to: Elsevier Scientific Publishing Company, P.O. Box 211, Amsterdam, The Netherlands.

Publication. The *Journal of Chromatography* (including *Chromatographic Reviews*) appears fortnightly and has 14 volumes in 1976. The subscription price for 1976 [Vols. 115–128 and Supplementary Vol. 5 (Bibliography of Paper and Thin-Layer Chromatography 1970–1973)] is Dfl. 1470.00 plus Dfl. 180.00 (postage) (total US$ 660.00). Subscribers in the U.S.A., Canada and Japan receive their copies by air mail. Additional charges for air mail to other countries are available on request. Back volumes of the *Journal of Chromatography* (Vols. 1 through 114) are available at Dfl. 100.00 plus postage.

Advertisements. Advertisement rates are available from the publisher on request. The Editor of the journal accepts no responsibility for the content of the advertisements.

DESAGA



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It is the final product from a screening of almost 30.000 individuals for hemoglobins with up to 1.200 analyses per day. It combines the high resolution of PAA-gel electrophoresis with the multiple handling of samples. Our system is 10 times faster in preparing samples and applying them to the gel and it separates up to 96 samples in one run. 4 $ for reagents are sufficient for 1000 samples to be tested for hemoglobins. Do you know anything more economical in this field?

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