ISSN 0378-4347

VOL. 272 NO. 1 JANUARY 14, 1983
(Biomedical Applications, Vol. 23, No. 1)

JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

JOURNAL OF CHROMATOGRAPHY

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

Submission of Papers. Papers in English, French and German may be submitted, in three copies. Manuscripts should be submitted to: The Editor of *Journal of Chromatography*, P.O. Box 681, 1000 AR Amsterdam, The Netherlands, or to: The Editor of *Journal of Chromatography, Biomedical Applications*, P.O. Box 681, 1000 AR Amsterdam, The Netherlands. Review articles are invited or proposed by letter to the Editors and will appear in *Chromatographic Reviews* or *Biomedical Applications*. An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation. Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere. For copyright regulations, see below.

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

Publication. The *Journal of Chromatography* (incl. *Biomedical Applications, Chromatographic Reviews* and *Cumulative Author and Subject Indexes, Vols. 251–260* and *261–270*) has 27 volumes in 1983. The subscription prices for 1983 are:

J. Chromatogr. (incl. *Chromatogr. Rev.* and *Cum. Indexes Vols. 251–260* and *261–270*) + *Biomed. Appl.* (Vols. 252–278):
Dfl. 4185.00 plus Dfl. 540.00 (postage) (total *ca.* US$ 1890.00)

J. Chromatogr. (incl. *Chromatogr. Rev.* and *Cum. Indexes Vols. 251–260* and *261–270*) only (Vols. 252–271):
Dfl. 3400.00 plus Dfl. 400.00 (postage) (total *ca.* US$ 1520.00)

Biomed. Appl. only (Vols. 272–278):
Dfl. 1120.00 plus Dfl. 140.00 (postage) (total *ca.* US$ 504.00).

Journals are automatically sent by airmail to the U.S.A. and Canada at no extra costs, and to Japan, Australia and New Zealand, with a small additional postal charge. Back volumes of the *Journal of Chromatography* (Vols. 1 through 251) are available at Dfl. 173.00 (plus postage). Claims for issues not received should be made within three months of publication of the issue. If not, they cannot be honoured free of charge. For customers in the U.S.A. and Canada, wishing additional bibliographic information on this and other Elsevier journals, please contact Elsevier Science Publishing Company Inc., Journal Information Center, 52 Vanderbilt Avenue, New York, NY 10017. Tel: (212) 867-9040.

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

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

Printed in The Netherlands

VOL. 272, NO.1 JOURNAL OF CHROMATOGRAPHY JANUARY 14, 1983

(Biomedical Applications, Vol. 23, No. 1)

CONTENTS

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

(Continued overleaf)

Contents (continued)

ELECTROPHORESIS

A Survey of Techniques and Applications

edited by Z. DEYL, *Czechoslovak Academy of Sciences, Prague*

JOURNAL OF CHROMATOGRAPHY LIBRARY, 18

PART A: TECHNIQUES

Z. DEYL *(editor)*
F. M. EVERAERTS, Z. PRUSÍK *and*
P. J. SVENDSEN *(co-editors)*

"... provides a sound, state-of-the-art survey of its subject".

– Chemistry in Britain

"... the editors have set out to bring everything together into a coherent whole... they have succeeded remarkably well... the book is bound to be well liked and appreciated by readers".

– Journal of Chromatography

This first part deals with the principles, theory and instrumentation of modern electromigration methods. Both standard procedures and newer developments are discussed and hints are included to help the reader overcome difficulties frequently arising from the lack of suitable equipment. Adequate theoretical background of the individual techniques is given and a theoretical approach to the deteriorative processes is presented to facilitate further development of a particular technique and its application to a special problem. In each chapter practical realisations of different techniques are described and examples are presented to demonstrate the limits of each method.

CONTENTS:
Introduction. Chapters: 1. Theory of electromigration processes *(J. Vacík)*. 2. Classification of electromigration methods *(J. Vacík)*. 3. Evaluation of the results of electrophoretic separations *(J. Vacík)*. 4. Molecular size and shape in electrophoresis *(Z. Deyl)*. 5. Zone electrophoresis (except gel-type techniques and immunoelectrophoresis) *(W. Ostrowski)*. 6. Gel-type techniques *(Z. Hrkal)*. 7. Quantitative immunoelectrophoresis *(P.J. Svendsen)*. 8. Moving boundary electrophoresis in narrow-bore tubes *(F.M. Everaerts and J.L. Beckers)*. 9. Isoelectric focusing *(N. Catsimpoolas)*. 10. Analytical isotachophoresis *(J. Vacík and F.M. Everaerts)*. 11. Continuous flow-through electrophoresis *(Z. Prusík)*. 12. Continuous flow deviation electrophoresis *(A. Kolin)*. 13. Preparative electrophoresis in gel media *(Z. Hrkal)*. 14. Preparative electrophoresis in columns *(P.J. Svendsen)*. 15. Preparative isoelectric focusing *(P. Blanický)*. 16. Preparative isotachophoresis *(P.J. Svendsen)*. 17. Preparative isotachophoresis on the micro scale *(L. Arlinger)*. List of frequently occurring symbols. Subject Index.

1979 xvi + 390 pp. US $72.25/Dfl. 170.00
ISBN 0-444-41721-4

PART B: APPLICATIONS

Z. DEYL *(editor)*
A. CHRAMBACH, F.M. EVERAERTS *and*
Z. PRUSÍK *(co-editors)*

Part B is an exhaustive survey of the present status of the application of electrophoretic techniques to many diverse compounds. Those categories of compounds most suited to these separations, such as proteins and peptides, are dealt with in detail, while the perspectives of the applications of these techniques to other categories of compounds less commonly electrophoresed are given. Special attention is paid to naturally occurring mixtures of compounds and their treatment. This is the first attempt to cover the field on such a broad scale and the book will be valuable to separation chemists, pharmacologists, organic chemists and those involved in biomedical research.

CONTENTS: 1. Alcohols and phenolic compounds *(Z. Deyl)*. 2. Aldehydes and ketones *(Z. Deyl)*. 3. Carbohydrates *(Z. Deyl)*. 4. Carboxylic acids *(F.M. Everaerts)*. 5. Steroids and steroid conjugates *(Z. Deyl)*. 6. Amines *(Z. Deyl)*. 7. Amino acids and their derivatives *(Z. Deyl)*. 8. Peptides and structural analysis of proteins *(Z. Prusík)*. 9. Gel electrophoresis and electrofocusing of proteins *(edited by A. Chrambach)*. Usefulness of second-generation gel electrophoretic tools in protein fractionation *(A. Chrambach)*. Membrane proteins, native *(L.M. Hjelmeland)*. Membrane proteins, denatured *(H. Baumann, D. Doyle)*. Protein membrane receptors *(U. Lang)*. Steroid receptors *(S. Ben-Or)*. Cell surface antigens *(R.A. Reisfeld, M.A. Pellegrino)*. Lysosomal glycosidases and sulphatases *(A.L. Fluharty)*. Heamocyanins *(M. Brenowitz et al.)*. Human haemoglobins *(A.B. Schneider, A.N. Schechter)*. Isoelectric focusing of immunoglobulins *(M.H. Freedman)*. Contractile and cytoskeletal proteins *(P. Rubenstein)*. Proteins of connective tissue *(Z. Deyl, M. Horáková)*. Microtubular proteins *(K.F. Sullivan, L. Wilson)*. Protein hormones *(A.D. Rogol)*. Electrophoresis of plasma proteins: a contemporary clinical approach *(M. Engliš)*. Allergens *(H. Baer, M.C. Anderson)*. 10. Glycoproteins and glycopeptides (affinity electrophoresis) *(T.C. Bøg-Hansen, J. Hau,)*. 11. Lipoproteins *(H. Peeters)*. 12. Lipopolysaccharides *(P.F. Coleman, O. Gabriel)*. 13. Electrophoretic examination of enzymes *(W. Ostrowski)*. 14. Nucleotides, nucleosides, nitrogenous constituents of nucleic acids *(S. Zadražil)*. 15. Nucleic acids *(S. Zadražil)*. 16. Alkaloids *(Z. Deyl)*. 17. Vitamins *(Z. Deyl)*. 18. Antibiotics *(V. Betina)*. 19. Dyes and pigments *(Z. Deyl)*. 20. Inorganic compounds *(F.M. Everaerts, Th. P.E.M. Verheggen)*. Contents of "Electrophoresis, Part A: Techniques" Subject Index. Index of compounds separated.

1982 xiii + 462 pp. US $104.75/Dfl. 225.00
ISBN 0-444-42114-9

ELSEVIER
P.O. Box 211, Amsterdam
The Netherlands
52 Vanderbilt Avenue
New York, NY 10017, U.S.A.

7255

JOURNAL OF CHROMATOGRAPHY

VOL. 272 (1983)

(Biomedical Applications, Vol. 23)

JOURNAL
of
CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY,
ELECTROPHORESIS AND RELATED METHODS

BIOMEDICAL APPLICATIONS

Vol. 23 1983

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

J. Chromatogr., Vol. 272 (1983)

Printed in The Netherlands

Journal of Chromatography, 272 (1983) 1—8
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1486

ANALYSIS OF SIALIC ACIDS BY GAS CHROMATOGRAPHY OF THE MANNOSAMINE DERIVATIVES RELEASED BY THE ACTION OF N-ACETYLNEURAMINATE LYASE

KAZUAKI KAKEHI, KAZUSHIGE MAEDA, MASAYUKI TERAMAE and SUSUMU HONDA*

Faculty of Pharmaceutical Sciences, Kinki University, Kowakae, Higashi-osaka (Japan)

and

TSUNEO TAKAI

Department of Internal Medicine, Yodogawa Christian Hospital, Awaji, Higashi-yodogawa-ku, Osaka (Japan)

(First received June 1st, 1982; revised manuscript received August 26th, 1982)

SUMMARY

A convenient method for the analysis of sialic acids is proposed, which is based on their dissociation into pyruvate and N-acylmannosamines by the action of N-acetylneuraminate lyase, followed by gas-chromatographic analysis of the latter products as trimethylsilylated diethyl dithioacetals. Conjugated sialic acids should be freed with neuraminidase before being subjected to the action of the lyase, but these sequential enzymic reactions may be performed in one pot. N-Acetyl-, N-glycolyl- and N,O-diacetylneuraminic acids gave the corresponding mannosamines, and the dithioacetal derivatives of these mannosamines were well separated on a column of silicone OV-1. Quantitation of this enzymic and gas chromatographic method indicated that the error and coefficient of variation for free N-acetylneuraminic acid were 1.1% and 2.5%, respectively, for ten determinations at the 100 nmol level. The values for conjugated N-acetylneuraminic acid in N-acetylneuramin-lactose were 2.9% and 5.9%, respectively. This method was applied to the analysis of sialic acids in some biological samples, and the results were compared with those obtained by the conventional colorimetric method. Preliminary data on urinary sialic acids indicated that cancerous patients gave significantly higher levels of urinary N-acetylneuraminic acid than normal subjects.

INTRODUCTION

Sialic acids occur widely in animals and bacteria as glycosidic components

0378-4347/83/0000—0000/$03.00

of various glycoconjugates [1]. N-Acetyl- and N-glycolylneuraminic acids are the most abundant sialic acids, and the occurrence of isomeric N,O-diacetylneuraminic acids in some tissues has been also reported [2]. Although several colorimetric methods [3, 4] are available for the determination of sialic acids, they are not very selective and the problem of interference is serious in applying them to biological samples, especially serum and urine. The recent enzymic method, based on the measurement of pyruvate released by the action of N-acetylneuraminate lyase (N-acetylneuraminate pyruvate lyase, EC 4.1.3.3) [5] has partially solved this problem, but it can only determine total sialic acids, because all sialic acids commonly produce pyruvate. In contrast with this, gas chromatographic analysis of the other products, namely N-acylmannosamines, by the trimethylsilylated dithioacetal method [6] allows good separation of their derivatives, and hence permits simultaneous analysis of individual sialic acid homologues without interference.

This paper is concerned with the optimization of this enzymic and gas chromatographic method. It also presents some applications of this method to various biological samples.

EXPERIMENTAL

Materials

Clostridium perfringens neuraminidase (mucopolysaccharide N-acetylneuraminidase, EC 3.2.1.18, type IV) and jack bean urease (type C-3) were obtained from Sigma (St. Louis, MO, U.S.A.). *Escherichia coli* N-acetylneuraminate lyase was purchased from Nakarai (Kyoto, Japan). The samples of N-acetyl- and N-glycolylneuraminic acids were from Nakarai, whereas those of N-acetylneuraminlactose, bovine submaxillary mucin (type 1), fetal calf serum fetuin (type III), human serum transferrin and human serum acid glycoprotein were from Sigma. Ethanethiol and chlorotrimethylsilane were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Trifluoroacetic acid and hexamethyldisilazane were from Wako Pure Chemicals (Osaka, Japan). All other chemicals, solvents and carbohydrate samples were of the highest grade commercially available.

Urine samples of normal subjects were collected in the early morning, before breakfast, from volunteers of both sexes and various ages ranging from twenties to fifties. Urine samples from cancerous patients were obtained in the same manner. All cancers had not been metastasized and all patients were not at the mortal stage. A 500-μl portion of each urine sample was submitted to sialic acid analysis by procedure 3 (see below). Urinary creatinine was assayed with alkaline picrate by the method of Bonsnes and Taussky [7].

Apparatus

Gas chromatography was performed on a Shimadzu 4BMPF instrument equipped with a hydrogen flame ionization detector. For the analysis of mannosamine derivatives by procedures 1 and 2 (see below), a glass column (1 m × 3 mm I.D.) packed with Chromosorb W AW DMCS (80—100 mesh) coated with 2% silicone OV-1 was used at 190°C, but for the analysis by procedure 3 a SCOT capillary column (50 m × 0.28 mm I.D.) coated with sili-

cone SF-96 was used at 225° C. The flow-rates of the carrier (nitrogen for both columns) were regulated at 50 and 1 ml/min, respectively. Peaks were integrated by a Shimadzu E1A Chromatopak integrator. Gas chromatography–mass spectrometry was carried out on a Hitachi M-70 spectrometer by using the silicone OV-1 column under the same conditions as described above. The ionization potential was 70 eV.

Procedure for the analysis of conjugated sialic acids in glycoprotein preparations (procedure 1)

Dissolve a sample (0.5—1 mg) of a glycoprotein preparation in water (500 μl), and mix the solution with 0.2 *M* phosphate buffer (pH 7.0, 200 μl). Add aqueous solutions of neuraminidase (0.1 U per 50 μl) and N-acetylneuraminate lyase (0.3 U per 60 μl), and incubate the mixture for 1 h at 37° C. Heat the mixture for 1 min at 100° C to inactivate the enzymes, and introduce it onto a column containing Amberlite CG-120 (H^+, 1 ml) and CG-400 (CH_3COO^-, 1 ml). Wash the column with water (30 ml), and evaporate the combined eluate and the washing fluids to dryness under reduced pressure below 40° C. Dissolve the residue in a 1×10^{-3} *M* aqueous solution (100 μl) of 3-O-methylglucose (internal standard), and transfer the solution to a small glass tube (5 cm × 5 mm I.D.) with a small volume of water washings. Evaporate the solution to dryness under reduced pressure by placing the tube in a desiccator containing sodium hydroxide. Dissolve the residue in a 2:1 (v/v) mixture (20 μl) of ethanethiol and trifluoroacetic acid, and keep the solution for 10 min at 25° C. Add pyridine (50 μl), hexamethyldisilazane (100 μl) and chlorotrimethylsilane (50 μl), and incubate the mixture for 30 min at 50° C. Centrifuge the mixture, and inject a 1-μl sample of the supernatant into the OV-1 column. Estimate the amounts of mannosamine derivatives from the relative peak responses to that of the internal standard. The amounts of mannosamine derivatives are equivalent to those of sialic acids.

N-Acetylneuraminic acid in N-acetylneuraminlactose was also determined by this procedure.

Procedure for the analysis of free sialic acids (procedure 2)

Free sialic acids were analyzed by the same procedure as procedure 1, except that the neuraminidase was omitted in the enzyme reaction.

Procedure for the analysis of urinary sialic acids (procedure 3)

Add 0.2 *M* phosphate buffer (pH 7.0, 200 μl) and aqueous solutions of neuraminidase (0.1 U per 50 μl), N-acetylneuraminate lyase (0.3 U per 60 μl) and urease (1 U per 100 μl) to a urine sample (500 μl), and incubate the mixture for 1 h at 37° C. Treat the reaction mixture as described for procedure 1, and analyze sialic acids using the SF-96 capillary column.

RESULTS AND DISCUSSION

Optimization

Free sialic acids were readily split into pyruvate and N-acylmannosamines by the action of N-acetylneuraminate lyase, but conjugated sialic acids were

resistent to this enzyme. However, the latter may be hydrolyzed with neuraminidase, and free sialic acids liberated were exposed to the action of the lyase. Because the optimum pH range (5.0—8.0) of neuraminidase was partially superimposed on that (6.5—8.5) of the lyase, proper choice of the common pH value, preferably 7.0, permitted concerted action of these two enzymes, making one-pot reaction possible. Both enzyme reactions were rapid, and almost complete in 1 h, when 0.1 and 0.3 U, respectively, were used for the 100 nmol amount of substrate. N-Acylmannosamines, formed by a combination of neuraminidase and the lyase (procedure 1), were quantitatively derivatized to their trimethylsilylated diethyl dithioacetals according to the procedure described in our previous paper [6], and the derivatives were analyzed by gas chromatography. From the data obtained, the total amount of free and bound sialic acids could be estimated for each sialic acid species. Schematic expression of these series of enzymic and chemical reations is shown in Fig. 1.

When neuraminidase was omitted from the above system (procedure 2), only free sialic acids could be determined.

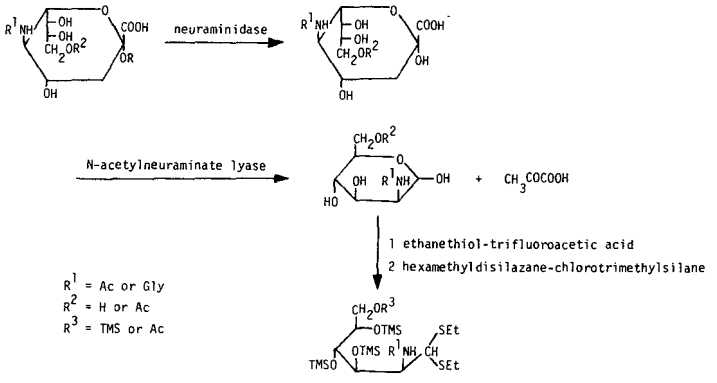


Fig. 1. Scheme of the enzymic and chemical reactions described.

Accuracy and precision

The recovery and coefficient of variation of the determination of free N-acetylneuraminic acid by procedure 2 were 101.1 and 2.5%, respectively, for ten determinations. For the determination of conjugated N-acetylneuramic acid in N-acetylneuraminlactose by procedure 1, the respective values were 97.1 and 5.9%. These data indicate that the present method is accurate and reproducible.

Analysis of sialic acids in glycoprotein preparations

Fig. 2 shows the results of gas chromatography—mass spectrometry of the products obtained by these sequential reactions on sialic acids in commercial bovine submaxillary mucin. The gas chromatogram shows the presence of three peaks (peaks 2, 3 and 4) of N-acylmannosamines, together with a peak (peak 1) of 3-O-methylglucose (internal standard). The retention times of peaks 2 and 4 were identical with those arising from the products obtained by treating N-acetyl- and N-glycolyl neuraminic acids, respectively, by procedure 1. All the

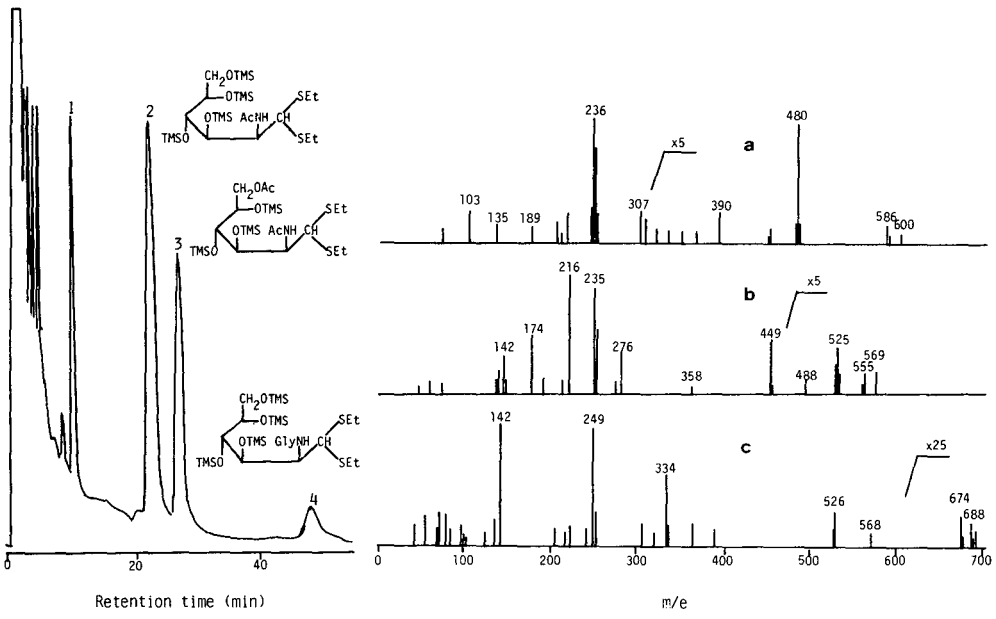


Fig. 2. Gas chromatography—mass spectrometry of the derivatives of N-acylmannosamines derived from the sialic acids in bovine submaxillary mucin by procedure 1. Assignment of gas chromatographic peaks: 1 = 3-O-methylglucose (internal standard); 2 = N-acetylmannosamine; 3 = N,O-diacetylmannosamine; 4 = N-glycolylmannosamine. The mass spectra (a), (b) and (c) represent those of the compounds of peaks 2, 3 and 4, respectively.

mass spectra of peaks 2, 3 and 4 gave no molecular ions, but they all had pairs of fragment ions assignable to $M^+ - CH_2CH_3$ and $M^+ - CH_3$ (586 and 600, respectively, for peak 2; 556 and 570, respectively, for peak 3; 674 and 688, respectively, for peak 4). The molecular weights of the compounds of peaks 2, 3 and 4, as estimated on the basis of these fragmentations, are 615, 585 and 703, respectively. Thus, the compounds giving peaks 2, 3 and 4 were identified as the trimethylsilylated diethyl dithioacetals of N-acetyl-, N,O-diacetyl- and N-glycolylmannosamines, respectively. Regarding the structure of the compound of peak 3, an additional evidence was provided; i.e. its mass spectrum gave fragment ions at *m/e* 175 and 277, assignable to $^5C^+HOTMS—^6CH_2OAc$ and $^4C^+HOTMS—^5CHOTMS—^6CH_2OAc$, respectively. These results suggest that the compound giving peak 3 has an O-acetyl group at C-6, together with an N-acetyl group, in the mannosamine residue, and accordingly the parent sialic acid is N-acetyl-9-O-acetylneuraminic acid. Although the presence of isomeric N,O-diacetylneuraminic acids in bovine submaxillary mucin has been reported in the literature [8], the commercial sample used in this work gave only one peak for diacetylmannosamine.

Table I summarizes the amounts of individual sialic acids in several commercial glycoprotein preparations, as estimated by procedure 1. It also gives the amounts of total sialic acids, as obtained by the conventional colorimetric method based on the reaction with periodate and barbiturate [3]. It is observed that fetal calf serum fetuin and human serum transferrin contained only N-acetylneuraminic acid and its amounts estimated by the present method

TABLE I

DETERMINATION OF SIALIC ACIDS IN VARIOUS GLYCOPROTEIN PREPARATIONS

Glycoprotein preparation	Amount of sialic acid (mg/g)			
	Present method			Colorimetric method [3]
	N-Acetyl-neuraminic acid	N,O-Diacetyl-neuraminic acid	N-Glycolyl-neuraminic acid	Total sialic acids
Fetal calf serum fetuin	53.3	0.0	0.0	53.1
Human serum transferrin	14.7	0.0	0.0	14.6
Human serum acid glycoprotein	105	0.0	0.0	80.0
Bovine submaxillary mucin	11.7	5.9	6.7	25.1

are in good agreement with those obtained by the colorimetric method. Human serum acid glycoprotein also gave only N-acetylneuraminic acid, but its amount exceeded that of the total sialic acids obtained by the colorimetric method. In this case the latter method should have underestimated the sialic acid due to interference by the accompanying protein. The amount of N-acetylneuraminic acid in bovine submaxillary mucin was 47% of that of total sialic acids. The amounts of N,O-diacetyl- and N-glycolylneuraminic acids were approximately one half of that of N-acetylneuraminic acid. The sum of the amounts of individual sialic acids agreed well with the amount of total sialic acids estimated by the colorimetric method. In estimating the amount of N,O-diacetylneuraminic acid the molar response was arbitrarily assumed to be the same as that of N-acetylneuraminic acid, because no authentic sample was available. For the estimation of the amount of N-glycolylneuraminic acid, the relative response of peak 4 to peak 2 was referred to that obtained by applying these sequential reactions on an equimolar mixture of N-acetyl- and N-glycolylneuraminic acids. The reference value was 0.61.

Analysis of urinary sialic acids

Application of procedure 1 to the analysis of urinary sialic acids met difficulty due to interference of the derivatization of N-acylmannosamines to trimethylsilylated diethyl dithioacetals and poor resolution of the gas chromatographic peaks from those of accompanying substances. Since the problem of interference is mainly due to large amounts of urea, this compound was eliminated by adding jack bean urease to the enzyme system of procedure 1. Under these conditions urea was decomposed nearly quantitatively to ammonia and carbon dioxide. On the other hand, the poor resolution of peaks was improved by using a SCOT capillary column coated with silicone SF-96. Based on these results a modified procedure (procedure 3) was devised for the analysis of urinary sialic acids. Fig. 3 shows an example of chromatograms obtained for normal urine samples by this procedure. Peak 6 is assignable to N-acetylmannosamine, but no peaks are detected for N,O-diacetyl- and N-glycolylneuraminic acid. Peaks 1, 2, 4 and 5 are of the derivatives of urinary aldoses, and peak 3 is assigned to 3-O-methylglucose (internal standard).

Table II presents some preliminary data on the urinary N-acetylneuraminic acid content of normal subjects and cancerous patients. The normal level

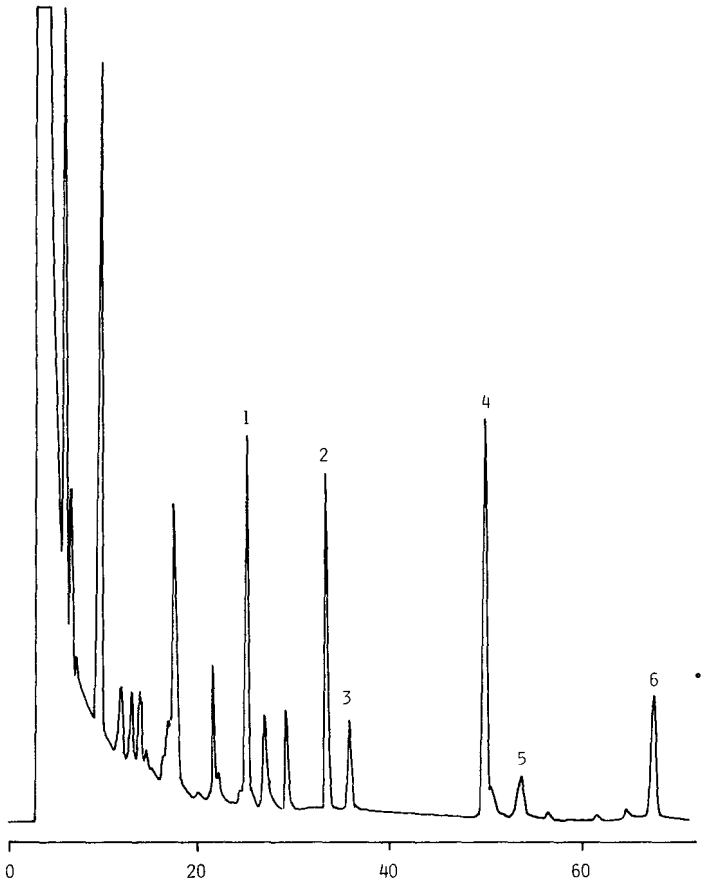


Fig. 3. Gas chromatogram of the derivatives of urinary carbohydrates, obtained by the enzyme reaction of a urine sample from a normal subject, followed by derivatization of the products to trimethylsilylated diethyl dithioacetals according to procedure 3. Peak assignment: 1 = xylose; 2 = fucose; 3 = 3-O-methylglucose (internal standard); 4 = glucose; 5 = galactose; 6 = N-acetylmannosamine.

obtained for eighteen subjects was 21.2 (mean, m) ± 9.7 (standard deviation, s) mg/g of creatinine, whereas nine of ten urine samples from patients with gastric cancer gave high values of N-acetylneuraminic acid that exceed $m + 2s$. The samples from patients with cancer of the lung, liver, pancreas and ovary showed much higher values of N-acetylneuraminic acid, though the number of cases was not sufficient to generalize these observations. There are findings that serum N-acetylneuraminic acid contents are elevated in cancer [9, 10], but reliable data on urinary N-acetylneuraminic acid content have not been published, probably because in the conventional colorimetric methods there is too much interference by accompanying substances. Our experiments indicate that the values obtained by the colorimetric method [3] were several times higher than those obtained by the present method, and in addition the former varied widely with sample size. Since these preliminary data showing the elevation of urinary N-acetylneuraminic acid levels in cancer is suggestive of diagnostic application, further studies are now in progress.

TABLE II

PRELIMINARY DATA ON URINARY N-ACETYLNEURAMINIC ACID LEVELS FOR NORMAL SUBJECTS AND CANCEROUS PATIENTS

No.	Age	Sex	Cancerous organ	Concentration of N-acetyl-neuraminic acid (mg/dl)	Concentration of creatinine (mg/dl)	N-Acetylneura-minic acid/ creatinine ratio (mg/g)
1	41	Female	Stomach	1.79	43.4	41.2
2	52	Male	Stomach	8.78	214	41.0
3	55	Male	Stomach	7.84	187	41.9
4	57	Female	Stomach	4.84	75.7	63.9
5	65	Male	Stomach	1.81	30.6	59.1
6	65	Female	Stomach	3.07	124	24.8
7	68	Female	Stomach	12.9	183	70.5
8	69	Male	Stomach	4.09	96.7	42.3
9	70	Male	Stomach	7.43	118	63.0
10	75	Female	Stomach	3.47	43.1	80.5
11	75	Male	Lung	5.29	70.3	75.2
12	75	Male	Lung	8.70	85.2	102
13	77	Female	Lung	6.03	49.0	123
14	65	Male	Liver	10.4	102	102
15	64	Male	Pancreas	8.27	55.4	149
16	54	Female	Ovary	8.84	56.7	156
Normal, mean ± standard deviation (n = 18)				2.83 ± 1.00	133 ± 54.6	21.2 ± 9.7

REFERENCES

1 A. Gottschalk, Glycoproteins, Their Composition, Structure and Function, Part A, Elsevier, Amsterdam, 1972, p. 403.
2 A.P. Confield, C. Ferreira do Amaral, M. Wember and R. Shauer, Eur. J. Biochem., 68 (1976) 597.
3 L. Warren, J. Biol. Chem., 234 (1959) 1971.
4 L. Svennerholm, Biochim. Biophys. Acta, 24 (1957) 604.
5 D.G. Comb and S. Roseman, Methods Enzymol., 5 (1962) 287.
6 S. Honda, N. Yamauchi and K. Kakehi, J. Chromatogr., 169 (1979) 287.
7 R.W. Bonsnes and H.H. Taussky, J. Biol. Chem., 158 (1945) 581.
8 J. Havercamp, R. Shauer, M. Wember, J.P. Farriaux, J.P. Karmaling, C. Vershuis and J.F.C. Viegenthart, Hoppe-Seyler's Z. Physiol. Chem., 357 (1976) 1699.
9 H.K.B. Silver, D.M. Rangel and D.L. Morton, Cancer, 41 (1976) 1497.
10 T.M. Kloppel, T.W. Keenan, M.J. Freeman and D.J. Morre, Proc. Nat. Acad. Sci. U.S., 74 (1977) 3011.

Journal of Chromatography, 272 (1983) 9—20
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1474

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC RESOLUTION OF *p*-NITROBENZYLOXYAMINE DERIVATIVES OF BRAIN GANGLIOSIDES

T. DENNIS TRAYLOR*

Oak Ridge Associated Universities, Comparative Animal Research Laboratory, P.O. Box 117, Oak Ridge, TN 37830 (U.S.A.)

and

DANIEL A. KOONTZ and EDWARD L. HOGAN

Department of Neurology, Medical University of South Carolina, Charleston, SC 29425 (U.S.A.)

(First received May 18th, 1982; revised manuscript received August 13th, 1982)

SUMMARY

A new quantitative procedure for the high-performance liquid chromatographic (HPLC) resolution of human brain gangliosides employing reversed-phase chromatography is described. To provide a derivative which can be determined by UV absorption techniques, *p*-nitrobenzyloxyamine was coupled to the gangliosides. Derivatization involves ozonation and cleavage of the ceramide double bond followed by oxime formation to the nascent aldehyde. Individual gangliosides, as they were resolved by HPLC, were collected. These fractions were then identified by thin-layer chromatography (TLC) and by gas chromatography of their monosaccharides. Quantitative results were obtained along with a marked increase in sensitivity over conventional resorcinol—hydrochloric acid quantitation of TLC-resolved gangliosides.

INTRODUCTION

The study of gangliosides is of wide interest among neurochemists since they appear to be actively involved in a number of plasma membrane functions that may include cell surface receptor mechanisms. Alterations in ganglioside content or distribution have often been associated with cell transformation [1] and hereditary gangliosidoses [2,3]. It is now known that the membrane receptor for cholera toxin is a specific ganglioside — GM1 [4]. Gangliosides are usually resolved by thin-layer chromatography (TLC) and determined by

0378-4347/83/0000—0000/$03.00

colorimetry or densitometry. TLC resolution is affected by plate-to-plate variations and by a lack of equilibrium conditions in the vapor phase. Quantitation following their resolution by scraping of silica gel and colorimetric determination of sialic acid is laborious and time consuming, while densitometry suffers considerable variability. These limitations render quantitation of a minor band difficult and further seriously limit studies where only small amounts of gangliosides are available, such as with some cell cultures, clinically relevant volumes of cerebrospinal fluid, micro-amounts of tissue, etc. Consequently, some investigations are simply reported as a photograph of a resorcinol-sprayed TLC plate or just as total lipid N-acetylneuraminic acid (NANA). Such reports suggest the problems inherent in TLC resolution and quantitation are compromising results and further suggest the need for improved methodology.

High-performance liquid chromatography (HPLC) offers an obvious solution to some of these difficulties since columns featuring a large number of theoretical plates are commercially available and since fast resolutions offered by HPLC should provide a separation of gangliosides that is more rapid and distinct than that by TLC. Because native gangliosides have little absorbancy in the ultraviolet and refractive index detectors do not offer sufficient sensitivity, we chose to derivatize the gangliosides with an adduct that absorbs in the ultraviolet and one that can be added rapidly and quantitatively. Our plan was to derivatize in the ceramide portion in order that subsequent structural characterization of the carbohydrate moiety would not be compromised. In this way it should be possible to study minor gangliosides and obtain adequate quantities to chemically characterize their structure.

MATERIALS AND METHODS

A Waters Assoc. (Milford, MA, U.S.A.) high-performance liquid chromatograph, composed of two Model 6000 pumps, Model 660 solvent programmer, Model U6K injector, and a Model 440 UV detector was used in this study. The reversed-phase column (Waters Assoc. μBondapak C_{18} 10 μm particle size, 30 cm $\times$ 2 mm) was run at ambient temperature with a flow-rate of 1 ml/min. The UV detector was operated at 254 nm. Solvent programming began with the initial solvent composition methanol—water (50:50) held constant for 1 min and then linearly adjusted over 15 min to methanol—water (70:30). Peak areas were quantitated by a Columbia Scientific Industries (Austin, TX, U.S.A.) Model CRS-208 integrator. Gangliosides were isolated from human brain by the method of Folch et al. [5], as modified by Suzuki [6]. Methanol and chloroform were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). *p*-Nitrobenzyloxyamine hydrochloride was obtained from Regis Chemical Company (Morton Grove, IL, U.S.A.), triphenylphosphine and N-acetylneuraminic acid were purchased from Supelco (Bellefonte, PA, U.S.A.), DEAE-Sephadex was obtained from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.), and other chemicals used were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Ozonolysis of gangliosides

Mixed human brain ganglioside was dissolved in methanol (1 mg/ml) and a 10-μl aliquot was retained for estimation of NANA by the method of Svennerholm [7] and Miettinen and Takki-Luukkainen [8]. Molar concentration of ganglioside was estimated from the pattern determined by TLC and total NANA. Ozonolysis at −70°C was performed with a Supelco Microozonizer at an oxygen flow-rate of 10 ml/min. Ozonation was promptly terminated when ozone began to evolve from the reaction tube and the tube was then flushed with nitrogen. Commonly, ozonation required about 2 min per mg of ganglioside. Triphenylphosphine was added to selectively cleave ozonide to aldehydes. Fuchsin test for aldehydes was routinely positive within 1 min (native ganglioside remained negative in the Fuchsin test after 1 h).

p-Nitrobenzyloxyamine derivatization of gangliosides

Extensive investigation of reaction conditions (see Results) led us to the adoption of standard conditions for derivatization of ganglioside-aldehyde without detectable by-product formation. We have routinely used a molar ratio of *p*-nitrobenzyloxyamine to the sum of ganglioside-aldehyde and long-chain fatty aldehyde of 1.3:1. The reaction mixture was incubated in methanol for 15 min at 40—45°C. Immediately following derivatization, chloroform was added to the reaction mixture, adjusting solvent composition to methanol—chloroform (7:2), and the mixture applied to a previously prepared DEAE-Sephadex column (25 cm × 2 cm) [9]. DEAE-Sephadex A-25 was washed four times with chloroform—methanol—0.8 *M* sodium acetate (30:60:8) and three times with chloroform—methanol—water (30:60:9). The column was prepared by suspension of the gel in methanol—chloroform (7:2) and then poured into a small glass column. The reaction mixture was applied to the column at a flow-rate of 1 ml/min and the column washed at that rate with 300 ml of methanol—chloroform (7:2). Labeled gangliosides were eluted with 200 ml of methanol—chloroform (7:2) that had been made 0.2 *M* in ammonium acetate. The column eluate was monitored at 254 nm by an Instrumentation Specialities (Lincoln, NE, U.S.A.) Model UA-5 absorbancy monitor equipped with a Type-6 optical unit. All peaks were examined for sialic acid by the resorcinol method of Svennerholm [7]. Following concentration, the sample was dialyzed to remove ammonium acetate and lyophilized.

Thin-layer chromatography of p-nitrobenzyloxyamine-labeled gangliosides

Individual peaks collected after resolution by HPLC were dried under a stream of nitrogen, dissolved in a small quantity of chloroform—methanol (1:1), and carefully applied to an E. Merck (Darmstadt, G.F.R.) precoated silica gel 60 HPTLC plate (Cat. No. 5641). The TLC plates were chromatographed twice in chloroform—methanol—water (60:35:8) containing 20 mg calcium chloride. Plates were then sprayed with resorcinol—hydrochloric acid, covered with a glass cover plate, and placed in a 110°C oven for 15 min. Gangliosides were quantitated by densitometry using a Kontes densitometer (Vineland, NJ, U.S.A.) with peak integration as before.

Gas chromatography of monosaccharides

Carbohydrate composition of individual *p*-nitrobenzyloxyamine-labeled gangliosides was determined by gas chromatography (GC). After drying, the labeled gangliosides were methanolyzed in 0.5 *M* methanolic hydrochloric acid (75°C, 24 h) and were transferred to microvials and dried under a stream of nitrogen. Trifluoroacetate derivatives were formed by the method of Zanetta et al. [10] in 0.2 ml of dichloromethane–trifluoroacetic anhydride (1:1). The reaction mixture was injected directly onto a 5% OV-21 6-ft. long column installed in a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 3920 gas chromatograph fitted with dual flame ionization detectors. Temperature programming, initiating at 90°C and ending at 210°C, at a rate of 4°C/min achieved good resolution of the monosaccharides. Peak areas were integrated electronically.

RESULTS

Gangliosides were prepared from human brain tissue and were stored at −45°C under nitrogen until use. Our experimental design to develop a sensitive assay for individual gangliosides by HPLC involved (see Fig. 1) ozonation of the double bond in the ceramide, cleavage of the ozonide by triphenylphosphine to aldehydes, oxime bond formation to *p*-nitrobenzyloxyamine, and resolution by reversed-phase chromatography.

Derivatization

Ozonation of human brain gangliosides proceeded rapidly with the end point, as determined by the starch—iodine test, dependent upon concentration of the gangliosides and on the oxygen–ozone flow-rate. The sharp appearance of color in this test occurred at about 2 min per mg of ganglioside with an oxygen flow-rate of 10 ml/min. After cleavage of the nascent ozonide by triphenylphosphine, derivatization of the aldehyde products was also observed to progress readily. Initial experiments involving greatly excessive molar amounts of *p*-nitrobenzyloxyamine and reaction at 60°C gave two successive sets of sialic acid positive peaks (Table I). Both sets on subsequent TLC gave the complete series of ganglioside derivatives. Evidence was obtained by using extended reaction times to suggest that once formed, the derivatives can undergo some further reaction or perhaps a degradation yielding another product (Table I). During incubation of the reaction mixture at 60°C, the total sialic acid content of the more slowly eluting set of peaks was found to increase with a parallel loss of neuraminic acid in the more rapidly eluting set of peaks. As outlined in the Materials and methods section, conditions were found that allowed a single set of derivatives to be formed, i.e., reaction at 40°C for 15 min with a *p*-nitrobenzyloxyamine—aldehyde ratio of 1.3:1. See Discussion for further details on this point.

Ion-exchange chromatography

Chromatography of the reaction mixture at this stage revealed that *p*-nitrobenzyloxyamine and triphenylphosphine elute with retention times in the middle of the ganglioside peaks. To remove impurities from the derivatized gangliosides, the ion-exchange chromatographic method of Ledeen et al. [9]

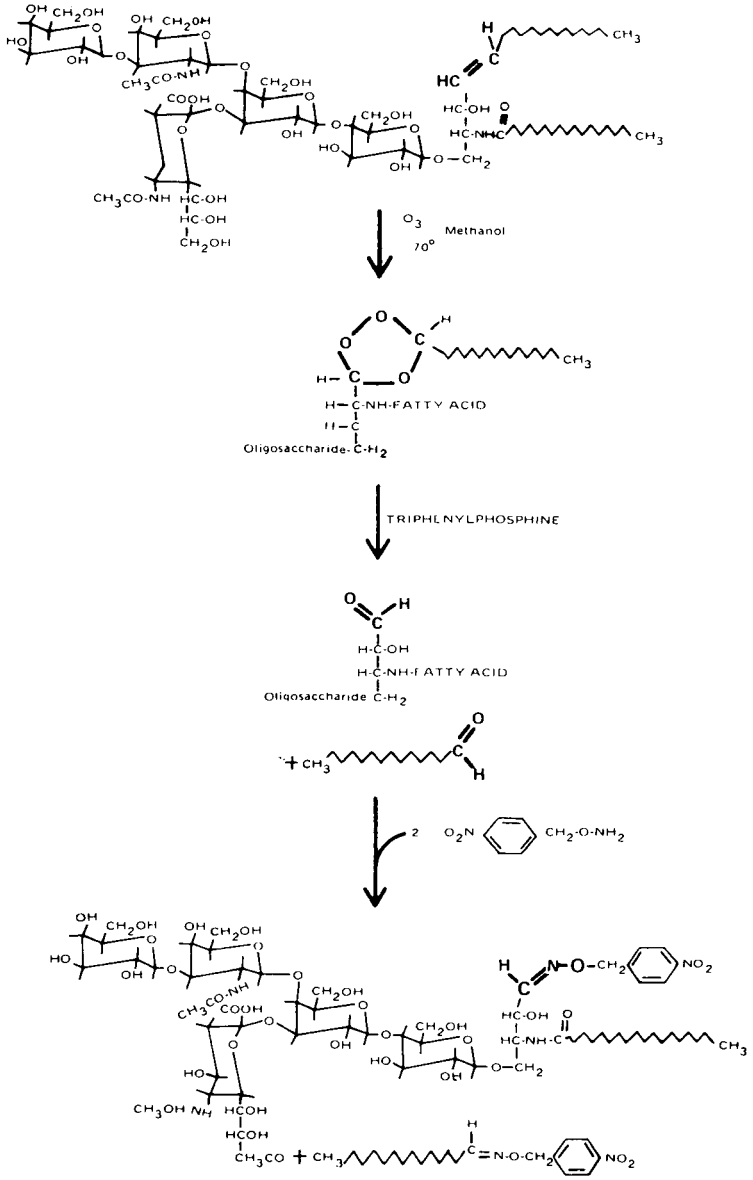


Fig. 1. Derivatization of ganglioside GM1 by *p*-nitrobenzyloxyamine. Other gangliosides are derivatized identically.

was employed. Free *p*-nitrobenzyloxyamine and *p*-nitrobenzyloxyamine-labeled long chain fatty aldehyde (primarily tetradecanal and hexadecanal) were eluted first since they are not strongly retained by the resin (Fig. 2). *p*-Nitrobenzyloxyamine-labeled gangliosides were eluted from the column following the addition of ammonium acetate to the mobile phase.

TABLE I

SECONDARY DERIVATIZATION OF HUMAN BRAIN GANGLIOSIDES IN EXCESS *p*-NITROBENZYLOXYAMINE AT 60°C

Aldehyde cleavage products of ozonolysed ganglioside (1 mg) were incubated at 60°C with a 30-fold molar excess of *p*-nitrobenzyloxyamine for the time indicated. Primary set of peaks were eluted from the HPLC system in 4—20 min. The secondary set was eluted from the column in 40—60 min. Data are presented as percent total sialic acid. Sialic acid was determined by the resorcinol–hydrochloric acid method [7].

	Time (min)			
	15	30	60	120
Primary set	83%	71%	58%	41%
Secondary set	17%	29%	42%	59%

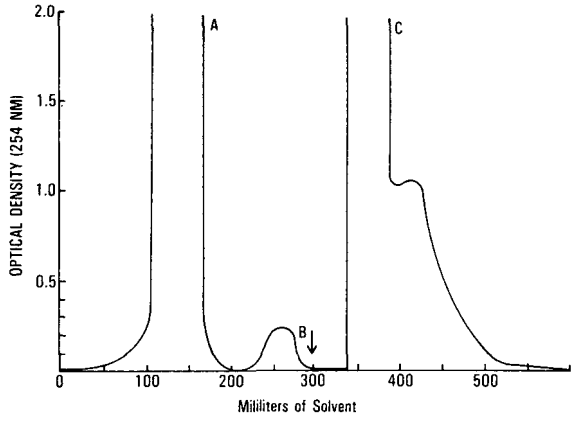


Fig. 2. DEAE-Chromatography of *p*-nitrobenzyloxyamine-labeled ganglioside, *p*-nitrobenzyloxyamine-labeled long-chain aldehyde, and other reaction components. A reaction mixture of 30 mg of ganglioside in methanol—chloroform (7:2) was placed onto a previously prepared DEAE-Sephadex column and the column was eluted with 300 ml of methanol—chloroform (7:2). *p*-Nitrobenzyloxyamine-labeled gangliosides were eluted by solvent change (at arrow) to methanol—water (7:2) that was made 0.2 *M* with ammonium acetate. Peaks: A = long-chain fatty aldehyde labeled with *p*-nitrobenzyloxyamine and unreacted *p*-nitrobenzyloxyamine; B = triphenylphosphine; C = *p*-nitrobenzyloxyamine-labeled ganglioside.

High-performance liquid chromatography

Labeled gangliosides were resolved by reversed-phase chromatography using methanol—water as mobile phase (Fig. 3). A gradient, beginning with methanol—water (50:50), held constant for 1 min, and then linearly adjusted over 15 min to methanol—water (70:30), gave good resolution. Four major peaks with retention times of A = 5.6, B = 6.6, C = 10.8, and D = 13.3 min were observed, and these were collected separately. Minor peaks C′ and D′ were also resolved but usually collected along with corresponding major peaks. Peak A consistently presented a leading shoulder.

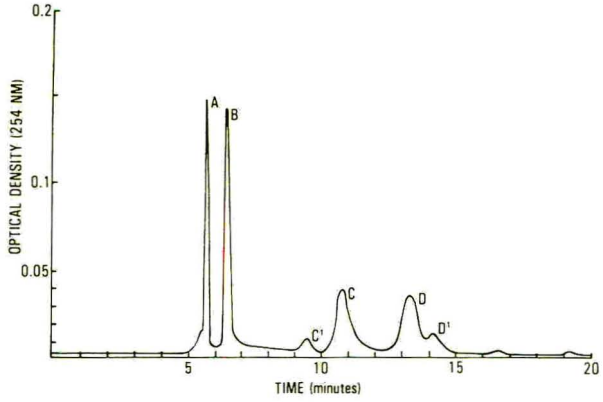


Fig. 3. Resolution of *p*-nitrobenzyloxyamine-labeled ganglioside by HPLC. Twenty-five μg NANA of *p*-nitrobenzyloxyamine-labeled ganglioside were injected onto a μBondapak C_{18} (30 cm × 2 mm) column. Initial solvent composition of methanol—water (50:50) at a flow-rate of 1 ml/min was used with linear programming over 15 min to methanol—water (70:30). For explanation of peaks see text.

Characterization of p-nitrobenzyloxyamine-labeled ganglioside

Identification of individual peaks as resolved by HPLC was undertaken by TLC and GC. *p*-Nitrobenzyloxyamine-labeled gangliosides migrated on TLC in a manner that closely resembled that of native gangliosides (Fig. 4). Indicated peaks were collected as they were eluted and were separately applied to the thin-layer plate. The sequence of elution by reversed-phase chromatography of *p*-nitrobenzyloxyamine-labeled gangliosides was the reverse of the sequence of migration on TLC. That is, on silica gel smaller monosialogangliosides migrate most rapidly, and larger more polar polysialogangliosides migrate more slowly, while on reversed phase the highly polar *p*-nitrobenzyloxyamine-labeled ganglioside GT1b is the first major ganglioside to elute from the column. GC analysis of monosaccharides [10] cleaved from labeled gangliosides after resolution by HPLC (Table II) further confirmed the ganglioside identification suggested by TLC. The monosaccharide ratios closely conform to those expected based upon the suggested identification provided by TLC.

Quantitative comparison of ganglioside analysis by HPLC with analysis by TLC

Quantitative analysis of the major *p*-nitrobenzyloxyamine-labeled gangliosides provided by UV absorption at 254 nm following their resolution by HPLC was compared (Table III) with analysis of the native ganglioside preparation as provided by TLC. Ganglioside determination after resolution by TLC involved the spraying of the plate with resorcinol—hydrochloric acid, heating, and densitometry. The two methods yielded essentially the same quantitative data.

DISCUSSION

The difficulty of consistent resolution and quantitation of the gangliosides by TLC [11–13] or by conventional column chromatography, as well as the

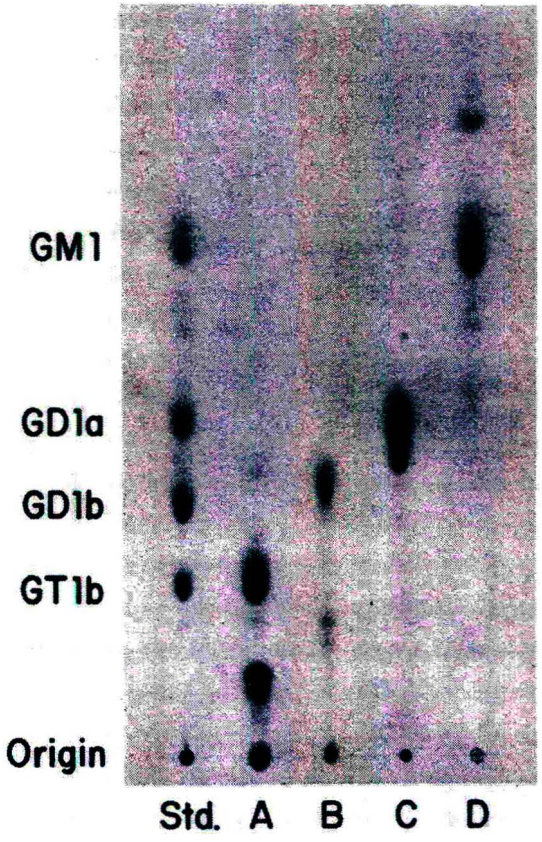


Fig. 4. Thin-layer chromatogram of *p*-nitrobenzyloxyamine-labeled gangliosides after resolution by HPLC. The standard lane was spotted with native brain gangliosides (14 μg N-acetylneuraminic acid). Lane A was spotted with the labeled ganglioside (23 μg N-acetylneuraminic acid) first to elute on HPLC (Peak A, Fig. 3). Lane B was spotted with 14 μg N-acetylneuraminic acid of *p*-nitrobenzyloxyamine-labeled ganglioside (Peak B, Fig. 3). Lane C was spotted with 21 μg N-acetylneuraminic acid of *p*-nitrobenzyloxyamine-labeled ganglioside (Peaks C and C′, Fig. 3). Lane D was spotted with 19 μg N-acetylneuraminic acid of *p*-nitrobenzyloxyamine-labeled ganglioside (Peaks D and D′, Fig. 3). The HPTLC plate was developed in chloroform—methanol—water (60:35:8) containing 20 mg $CaCl_2 \cdot 2H_2O$. The plate was sprayed with resorcinol—hydrochloric acid and placed in an 110°C oven for 15 min [6].

number of known different ganglioside species [14], make use of the superior resolving power of HPLC attractive for the resolution and quantitation of these glycolipids. Tjaden et al. [15] demonstrated the feasibility of resolving native gangliosides by HPLC expanding on methods developed for TLC, that is a silica gel column and a similar solvent system were used along with a moving wire detector. It was our goal to develop an assay system for gangliosides that would rival or exceed the sensitivity of the resorcinol—hydrochloric acid assay and so be applicable to clinical samples having low levels of gangliosides and to general experimental applications. To accomplish this goal, we chose to add a UV-absorbing compound with a high extinction coefficient to the ganglioside. We desired a method that would not alter the carbohydrate moiety in order to

TABLE II

MONOSACCHARIDE COMPOSITION OF LABELED GANGLIOSIDES AFTER RESOLUTION BY HPLC

Molar ratios of individual saccharides (relative to galactose) are given as determined by GC of trifluoracetate derivatives by the method of Zanetta et al. [10].

Peak	Retention time (min)	Major ganglioside	Monosaccharides		
			Galactose	*n*-Acetylgalactosamine	NANA
A	5—6	GT1b	2	0.91	3.20
B	6—7	GD1b	2	0.98	2.14
C, C′	9—12	GD1a	2	1.05	2.25
D, D′	12—15	GM1	2	1.12	1.26

TABLE III

COMPARISON OF HPLC QUANTITATION OF *p*-NITROBENZYLOXYAMINE-LABELED GANGLIOSIDES WITH TLC OF GANGLIOSIDES FOLLOWED BY RESORCINOL ASSAY FOR N-ACETYLNEURAMINIC ACID

Data are presented as mole percent (± S.D.) of ganglioside. After TLC, the plates were sprayed with resorcinol–hydrochloric acid [6] and quantitated by densitometry coupled with electronic integration of peak areas. For HPLC, the eluate was monitored at 254 nm using a fixed-wavelength detector, the output of which was quantitated by electronic integration of peak areas. Peaks C′ and D′ are not included. Twelve determinations were made by TLC of native human brain gangliosides. Similarly twelve determinations were made by HPLC of the same preparation of gangliosides which had been derivatized with *p*-nitrobenzyloxyamine.

Ganglioside	TLC	HPLC
GM1	30.5 ± 2.2	30.0 ± 1.6
GD1a	20.5 ± 1.5	20.4 ± 1.2
GD1b	23.7 ± 2.1	23.6 ± 1.8
GT1b + GQ	25.3 ± 3.3	25.9 ± 2.0

permit later chemical characterization of unknown or unusual peaks. Double bonds characteristically add ozone rapidly and quantitatively form ozonides (see Fig. 1). Although ozonides are unstable and give rise to multiple oxidation products when allowed to degrade randomly [16], the product can be selected by use of the appropriate reducing agent. For specific cleavage to aldehydes, we employed triphenylphosphine [17]. The nascent aldehyde group appeared to fulfill our requirements for a site for the addition of a UV label since there are no aldehyde groups on the native ganglioside molecule. The property of aldehydes to react quickly and quantitatively with hydroxylamines to form oximes is well known. A wide variety of aromatic hydroxylamines are available that absorb at 254 nm; *p*-nitrobenzyloxyamine was chosen since its oximes possess high extinction coefficients [18].

Experimental verification of the soundness of this approach was quickly attained. Ozonation in methanol proceeded quickly. Derivatization after cleavage with triphenylphosphine was carried out in the same vessel without

solvent change by simply adding an aliquot of a methanolic solution containing a carefully weighed quantity of *p*-nitrobenzyloxyamine. Derivatization conditions detailed herein should be carefully adhered to. In our initial attempts, it was found that in the presence of excessive molar amounts of *p*-nitrobenzyloxyamine (30-fold), some secondary derivatization products of the gangliosides were formed. It is not clear whether these are degradation products or follow a second addition of *p*-nitrobenzyloxyamine. In either case, it was found that the secondary derivatization proceeds slowly below 50°C. Closely controlling the molar ratio of *p*-nitrobenzyloxyamine to the aldehyde and lower temperatures eliminated detectable levels of the secondary set of peaks. The chemical nature of the secondary derivatives was not determined, but if it is assumed that a second addition is occurring, then some other carbonyl must be present since the reagent is specific for these. Perhaps an alternative canonical form of the aldose sugars exists momentarily and is able to add hydroxylamine. To remove *p*-nitrobenzyloxyamine following derivatization, the reaction mixture is immediately applied to a DEAE-Sephadex column. Triphenylphosphine, its oxidized product (both of which absorb in the ultraviolet), and *p*-nitrobenzyloxyamine-labeled long-chain fatty aldehydes are also removed in this step.

To apply this methodology to the assay of individual gangliosides, it is mandatory that their lipid structure be known. Although C_{18}-sphingosine and C_{20}-sphingosine will give rise to the same polar cleavage product, dihydrosphingosine contains no double bond and will fail to react. Fortunately, human brain gangliosides contain little reduced ganglioside sphingosine [19—21] and the amount present appears to be relatively constant. Fatty acids in human brain ganglioside are largely saturated [21—23] (stearic acid is the dominant component), but small amounts of unsaturated fatty acids have been detected. Undoubtedly, these double bonds also readily ozonate and derivatize. It is unlikely that a ganglioside derivatized once — either from an aldehyde derived from a sphingosine or from an unsaturated fatty acid — would be distinguished by the reversed-phase packing; ganglioside molecules unsaturated both in the sphingosine and the fatty acid surely add two molecules of *p*-nitrobenzyloxyamine. If such double-labeling is occurring in this procedure, then either the products do chromatograph identically with the mono-derivatized products or occur at such a low incidence they have escaped detection. Since quantitation by HPLC and by conventional TLC gave closely comparable results (Table III), it is evident that the problems just described do not hinder the application of the procedure to the assay of human brain gangliosides. The olefinic structure of the gangliosides from other sources must be known before this method can be applied with confidence for their assay. Fortunately, many studies have already been reported detailing the olefinic composition of the gangliosides isolated from various sources, e.g., the fatty acid and/or sphingosine structure of gangliosides isolated from brain of many species have been reported [23—27]; from human plasma [28]; from human adrenal [29]; from human lens [30]; from human, bovine, and rabbit retina [31]; from bovine [32] and human kidney [33]; from bovine mammary gland [34, 35]; and from rat liver [36].

Sensitivity of our assay exceeds that of TLC resolution of the gangliosides

followed by resorcinol—hydrochloric acid assay from the plate [6]. In our experience, a minimum of 10 μg of ganglioside NANA is required (15—25 μg is desirable) in order to resolve and quantitate individual gangliosides by TLC. We have resolved and quantitated less than 1 μg ganglioside NANA using this methodology; however, the ultimate sensitivity was not examined. Preliminary work (unpublished) on a derivatization method similar to that reported here but using dansyl hydrazine as a label, gave a similar resolution of gangliosides. Although we monitored UV absorption, this compound is strongly fluorescent at 525 nm, suggesting fluorescence monitoring should offer sensitivity to allow ganglioside resolution and quantitation of less than 100 ng of ganglioside NANA. Such a procedure would allow resolution and quantitation of gangliosides on 200 μg of brain tissue, which might prove useful in examining local minute areas of brain for anatomical distribution of gangliosides or following physiological or pharmacological experimentation.

Derivatization at the oxidized double bond of the ceramide by *p*-nitrobenzyloxyamine or by dansyl hydrazine can be generalized to other glycolipids. Cerebrosides, globosides, and other neutral glycolipids can be derivatized by a straightforward extension of the procedures detailed herein. It must be remembered, however, that foreknowledge of the olefinic structures of the molecules of interest must be available. Preliminary experiments suggest the retention times of *p*-nitrobenzyloxyamine-labeled neutral glycolipids are longer, and appropriate changes in the mobile phase are indicated.

HPLC of *p*-nitrobenzyloxyamine-labeled gangliosides offers a procedure that is subject to less variation than TLC, is more sensitive than the resorcinol assay (fluorescence labeling offering an even greater sensitivity), and is non-destructive of the oligosaccharide structure. The method is applicable to routine assay of ganglioside, gangliosides isolated from slow-growing cell cultures, and can also be applied to clinical analyses. The procedure is relatively rapid, the slowest steps are the ion-exchange chromatography and dialysis. Miniaturization should offer no obstacle since ozonation and derivatization can be performed in dilute solution and appropriately sized ion-exchange columns utilized. The potential of derivatization of the initial lipid extract by our procedures and then isolation by an adaption to HPLC of the ion-exchange chromatographic method of Ledeen et al. [9] has not been evaluated. Successful application, however, should provide a means to isolate and characterize gangliosides in a matter of hours rather than days or weeks as presently required.

ACKNOWLEDGEMENTS

Our studies were supported in part by the Institute Grants Program of the American Cancer Society, the Koebig Foundation and the South Carolina Appropriation for Medical Research.

REFERENCES

1 R.O. Brady and P.H. Fishman, Biochim. Biophys. Acta, 355 (1974) 122.
2 K. Sandhoff and H. Jatzkewitz, Adv. Exp. Biol. Med., 19 (1971) 305.

3 N.K. Gonatas and J. Gonatas, J. Neuropathol. Exp. Neurol., 24 (1965) 318.
4 J. Holmgren, Nature (London), 292 (1981) 413.
5 J. Folch, M. Lees and G.H. Sloane-Stanley, J. Biol. Chem., 226 (1957) 497.
6 K. Suzuki, J. Neurochem., 12 (1965) 629.
7 L. Svennerholm, Biochim. Biophys. Acta, 24 (1957) 604.
8 T. Miettinen and I. Takki-Luukkainen, Acta Chem. Scand., 13 (1959) 856.
9 R.W. Ledeen, R.K. Yu and L.F. Eng, J. Neurochem., 21 (1973) 829.
10 J.-P. Zanetta, W.C. Breckenridge and G. Vincendon, J. Chromatogr., 69 (1972) 291.
11 J.-P. Zanetta, F. Vitiello and J. Robert, J. Chromatogr., 137 (1977) 481.
12 K. Eberlein and G. Gercken, J. Chromatogr., 106 (1975) 425.
13 R.J. Penick, M.H. Meisler and R. McCluer, Biochim. Biophys. Acta, 116 (1966) 279.
14 M. Iwamori and Y. Nagai, Biochim. Biophys. Acta, 528 (1978) 257.
15 U.R. Tjaden, J.H. Krol, R.P. van Hoeven, E.P. Oomen-Meulemans and P. Emmelot, J. Chromatogr., 136 (1977) 233.
16 P.S. Bailey, Chem. Rev., 58 (1958) 925.
17 M. Beroza and B.A. Bierl, Anal. Chem., 39 (1957) 1131.
18 D.R. Knapp and S.A. Kruger, Anal. Lett., 8 (1975) 603.
19 T. Taketomi and N. Kawamura, J. Biochem. (Tokyo), 75 (1974) 1403.
20 M. Naoi and E. Klenk, Hoppe-Seyler's Z. Physiol. Chem., 353 (1972) 1677.
21 T. Abe and W.T. Norton, J. Neurochem., 23 (1974) 1025.
22 G. Rouser and A. Yamamoto, J. Neurochem., 19 (1972) 2697.
23 K. Sambasivarao and R.H. McCluer, J. Lipid Res., 5 (1964) 103.
24 E.G. Trams, L.E. Giuffrida and A. Karmen, Nature (London), 193 (1962) 680.
25 N.Z. Stanacev and E. Chargaff, Biochim. Biophys. Acta, 59 (1962) 733.
26 N.Z. Stanacev and E. Chargaff, Biochim. Biophys. Acta, 98 (1965) 168.
27 N.F. Avrova and S.A. Zabelinskii, J. Neurochem., 18 (1971) 675.
28 R.K. Yu and R.W. Ledeen, J. Lipid Res., 13 (1972) 680.
29 M. Igarashi, H.H. Schaumburg, J. Powers, Y. Kishimoto, E. Kolodny and K. Suzuki, J. Neurochem., 26 (1976) 851.
30 G.L. Feldman and L.S. Feldman, Lipids, 1 (1966) 21.
31 M. Holm, J.E. Mansson, M.T. Vanier and L. Svennerholm, Biochim. Biophys. Acta, 280 (1972) 356.
32 K. Puro and A. Keranen, Biochim. Biophys. Acta, 187 (1969) 393.
33 H. Rauvala, J. Biol. Chem., 251 (1976) 7517.
34 T.W. Keenan, Biochim. Biophys. Acta, 337 (1974) 255.
35 A.A. Bushway and T.W. Keenan, Lipids, 13 (1978) 59.
36 E.V. Dyatlovitskaya, A.M. Novikov and L.D. Bergel'son, Biokhimiya, 39 (1974) 552.

Journal of Chromatography, 272 (1983) 21—27
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1477

SPHINGOSINE DETERMINATION AT THE PICOMOLE LEVEL USING DIMETHYLAMINOAZOBENZENE SULPHONYL CHLORIDE

GÜNTER ROSENFELDER*, JUI-YOA CHANG and DIETMAR G. BRAUN

Pharmaceuticals Research Laboratories, Ciba-Geigy Ltd., CH-4002 Basle (Switzerland)

(First received June 1st, 1982; revised manuscript received August 18th, 1982)

SUMMARY

A sensitive method (lower detection limit 5 picomoles) is proposed for the determination of sphingoid bases liberated from mammalian glycosphingolipids by acid hydrolysis. The azo dye 4-dimethylaminoazobenzene-4′-sulfonyl chloride reacts with the primary amino group of the sphingosine bases, forming a stable derivative. Excess reagent, which is degraded during the derivatization, and free amino sugars as common hydrolysis products of glycosphingolipids are completely separated by reversed-phase high-performance liquid chromatography. This method was applied to the determination of the glycosphingolipid content of mouse spleen and thymus.

INTRODUCTION

Sphingoid bases are backbone constituents of sphingolipids, which occur in the form of three major classes: the sphingomyelins, and neutral and acidic glycosphingolipids. The quantitative determination of these amino alcohols may be achieved by spectrometric or fluorospectrometric methods after coupling a chromophore to the primary amino group [1—6]. Alternatively, gas—liquid chromatography of either the trimethylsilyl derivative of sphingosine bases [7] or of the fragments obtained after cleavage of the 4-sphingenine double bond may be used [8]. Because of their relatively low sensitivity levels (nanomole range), these methods are not applicable to the determination of the glycosphingolipid content of small amounts of murine lymphoid tissues and isolated lymphocytes.

Recently, high-performance liquid chromatographic (HPLC) separation and determination of amino acids and aliphatic amines at the picomole level has been attained by their precolumn derivatization with the azo dye 4-dimethylaminoazobenzene-4′-sulfonyl chloride (dabsyl-Cl) [9, 10]. Dabsyl-Cl,

0378-4347/83/0000—0000/$03.00

like dansyl chloride, only reacts with primary and secondary amino groups, thiols, imidazoles and phenols under the dabsylation conditions described in this paper. Here, we describe a sensitive method (lower detection limit 5 pmoles) for the determination of sphingoid bases liberated from mammalian glycosphingolipids by acid hydrolysis. Excess reagent, which is degraded during the derivatization, and free amino sugars as common hydrolysis products of glycosphingolipids are completely separated by reversed-phase HPLC. This method was applied to the determination of the glycosphingolipid content of mouse spleen and thymus.

EXPERIMENTAL

Materials

Dabsyl-Cl was purchased from Fluka (Buchs, Switzerland). 4-Spingenine*, sphinganine*, cerebrosides and GD_{1a} ganglioside, all from bovine brain, were obtained from Supelco (Crans, Switzerland). Synthetic sphingoid bases were kindly provided by Prof. E. Jenny (Ciba-Geigy Ltd., Basle, Switzerland).

Mice

AKR and CBA/J mice (4—6 weeks old) were purchased from Bomholtgard (Ry, Denmark); BALB/c and C57BL/6 strains were bred at Ciba-Geigy animal facilities (Sisseln, Switzerland).

HPLC system

The components of the HPLC system were: a Zorbax CN column, particle size 10 μm (25 cm × 4.6 mm I.D.; DuPont Instruments, Wilmington, DE, U.S.A.), a Model U6K injector (Waters Assoc., Milford, MA, U.S.A.), 2 Model 110A pumps (Altex, Berkeley, CA, U.S.A.), an Altex Model 420 programmer, a Kontron Uvikon LCD 725 variable-wavelength detector (Kontron Analytic, Zürich, Switzerland), an SP 4020 interface and an SP 4000 integrator (Spectra-Physics, Darmstadt, G.F.R.), and a W+W Model 314 recorded (W+W Electronics, Basle, Switzerland).

A 5—10-μl sample volume was injected. The flow-rate was 1.0 ml/min. The solvent program was as follows: 60% acetonitrile, 40% 0.0175 *M* sodium acetate, pH 6.0. Isocratic conditions were maintained for 10 min, followed by a linear increase of the acetonitrile concentration up to 90% within 5 min, a linear decrease to initial conditions in 5 min, and isocratic conditions for another 10 min. The detector wavelength was 430 nm, and the sensitivity range was 0.01 a.u.f.s. The integrator was programmed to calculate area per cent.

Mass spectrometry

A ZAB fast atom bombardment mass spectrometer (Vacuum Generators, Altrincham, Great Britain) was used. The sample was dissolved in glycerol—methanol and bombarded by a 8 kV xenon source.

---

*Abbreviations: 4-sphingenine = D-*erythro*-2-amino-4-*trans*-1,3-dihydroxy-octadecane; 4-sphinganine = D-*erythro*-2-amino-1,3-dihydroxy-octadecane.

Preparation of mouse spleen and thymus glycosphingolipids

The spleen and thymus from 25 mice were excised and homogenized in 50 ml of cold chloroform–methanol (2:1, v/v) using a Sorvall Omnimixer (DuPont Instruments). The tissue extract was passed through glass filter discs. This procedure was repeated twice. The combined extracts were dried in a rotary evaporator. The further fractionation was done as described by Saito and Hakomori [11]. Briefly, gangliosides were obtained from the upper phase of a modified Folch partition. The lower phase lipids were acetylated and fractionated on a Florisil column. Thus, sphingomyelin and neutral glycosphingolipids were completely separated. The glycosphingolipid fractions were checked for purity by high-performance thin-layer chromatography on silica gel plates [12]. Gangliosides were stained with resorcinol, and neutral glycosphingolipids with orcinol [13].

Hydrolysis of glycosphingolipids

Sphingoid bases were liberated by hydrolysis in 150 μl of methanol–water–conc. hydrochloric acid (29:4:3, v/v) in 200-μl Reactivials (Pierce, Rockford, IL, U.S.A.) at 79°C for 18 h [14]. Samples were dried in vacuo over KOH pellets, and used directly for dabsylation.

Dabsylation

Hydrolyzed glycosphingolipids were added with 20 μl of 0.2 *M* $NaHCO_3$ – NaOH solution (pH 8.75) and 40 μl of dabsyl-Cl solution (5 nmoles/μl in acetone). The sample was heated at 70°C for 10 min with constant shaking. The dabsylated sample was diluted, if necessary, with methanol and injected directly for HPLC analysis.

RESULTS AND DISCUSSION

The proposed structure of dabsyl-sphingosine (DABS-Sph) is shown in Fig. 1. Fast atomic bombardment mass spectrometry showed the prominent molecular ion MH^+ at m/e = 587. There was no evidence of mass fragments indicating the reaction of the dabsyl-Cl with free sphingosine hydroxyl groups.

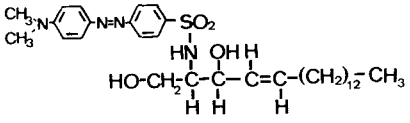


Fig. 1. Structure of dabsyl-sphingosine (dabsyl derivative of D-*erythro*-2-amino-4-*trans*-1,3-dihydroxy-octadecene).

Dabsylation of sphingosine was carried out in acetone–sodium bicarbonate, pH 8.75 (2:1, v/v) solution. The extent of dabsylation is dependent upon the initial concentration of dabsyl-Cl. However, like amino acid analysis using dabsyl-Cl [9], at any fixed concentration of dabsyl-Cl, there is a linear relationship between the peak response of DABS-Sph and the amount of sphingosine subjected to dabsylation (Fig. 2). This linearity comprises the basic and most important requisite for a quantitative analytical method using pre-

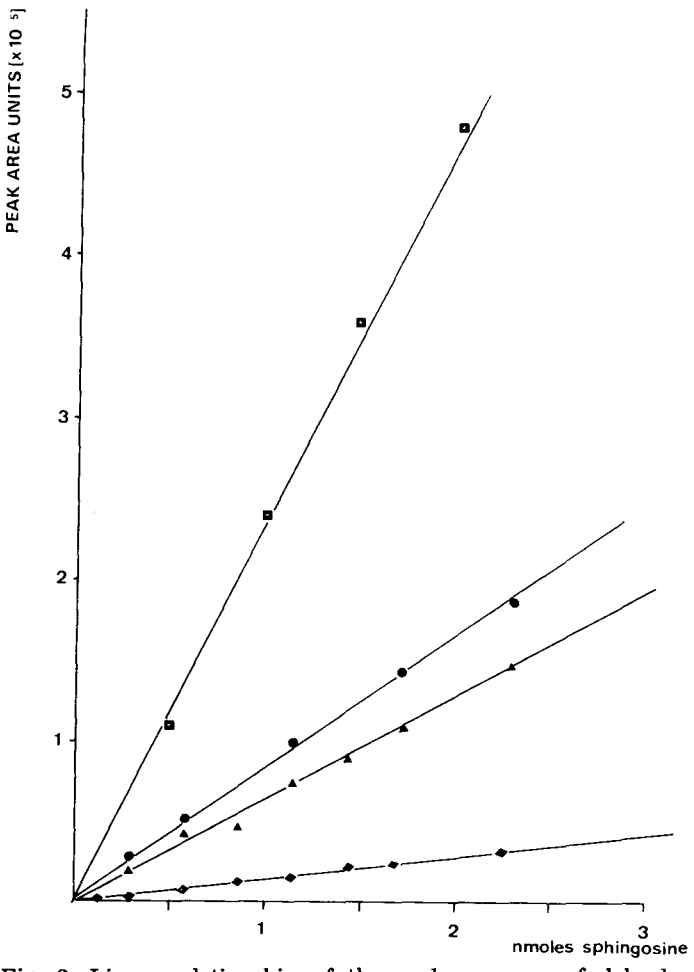


Fig. 2. Linear relationship of the peak response of dabsyl-sphingosine as a function of amount of sphingosine subjected to dabsylation at different dabsylation concentrations: (♦) 0.67 m*M*, (▲) 1.67 m*M*, (●) 3.33 m*M*, (■) 16.7 m*M*. For other experimental details see text.

column derivatization technique. The concentration 3.33 nmoles of dabsyl-Cl per μl of reaction volume (i.e. 5 nmoles dabsyl-Cl per μl of acetone) has been selected throughout this study on the basis that it gave the optimal sensitivity at tolerable level of excess reagent.

Fig. 3 shows the HPLC separation of DABS-Sph (Fig. 3b). The DABS-Sph which clearly separated from the excess reagent and by-products was eluted at 7 min. With the detector sensitivity set at 0.01 a.u.f.s., the integrator gave 930,000 area units per nanomole of DABS-Sph. Since 3000 area units can be easily recognized, the detection limit of dabsyl-Cl is thus around 5 pmoles.

Generally, in glycosphingolipids from distinct mammalian tissues only a few sphingosine bases occur at substantial levels. In most cases 4-sphingenine is predominant, but also sphinganine, isocasphingosine and 4-hydroxy-sphinganine are found [14]. In order to establish the contribution of the chemi-

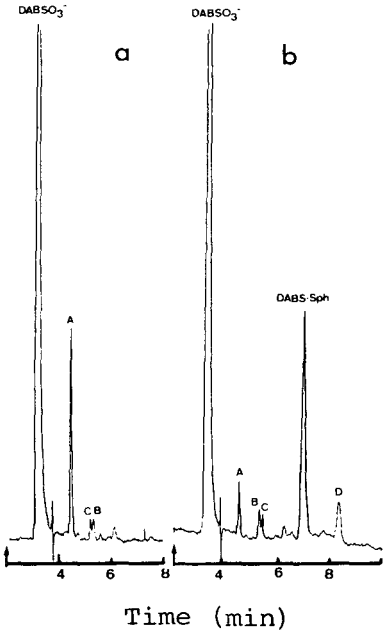


Fig. 3. (a) Blank run (dabsyl-Cl heated for 10 min at 70°C). (b) HPLC separation of dabsyl-sphingosine (Supelco). Peaks A, B and C represent reagent-derived by-products. Peak D is probably C20-4-sphingenine present in Supelco bovine brain sphingosine. For experimental details see text.

cal structure of sphingosines (i.e. number of carbon atoms or chain length, presence of double bonds and of additional hydroxyl groups) to the retention behaviour upon reversed-phase HPLC, a number of naturally occurring and synthetic sphingosines were dabsylated and submitted to the system.

Table I summarizes the sphingoid bases chromatographed and the retention times relative to 4-sphingenine. It is obvious that the separation behaviour of sphingosines is primarily according to their carbon atom chain length. A one C-atom difference will result in complete separation. Saturation of double bonds does not significantly change retention times. Thus, the sphinganine peak chromatographs as a shoulder of the 4-sphingenine peak. The configuration at asymmetric C-atoms, i.e. *erythro-* or *threo*-configuration and *cis-* or *trans*-isomerism of the double bond, does not contribute essentially to the separation on this column. Separation of isomers remains incomplete. With the exception of isocasphingenine which overlaps with a minor peak there is no interference of sphingoid base retention times with peaks of reagent-derived origin. Preliminary results indicate that also the 4-hydroxy-sphinganine (relative retention time = 0.84) is clearly separated from 4-sphingenine in our HPLC system.

Next this method was applied to the determination of the glycosphingolipid content of purified lipid fractions from mouse lymphoid organs. The average total lipid weight of a mouse spleen containing in the order of 10^8 lymphocytes is about 3 mg (G. Rosenfelder, unpublished results).

From human and pig lymphocytes it is known that glycosphingolipids

TABLE I

RETENTION TIMES OF SYNTHETIC AND NATURALLY OCCURRING SPHINGOID BASES SEPARATED ON A ZORBAX CN REVERSED-PHASE HPLC COLUMN

Sphingoid base (systematic denomination)	Origin	Relative retention time* (*T*)
D-*erythro*-2-Amino-4-*trans*-1,3-dihydroxy-octadecene	Bovine brain**	1.00
D-*erythro*-2-Amino-1,3-dihydroxy-octadecane	Bovine brain**	1.04
DL-*threo*-2-Amino-1,3-dihydroxy-octadecane	Synthetic***	1.04
DL-*erythro*-2-Amino-4-*cis*-dihydroxy-nonadecene	Synthetic***	1.10
DL-*threo*-2-Amino-4-*cis*-1,3-dihydroxy-nonadecene	Synthetic***	1.11
DL-*threo*-2-Amino-4-*trans*-1,3-dihydroxy-nonadecene	Synthetic***	1.07
DL-*threo*-2-Amino-1,3-dihydroxy-nonadecane	Synthetic***	1.14
D-*erythreo*-2-Amino-4-*trans*-1,3-dihydroxy-eicosene	Bovine brain ganglioside GD_{1a}**	1.17
DL-*threo*-2-Amino-1,3-dihydroxy-tricosane	Synthetic***	1.71

*Relative to the major bovine brain sphingosine 4-sphingenine = 1.00.
**Supelco.
***Synthesized by Prof. E. Jenny, Ciba-Geigy Ltd.

constitute up to 10% of total lipids [15, 16]. Therefore, a content of 30—300 μg of glycosphingolipids was expected in one mouse spleen. This equals 30—300 nmoles if an average molecular weight of 1000 is assumed. Our results (Table II) show a range of 60—166 nmoles of glycosphingolipids per spleen and of 24—62.4 nmoles per thymus, depending on the inbred strain that was used.

The ganglioside concentration is very low compared to the neutral glycosphingolipid concentration, with the lowest value (1.1. nmoles) in the C57BL/6 thymus.

TABLE II

GLYCOSPHINGOLIPID CONTENT OF SPLEEN AND THYMUS FROM MICE OF DIFFERENT INBRED STRAINS

Mouse strain	Glycosphingolipid content per mouse				Relative ganglioside content***	
	Gangliosides*		Neutral glycosphingolipids**			
	Spleen (nmoles)	Thymus (nmoles)	Spleen (nmoles)	Thymus (nmoles)	Spleen (%)	Thymus (%)
BALB/c	13.5 ± 0.48	1.7 ± 0.1	166.0 ± 7.2	26.4 ± 2.3	7.5	6.1
C57BL/6	7.2 ± 0.36	1.1 ± 0.1	60.9 ± 3.0	62.4 ± 4.0	10.6	1.7
AKR	13.4 ± 0.37	4.6 ± 0.3	63.3 ± 3.9	49.6 ± 1.8	1.8	8.5
CBA/J	6.0 ± 0.42	1.8 ± 0.2	60.0 ± 4.2	24.0 ± 1.4	9.1	7.0

*Dialyzed Folch upper phase lipids.
**Obtained by peracetylation and Florisil chromatography of Folch lower phase lipids [11].
***Gangliosides + neutral glycosphingolipids = 100%.

These low concentrations stress the necessity to use a quantitative glycosphingolipid determination method working at the picomole level if fractions of glycosphingolipids from separated lymphoid cell populations are studied in the mouse system. The dabsylation method described can be applied to the study of 10^5 or less lymphoid cells, i.e. of minor lymphocyte subpopulations. Moreover, it may be useful to study the total glycosphingolipid content of other extraneural cells where low concentrations of glycosphingolipids may play an important role in physiological processes like cell—cell recognition, receptor function or malignant transformation [17].

ACKNOWLEDGEMENTS

The skilful technical assistance of Mrs. A. Bordmann and secretarial assistance of Mrs. V. Forster are gratefully acknowledged. We thank Mr. F. Raschdorf for the mass spectrometric confirmation of the DABS-Sph structure.

REFERENCES

1 C.J. Lauer and E.G. Trams, J. Lipid Res., 3 (1962) 136.
2 K.A. Karlsson, Chem. Phys. Lipids, 5 (1970) 643.
3 L. Coles and G.M. Gray, J. Lipid Res., 11 (1970) 164.
4 M. Naoi, Y.C. Lee and S. Roseman, Anal. Biochem., 58 (1974) 571.
5 J.L. Ryan and M. Schinitzky, Eur. J. Immunol., 9 (1979) 171.
6 F.B. Jungalwala, B. Hayssen, E. Bremer and R.H. McCluer, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 37 (1978) 1644.
7 H.E. Carter and R.C. Gaver, J. Lipid Res., 8 (1967) 391.
8 C.C. Sweeley and F. Moscatelli, J. Lipid Res., 1 (1959) 40.
9 J.Y. Chang, R. Knecht and D.G. Braun, Biochem. J., 199 (1981) 547.
10 J.K. Lin and C.C. Lai, Anal. Chem., 52 (1980) 630.
11 T. Saito and S. Hakomori, J. Lipid Res., 12 (1971) 257.
12 G. Rosenfelder, H. Herbst and D.G. Braun, FEBS Lett., 114 (1980) 213.
13 R.A. Laine, K. Stellner and S. Hakomori, in E.D. Korn (Editor), Methods in Membrane Biology, Vol. 2, Plenum Press, London, New York, 1974, p. 205.
14 C.C. Sweeley and B. Siddiqui, in M.I. Horowitz and W. Pigman (Editors), The Glycoconjugates, Vol. 1, Academic Press, New York, San Francisco, London, 1977, p. 459.
15 B.A. Macher and J.C. Klock, J. Biol. Chem., 255 (1980) 2092.
16 G.M. Levis, G.P. Evangelatos and M.J. Crumpton, Biochem. J., 156 (1976) 103.
17 T. Yamakawa and T. Nagai, Trends Biochem. Sci., 6 (1978) 128.

Journal of Chromatography, 272 (1983) 29—41
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1475

FLUORESCENCE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FREE AND CONJUGATED BILE ACIDS IN SERUM AND BILE USING 1-BROMOACETYLPYRENE AS A PRE-LABELING REAGENT

SATORU KAMADA, MASAKO MAEDA and AKIO TSUJI*

School of Pharmaceutical Sciences, Showa University, 1-5-8, Hatanodai, Shinagawa, Tokyo (Japan)

(First received May 27th, 1982; revised manuscript received August 11th, 1982)

SUMMARY

A fluorescence high-performance liquid chromatographic method is described for the determination of free and conjugated bile acids in serum and bile. Free and conjugated bile acids are extracted from serum or bile using a Sep-Pak C_{18} cartridge and then fractionated on a piperidinohydroxypropyl Sephadex LH-20 column. Free and glycine-conjugated bile acids are labeled with 1-bromoacetylpyrene in acetonitrile using dicyclohexyl-18-crown-6-ether as catalyst. Taurine-conjugated bile acids are hydrolyzed by cholylglycine hydrolase and then derivatized by the same reagent. Derivatized bile acids are separated stepwise on a reversed-phase column (Radial Pak A) using acetonitrile—methanol—water (A) (100 : 50 : 40) and (B) (100 : 50 : 20) as mobile phase. The eluate is monitored by a fluorophotometer at 370 nm (excitation) and 440 nm (emission). Linearities of fluorescence intensities (peak heights) with the amounts of free and conjugated bile acids were obtained between 50 pmol and 200 pmol for free bile acids and between 25 pmol and 100 pmol for glycine-conjugated bile acids, respectively. Recoveries from serum and bile samples are not less than 90%. This method is sensitive, reliable and useful for the simultaneous determination of free and conjugated bile acids in serum and bile.

INTRODUCTION

The identification and determination of individual bile acids and their conjugates in serum and bile are of value in the diagnosis of liver disease [1]. A variety of methods for the simultaneous determination of individual free and conjugated bile acids in human serum and bile has been described, in which thin-layer chromatography [2,3], gas chromatography [4,5], and gas chromatography—mass spectrometry [6—8] were used. In recent years, high-

0378-4347/83/0000—0000/$03.00

performance liquid chromatography (HPLC) [9—17] has been developed, but the most common bile acids, which have no UV light-absorbing properties, could not be detected with high sensitivity by a photometric detector. Therefore, bile acids should be derivatized before chromatographic separation with a UV-absorbing prelabeling reagent, such as 1-*p*-nitrobenzyl-3-*p*-tolyltriazine [12], phenacyl bromide [13,14], *p*-chlorobenzoyl chloride [15], O-(*p*-nitrobenzyl)-N,N-diisopropylisourea [15,16], and 1-naphthyldiazomethane [17]. These labeling reagents are the derivatization reagents for carboxylic acids. Though these methods [12—17] are useful for the assay of bile acids in bile, their sensitivities are not enough to determine small amounts of bile acids in human serum samples. Therefore, in order to improve the detectability for bile acids, fluorescent derivatization reagents such as 4-bromomethyl-7-methoxycoumarin [18] and 9,10-diaminophenanthrene [19] have been used for the assay of bile acids by HPLC. Baba et al. [20] reported a different fluorescence HPLC system in which bile acids and their conjugates eluted from the column were converted to 3-oxo bile acids and NADH using 3α-hydroxysteroid dehydrogenase (3α-HSD) and NAD^+ solution, and NADH was monitored by a fluorophotometric detector. However, this method consumes considerable amounts of expensive enzyme. Okuyama et al. [21] and Arisue et al. [22] devised a modified method using an immobilized enzyme column instead of the enzyme solution. In the previous paper [23], we used a voltammetric detector to measure NADH instead of a fluorophotometric detector in an HPLC method for the determination of bile acids using an immobilized 3α-HSD column and NAD^+ solution. Though each bile acid was measurable at the 20 pmol level by this method, a more highly sensitive HPLC method was required because the amounts of bile acids in human serum samples are as low as a few picomoles. In this paper, we have attempted to develop a new fluorescent derivatizing reagent for a highly sensitive method for determining free and conjugated bile acids.

EXPERIMENTAL

Materials

Cholic acid, chenodeoxycholic acid, deoxycholic acid, ursodeoxycholic acid, lithocholic acid, and their glycine and taurine conjugates were purchased from Sigma (St. Louis, MO, U.S.A.), Wako (Osaka, Japan) and PL Biochemicals (Milwaukee, WI, U.S.A.). Dicyclohexyl-18-crown-6-ether was obtained from Nippon Soda (Tokyo, Japan), and Sep-Pak C_{18} cartridge from Waters Assoc. (Milford, MA, U.S.A.). 1-Bromoacetylpyrene and piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) were gifts from Dr. Y. Kawahara (Product development laboratories of Sankyo, Tokyo, Japan) and from Professor T. Nambara (Pharmaceutical Institute, Tohoku University, Sendai, Japan), respectively. Cholylglycine hydrolase (EC 3.5.1.24) was purchased from Sigma. All the other reagents employed were of analytical grade. Solvents were purified by distillation prior to use.

Reagent solutions

Stock solutions of bile acids: each bile acid was dissolved in methanol and made up to 10 μmol/ml with methanol.

Standard mixture solution of bile acid: standard mixture solutions of free, glycine-conjugated, and taurine-conjugated bile acids were prepared by mixing the bile acid stock solutions, and their concentrations were adjusted to 100 nmol/ml (free acids), 50 nmol/ml (glycine conjugates) and 100 nmol/ml (taurine conjugates).

Lauric acid standard solution (internal standard); lauric acid was used as internal standard. Purified lauric acid was dissolved in methanol and made up to 100 nmol/ml with methanol.

1-Bromoacetylpyrene solution: this solution was prepared by dissolving 1-bromoacetylpyrene in acetonitrile (25 μmol/ml).

Dicyclohexyl-18-crown-6-ether solution: this solution was prepared by dissolving dicyclohexyl-18-crown-6-ether in acetonitrile (1 mg/ml).

KOH–methanol solution (0.01%): this solution was prepared by dissolving KOH in methanol (0.1 mg/ml).

Instruments

A Shimadzu (Kyoto, Japan) Model LC-3A high-performance liquid chromatograph equipped with a Waters Assoc. Model RCM-100 Radial-Pak A column (100 mm × 8 mm I.D.; particle size 10 μm) was utilized for HPLC. A Shimadzu Model RF-500 spectrofluorophotometer equipped with an 8-μl micro flow cell was used as a monitor.

Derivatization procedure

A standard mixture solution of bile acids or sample solution (50 μl), a lauric acid standard solution (internal standard) (50 μl) and 0.01% KOH—methanol solution (50 μl) were mixed in a vial and evaporated to dryness under a stream of nitrogen gas. A dicyclohexyl-18-crown-6-ether solution (100 μl) and 1-bromoacetylpyrene solution (100 μl) were then added to the tube. The reaction mixture was heated at 40°C for 30 min. After cooling, an aliquot of 8 μl of the solution was injected into the chromatograph.

Chromatographic conditions

Separation of free and glycine-conjugated bile acids. After derivatization with 1-bromoacetylpyrene, the separation of derivatives of bile acids was carried out on a Radial-Pak A column at room temperature using acetonitrile–methanol—water [100 : 50 : 40 (A) and 100 : 50 : 20 (B)] as the mobile phase at a flow-rate of 2 ml/min. The mobile phase was changed by a solvent exchanger from A to B at 30 min after injection of the sample solution. The effluent from the column was monitored by a fluorophotometric detector at an excitation wavelength of 370 nm and an emission wavelength of 440 nm.

Taurine-conjugated bile acids. After derivatization of enzymatically hydrolyzed taurine-conjugated bile acids, the separation was carried out by the same column described above using acetonitrile–methanol–water (100 : 50 : 22) as the mobile phase at a flow-rate of 2 ml/min.

Procedure for the determination of free and glycine-conjugated bile acids in human serum

A serum sample (100 or 200 μl) was mixed with methanol (1 ml) and ultrasonicated for 15 min. The supernatant (600 μl) was transferred into a micro test tube and evaporated to dryness under nitrogen gas stream. The residue was dissolved by adding 0.05 *M* phosphate buffer (pH 7.0) (1 ml), and then percolated through a Sep-Pak C_{18} cartridge. The cartridge was washed with 20% (v/v) methanol (2 ml), and bile acids were then eluted with 80% (v/v) methanol (4 ml). After evaporation of methanol under reduced pressure at 40°C, the residue was redissolved by addition of methanol (1 ml), and an aliquot of 500 μl of the resultant solution was then used for the derivatization step described above.

Procedure for the determination of free and glycine-conjugated bile acids in human bile

A bile sample (10 μl) was diluted with 0.05 *M* phosphate buffer (pH 7.0) (10 ml) and the diluted solution (1 ml) was then applied to a Sep-Pak C_{18} cartridge and assayed as described above.

Procedure for the determination of taurine-conjugated bile acids in human serum

The bile acid fraction was extracted from the serum sample (100 or 200 μl) by a Sep-Pak C_{18} cartridge as described above. The evaporated bile acid fraction was dissolved in 90% (v/v) ethanol (1 ml) and applied gently to a column (115 × 7.5 mm I.D.) of PHP-LH-20 (acetate) (ca. 100 mg) at a flow-rate of 7—8 drops/min. After washing with 90% (v/v) ethanol (4 ml), free, glycine-conjugated and taurine-conjugated bile acids were eluted stepwise with 0.1 *M* acetic acid in 90% (v/v) ethanol (4 ml), 0.2 *M* formic acid in 90% (v/v) ethanol (4 ml), and 0.3 *M* acetic acid--potassium acetate in 90% (v/v) ethanol (pH 6.3--6.5) (4 ml). The fraction of taurine-conjugated bile acids was evaporated to dryness under a nitrogen gas stream. The residue was dissolved in water (1 ml) and percolated through a Sep-Pak C_{18} cartridge. After washing the column with 2% (v/v) methanol (2 ml), taurine-conjugated bile acids were eluted with 80% (v/v) methanol (4 ml). After evaporation of methanol, the residue was redissolved by the addition of water (500 μl) and 0.025 *M* phosphate buffer (200 μl), and then mixed with cholylglycine hydrolase solution (30 units/ml) (100 μl). After incubation at 37°C for 15 min, the reaction mixture was cooled in an ice bath and mixed with 0.25 *M* phosphate buffer (pH 7.0) (200 μl). The free bile acids in the resultant solution were extracted using a Sep-Pak C_{18} cartridge and then derivatized with 1-bromoacetylpyrene as mentioned above.

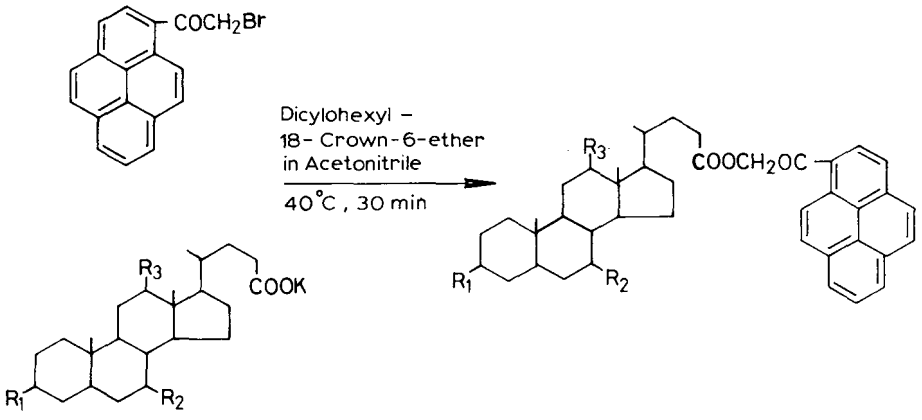
Recovery test

A synthetic mixture of 50 nmol of each free bile acid and 25 nmol of each glycine-conjugated bile acid was added to 1.0 ml of serum and 1 μl of bile, respectively, and then assayed by the procedure.

RESULTS AND DISCUSSION

Derivatization

Phenacyl bromide type derivatives are the favorite reagents for the formation of carboxylic acid esters because of their high reactivity under mild conditions. Therefore, 1-bromoacetylpyrene was synthesized as a fluorescent derivatization reagent [24]. It was relatively easy to synthesize this reagent from readily available compounds. The free and glycine-conjugated bile acids were esterified with 1-bromoacetylpyrene using dicyclohexyl-18-crown-6-ether as catalyst in acetonitrile after converting them to their potassium salts, as shown in Fig. 1.



R_1	R_2	R_3	
OH	OH	OH	Cholic acid
OH	H	OH	Deoxycholic acid
OH	OH	H	Chenodeoxycholic acid
OH	OH(β)	H	Ursodeoxycholic acid
OH	H	H	Lithocholic acid

Fig. 1. Esterification of bile acids with 1-bromoacetylpyrene.

Taurine-conjugated bile acids were separated from free and glycine-conjugated bile acids by column chromatography with PHP-LH-20 according to the method of Goto et al. [25]. After collecting the taurine-conjugated bile acid fraction and enzymatic hydrolysis with cholylglycine hydrolase, esterification was conducted with 1-bromoacetylpyrene as well as the free and glycine-conjugated acids.

As shown in Fig. 2, the reaction of all bile acids tested with the reagent at 40°C was completed within 20 min. When heated at 80°C, the reaction was completed within about 10 min and the fluorescence intensity increased slightly, but unknown subpeaks appeared and overlapped some bile acid peaks.

The peak height of bile acids increased with increasing the amount of reagent added and reached a constant value at 10 μmol/ml. Though bile acids were esterified in the absence of dicyclohexyl-18-crown-6-ether, the peak height of free bile acids was very low compared with that of glycine-conjugated bile

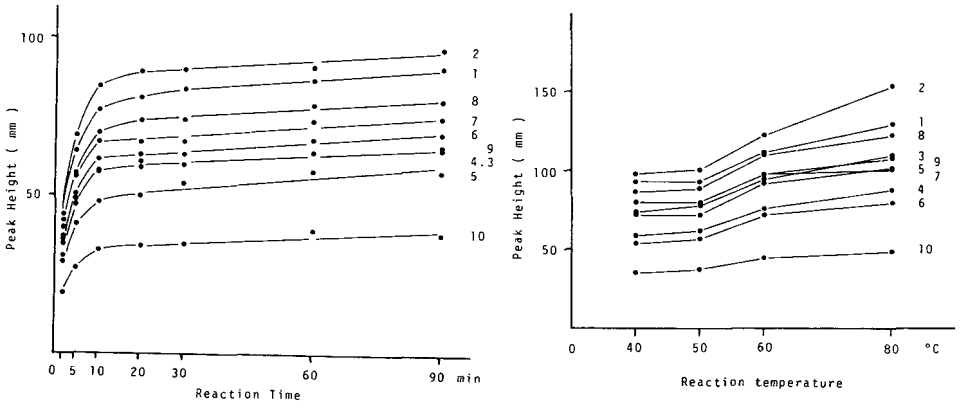


Fig. 2. Reaction conditions of derivatization. 1 = Glycoursodeoxycholic acid, 2 = glycocholic acid, 3 = glycochenodeoxycholic acid, 4 = glycodeoxycholic acid, 5 = ursodeoxycholic acid, 6 = cholic acid, 7 = glycolithocholic acid, 8 = chenodeoxycholic acid, 9 = deoxycholic acid, 10 = lithocholic acid.

acids. By adding 50 μl of 0.01% KOH–methanol solution the peak height of free bile acids increased and the peak height ratio with the internal standard peak of free and glycine-conjugated bile acids became nearly equal to each other.

From these results, the derivatization procedure was decided as described in Experimental.

Hydrolysis of taurine conjugated bile acids

Taurine-conjugated bile acids are unable to be directly esterified with 1-bromoacetylpyrene. Therefore, prior to derivatization, an hydrolysis step is inevitable. In a preliminary experiment, we examined alkaline hydrolysis of taurine-conjugated bile acids but could not obtain satisfactory reproducibility. Enzymatic hydrolysis with cholylglycine hydrolase from *Clostridium perfringens* (EC 3.5.1.24) was examined according to the literature reported by Karlaganis et al. [6]. In order to obtain quantitative hydrolysis conditions, the amount of enzyme, pH of buffer and incubation time were examined. From the results, the enzymatic hydrolysis conditions were decided as the procedure described in Experimental.

Chromatographic separation

The chromatographic separation of the derivatized free and glycine-conjugated bile acids was carried out under a variety of conditions. The reversed-phase type column, Waters Radial-Pak A, and the stepwise elution system using acetonitrile–methanol–water mixtures (A) and (B) were used as described in Experimental. A typical chromatogram obtained by synthetic mixture of free and glycine-conjugated bile acids is shown in Fig. 3. Though glycoursodeoxycholic acid was not separated completely from the peaks of excess or decomposed reagent, baseline separation was obtained between other bile acids.

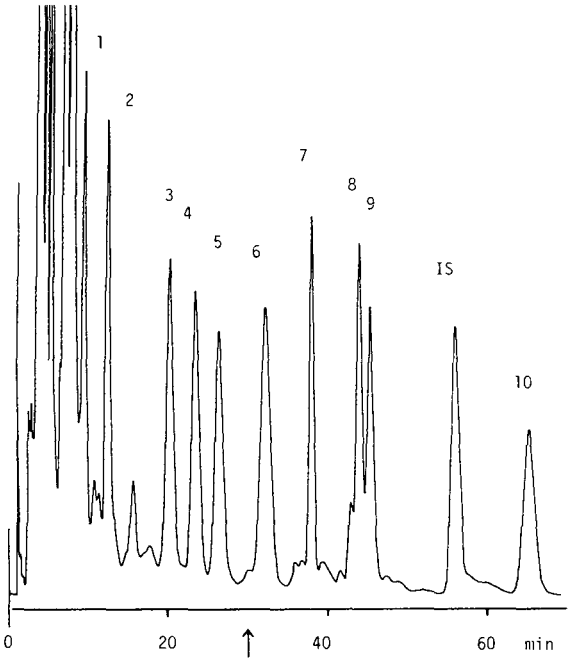


Fig. 3. Chromatogram of standard free and glycine conjugated bile acids. Peaks: 1 = glycoursodeoxycholic acid, 2 = glycocholic acid, 3 = glycochenodeoxycholic acid, 4 = glycodeoxycholic acid, 5 = ursodeoxycholic acid, 6 = cholic acid, 7 = glycolithocholic acid, 8 = chenodeoxycholic acid, 9 = deoxycholic acid, 10 = lithocholic acid.

In the assay of taurine-conjugated bile acids, derivatization was carried out after enzymatic hydrolysis and Sep-Pak C_{18} cartridge clean-up. As shown in Fig. 4, the derivatives of liberated free bile acids were satisfactorily separated from each other when acetonitrile—methanol—water (100 : 50 : 22, v/v) was used as the mobile phase.

Pyrenacyl esters of bile acids were eluted in the same definite order as underivatized bile acids. This suggests that the pyrene group bound to the side-chain carboxylic acid would not affect the elution pattern of bile acids.

Calibration graphs

Calibration graphs were constructed by plotting the ratio of the peak height of bile acid to that of an internal standard, lauric acid, against the amount of the bile acid. Typical calibration graphs are shown in Fig. 5. Linearity of the relationship between peak height ratio and the amount of bile acids was obtained in the range 50—200 pmol for free bile acids and 25—100 pmol for glycine-conjugated bile acids. From these graphs the detection limits were 10 pmol for free bile acids, 5 pmol for glycine-conjugated bile acids and 10 pmol for taurine-conjugated bile acids, depending on the efficiency of the detector and the final volume of sample solution.

As shown in Fig. 5, the responses of free bile acids were about half those of glycine-conjugated bile acids. In order to elucidate the reason for this

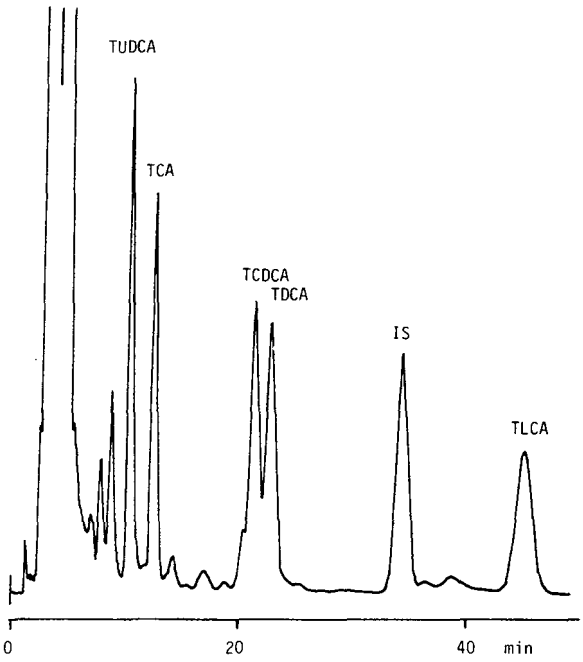


Fig. 4. Chromatogram of taurine-conjugated bile acids after enzymatic hydrolysis. TUDCA = tauroursodeoxycholic acid, TCA = taurocholic acid, TCDCA = taurochenodeoxycholic acid, TDCA = taurodeoxycholic acid, IS = internal standard, TLCA = taurolithocholic acid.

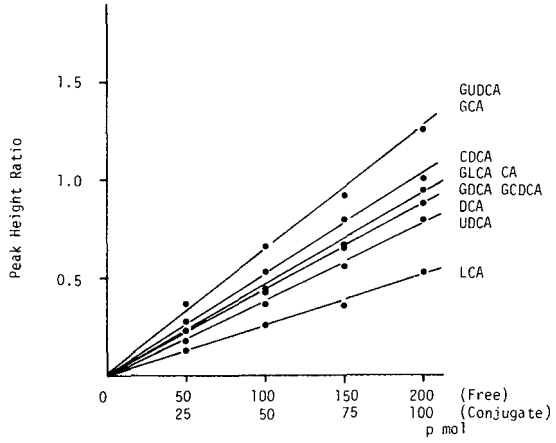


Fig. 5. Calibration curves obtained by standard bile acid mixture. GUDCA = glycoursodeoxycholic acid, GCA = glycocholic acid, CDCA = chenodeoxycholic acid, GLCA = glycolithocholic acid, CA = cholic acid, GDCA = glycodeoxycholic acid, GCDCA = glycochenodeoxycholic acid, DCA = deoxycholic acid, UDCA = ursodeoxycholic acid, LCA = lithocholic acid.

phenomenon, [^{3}H]glycochenodeoxycholic acid and [^{14}]cholic acid were diluted with carrier. After derivatization with 1-bromoacetylpyrene, the resultant reaction solution was divided into halves, one half being separated by HPLC as described in Experimental. The radioactivities of the separated fractions of cholic acid and glycochenodeoxycholic acid were measured and compared with those of the original derivatized reaction solution. After separation by HPLC, the recovery of [^{3}H]glycochenodeoxycholic acid was about 70%, and the recovery of [^{14}C]cholic acid was about 35%. Therefore, the derivatization yield of free bile acids appeared to be half that of the glycine-conjugated bile acids.

Recovery tests

In order to determine the recoveries, the standard bile acid mixture of each five free and glycine-conjugated bile acids was added to human serum and bile, and then subjected to the HPLC method after extraction and derivatization as described in Experimental. As shown in Table I, the recoveries of free and glycine-conjugated bile acids were satisfactory. The mean recoveries and coefficients of variation (C.V.) from serum samples ranged from 90.3 to 104.7% (C.V. = 2.9—18.0%) for intra-assay and from 96.8 to 108.5% (C.V. = 3.4—11.1%) for inter-assay. In the case of bile, the mean recoveries and C.V., intra-assay and inter-assay, ranged from 88.0 to 106.4% (C.V. = 1.0—2.5%) and from 94.7 to 107.7% (C.V. = 1.7—9.8%), respectively.

The recoveries of taurine-conjugated bile acids from serum were also determined. The results are shown in Table II. The recoveries and coefficients of variation were not good compared with those of free and glycine-conjugated bile acids.

TABLE I

RECOVERIES OF FREE AND GLYCINE-CONJUGATED BILE ACIDS FROM HUMAN SERUM AND BILE

Bile acids*	Serum				Bile			
	Intra-assay (n=4)		Inter-assay (n=4)		Intra-assay (n=4)		Inter-assay (n=4)	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
GUDCA	95.9	7.3	96.8	9.4	105.2	2.4	107.7	6.2
GCA	90.3	18.0	97.9	11.1	98.4	2.5	103.6	9.7
GCDCA	98.1	10.8	98.3	3.9	104.4	1.3	104.4	5.6
GDCA	98.1	10.9	101.9	7.7	106.4	1.0	103.8	6.6
UDCA	104.7	9.7	109.1	8.0	103.4	2.2	103.2	3.6
CA	99.1	6.1	108.5	5.6	106.4	1.4	101.1	3.9
GLCA	102.0	5.9	98.4	3.4	103.0	1.6	98.6	3.6
CDCA	91.7	5.1	105.4	5.1	99.2	1.1	97.9	1.7
DCA	102.9	2.9	109.4	5.5	100.6	1.4	98.6	2.8
LCA	96.0	5.8	104.0	10.0	88.0	1.4	94.7	9.8

*For abbreviations see legend to Fig. 5.

TABLE II

RECOVERIES OF TAURINE-CONJUGATED BILE ACIDS FROM HUMAN SERUM

Bile acids*	Intra-assay ($n=5$)		Inter-assay ($n=5$)	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
TUDCA	73.9	9.0	85.9	7.2
TCA	75.7	6.8	88.9	11.2
TCDCA	80.2	9.7	85.3	4.4
TDCA	78.7	11.5	82.5	4.3
TLCA	74.6	9.7	73.8	11.3

*For abbreviations see legend to Fig. 4.

The peak height ratios of the same amount of bile acids varied between different days and columns; therefore, proper calibration curves are required for each day and each column.

Application

In order to investigate the applicability of the present method the simultaneous determination of free and glycine-conjugated bile acids in serum and bile was carried out on eleven sera of patients and one bile sample from a normal subject. The results are given in Table III. The typical chromatograms of normal human serum and bile are shown in Fig. 6. The chromatograms of patients with liver cirrhosis and primary biliary cirrhosis are shown in Fig. 7. Appreciable differences were found among individual bile acids with each sample. Further studies on a large number of samples are necessary to elucidate

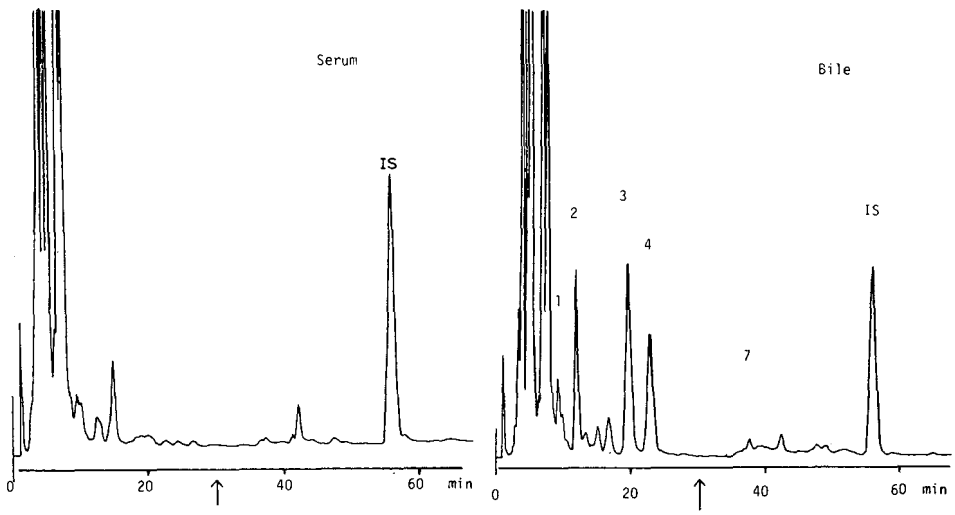


Fig. 6. Typical chromatograms of normal human serum and bile. Peaks: 1 = glycoursodeoxycholic acid, 2 = glycocholic acid, 3 = glycochenodeoxycholic acid, 4 = glycodeoxycholic acid, 7 = glycolithocholic acid, IS = internal standard.

TABLE III

INDIVIDUAL BILE ACIDS* IN SERUM AND BILE OF ONE HEALTHY SUBJECT AND PATIENTS BY FLUORESCENCE HPLC

Values are expressed as μmol/dl for serum and μmol/ml for bile.

Disease		Glycine-conjugated					Free				
		UDCA	CA	CDCA	DCA	LCA	UDCA	CA	CDCA	DCA	LCA
Liver cirrhosis		0.71	1.34	3.53	0.10	0.20	0.22	0.33	ND	0.25	0.54
Acute hepatitis	(1)	0.75	2.70	3.34	0	ND**	0	0	0	0.33	ND
	(2)	0.54	0.21	1.03	0	0.15	0	1.02	0.70	0.42	0.49
Primary biliary cirrhosis	(1)	0.33	7.10	5.51	0.14	0.25	ND	0.30	ND	13.32	3.98
	(2)	0.58	5.31	5.28	0.12	0.22	0.14	0.42	0.07	24.95	3.18
Fulminant		2.37	1.00	5.98	0.48	ND	1.25	ND	0	0	0.80
Pancreas cancer	(1)	1.17	0.33	0.21	0.09	0	ND	0.24	ND	0.23	0.39
	(2)	0.57	1.01	0.99	0.10	0.22	ND	ND	0	0.76	0.48
Breast cancer		0.61	0.13	0.41	0.18	0.32	0.19	0	0	0.70	7.36
Uterine cancer		0.93	0	0.19	0.12	0	0.23	0.64	ND	0.26	1.47
Hepatitis ?		0	0.81	1.73	0.24	0	0	0	0	0.39	0
Normal bile		2.33	8.35	10.00	7.68	0.88	ND	0	0	0	ND

*For abbreviations see legend to Fig. 5.
**ND = not detectable.

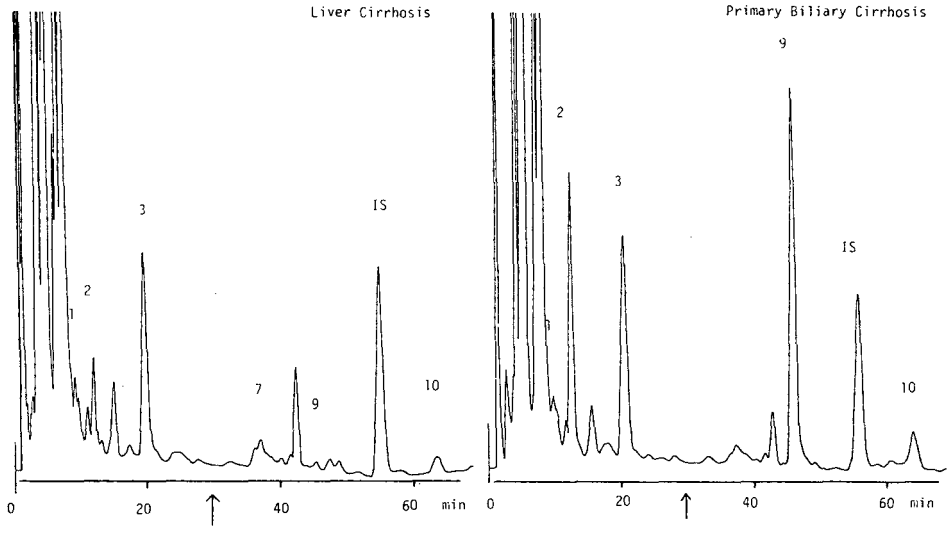


Fig. 7. Typical chromatograms of serum from patients. Peaks: 1 = glycoursodeoxycholic acid, 2 = glycocholic acid, 3 = glycochenodeoxycholic acid, 7 = glycolithocholic acid, 9 = deoxycholic acid, IS = internal standard, 10 = lithocholic acid.

this biological variability and to gain information in relation to physiological and/or pathological events. In this connection, the present method facilitates a much more accurate study of bile acids, allowing the investigation of a possible relationship between states of liver function and bile acid patterns.

ACKNOWLEDGEMENTS

This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. The authors are grateful to Dr. Y. Kawahara of Sankyo Co. Ltd. for his generous gift of 1-bromoacetylpyrene and also to Professor T. Nambara and Dr. J. Goto of Tohoku University for a gift of PHP-LH-20. The authors wish to thank Dr. E. Araki of the National Cancer Center for supplying serum samples of patients.

REFERENCES

1 M.A. Goldman, C.C. Schwartz, L. Swell and Z.R. Vlahcevic, in H. Popper and F. Schaffner (Editors), Progress in Liver Disease, Vol. 6, Grune and Stratton, New York, San Francisco, London, 1979, p. 225.
2 R.W. Shepherd, P.S. Bunting, M. Khan, J.G. Hill, S.J. Soldin and D.G. Gall, Clin. Biochem., 11 (1978) 106.
3 A.K. Batta, G. Salen and S. Shefer, J. Chromatogr., 168 (1979) 557.
4 G. Karlaganis and G. Paumgartner, Clin. Chim. Acta, 92 (1979) 19.
5 P.T. Clayton and D.P.R. Muller, Clin. Chim. Acta, 105 (1980) 401.
6 G. Karlaganis, R.P. Schwarzenbach and G. Paumgartner, J. Lipid Res., 21 (1980) 377.

7 J. Yanagisawa, M. Itoh, M. Ishibashi, H. Miyazaki and F. Nakayama, Anal. Biochem., 104 (1980) 75.
8 M. Tohma, Y. Nakata, Y. Yamada, T. Kurosawa, I. Makino and S. Nakagawa, Chem. Pharm. Bull., 29 (1981) 137.
9 K. Shimada, M. Hasegawa, J. Goto and T. Nambara, J. Chromatogr., 152 (1978) 431.
10 J. Goto, H. Kato, Y. Saruta and T. Nambara, J. Liquid Chromatogr., 3 (1980) 991.
11 G. Mingrone, A.V. Greco and S. Passi, J. Chromatogr., 183 (1980) 277.
12 S. Okuyama, D. Uemura and Y. Hirata, Chem. Lett., (1976) 679.
13 F. Stellaard, D.L. Hachey and P.D. Klein, Anal. Biochem., 87 (1978) 359.
14 G. Mingrone, A.V. Greco and S. Passi, J. Chromatogr., 183 (1980) 277.
15 B. Shaikh, N.J. Pontzer, J.E. Molina and M.I. Kelsey, Anal. Biochem., 85 (1978) 47.
16 S. Okuyama, D. Uemura and Y. Hirata, Bull. Chem. Soc. Jap., 52 (1979) 124.
17 D.P. Matter and W.C. Purdy, Anal. Chim. Acta, 109 (1979) 61.
18 S. Okuyama, D. Uemura and Y. Hirata, Chem. Lett., (1979) 461.
19 J.B.F. Lloyd, J. Chromatogr., 189 (1980) 359.
20 S. Baba, R. Uenoyama, K. Suminoe, F. Takeda, S. Hasegawa and Y. Kameno, Kobe J. Med. Sci., 26 (1980) 89.
21 S. Okuyama, N. Kokubun, S. Higashidate, D. Uemura and Y. Hirata, Chem. Lett., (1979) 1443.
22 K. Arisue, Z. Ogawa, K. Kohda, C. Hayashi and Y. Ishida, Jap. J. Clin. Chem., 9 (1980) 104.
23 S. Kamada, M. Maeda, A. Tsuji, Y. Umezawa and T. Kurahashi, J. Chromatogr., 239 (1982) 773.
24 Y. Kawahara, A. Inage, T. Morioka, Y. Shibano and N. Kasuga, The 101th Annual Meeting of Pharmaceutical Society of Japan, 1980, Abstract p. 568.
25 J. Goto, M. Hasegawa, H. Kato and T. Nambara, Clin. Chim. Acta, 87 (1978) 141.

Journal of Chromatography, 272 (1983) 43—50
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1491

DETERMINATION OF 6-N-TRIMETHYLLYSINE IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

CHARLES L. HOPPEL*, DOUGLAS E. WEIR, AUSTIN P. GIBBONS, STEPHEN T. INGALLS, ALICE T. BRITTAIN and FREDERIC M. BROWN

Medical Research Service, Veterans Administration Medical Center, Cleveland, OH 44106 (U.S.A.) and Departments of Pharmacology and Medicine, Case Western Reserve University School of Medicine, Cleveland, OH 44106 (U.S.A.)*

(First received June 14th, 1982; revised manuscript received August 30th, 1982)

SUMMARY

A method for determination of 6-N-trimethyllysine in urine is described. Trimethyllysine and the chemically analogous 6-N-triethyllysine internal standard were isolated from aqueous samples by microcolumn ion-exclusion chromatography. The specimens were derivatized by reaction with 1-fluoro-2,4-dinitrobenzene and reaction byproducts extracted by organic solvents. The trimethyllysine and internal standard derivatives were separated easily from other sample constituents by reversed-phase paired-ion high-performance liquid chromatography with spectrophotometric detection at 405 nm. Standard curves were linear over a sample concentration range of 10—150 nmol/ml; the detection limit corresponded with 0.1 nmol trimethyllysine injected into the chromatograph. The procedure was used for determination of trimethyllysine in human urine.

INTRODUCTION

The amino acid 6-N-trimethyllysine occurs as a minor constituent of proteins in a variety of organisms [1]. Trimethyllysine has been identified in cytochrome *c* of yeast [2] and wheat germ [3], myosin [4], histone [5], and is present in the plasma and urine of human subjects [6]. Trimethyllysine arises in mammals via post-translation protein-specific methyltransferase mediated reactions in which S-adenosylmethionine functions as the methyl group donor [7].

The metabolism of trimethyllysine following proteolysis has been the subject of recent study. The mitochondrial fatty acyltransferase system cofactor carnitine [3-hydroxy-4-(N,N,N-trimethylammonio)-butanoate] has been shown to be synthesized from lysine in the rat [8, 9], and specifically from 6-N-trimethyllysine in that animal [10]. Several other trimethyllysine metabolites

0378-4347/83/0000—0000/$03.00

have been identified; these include the immediate carnitine precursor 4-(N,N,N-trimethylammonio)-butanoate [10], 5-(N,N,N-trimethylammonio)-pentanoate [10], 3-hydroxy-6-N-trimethyllysine [11—13], 2-N-acetyl-trimethyllysine [12], and 2-oxo-6-(N,N,N-trimethylammonio)-hexanoate [14].

Chromatographic identification and determination of trimethyllysine has been accomplished by paper chromatography [6], paper electrophoresis [15], thin-layer chromatography [16], and column cation-exchange chromatography [5, 17]. A number of separations of the three possible 6-N-methylated lysines from basic amino acids have been achieved by cation-exchange chromatography in amino acid analysis systems [18—24]. Extremely long separation times are required, with trimethyllysine eluting after 9 h and more than 24 h required for complete chromatographic development in the system which best resolves the three 6-N-methylated lysines [23]. These methods provide poor sensitivity owing to the large peak elution volumes obtained after 6—9 h. A more rapid separation requiring only 90 min has been reported recently [25], but 6-N-trimethyllysine is not resolved completely from the other 6-N-methylated lysines by this system.

Our interest in the details of the metabolic disposition of trimethyllysine prompted us to develop a method for its automated determination in large numbers of urine samples. The procedure reported below consists of a rapid and specific isolation of trimethyllysine and an analytically appropriate internal standard from urine specimens by ion-exclusion chromatography, pre-column derivatization by reaction with 1-fluoro-2,4-dinitrobenzene, and chromatographic determination by reversed-phase paired-ion high-performance liquid chromatography (HPLC) with spectrophotometric detection.

EXPERIMENTAL

Equipment

The liquid chromatograph consisted of a Model 6000A solvent delivery system, Model U6K syringe loading injection valve, RCM-100 radial compression module, and Model 440 fixed-wavelength absorbance detector purchased from Waters Assoc. (Milford, MA, U.S.A.). A precolumn constructed of zero dead volume chromatographic fittings (Crawford Fitting Co., Solon, OH, U.S.A.) and packed with Co:Pell ODS reversed-phase pellicular chromatographic medium (Whatman, Clifton, NJ, U.S.A.) preceeded the column compression unit in the eluent stream. The chromatographic separation was accomplished on a 10 × 0.8 cm cartridge of 10 μm nominal particle diameter Radial-Pak C_{18} (Waters), and the detector output signal was recorded by a Linear Instruments (Irvine, CA, U.S.A.) chart recorder. A Waters Model 710A automatic sample injection unit and Hewlett-Packard (Avondale, PA, U.S.A.) Model 3354C chromatographic data system were used during routine determination of trimethyllysine in large numbers of samples. In early stages of the project a Waters μBondapak C_{18} reversed-phase column was employed for separations related to the development of the sample isolation procedure and optimization of the derivatization reaction.

Materials

Trimethyllysine and [Me-^{14}C]trimethyllysine were synthesized and purified as described [10]. Triethyllysine was prepared and purified for use as a chromatographic internal standard by a modification of the same procedure in which iodoethane replaced iodomethane as the alkylating agent. The methylated amino acids 1-methylhistidine, 3-methylhistidine, N^G-methylarginine, N^G,N^G-dimethylarginine, N^G,N'^G-dimethylarginine, N^6-methyllysine and N^6,N^6-dimethyllysine were purchased from Calbiochem-Behring (San Diego, CA, U.S.A.). Aqueous stock solutions of trimethyllysine and triethyllysine were prepared and standardized spectrophotometrically [26]. Aqueous trimethyllysine standard solutions were prepared by serial dilution of the stock solution. A working internal standard solution was prepared at 200 nmol/ml concentration. Dowex 1-X8 (200—400 mesh, Cl^- form) anion-exchange resin was purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.), converted to the OH^- form according to the vendor's instructions, and rinsed with water until the effluent pH was neutral. 1-Fluoro-2,4-dinitrobenzene was purchased from Koch-Light Labs. (distributed by Research Products International, Elk Grove Village, IL, U.S.A.); a 2% (w/v) ethanolic solution was prepared daily for use in the derivatization procedure. 1-Heptanesulfonic acid sodium salt was obtained from Eastman Organic Chemicals (Rochester, NY, U.S.A.). Acetonitrile (non-spectro grade) was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Distilled water was prepared for use as a chromatographic eluent constituent by passage through the mixed bed ion-exchange and activated carbon columns of a Milli-Q water purification unit (Millipore, Bedford, MA, U.S.A.).

Sample preparation

In a plastic microcentrifuge tube were combined 500 μl of aqueous standard solution or urine specimen and 200 μl of the triethyllysine internal standard solution. The tube was vortexed briefly, and 500 μl of the contents applied to a 7 × 0.5 cm water washed column of Dowex 1-X8 (200—400 mesh, OH^- form) anion-exchange resin contained by a pasteur pipette. Excluded species were eluted from the column by 2 ml of distilled water. The effluent was collected in 13 × 100 mm disposable glass test tubes which subsequently were transferred to a 50°C water bath and their contents brought to dryness by a gentle stream of oil-free compressed air. Samples so prepared may be stored in a refrigerator prior to derivatization and chromatography.

Derivatization

The dry residue from the isolation procedure was reconstituted in 150 μl of distilled water. To this were added in sequence 250 μl of 1 *M* sodium bicarbonate and 1 ml of the 1-fluoro-2,4-dinitrobenzene reagent solution. The tubes were vortexed, covered by a marble, and placed within a shaking water bath at 37°C for 1 h. At the conclusion of the procedure, the tubes were transferred to a 50°C water bath and their contents evaporated to dryness under a compressed air stream.

To the dried residue were added 4 ml of diethyl ether and the tubes vortexed for 10 sec. The ether was removed by vacuum aspiration, with care taken to

avoid removal of any insoluble material. This extraction step was repeated. The solid residues were then reconstituted in 300 μl 1.4 *M* hydrochloric acid by vortexing for 10 sec. Diethylether (4 ml) was added, the tubes vortexed, and the ether layer removed. Then 2 ml of *n*-butyl acetate were added, the tubes vortexed, and phase separation facilitated by centrifugation in a table top unit for 5 min at 1250 *g*. After aspiration of the organic layer, the tubes were covered and wrapped with aluminum foil for protection against light. Samples were stored in a freezer at −20°C prior to chromatography.

Chromatographic conditions

The chromatographic mobile phase was 0.01 *M* sodium heptanesulfonate in acetonitrile—water (50:50). In 500 ml of purified water were dissolved 2.20 g (0.01 mol) 1-heptanesulfonic acid sodium salt and the solution filtered through a 0.2-μm pore diameter cellulose nitrate membrane. To this filtered solution were added 500 ml filtered acetonitrile with thorough magnetic stirring. The eluent was pumped at 2 ml/min; absorbance was monitored at 405 nm. The sample injection volume was 50 μl in all cases.

Quantitation

Standard curves of trimethyllysine:triethyllysine peak height or area ratios vs. concentration were established daily over a trimethyllysine standard solution concentration range of 10—150 nmol/ml. Trimethyllysine concentrations in experimental samples were interpolated from the least squares regression line through the standard data points. All standard and experimental samples were analyzed in duplicate.

RESULTS AND DISCUSSION

It was our purpose in this work to develop a simple and specific method for determination of urinary 6-N-trimethyllysine. We discovered immediately that accurate measurement of the small urinary quantities of trimethyllysine was complicated by comparatively enormous accompanying quantities of amino acids of all acidity categories. The necessary degree of sample simplification was achieved by ion-exclusion chromatography on small columns of the strong anion-exchange resin Dowex 1-X8 (OH^- form), by which anions are quantitatively adsorbed. Cations and polar species without net charge at the high effective pH of the resin surface are unretained and eluted by distilled water. Recovery of trimethyllysine was investigated by application of [Me-^{14}C] trimethyllysine to the sample preparation columns; 96% of the applied radioactivity was recovered in the 2 ml of distilled water used for elution of excluded species.

A series of experiments intended to verify the removal of nonquaternary N-methylated amino acids by the Dowex-1 column were performed. Aqueous solutions of 1-methylhistidine, 3-methylhistidine, N^G-methylarginine, N^G,N^G-dimethylarginine, N^G,N'^G-dimethylarginine, N^6-methyllysine and N^6,N^6-dimethyllysine were prepared. Each was subjected to the derivatization procedure and the k' value for each derivative determined on the μBondapak C_{18} column used in early stages of the project. Only the N^G-methylarginine 2,4-

dinitrophenylamino derivative was found to be retained similarly to the trimethyllysine derivative by this column; the two peaks eventually were resolved with considerable experimental difficulty. One micromole of each amino acid was then applied to the Dowex-1 (OH^- form) resin sample preparation columns, the columns eluted with distilled water, and the collected effluent derivatized and chromatographed. The chromatograms obtained in experiments with all of the tested compounds except N^G-methylarginine were indistinguishable from those obtained upon derivatization of an identically treated sample of water. Some N^G-methylarginine was found to be eluted from the Dowex-1 column by distilled water, but none of this compound was detected in the effluent of sample preparation columns eluted with dilute ammonium hydroxide at pH 12.5.

[Me-^{14}C]Trimethyllysine was subjected to a series of derivatization reaction optimization experiments in which the labelled precursor and its 2,4-dinitrophenylamino derivative were separated by HPLC. The column effluent was collected in 0.5-ml fractions and the radioactivity determined in each fraction by liquid scintillation counting. Two radioactive peaks were detected. The first was barely retained by the reversed-phase column and co-chromatographed with injected [Me-^{14}C]trimethyllysine. The second peak of radioactivity appeared coincidentally with a single spectrophotometrically detectable peak, which in turn was shown to vary in size directly with sample trimethyllysine concentration. The reaction was found to proceed to 85% (± 3%, n=5) completion with manageable chromophoric byproduct formation under the reaction conditions described.

A series of extraction steps were developed for removal of residual reagent and chromophoric side reaction products. Recovery of [Me-^{14}C]trimethyllysine was verified: 3% of the radioactivity present in samples after the ion-exclusion sample preparation step was found in the organic solvents employed for the extractions, while the remaining 97% was found in the final aqueous acid specimen ready for chromatography. Since 96% of applied [Me-^{14}C]trimethyllysine was recovered from the sample preparation columns, the recovery of trimethyllysine throughout the preparation sequence prior to chromatography was 93%. The photosensitivity of amino acid 2,4-dinitrophenylamino derivatives has been described [27]. It was found that [Me-^{14}C]trimethyllysine 2,4-dinitrophenylamino derivatives did undergo slow decomposition to another chromatographically distinguishable labelled species upon either exposure to light or storage at room temperature. Specimens prepared for chromatography therefore were protected against exposure to light and stored overnight at low temperature prior to analysis.

Application of the developed sample isolation and derivatization procedure to urine specimens revealed the presence of an interfering endogenous sample constituent which was both unretained by the ion-exclusion columns used for sample preparation and reactive toward 1-fluoro-2,4-dinitrobenzene. Systematic manipulation of the eluent counter-ion concentration, pH, and organic modifier concentration and identity did not adequately resolve the trimethyllysine derivative and the sample contaminant on the μBondapak C_{18} column initially used in this work. Further experimentation with higher and lower chain length alkanesulfonate counter-ions also did not provide the required

selectivity. The necessary separation was achieved on the highly retentive reversed-phase medium Radial-Pak C_{18}, upon which all urine specimens were then chromatographed during the course of this study.

Fig. 1 is a chromatogram of an aqueous blank carried through the analytical scheme. A chromatogram of a processed aqueous trimethyllysine standard solution at 50 nmol/ml which included triethyllysine internal standard is shown in Fig. 2. Fig. 3 is a chromatogram of a derivatized urine specimen containing 21 nmol/ml trimethyllysine. Although the methylhistidines, methyllysines, and methylarginines (other than N^G-methylarginine) tested were retained during the ion-exclusion chromatographic sample preparation step, their 2,4-dinitrophenylamino derivatives were shown to be separated from those of trimethyllysine and the internal standard under these chromatographic conditions. The trimethyllysine metabolite 3-hydroxy-6-N-trimethyllysine was not retained by the anion-exchange resin and therefore in principle could be determined if present in samples at sufficient concentration. The time required for the analysis may be reduced by operating the instrument at solvent flow-rates as high as 4 ml/min without unacceptable loss of resolution of the trimethyllysine derivative and the preceeding more polar endogenous sample constituent.

Standard curves of trimethyllysine:triethyllysine peak height ratios vs. sample trimethyllysine concentration were found to be linear over a sample concentration range of 10—150 nmol/ml trimethyllysine (r^2=0.99) and to pass through the graphic origin. The slopes of 50 standard curves varied with a relative standard deviation of 6% during the one-year course of the study. The detection limit at a signal-to-noise ratio of 5:1 was found to correspond

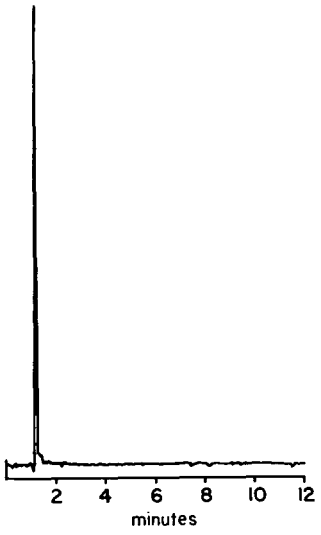


Fig. 1. Chromatogram obtained after complete preparation and derivatization of an aliquot of distilled water according to the described procedure. The column was a 10 × 0.8 cm cartridge of 10-μm nominal particle diameter Radial-Pak C_{18} (Waters Assoc.). The chromatographic eluent was $1 \cdot 10^{-2}$ *M* 1-heptanesulfonic acid sodium salt in acetonitrile—water (50:50) and was pumped at 2.0 ml/min. The spectrophotometric detector was operated at 405 nm. The full scale of the ordinate is 0.02 absorbance units.

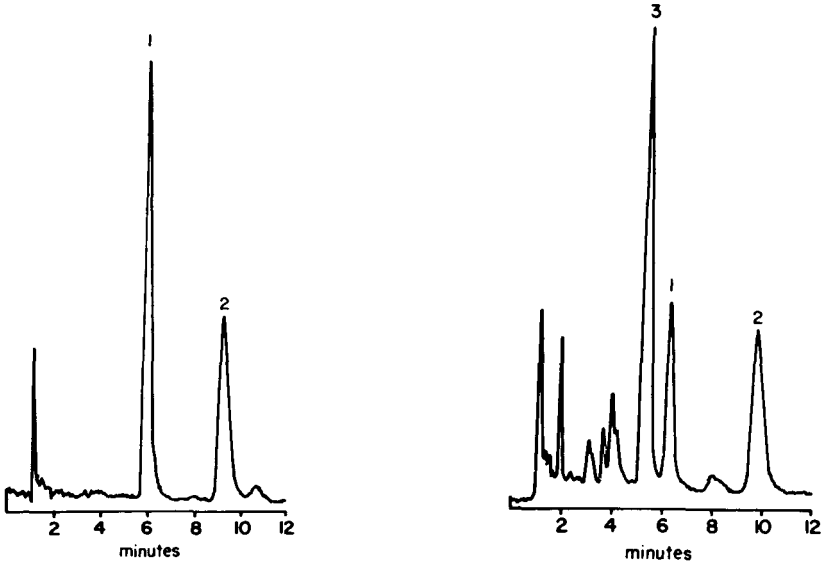


Fig. 2. Chromatogram of a derivatized aqueous standard solution of trimethyllysine at 50 nmol/ml which included triethyllysine the internal standard. The chromatographic conditions were as described in Fig. 1. Peaks: 1 = trimethyllysine 2,4-dinitrophenylamino derivative; 2 = triethyllysine 2,4-dinitrophenylamino derivative.

Fig. 3. Chromatogram of a prepared and derivatized urine specimen containing 21 nmol/ml trimethyllysine. The chromatographic conditions were as described in Fig. 1. Peaks: 1 = trimethyllysine 2,4-dinitrophenylamino derivative; 2 = triethyllysine 2,4-dinitrophenylamino derivative, internal standard; 3 = urine sample constituent unretained by ion-exclusion sample preparation columns.

with 0.1 nmol of trimethyllysine injected into the chromatograph. Mean deviations for duplicate sample determinations were typically less than 5%. A dependence of the absolute retention of trimethyllysine and the internal standard upon injection volume was noted. When the injection volume was fixed at 50 μl, absolute retention of the derivative peaks varied by less than 1% from injection to injection. More than 2500 trimethyllysine determinations were performed in the course of this investigation; throughout the project, the chromatographic performance of the Radial-Pak C_{18} cartridge was satisfactory.

Urinary excretion of trimethyllysine by human subjects

A healthy female volunteer of normal body weight ingested Ensure brand isocaloric liquid diet for two days and then fasted for three days. Urinary trimethyllysine excretion was found to be 36.4 μmol per 24 h on the day prior to fasting. The values obtained during the fasting period were 38.4, 39.2, and 37.5 μmol per 24 h on the first, second, and third days of fasting, respectively. Although these values fall below the ranges reported by Kakimoto and Akazawa [6], Löwer et al. [28] and Lou and Siena [29], our test subject consumed a diet (Ensure) which has a very low content of trimethyllysine. Löwer et al. [28] reported a mean daily urinary excretion of 64 μmol tri-

methyllysine with wide variation among individuals in both total excretion of methylated lysines and in trimethyllysine as a fraction of total excreted methylated lysines. Kakimoto and Akazawa [6] reported no change in urinary trimethyllysine excretion by human subjects ingesting a protein-free diet for 30 h. Our own comparison of trimethyllysine excretion by normal-weight and obese subjects will be reported elsewhere [30].

ACKNOWLEDGEMENTS

We wish to thank Ms. Lauri L. Albers for her excellent and precise technical assistance and Ms. Sandra L. Evans for preparation of this manuscript. The human urine specimens were provided by Dr. Saul M. Genuth, Director of the Saltzman Institute for Clinical Investigation at Mount Sinai Hospital in Cleveland. This work was supported by funds provided by the United States National Institutes of Health (AM 15804 and AM 21009) and the Veterans Administration Medical Research Service.

REFERENCES

1 W.K. Paik and S. Kim, Protein Methylation, Wiley, New York, 1980, pp. 13—14.
2 A.-M. Becam and F. Lederer, Eur. J. Biochem., 118 (1981) 295.
3 R.J. Delange, A.N. Glazer and E.L. Smith, J. Biol. Chem., 244 (1969) 1385.
4 V.K. Hempel, H.W. Lange and L. Birkofer, Hoppe-Seyler's Z. Physiol. Chem., 349 (1968) 603.
5 V.K. Hempel, H.W. Lange and L. Birkofer, Z. Naturforsch., 1 (1968) 37.
6 Y. Kakimoto and S. Akazawa, J. Biol. Chem., 245 (1970) 5751.
7 W.K. Paik and S. Kim, J. Biol. Chem., 245 (1970) 6010.
8 D.W. Horne, V. Tanphaichitr and H.P. Broquist, J. Biol. Chem., 246 (1971) 4373.
9 R.A. Cox and C.L. Hoppel, Biochem. J., 136 (1973) 1075.
10 R.A. Cox and C.L. Hoppel, Biochem. J., 136 (1973) 1083.
11 C.L. Hoppel, R. Novak and R.A. Cox, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 35 (1976) 1478 (abstract 623).
12 C.L. Hoppel, R.A. Cox and R. Novak, Biochem. J., 188 (1980) 509.
13 R. Novak, T.J. Swift and C.L. Hoppel, Biochem. J., 188 (1980) 521.
14 V.R. Villanueva and E. Lederer, FEBS Lett., 52 (1975) 308.
15 M. Reporter and J.L. Corbin, Biochem. Biophys. Res. Commun., 43 (1971) 644.
16 C.J. Rebouche and H.P. Broquist, J. Bacteriol., 126 (1976) 1207.
17 R.T. Markiw, Biochem. Med., 13 (1975) 23.
18 C.F. Crampton, W.H. Stein and S. Moore, J. Biol. Chem., 225 (1957) 363.
19 R.P. Ambler and M.W. Rees, Nature (London), 184 (1959) 56.
20 S. Kim and W.K. Paik, J. Biol. Chem., 240 (1965) 4629.
21 W.K. Paik and S. Kim, Biochem. Biophys. Res. Commun., 27 (1967) 479.
22 G.E. Deibler and R.E. Martenson, J. Biol. Chem., 248 (1973) 2387.
23 C.G. Zarkadas, Can. J. Biochem., 53 (1975) 96.
24 C.G. Zarkadas, Can. J. Biochem., 56 (1978) 952.
25 M.J. Dognin and B. Wittman-Liebold, Hoppe-Seyler's Z. Physiol. Chem., 361 (1980) 1697.
26 S.J. Moore and W.H. Stein, J. Biol. Chem., 211 (1954) 907.
27 T. Okuyama and K. Satake, J. Biochem., 47 (1960) 454.
28 R. Löwer, H.W. Lange and V.K. Hempel, Clin. Chim. Acta, 58 (1975) 155.
29 M.E. Lou and M. Siena, Biochem. Med., 25 (1981) 309.
30 C.L. Hoppel and S.M. Genuth, in preparation.

Journal of Chromatography, 272 (1983) 51—65
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1482

INVESTIGATION OF THE HETEROGENEITY OF HEMOGLOBIN BY CATION-EXCHANGE CHROMATOGRAPHY ON BIO-REX 70

MASSIMO CASTAGNOLA*

Istituto di Chimica e C.N.R. Centro di Studio per la Chimica dei Recettori e delle Molecole Biologicamente Attive, Università Cattolica del Sacro Cuore, Via Pineta Sacchetti 644, 00168 Rome (Italy)

PAOLO CARADONNA

Istituto di Patologia Medica, Facoltà di Medicina e Chirurgia, Università Cattolica del Sacro Cuore, Via Pineta Sacchetti 644, 00168 Rome (Italy)

and

MARIA LETIZIA SALVI and DIANA ROSSETTI

Istituto di Chimica e C.N.R. Centro di Studio per la Chimica dei Recettori e delle Molecole Biologicamente Attive, Università Cattolica del Sacro Cuore, Via Pineta Sacchetti 644, 00168 Rome (Italy)

(First received March 4th, 1981; revised manuscript received July 29th, 1982)

SUMMARY

The use of Bio-Rex 70 cation-exchange resin for chromatography of normal and diabetic hemoglobin provides a reproducible pattern of the "fast components". Particular attention to the choice of sample preparation, pH of elution, and the increase of ionic strength by sodium chloride linear gradients results in the separation of Hb-A_{1b} into two components and in the isolation of a new component eluting between Hb-A_{1c} and Hb-A_0. Experiments with [^{3}H]glucose and the colorimetric test (thiobarbituric acid) normally used to determine the extent of non-enzymatic glycosylation, as well as an increase of this component in diabetic samples compared with normoglycemic ones and a significant linear correlation with Hb-A_{1c}, indicate that this component should be a part of the hemoglobins glucosylated on the ϵ-NH_2 group of the lysines of both chains and/or the hemoglobin glucosylated on the α-NH_2 of the valine of the α-chain. We propose to call this component Hb-A_{1x}, pending confirmation of its identity.

Normally Hb-A_{1x} accounts for about 3% of Hb-A, but up to 5—7% of glucosylated hemoglobins should be confined to the early part of Hb-A_0. In diabetics, the percentage of Hb-A_{1x} rises to 4—5% and that of the other glucosylated hemoglobins increases to 12—16%.

0378-4347/83/0000—0000/$03.00

INTRODUCTION

It has already been shown that cation-exchange chromatography on Bio-Rex 70 (or Amberlite IRC 50) of normal hemoglobin allows the separation of a number of "minor fast components", namely Hb-A_{1a-e}, with respect to the main Hb-A_0, and to a slower component designated Hb-A_2 [1]. Accurate determination of the identity of these minor fast components presents serious difficulties; at the moment not all have been structurally defined [2].

Briefly, Hb-A_{1a}, originally supposed to be a unique component, now appears to result from two components named [3] Hb-A_{1a1} and Hb-A_{1a2}. It is supposed that they derive from a slow, non-enzymatic, post-transcriptional hemoglobin modification due to the action, respectively, of fructose 1,6-diphosphate and glucose 6-phosphate on the α-NH_2 group of the terminal valines of both β-chains of hemoglobin [3]. Their identity has not been completely determined due to experimental disagreement [4]. The identity of Hb-A_{1b} is still unknown, even though its characteristics have been partially elucidated [5].

Hb-A_{1c} is the most characterized component and also the most abundant one; it is the result of the attachment by glucose on hemoglobin, described above for Hb-A_{1a1} and Hb-A_{1a2}. The aldimine adduct formed subsequently undergoes an Amadori rearrangement to form a more stable ketoamine linkage [6].

Determination of the percentage of Hb-A_{1c} in a hemoglobin sample is important in some pathological alterations of the glucosidic metabolic pathways. In red cells of patients with overt diabetes mellitus there is a two- to threefold increase in the percentage of Hb-A_{1c} (4—6% to 10—15% of total hemoglobin) [2]. It has therefore been proposed that the percentage of Hb-A_{1c} could be used in assessing the degree of diabetes, by providing an integrated measurement of blood glucose according to the red cell life span.

The Hb-A_{1c} component is not separable from Hb-F by cation-exchange chromatography [1]; hemoglobin samples with a high value of Hb-F give a falsely elevated percentage of Hb-A_{1c}. Hb-A_{1d} is probably an artifact which forms in vitro when one sulphydryl group of each of the two β-chains of Hb-A has reacted with oxidized gluthathione, and Hb-A_{1e} seems to be an artifact formed as a result of the incubation of hemoglobin at high temperatures [1].

This is further complicated by the observation [7] that the glucosylation products on the ϵ-NH_2 of the lysines of both chains and on the α-NH_2 of the valine of the α-chains, are not separable by this chromatographic technique, but remain with the early part of the Hb-A_0 peak. For this reason we have tried to perform accurate chromatographic separation to achieve good resolution of the early part of the Hb-A_0 peak. We used the preparative method of McDonald et al. [3] for analytical purposes. In fact, the exact determination of the complete glycosylation pattern of hemoglobin, including the components glucosylated on the ϵ-NH_2 of the lysines and on the α-NH_2 of the valine of the α-chain, should provide a more accurate integrated measurement of blood glucose.

MATERIALS AND METHODS

Reagents

All reagents used were of analytical grade; the common reagents were Merck products, (Merck, Darmstadt, G.F.R.). Thiobarbituric acid was from Merck and Ega Chemie (Steinheim/Albuch, G.F.R.). Bio-Rex 70 (200—400 mesh); Na^+ (carboxylic resin, pK_a = 6.1, copolymeric methacrylic acid—divinylbenzene matrix) was from Bio-Rad Labs. (Richmond, CA, U.S.A.). [^{3}H]Glucose was from The Radiochemical Centre (Amersham, Great Britain). YM 10 membranes were from Amicon, (Lexington, MA, U.S.A.). Instagel was from Packard, (Downers Grove, IL, U.S.A.).

Preparation of hemoglobin samples

Venous blood was collected in EDTA tubes from diabetic and normoglycemic volunteers and processed within 1 h of collection. All samples were tested for the presence of Hb-F by the alkali denaturation method [8]. Samples with a percentage of Hb-F greater than 1.0% were not submitted to chromatography. The red cells were washed four times with saline and lysed with 3 volumes of distilled water to 1 volume of packed erythrocytes and then stored at 4°C for 30 min. During mild stirring at 4°C, sodium chloride was added to each sample to a final concentration of about 0.4 mol/l. The solution was gently stirred for 15 min at 4°C, then centrifuged for 30 min at 4°C, 15,000 *g*, to discard the large pellet. After exhaustive dialysis against distilled water (four changes of 200 volumes), which eliminates the glucose reversibly linked to hemoglobin (aldimine adduct), the supernatant was submitted to a second centrifugation at 60,000 *g*, 60 min, 4°C. The sample was stored in lots of 3.0 ml, frozen at —20°C and chromatographed within two months.

Some hemoglobin samples, before Bio-Rex 70 chromatography, were stripped of 2,3-diphosphoglycerate by the dialysis method reported by Jelkmann and Bauer [9].

Chromatographic procedure

Eighty grams of Bio-Rex 70 were suspended in distilled water; after vigorous stirring and sedimentation, the water was aspirated off and the resin poured into a column of large diameter and equilibrated with a 0.05 mol/l potassium phosphate buffer at a pH ranging from 6.45 to 6.65 (determined at 20°C with a precision of ± 0.01 pH units). One lot of 3.0 ml of hemolysate (about 8 μmol/l in heme, equivalent to 150 mg of hemoglobin), dialysed at the chosen pH, in oxy form or with prior conversion to the carbon monoxide form, was loaded onto a 50 cm × 2 cm^2 column. The flow-rate was 40 ml/h and the fraction volume was 3.5 ml. The whole chromatographic procedure was performed at 4°C. After the exit of the non-hemoglobin protein and of Hb-A_{1a1} and Hb-A_{1a2} components (approximately 50 fractions), linear gradients of sodium chloride (0--0.10 mol/l, 0.10—0.20 mol/l, and 0.20—0.30 mol/l of sodium chloride in equilibrating buffer; 120 ml of each gradient) were applied variously as a function of pH, to elute the other components (Fig. 1). The elution pattern was determined by monitoring each fraction at 415 nm, and a few fractions, as control, at 280 nm; when the absorbance (*A*) was too high

for direct determination, the exact value was determined by appropriate dilution. The percentage of each component was computed as described by Schroeder and Huisman [1]. The ϵ_{max} values are those obtained from Antonini and Brunori [10]. Gradient linearity was controlled by checking the refractive index of the fractions eluted.

It should be noted that the sodium chloride-buffered solutions used for the gradients showed a pH slowly decreasing in function with concentration; this is probably due to the sensitivity of the electrode (Beckman 39501) to Na^+; correction of the pH of the sodium chloride buffers to the exact pH value of the starting buffer results in a poor separation pattern. We then left them uncorrected and, sometimes, we used the pH of the fractions as a further control of the linearity of the gradients.

Incubation of Hb-A_0 with labelled glucose

Samples of HbCO were chromatographed as described above. The central part of the Hb-A_0 peak was collected, concentrated by ultrafiltration on YM 10 membranes up to a concentration of about 10 mg/ml; 3.0 ml of this concentrated pool, after dialysis against a sodium phosphate buffer, 0.02 mol/l, pH 7.4, 0.15 mol/l in sodium chloride, were incubated with an appropriate quantity of labelled glucose and with unlabelled glucose to reach a total concentration of 25 mmol/l. The incubation was performed in an atmosphere of carbon monoxide.

At the chosen time (normally five days, as in the case reported under Results), a 1.0-ml volume of the solution was collected and filtered through a column of Sephadex G-50 fine (Pharmacia, Uppsala, Sweden), (25 cm × 2 cm^2), equilibrated with the buffer (pH 6.55) used for the Bio-Rex 70 column, both to eliminate the free glucose from the incubation solution and to bring the sample to the exact pH required for chromatography. The labelled hemoglobin was then added to 1.0 ml of the starting whole HbCO sample and chromatographed on Bio-Rex 70 at pH 6.55 (± 0.01) as described under Chromatographic procedure.

After the spectrophotometric readings, 10 ml of Instagel were added to each fraction and the radioactivity was monitored by a Packard scintillation counter (Model 460 C D).

Colorimetric (thiobarbituric acid) test

Colorimetric analysis of the components was carried out using the thiobarbituric acid test of Fluckiger and Winterhalter [11], slightly modified as follows. The chosen hemoglobin pool was obtained by ultrafiltration on YM 10 membranes. The small quantity of the HbA_{1a-b} components never allowed their determination by this method.

To 1.0 ml of the pooled components, 1.0 ml of 1.0 mol/l oxalic acid was added; after incubation at 100° C (4.5 h), 1.0 ml of 40% (w/w) trichloroacetic acid was added to each sample and standard at 0° C. The solution was centrifuged (3000 *g*) and to 1.5 ml of the supernatant 0.5 ml of 0.05 mol/l thiobarbituric acid was added; after incubation at 40°C for 30 min and for 10 min at room temperature, the absorbance of each sample and standard at 445 nm was determined against blanks of distilled water.

RESULTS

Fig. 1 shows elution patterns for three chromatograms of the same diabetic HbCO sample at different pH values: 6.60, 6.55, 6.50 (± 0.01). By lowering the pH, the interactions between the cation-exchange resin and the hemoglobin components obviously increase. To obtain a reasonable elution time it was necessary to increase the ionic strength with the use of sodium chloride to allow good elution of each component.

The effect of the pH is different on each component; Fig. 2 illustrates the changes in $V_{e(max)}$ as a function of pH. The best resolution was at pH 6.55; therefore all analytical chromatograms were made at pH 6.55 ± 0.01. The percentages of each component at different pH values are reported in Table I; only small variations are present, probably due only to overlapping of some components.

Considering each component singly, the following can be deduced:

(1) The first chromatographic peak can be attributed to non-heme proteins or hemoglobin degradation products, as pointed out also by McDonald et al. [3], for a A_{415}/A_{280} ratio very different from that of HbCO (not reported).

(2) The Hb-A_{1a1} and Hb-A_{1a2} components, with a clear profile at pH 6.60, at lower pH have a broad elution, thus, because of their low percentage, it is difficult to recognize them precisely; nevertheless, a lower pH allows a better separation when bulky preparative samples are used.

(3) The Hb-A_{1b} component, under all the chromatographic conditions, is

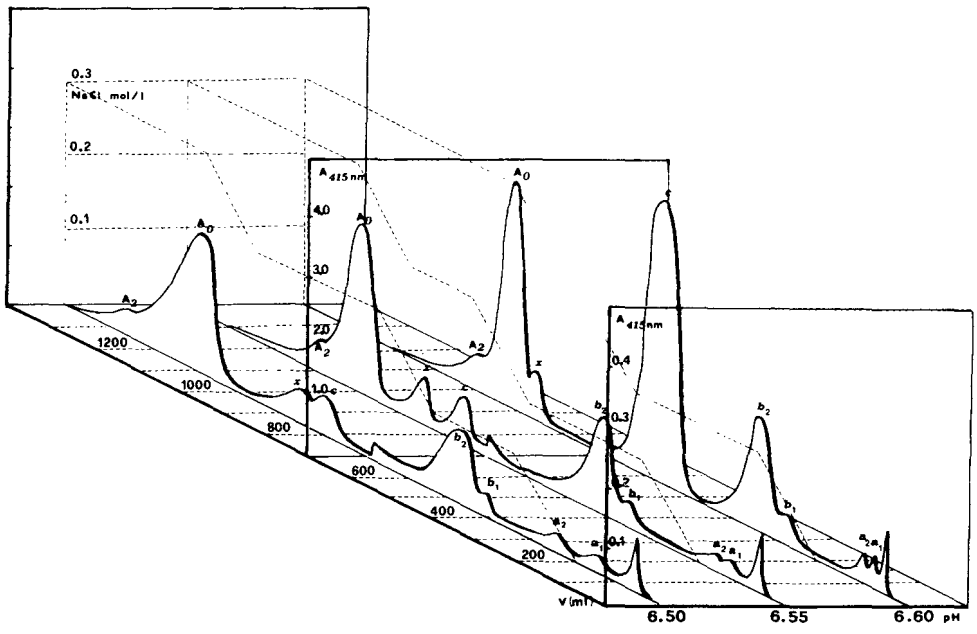


Fig. 1. Elution patterns of the same diabetic HbCO sample at different pH values. At 700 ml there is a change of absorbance scale (10 ×) to include the Hb-A_0 component. Thus, the Hb-A_{1c} component which elutes at pH 6.60 with a volume of about 500 ml, at lower pH increases its elution volume with a resulting tenfold reduction in peak area.

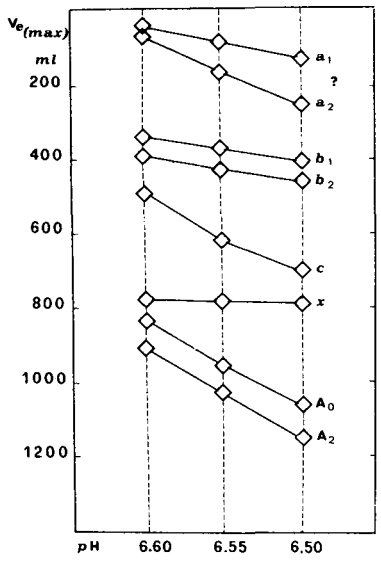


Fig. 2. Elution volume corresponding to the maximum absorbance value ($V_{e(max)}$) of each chromatographic peak as a function of pH. For Hb-A_{1a_1} and Hb-A_{1a_2} the question mark indicates difficulties in determining their exact position.

TABLE I

PERCENTAGE OF EACH COMPONENT OBTAINABLE FROM THE DIFFERENT CHROMATOGRAPHIC RUNS OF FIG. 1

More accurate values than those reported are not significant.

Component	pH 6.60	pH 6.55	pH 6.50
a_1	0.1	0.1	0.1
a_2	0.1	0.1	0.1
b_1	0.2	0.2	0.2
b_2	0.9	0.8	0.8
c	5.4	5.6	5.4
x	6.3	5.5	6.1
A_0+A_2	87.0	87.7	87.3

split into two peaks, a smaller peak always preceding a larger one. These two components are named Hb-A_{1b1} and Hb-A_{1b2}. The chromatographic resolution is poor and we did not succeed in improving it.

(4) The Hb-A_{1c} component is influenced to the greatest extent by change in pH (Fig. 2). It is well separated at pH 6.60 and 6.55, but at pH 6.50 it partially overlaps the Hb-A_{1x} component. At pH 6.60 the elution of this component is favoured by a concentration of 0.1 mol/l sodium chloride. Thus, for the elution of the last components it is advisable to apply a linear gradient increasing directly from 0.1 mol/l to 0.3 mol/l. When the pH is lowered because of the higher interaction of the Hb-A_{1c} component with Bio-Rex 70, it is convenient to apply an intermediate plateau of 0.2 mol/l sodium chloride.

(5) Between Hb-A_0 and Hb-A_{1c} there is a new component which at pH 6.60

is eluted just before the Hb-A_0 peak and at a lower pH practically does not change its $V_{e(max)}$. Therefore, pH 6.55 is the best resolutive condition for this component, because at pH 6.50 the greater retardation of Hb-A_{1c} causes overlapping of these two components. This is why it is difficult to calculate the percentage of this new component at extreme pH values (see Table I).

We propose to name this new component Hb-A_{1x}, until its identity is clearly determined.

(6) Finally, at the end of the Hb-A_0 peak eluted by 0.3 mol/l sodium chloride, the Hb-A_2 component is present, which is so overlapped with Hb-A_0 that it is difficult to separate [1].

As regards sample preparation and chromatographic conditions the following can be observed:

(1) Different sample preparations can cause very slight differences in the percentage of the components but do not change their position ($V_{e(max)}$). After comparing different sample preparations we chose the conditions described under Materials and methods, with a salting-out step after hemolysis of the erythrocytes. This step restricts the possibility of contamination by artifacts.

(2) The use of HbCO or HbO_2 does not cause noticeable variations in the percentages and in the $V_{e(max)}$ of the components, but only a slight enlargement of the peak base of the fast components and a greater one of the Hb-A_0 in HbO_2 samples. This fact, together with the well-known instability of HbO_2, advises the use of HbCO.

(3) The removal of 2,3-diphosphoglycerate from hemoglobin by dialysis [9] does not cause any changes in the chromatographic patterns, especially with regard to the major fast components. There is only a small variation in the initial part of the chromatogram with a decrease in the percentage of the non-heme peak. Although it does mean that the sample is contaminated, it is not worthwhile including this long step for a percentage determination. On the other hand, if study of Hb-A_{1a1} and Hb-A_{1a2} is the object of the experiment, it is advisable to remove the 2,3-diphosphoglycerate.

Fig. 3 shows the elution pattern of Hb-A_0 incubated with labelled glucose and chromatographed together with the starting unlabelled normal HbCO sample. The incubation generates various peaks; the first elutes at the position attributable to non-heme proteins or hemoglobin degradation products. After this, two peaks, approximately at the position of Hb-A_{1a1} and Hb-A_{1a2}, are present. A well-defined peak is present just before Hb-A_{1c}, and in the position of the Hb-A_{1x} component a broad labelled peak is evident; this peak also overlaps with the early part of Hb-A_0, where, in the leading fractions, there is a net increase of radioactivity.

The experiment with labelled glucose offers only a qualitative picture of glucosylation in vitro and the results obtained must be discussed with due consideration to the radioactive impurities probably present in the labelled glucose, as pointed out by Trueb et al. [12].

In Fig. 3 are also indicated the fractions that are usually pooled prior to concentration on Amicon YM 10 membranes for the thiobarbituric acid test. The pools are collected in accordance with the labelling experiments. The Hb-A_0 component is divided into three pools: two small ones in the leading

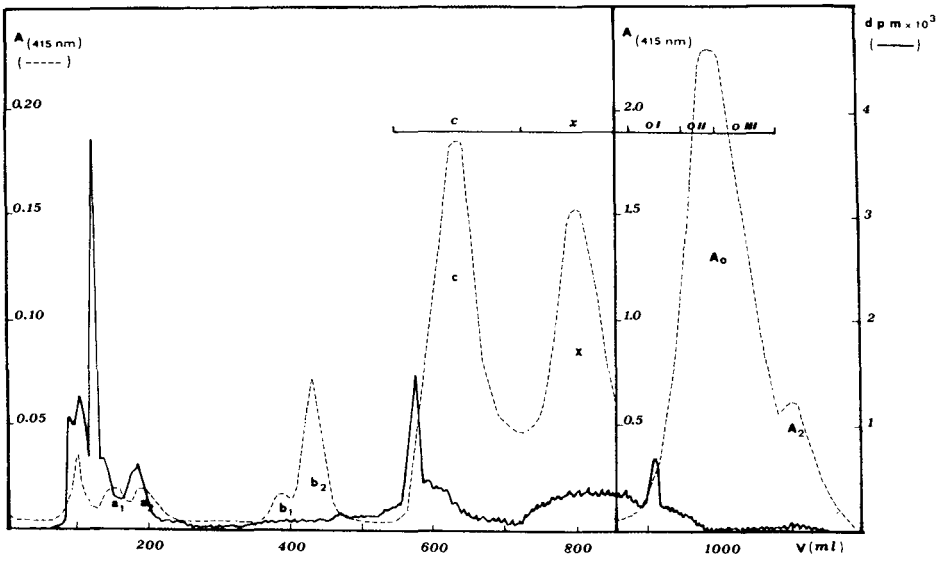


Fig. 3. Elution pattern of a normal HbCO sample at pH 6.55 ± 0.01; the dashed line (— — —) indicates absorbance (415 nm) and the solid line (——) indicates dpm obtained by incubation experiments with [^{3}H]glucose. On the top right-hand side of the figure are also indicated the pools collected for the thiobarbituric acid test.

edge of the peak and a large one at the end. Hb-A_2 is not considered in the pool collection.

The pools were named c, x, 0I, 0II, 0III and the results obtained with the thiobarbituric acid test are reported in Table II. The 0III pool shows a low positivity and other experiments (not reported) suggest that it may derive from some heme contaminants. In fact, the labelling experiments show that no glucose is linked in this part of the Hb-A_0; thus the A_{445} reading obtained from this component must be subtracted from the value of the other pools, not as an absolute value but as A_{445}/mol hemoglobin, assuming that the contaminant release is proportional to the total hemoglobin content.

Calculation of the other pools is made in two different ways. In the first method it is assumed that the conversion to 5-hydroxymethylfurfural of the Glu-Hb adduct (Amadori rearrangement) is equivalent to that of fructose. In effect, using fructose and 5-hydroxymethylfurfural standards, it can be observed that, under the conditions of the test, the fructose conversion is about 30%. Thus the results, expressed as mol fructose equivalent per mol Hb (tetramer), are reported for the pools in the first column of Table II. These results are a little higher than expected, as can be seen from the c pool value (2.3 mol fructose per mol Hb). Therefore, there is no reason to expect a conversion of the Glu-Hb adduct (ketoaminic form) with the same yield of fructose.

In effect, assuming that, in the second type of calculation, there is a conversion of about 36% of the ketoaminic adduct in 5-hydroxymethylfurfural, the values reported in the second column are obtained, expressed as mol Glu → Amadori per mol Hb (tetramer). Clearly, Hb-A_{1c} can be supposed to be a diglucosylated form of hemoglobin, while Hb-A_{1x} and the Hb-A_{0I} pools are

TABLE II

VALUES OBTAINED FROM THE THIOBARBITURIC ACID TEST RESULTS ON THE POOLS OF FIG. 3 (COLLECTED FROM CHROMATOGRAPHIC FRACTIONS)

In the first column the calculation is made assuming a conversion of the ketoaminic form of hemoglobin (in Amadori rearrangement) comparable with that of fructose standards (mol fructose per mol hemoglobin). In the second column the calculation is made assuming a conversion of the ketoaminic form of hemoglobin of about 36%, choosing for Hb-A_{1c} a value of 2.0 mol/mol hemoglobin (mol Amadori per mol hemoglobin). On the right hand side of the table are reported the percentage ranges for normal and diabetic samples obtained from the thiobarbituric acid test and the quantity of hemoglobin present in the pools collected.

Pool	mol fructose / mol hemoglobin	mol Amadori / mol hemoglobin	Percentage	
			Normal	Diabetic
c	2.3	2.0	4—5	8—9
x	1.4	1.2	2—3	4—5
0I	1.3	1.1	5—7	12—16
0II	0.7	0.6		
0III	—	—	—	—

monoglucosylated forms of hemoglobin. Hb-A_{0II} is a mixture in which about 60% of monoglucosylated hemoglobin is still present.

The mean value of 1.2 for Hb-A_{1x} could derive from a partial overlapping with the Hb-A_{1c} component. Obviously, the second method of calculation takes into account that Hb-A_{1c} is a diglucosylated form. Thus, considering a conversion of the ketoaminic adduct of 18%, the values obtained should be all halved.

On the right-hand side of Table II percentages of glucosylated hemoglobin in normoglycemic and diabetic samples are reported as obtained from the thiobarbituric acid test and from the total quantity of hemoglobin in each pool.

In Table III the statistical inter-assay data for the column chromatography are presented ($n = 10 \times 2$), intra-assay statistical computation is very difficult due to the length of time required for the chromatography, making it difficult to perform many experiments on the same sample.

In addition, the mean values for diabetics ($n = 28$) and for normoglycemics ($n = 15$) of each fast component are presented; Hb-A_{1b1} and Hb-A_{1b2} are computed as a single component because of difficulties in quantifying the two peaks. While the normoglycemic ranges are calculated with the aid of standard deviation and Student's t test, for the diabetic ranges, in which the distribution seems not to be of normal type, those of Hb-A_{1c} and Hb-A_{1x} are computed as the differences between the mean and the highest value and the mean and the lowest value of the range.

Also in Table III are presented the crossed correlation coefficients together with linear regression analysis among all the fast components. Hb-A_{1a1} correlates with Hb-A_{1a2}, and Hb-A_{1b}' Hb-A_{1c} and Hb-A_{1x} correlate among themselves. Our previous data on a smaller number of samples [13] did not show a significant correlation of Hb-A_{1b} with the other components, so for

TABLE III

STATISTICAL DATA OBTAINED FOR EACH COMPONENT FROM THE CHROMATOGRAPHY OF 43 SAMPLES

In the first column are shown the inter-assay coefficients of variation ($n = 10 \times 2$). In the second and third columns the normoglycemic and diabetic ranges are shown. On the right-hand side the crossed linear regression parameters are presented together with the correlation coefficient. $t \times$ S.D. is the standard deviation multiplied by the t value from the "t test" table for "95% limits".

Fast component	Inter-assay C.V. ($n = 10 \times 2$)	Normal mean ± ($t \times$ S.D.) ($n = 15$)	Diabetic mean ± ($t \times$ S.D.) ($n = 28$)	Linear regression* and correlation				
				a_1	a_2	b_{1+2}	c	x
a_1	14.4	$0.12^{+0.24}_{-0.12}$	$0.14^{+0.27}_{-0.14}$	—				
a_2	7.3	0.14 ± 0.12	0.18 ± 0.18	$a = +0.27$ $b = +0.12$ $r = +0.706$ **	—			
b_{1+2}	3.0	1.51 ± 0.66	1.81 ± 0.79	$a = -0.79$ $b = +1.83$ $r = -0.194$	$a = +0.31$ $b = +1.67$ $r = +0.029$	—		
c	4.0	4.55 ± 1.71	$8.57^{+5.95}_{-3.02}$	$a = -1.58$ $b = +6.88$ $r = -0.104$	$a = +4.54$ $b = +5.93$ $r = +0.114$	$a = +2.48$ $b = +2.37$ $r = +0.668$ **	—	
x	3.9	2.86 ± 0.91	$5.21^{+4.53}_{-2.04}$	$a = -0.79$ $b = +4.55$ $r = -0.078$	$a = +3.04$ $b = +3.96$ $r = +0.115$	$a = +1.50$ $b = +1.86$ $r = +0.687$ **	$a = +0.48$ $b = +1.28$ $r = +0.714$ **	—

*$Y = aX + b$; $n = 43$.
**$p < 0.001$.

this component it is important that the statistical analysis is performed on a larger number of samples in order to confirm the present data.

DISCUSSION

The use of Bio-Rex 70 cation-exchange chromatography to demonstrate the heterogeneity of Hb has been described [1, 3]. In fact, the preparative chromatography used by McDonald et al. [3] did not allow the separation of some components, probably due to the too high elution pH and the too steep sodium chloride gradient used in the last chromatographic step. A smoother final sodium chloride gradient and a lower pH value (6.55 ± 0.01), as well as smaller sample volumes compared to the total volume of the column, result in a more accurate separation. The interpretation of the results can be summarized as follows:

(1) The first chromatographic peak is clearly attributable to non-heme proteins because it shows a different A_{415}/A_{280} ratio to that of HbCO. In this part of the elution pattern probably a small quantity of 2,3-diphosphoglycerate hemoglobin is present along with the free glucose of the sample which has not been retained by the resin. This interpretation comes from the comparison between samples free and not free from 2,3-diphosphoglycerate and from the experiments with labelled glucose. After incubation the mixture is immediately filtered on a Sephadex G-50 column both to eliminate free glucose and to bring the labelled sample to the subsequent column pH. At this point and before the Bio-Rex column there is a lag of about 1 h which is needed for sample adjustments (addition of unlabelled hemoglobin and readings). In the meantime the reversible glucose aldimine adduct separates from hemoglobin to yield a separate labelled peak at one void volume of the column. Thus the initial peak and/or peaks observed could derive from this glucose. If true, this step could be proposed as a method for measuring the glucose reversibly linked to hemoglobin (aldimine form) which, from the results, seems to be more than previously supposed [14]. Furthermore, the possible presence of some radioactive contaminants must be considered [12]. We are performing other labelling incubation experiments to elucidate these problems.

(2) Hb-A_{1a1} and Hb-A_{1a2} are characterized with some difficulty because of their low absolute amounts. Larger samples overload the columns and the use of larger columns would be too time-consuming.

Little can be said about these two components. By lowering the pH their resolution improves. Their carbohydrate moieties [3] — fructose 1,6-diphosphate (Hb-A_{1a1}) and glucose 6-phosphate (Hb-A_{1a2}) — cannot be elucidated from our data. The fact that two labelled peaks are present at approximately the same elution volumes of these components may be explained by the above-mentioned presence of radioactive contaminants or by the presence of free labelled glucose. It is also possible that there is at this elution volume a triglucosylated form of hemoglobin which, though of little statistical importance, may be highly radioactive.

These two components correlate significantly between themselves but not with the others. This fact can be a point in favour of the given interpretation. In fact, the intra-erythrocyte concentration of the two metabolic products,

fructose 1,6-diphosphate and glucose 6-phosphate, is enzymically mediated and an increase in one of them may reflect an increase in the other.

The lack of correlation with the other components could, on the other hand, be due to a non-enzymically mediated glucose concentration. Furthermore, the mean values for normoglycemic and diabetic samples of these two components do not vary appreciably. For the very low absolute quantities of these components the results obtainable with the thiobarbituric acid test are below the sensitivity range of the system. The positive data obtained by others [3] may be artifactual contaminants from heme degradation products and, in any case, it is not clear whether the ketoaminic reversible adduct of hemoglobin with fructose 1,6-diphosphate can convert into a more stable ketoaminic form (Amadori rearrangement) sensitive to the thiobarbituric acid test.

(3) Also, very little about the identity of Hb-A_{1b} emerges from our data. This component, previously thought to be a minor homogeneous hemoglobin component, is probably the sum of two minor hemoglobin forms very similar in chromatographic properties, as shown by their parallel $V_{e(max)}$ variation as a function of pH. No labelled glucose is linked to them. From the statistical analysis no significant differences between the two mean values of normoglycemic and diabetic samples can be observed, but there is a significant linear correlation with Hb-A_{1c} and Hb-A_{1x}. An earlier statistical analysis on a smaller number of samples (n = 21) did not show a significant correlation [13]. In any case, it is not sure if the Hb-A_{1b} component(s) derive from hemoglobin modification as postulated for the other components.

(4) Hb-A_{1c} has already been mentioned as the most studied component. Our data are all in agreement with the known properties: (A) the high sensitivity of its $V_{e(max)}$ to variations in pH should indicate a pK_a of the amino group of the ketoaminic glucosidic moiety in the same range as the pH of elution; (B) the net labelled peak present in the leading edge of the Hb-A_{1c} peak in the incubation experiments. The non-exact overlapping is probably due to a partial denaturation of the glucosylated hemoglobin form during the incubation; (C) the large increase in its percentage mean value in diabetic samples compared to normoglycemic ones. It is important to note that, while in normoglycemics the percentage distribution is normal (Gaussian), in diabetics the distribution is asymmetrical and it is advisable not to use the common statistical analysis to obtain the pathological range; (D) finally, its identity is also confirmed by a high positivity in the thiobarbituric acid test; the values observed lead one to assume, in comparison with fructose standards, the presence of about 2 mol of glucose in Amadori rearrangement for 1 mol of hemoglobin, both for normal and diabetic samples; this fact shows that Hb-A_{1c} is a diglucosylated form of hemoglobin, as previously demonstrated by Bookchin and Gallop [15].

(5) Hb-A_{1x} is the new component obtained. The $V_{e(max)}$ of elution is very similar to that described for Hb-A_{1d} and Hb-A_{1e} [1]. However, we do not believe that Hb-A_{1x} is one of these two known components. Hb-A_{1d} is assumed to be an artifact formed in vitro when undialyzed hemolysates are stored and in which one sulphydryl group of each of two β-chains of Hb-A has reacted with oxidized glutathione. This is not so in our case. Hb-A_{1e} apparently is formed as a result of the incubation at 37° C, and its chromatographic properties are

different from those of Hb-A_{1d}. We do not use experimental steps in which the sample undergoes incubation at 37°C, except in the experiments with labelled glucose, where the broad peak, corresponding to the Hb-A_{1x} component, could also be thought of as a labelled Hb-A_{1e} contaminant. Hence, we indicate this component with the subscript "x" until its identity is clearly demonstrated.

We suggest, on the basis of our results, that the Hb-A_{1x} component is a glucosylated hemoglobin with blocked ϵ-NH_2 group(s) of the lateral lysines, or a hemoglobin glucosylated on the α-NH_2 of the valine of the α-chain. It has chromatographic properties ranging between those of Hb-A_{1c} and Hb-A_0, showing, therefore, that it has a positive charge lower than that of Hb-A_0 and greater than that of Hb-A_{1c}. According to the thiobarbituric acid test, the Hb-A_{1x} component shows that about 1 mol of glucose is converted in Amadori rearrangement per 1 mol of hemoglobin (tetramer), both in normal and diabetic samples. The [^{3}H]glucose incubation results in a broad peak in the position of Hb-A_{1x} (also present in the early part of the Hb-A_0 peak), so that it may correspond to a not strictly homogeneous component. Last, but not least, there is an increase in the percentage of Hb-A_{1x} in diabetic samples almost comparable to that of Hb-A_{1c} and, furthermore, the linear correlation between these two components is significant. The correlation observed is highly significant, but not in absolute value ($r = 0.714$). This is not surprising because the glucose concentration could not be the only variable affecting the percentage of these components, but it can be supposed that, owing to a very different pK_a between the amino group of the terminal valine of the β-chain (ca. 7) and the ϵ-NH_2 of lateral lysines (ca. 10), the rate of glucosylation has varying sensitivity to slight pH changes [16]. Indeed an increase of the intra-erythrocyte pH should be reflected in a variation of the rate of glucosylation more for Hb-A_{1c} than for Hb-A_{1x}, because of the greater increase in non-protonated NH_2 of the valine. Thus, the non-exact correlation may be the result of these different behaviours. These results seem to dispel every doubt on the given interpretation of the Hb-A_{1x} component, but further discussion leads to some uncertainties. The very small variation in the $V_{e(max)}$ of Hb-A_{1x} as a function of Ph shows that this component is modified on those groups interacting with Bio-Rex 70 that are particularly sensitive to pH changes, and which are present in Hb-A_0, Hb-A_{1c} and Hb-A_2. This fact seems contrary to a modification on the ϵ-NH_2 of the lateral lysines. However, as has already been said, Hb-A_{1x} could represent a peak resulting from non-homogeneous hemoglobins, so a chromatographic property which varies with pH must be justified in this respect.

Furthermore, the interpretation of the labelling experiments may be criticized for the possible presence of radioactive contaminants and for the possible presence of labelled Hb-A_{1e} in the same elution range as Hb-A_{1x}, as previously noted.

However, even with due allowance given for these uncertainties, the interpretation afforded by the other experimental results, such as the statistical analysis on normoglycemic and diabetic samples as well as the values obtained through the thiobarbituric acid test, provides a sufficiently strong demonstration.

(6) Not all the glucosylation products are confined to the Hb-A_{1c} and Hb-A_{1x} components. The labelling experiments and the thiobarbituric acid test

indicate that appreciable glucosylation is still present in the early part of the Hb-A_0 peak. Shapiro et al. [7] found that about 8—10% of normal Hb-A_0 is glucosylated at the NH_2-terminus of the α-chains or at lysine amino groups, and our mean percentage value of Hb-A_{1x} for normal samples is about 3%, so that 5—7% of glucosylated hemoglobins should still be located in the early part of the Hb-A_0 peak. From calculations of the thiobarbituric acid test, the percentage glucosylation obtained is in very good agreement with the percentage values postulated above: the leading edge of the Hb-A_0 peak contains about 5—7% of glucosylated hemoglobins, while in diabetic samples the percentage increases to about 12—16% of the whole Hb-A.

However, it is not easy to define which type of glucosylated hemoglobin is present in the early part of the Hb-A_0 peak or in the Hb-A_{1x} component. Once it is clearly established that Hb-A_{1c} is the diglucosylated form of Hb-A on the NH_2-terminus of both the β-chains, it is important to obtain information on the presence, position and percentage of the monoglucosylated form. In effect, a simple non-enzymic, non-cooperative attack of glucose should account for about 20% of the monoglucosylated form when 4% of the diglucosylated component (Hb-A_{1c}) is present. This percentage is much higher than the sum of the Hb-A_{1x} percentage and the percentage of glucosylated hemoglobins confined to the early part of the Hb-A_0 peak.

In the experiments with labelled glucose a very distinct peak can be noted in the position of the first 6—10 fractions of the Hb-A_0 peak: if the monoglucosylated form is present in this peak it should account for about the same percentage as the diglucosylated form (Hb-A_{1c}), since it has twice the specific activity of the latter. Again, this interpretation does not comply with expectations. In any case, to date there is not clear proof of a consistent presence of a monoglucosylation step for Hb-A_{1c}. This could be explained not as a demonstration of enzymic or high cooperative reaction, but rather on the assumption that this expected hemoglobin, monoglucosylated on the NH_2-terminus of the β-chains, could have a higher dimeric dissocation constant ($K_{4,2}$ splitting constant [17]) than those of Hb-A_{1c} and Hb-A.

REFERENCES

1 W.A. Schroeder and T.H.J. Huisman, in M.K. Schwartz (Editor), Clinical and Biochemical Analysis, Vol. IX, Marcel Dekker, New York, Basel, 1980.
2 H.F. Bunn, K.H. Gabbay and P.M. Gallop, Science, 200 (1978) 27.
3 M.J. McDonald, R. Shapiro, M. Bleichman, J. Solway and H.F. Bunn, J. Biol. Chem., 253 (1978) 2327.
4 M.J. McDonald, M. Bleichman, H.F. Bunn and R.W. Noble, J. Biol. Chem., 254 (1979) 702.
5 R. Krishnamoorthy, G. Gacon and D. Labie, FEBS Lett., 77 (1977) 99.
6 H.F. Bunn, D.N. Haney, K.H. Gabbay and P.M. Gallop, Biochem. Biophys. Res. Commun., 67 (1975) 103.
7 R. Shapiro, M. McManus, C. Zalut and H.F. Bunn, J. Biol. Chem., 255 (1980) 1406.
8 K. Betke, H.R. Marti and I. Schlicht, Nature (London), 184 (1959) 1877.
9 W. Jelkmann and C. Bauer, Anal. Biochem., 75 (1976) 382.
10 E. Antonini and M. Brunori, Annu. Rev. Biochem., 39 (1970) 977.
11 R. Fluckiger and K.H. Winterhalter, FEBS Lett., 71 (1976) 356.
12 B. Trueb, C.G. Holenstein, R.W. Fisher and K.H. Winterhalter, J. Biol. Chem., 255 (1980) 6717.

13 M. Castagnola, P. Caradonna, M.L. Salvi, R. Pellicano and D. Rossetti, J. Clin. Chem. Clin. Biochem., in press.
14 P.J. Higgins and H.F. Bunn, J. Biol. Chem., 256 (1981) 5204.
15 R.M. Bookchin and P.M. Gallop. Biochem. Biophys. Res. Commun., 32 (1968) 86.
16 M.H. Garner, R.A. Bogart, Jr. and F.R.N. Gurd, J. Biol. Chem., 250 (1975) 4398.
17 E. Antonini and M. Brunori, Hemoglobin and Myoglobin in Their Reactions with Ligands, North-Holland, Amsterdam, 1971, pp. 110—119.

Journal of Chromatography, 272 (1983) 67—73
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1485

DETERMINATION OF 4-METHYLUMBELLIFERONE AFTER SEPARATION FROM ITS CONJUGATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

APPLICATION TO LYSOSOMAL ENZYME ACTIVITY ASSAYS

ROBERT SANDMAN

Department of Ophthalmology, 490-U, University of California at San Francisco, San Francisco, CA 94143 (U.S.A.)

(First received May 24th, 1982; revised manuscript received August 24th, 1982)

SUMMARY

A high-performance liquid chromatographic method is described for the separation and estimation of 4-methylumbelliferone in the presence of its conjugates. The technique utilizes a simple, isocratic eluent and fluorometric detection. Percentages of 4-methylumbelliferone in the conjugates and fluorescences of the conjugates are reported. 4-Methylumbelliferone, liberated under currently used conditions for enzyme activity in urine and fibroblasts, can be measured by this procedure because these materials contain no substances interfering with the 4-methylumbelliferone peak. Applied to lysosomal enzymatic activity analyses, this procedure eliminates substrate background fluorescence.

Determinations of eight lysosomal enzymes in urine and fibroblasts are presented in bar graph form.

INTRODUCTION

The measurement of lysosomal hydrolases in tissues, cultured fibroblasts, leucocytes, macrophages and body fluids is important for a large number of studies involving both normal and pathological conditions ranging from the role of acrosomal hydrolases in fertilization to diagnosis of genetic deficiency diseases and rejection of a transplanted kidney [1—5]. Following their introduction [6—8], 4-methylumbelliferone (4-MU) conjugates have been used extensively for the estimation of activities of lysosomal glycosidases, lipases, acid phosphatases and arylsulfatases. The determinations are based on the ability of these enzymes to hydrolyze compounds consisting of carbohydrates, lipids, phosphate or sulfate conjugated with 4-MU. Free 4-MU released by enzymatic action is readily estimated by fluorometry, a technique which

0378-4347/83/0000—0000/$03.00

possesses the advantage of greatly increased sensitivity over spectrophotometric methods. Aqueous solutions of 4-MU conjugates, however, exhibit considerable fluorescence due in part to the presence of free 4-MU resulting from its incomplete removal after synthesis and/or from spontaneous decomposition of the compounds during storage and in part to native fluorescence of the conjugate itself. The fluorescence of the latter is usually low relative to 4-MU but, as a substrate, it is present in high concentrations and, consequently, makes an appreciable contribution to the blank correction of the assay. Total background fluorescence can exceed fluorescence of 4-MU liberated by the enzyme.

This paper describes a technique for elimination of that part of the background problem due to fluorescence of the conjugate. This is accomplished by separation of 4-MU from the conjugate using reversed-phase high-performance liquid chromatography (HPLC) on a column of alkali-stable adsorbent and a simple alkaline eluent system for maximizing fluorescence of 4-MU.

MATERIALS AND METHODS

4-MU, 4-MU conjugates and ammonia-free glycine were obtained from Sigma (St. Louis, MO, U.S.A.); methanol was supplied by Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Isocratic reversed-phase HPLC was used with a Hamilton 15 × 0.4 cm PRP-1 column (Hamilton, Reno, NV, U.S.A.). The column packing is a 10-μm spherical styrene—divinylbenzene copolymer which is stable at pH values from 1—13 and in salt concentrations up to 0.5 *N*. A Brownlee 3 cm × 4.6 mm RP 8 guard column (Rheodyne, Berkeley, CA, U.S.A.) was placed in line before the PRP-1 column.

The detector was a Gilson Spectra/Glo Model 901 fluorometer with a standard 45-μl flow cell (Beckman Instruments, Mountain View, CA, U.S.A.). The primary filter had a band pass of 330—380 nm with a peak of 360 nm. The band pass of the secondary filter was 430—600 nm peaking at 455 nm. A linear Model 585 recorder was used (Tegal Scientific, Concord, CA, U.S.A.). Relative fluorescence is expressed in arbitrary units derived from peak heights rather than areas.

Eluent A, used for all conjugates except the sulfate, consisted of 0.04 *M* glycine—sodium hydroxide buffer (pH 10.32) in aqueous methanol. This was prepared by adding 300 ml of a stock 0.4 *M* glycine buffer (pH 10.32) to 1700 ml of water, mixing well, then adding 1 l of spectral-grade methanol. The pH was readjusted if necessary. This system did not separate 4-MU from its sulfate. Eluent B, the same concentration of the glycine buffer in a more aqueous solution (20% methanol in water) achieved this separation. Degassed eluent was pumped at the rate of 1 ml/min at room temperature.

The standard curve was prepared with 4-MU dissolved in water. For the determination of free 4-MU, retention times and relative fluorescence, the conjugates were dissolved in water with warming if necessary. Concentrations ranged from 0.5 m*M* for 4-MU-β-galactoside to 5.0 m*M* for 4-MU-sulfate; the remaining concentrations were 1.0 m*M*. Samples were applied to the column by filling a 20-μl stainless-steel injector loop using a plastic syringe.

For estimation of bound 4-MU, the above solutions were diluted 100-fold

with 0.1 *N* hydrochloric acid and aliquots were sealed in glass ampoules which were placed in a heating block at 100°C for the indicated time periods, then cooled and diluted to 0.2 μM for chromatography.

Normal human fibroblasts were grown to confluency in Falcon T-75 flasks in Dulbecco's MEM H21 medium 1 with 10% fetal calf serum. Cell harvest and preparation of the enzyme solution are described in a previous communication [3]. Protein content of the cell supernatant after the final centrifugation was estimated using the method of Lowry et al. [9].

Overnight urine samples were diluted 5-fold or greater in distilled water containing 1 mg/ml bovine serum albumin. Creatinine determinations were performed by the hospital clinical laboratories using a standard automated procedure based on the reaction with picric acid.

Conditions for enzyme analysis are given in Table I.

TABLE I

CONDITIONS OF ENZYME ANALYSIS

Abbreviations used: β-Glu, β-D-glucuronidase (E.C. 3.2.1.31); β-gal, β-D-galactosidase (E.C. 3.2.1.23); α-Gal, α-D-galactosidase (E.C. 3.2.1.22); NAG, β-N-acetyl-D-glucosaminidase (E.C. 3.2.1.30); AS, arylsulfatase (E.C. 3.1.6.1); AP, acid phosphatase (E.C. 3.1.3.2); α-Man, α-D-mannosidase (E.C. 3.2.1.24); α-Fuc, α-L-fucosidase (E.C. 3.2.1.51); 4-MU-β-glu, 4-methylumbelliferyl β-D-glucuronide; 4-MU-β-gal, 4-methylumbelliferyl β-D-galactoside; 4-MU-α-gal, 4-methylumbelliferyl α-D-galactoside; 4-MU-NAG, 4-methylumbelliferyl N-acetyl-β-D-glucosaminide; 4-MU-SO_4, 4-methylumbelliferyl sulfate; 4-MU-PO_4, 4-methylumbelliferyl phosphate; 4-MU-α-man, 4-methylumbelliferyl α-D-mannoside; 4-MU-α-fuc, 4-methylumbelliferyl α-L-fucoside.

Enzyme	Buffer	pH	Substrate
β-Glu	0.1 *M* acetate	4.75	1.0 m*M* 4-MU-β-glu
β-Gal	0.1 *M* acetate	4.50	0.5 m*M* 4-MU-β-gal
α-Gal	0.1 *M* acetate	4.20	1.0 m*M* 4-MU-α-gal
NAG	0.2 *M* citrate phosphate*	4.60	1.0 m*M* 4-MU-NAG
AS	0.1 *M* acetate	5.00	1.0 m*M* 4-MU-SO_4
AP	0.1 *M* citrate	4.00	1.0 m*M* 4-MU-PO_4
α-Man	0.1 *M* citrate	4.75	1.0 m*M* 4-MU-α-man
α-Fuc	0.1 *M* citrate	5.25	1.0 m*M* 4-MU-α-fuc

*See ref. 10.

The total volume of the reaction mixture was 0.5 ml including the 0.05 ml of diluted urine or homogenate used to initiate the reaction. In addition, bovine serum albumin was present at a concentration of 1 mg/ml. Incubations were carried out at 37°C for 15, 30, or 60 min. The reaction was stopped by the addition of 2 ml of 100% methanol which has an advantage over the generally used alkaline glycine-sodium hydroxide buffer in that it precipitates proteins of the reaction mixture thus avoiding the possibility of their adsorption on the chromatography column. Blank values were obtained by adding the 0.05 ml of enzyme source after the methanol. Precipitated protein was removed by centrifuging at 570 *g* for 10 min and 20 μl of the supernatant were chromatographed using eluent A.

RESULTS AND DISCUSSION

Using eluent A, a standard curve was prepared by chromatographing 0.24 to 36 pmol of 4-MU. A linear correlation between relative fluorescence based on peak height and concentration was observed at all levels. Relative fluorescence by least squares linear regression analysis was given by the equation: $3858 \times [4\text{-MU}] + 10$; r^2 was 0.999. The minimum detectable amount having a peak height two times the short-term baseline noise is 6 fmol.

Table II shows retention times of 4-MU and its conjugates using eluent A. Because this solution did not separate 4-MU from its sulfate, retention times of both these compounds are given for eluent B. Table II also lists relative fluorescences of the conjugates. These values, based on peak heights, are given only to indicate that they will add to background fluorescence. They are not equivalents of 4-MU fluorescence because peak height at a given concentration varies inversely with its retention time. The low fluorescent intensity of the superimposed peaks of 4-MU and its sulfate in eluent A should be noted.

Free and bound 4-MU was determined chromatographically before and after acid hydrolysis of freshly prepared solutions of the conjugates. These values are given in Table III as molar percentages based on weight of the conjugate. The quantity of free 4-MU is very low; however, the compound is much more fluorescent than the substrates and these small percentages in the relatively high substrate concentrations employed in an assay make a considerable contribution to blank fluorescence.

TABLE II

RETENTION TIMES AND RELATIVE FLUORESCENCE OF 4-METHYLUMBELLIFERONE AND SOME CONJUGATES

Reversed-phase HPLC with fluorometric detection. All samples were dissolved in distilled water. To hasten solution 4-MU-β-gal and 4-MU-α-fuc were heated to 65°C; 4-MU-NAG to 50°C; and 4-MU-α-gal, 4-MU-α-man and 4-MU-β-glu to approximately 40°C. Unless otherwise noted eluent A was used. The compositions of eluents A and B are given in the Materials and methods section. 4-Methylumbelliferone is abbreviated 4-MU. See Table I for other abbreviations.

Compound	Retention time (min)	Relative fluorescence/nmol
4-MU	6.4	195,800
4-MU-β-glu	3.3	130.5
4-MU-β-gal	9.9	36.5
4-MU-α-gal	10.4	57.9
4-MU-NAG	15.0	20.1
4-MU-SO_4*	6.4	7.3
4-MU-PO_4	1.8	2650
4-MU-α-man	30.5	14.9
4-MU-α-fuc	58.7	10.3
4-MU**	15.8	69,200
4-MU-SO_4**	19.4	0.75

*Peaks of 4-MU and 4-MU-SO_4 are superimposed.
**Eluent B.

TABLE III

FREE AND BOUND 4-MU IN 4-MU CONJUGATES

All compounds were dissolved in distilled water for determination of free 4-MU. To aid solution, conjugates were heated as noted in Table II. Bound 4-MU was determined after hydrolysis as described in the Materials and methods section. Eluent A was used for all compounds except 4-MU-SO_4 which was chromatographed in eluent B. Composition of these eluents is given in the Materials and methods section. Explanations of abbreviations are given in Tables I and II.

Conjugate	Concentration (m*M*	Molar % free 4-MU	Molar % of theoretical 4-MU recovered after hydrolysis for							
			4 h	5 h	6 h	21 h	24 h	28 h	29 h	30 h
4-MU-β-glu	1.0	0.0066	74.4	82.2	87.1		103.7		104.3	105.2
4-MU-β-gal	0.5	0.0009		99.4			103.0	103.8		
4-MU-α-gal	1.0	0.0103*	92.8	94.1			95.6			
4-MU-NAG	1.0	0.0194	92.3	94.5			95.1			
4-MU-SO_4	5.0	0.0036	92.6				99.3		100.3	
4-MU-PO_4	1.0	0.0723	91.6			97.6	93.8			
4-MU-man	1.0	0.0272		101.2			99.7		101.8	
4-MU-β-fuc	1.0	0.0023	96.5				96.1		94.1	

*At room temperature the percentage of free 4-MU in the 4-MU-α-gal solution gradually increased to 0.0199% over a 5-h period.

Following acid hydrolysis of the conjugates, the expected one-to-one molar ratio of 4-MU to conjugate was found for all compounds. With the exception of the β-glucuronide, over 90% of the theoretical amount of bound 4-MU was liberated after a 4—5 h hydrolysis period. By 24 h hydrolysis was essentially complete for all compounds. Results of the hydrolysis experiments show that 4-MU is stable for at least 30 h at 100°C in 0.1 *N* hydrochloric acid.

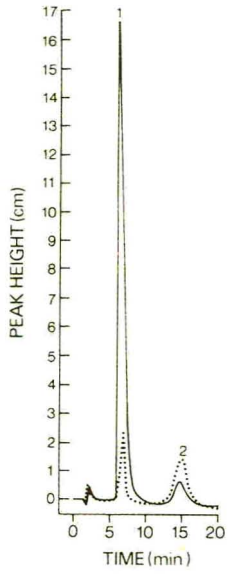


Fig. 1. Chromatogram of urinary NAG assay reaction mixture (——) and assay blank (····). Conditions of analysis and chromatography are described under Materials and methods. Peaks: 1 = 4-MU, 2 = 4-MU-NAG. Enzyme source: 10-fold diluted urine. Detector sensitivity for the assay blank was twice that of the assay reaction mixture.

After a preliminary experiment demonstrated that neither diluted urine nor a fibroblast enzyme preparation in commonly used reaction mixtures would interfere with the chromatography of 4-MU, analyses of eight lysosomal enzymes in urine or fibroblasts were carried out. Fig. 1 shows a typical assay and blank chromatogram from one of these. With the exception of the sulfate, blank corrections involved only fluorescence of free 4-MU in the substrates. From Tables II and III it can be calculated that the 0.0036 molar percent free 4-MU in the sulfate conjugate is responsible for 96% of the fluorescence of their superimposed peaks emerging with eluent A. The remaining 4% was due to fluorescence of 4-MU-sulfate. Therefore, separation of product and substrate with eluent B was not deemed necessary and a blank correction based on their superimposed peaks was used.

Fig. 2 shows, in bar graph form, results of the activity determinations of the enzyme in urine and fibroblasts. The results are in general agreement with those reported previously even though substrate concentrations and pH values of the reaction mixtures differed in most instances [2, 3, 5, 11—14]. The value for urinary acid phosphatase is high reflecting the presence of prostatic enzyme [15, 16]. This activity decreased rapidly over the course of several days when the urine was stored at —20°C.

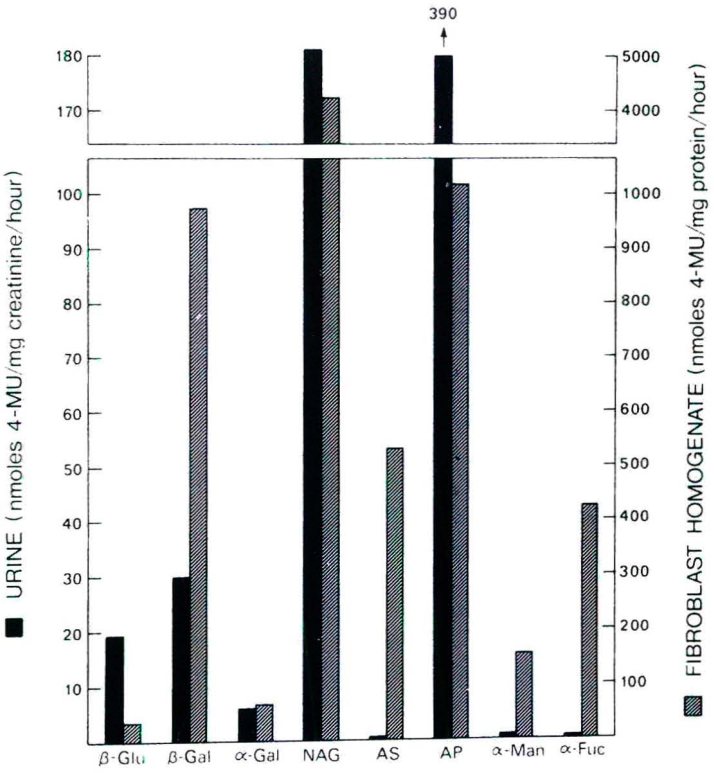


Fig. 2. Activity of eight lysosomal enzymes in human urine (solid bars) and in a fibroblast homogenate (cross-hatched bars). The urinary acid phosphate value given in parentheses above the broken bar includes the prostatic enzyme present in adult male urine. For explanation of abbreviations, see in Tables I and II.

When a long time lapse occurs between the emergence of the 4-MU and a substrate peak, the assay can be shortened by injecting samples during that interval. This technique does not alter the peak height of 4-MU. The timing for these multiple injections can be calculated from retention times of 4-MU and conjugates. As an alternative, late-eluting peaks can be eliminated by substituting a short (3 × 0.46 cm) column for the sample loop. When 4-MU moves to the long column, conjugate retained by the short column is removed with a strong eluent and the column re-equilibrated.

In summary, a method is presented for the estimation of 4-MU in enzyme reaction mixtures commonly used for the determination of lysosomal enzyme activities. The technique is accurate, relatively rapid and eliminates part of the background fluorescence encountered in analyses where 4-MU conjugates are used as substrates. Because only 20 μl or less of reaction mixture need be chromatographed, the method can easily be adapted for use as a microassay.

ACKNOWLEDGEMENT

This research was supported by grants EY-02706 and EY-02162 from the National Eye Institute.

REFERENCES

1 A.C. Allison and E.F. Hartree, J. Reprod. Fert., 21 (1970) 501.
2 M.W. Ho, S. Beutler, L. Tennant and J.S. O'Brien, Amer. J. Hum. Genet., 24 (1972) 256.
3 R. Sandman, S. Yatsiv, W.v.B. Robertson and R.P. Erickson, Clin. Chim. Acta, 80 (1977) 471.
4 S. Kelly and R. Bakhru-Kishore, Clin. Chim. Acta, 97 (1979) 239.
5 R. Sandman, R.M. Margules and S.L. Kountz, Clin. Chim. Acta, 45 (1973) 349.
6 J.A.R. Mead, J.N. Smith and R.T. Williams, Biochem. J., 61 (1955) 569.
7 D. Robinson, Biochem. J., 63 (1956) 39.
8 D.H. Leaback and P.G. Walker, Biochem. J., 78 (1961) 151.
9 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
10 P.J. Elving, J.M. Markowitz and I. Rosenthal, Anal. Chem., 28 (1956) 1179.
11 J. Butterworth, F. Scott, W.M. McCrae and A.D. Bain, Clin. Chim. Acta, 40 (1972) 139.
12 W.G. Ng, G.N. Donnell, R. Koch and W.R. Bergren, Birth Defects: Original Article Series, Disorders of Connective Tissue, Vol. 11, No. 6, Stratton, New York, 1975, p. 335.
13 R.G. Price, R. Thorpe, S.M. Tucker and N.C. Phillips, Biochem. Soc. Trans., 5 (1977) 248.
14 B. Hultberg, J. Clin. Chem. Clin. Biochem., 17 (1979) 795.
15 M.D. Altschule, B.H. Parkhurst and G.R. Zager, Amer. J. Clin. Path., 21 (1951) 480.
16 H.J. Kramer, J.E. Wight, W.L. Paul and H.C. Gonick, Enzymol. Biol. Clin., 11 (1970) 435.

Journal of Chromatography, 272 (1983) 75—85
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1472

SCREENING PROCEDURE FOR DETECTING BUTYROPHENONE AND BISFLUOROPHENYL NEUROLEPTICS IN URINE USING A COMPUTERIZED GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC TECHNIQUE*

HANS MAURER and KARL PFLEGER*

Institut für Pharmakologie und Toxikologie der Universität des Saarlandes, D-6650 Homburg/Saar (G.F.R.)

(First received March 5th, 1982; revised manuscript received August 11th, 1982)

SUMMARY

A method for the identification of butyrophenone and bisfluorophenyl neuroleptics and their predominant basic metabolites in urine after acid hydrolysis is described. The acetylated extract is analysed by computerized gas chromatography—mass spectrometry. An on-line computer allows rapid detection using mass fragmentography with the masses *m/e* 112, 123, 134, 148, 169, 257, 321 and 189, 191, 223, 233, 235, 245, 287, 297. The identity of positive signals in the reconstructed mass fragmentograms is established by a comparison of the entire mass spectra with those of standards. The mass fragmentograms and the underlying mass spectra are documented.

INTRODUCTION

Screening for butyrophenone and bisfluorophenyl neuroleptics is necessary in analytical toxicology to diagnose a probable intoxication. Only an assay for differentiation of some of these neuroleptics employing thin-layer chromatography and gas—liquid chromatography [2] has been described. However, this assay does not allow the rapid and exact identification of all drugs or their metabolites. This is important in clinical or forensic estimations because the various neuroleptics have very different pharmacological potencies. These demands are met by the computerized gas chromatographic—mass spectro-

*This work is part of the Thesis of H. Maurer at the Universität des Saarlandes, Saarbrücken, G.F.R. Part of these results were reported at the 23. Frühjahrstagung der Deutschen Pharmakologischen Gesellschaft (DPhG), in Mainz, G.F.R., March 16th, 1982 [1].

0378-4347/83/0000—0000/$03.00

metric (GC—MS—DS) technique described below. If necessary plasma levels of the identified drugs can be determined using a GC procedure [3—7] or a radioimmunoassay [8—10] described in the literature.

EXPERIMENTAL

Apparatus

The apparatus used for this study has been previously described [11].

Hydrolysis and extraction procedure

Ten millilitres of urine were refluxed with 3 ml of hydrochloric acid (37%) for 15 min, then made alkaline with about 3 g of potassium hydroxide pellets and mixed with 10 ml of 30% aqueous ammonium sulfate to obtain a pH of between 8 and 9. The extraction procedure [11] was modified so that samples were extracted twice with 10 ml each of a mixture of two parts dichloromethane, two parts isopropanol and six parts ethyl acetate. After phase separation by centrifugation the combined organic extracts were evaporated to dryness under vacuum. The residue was redissolved in 0.1 ml of methanol.

Acetylation

Forty microlitres of extract were evaporated and then acetylated for 30—60 min at 60°C with 40 μl of a mixture of three parts acetic anhydride in two parts pyridine. After evaporation of the acetylation mixture the residue was redissolved in 40 μl of ethyl acetate [12]. One to four microlitres of this were injected into the gas chromatograph.

Gas chromatographic—mass spectrometric analysis

The GC—MS—DS analysis procedure used in this study has been previously described [11].

RESULTS AND DISCUSSION

Some of the butyrophenone and bisfluorophenyl neuroleptics are excreted in urine completely metabolized and conjugated. Therefore conjugates were decomposed by acid hydrolysis which can be completed more quickly than enzymatic hydrolysis. The former extraction procedure [11] was modified to improve the extraction of polar metabolites. In addition, hydroxy and amino groups were acetylated to improve the GC characteristics. The acetylated extract was redissolved in ethyl acetate to avoid solvolysis of the acetylated compounds by methanol.

The results of our investigations are shown in Table I. The two mass fragmentograms with eight masses each allow the detection of twelve butyrophenone and bisfluorophenyl neuroleptics or their predominant basic metabolites. Some of the metabolites are acetylated.

The retention indices were determined using a gas chromatograph combined with flame ionization detection (FID) and nitrogen-sensitive FID with a temperature program [11]. In our experience retention indices are not necessary when employing the GC—MS technique but give preliminary indications

TABLE I

MONITORING PROGRAMS FOR BUTYROPHENONE AND BISFLUOROPHENYL NEUROLEPTICS

MS No.	Drug/metabolite	*m/e* (relative intensity, %)								Retention index
		112	123	134	148	169	257	259	321	
01	Benperidol M*			60				100		2750
02	Droperidol M*			100						1730
03	Fluanisone		60	10						2794
04	Fluanisone M I*			10	100					2140
05	Fluanisone M II*		100	10	40					2830
06	Melperone	100	30							1889
07	Melperone M I*	100								1837
08	Melperone M II*	100								2163
09	Moperone		95			40				2828
10	Moperone M					100				1600
11	Moperone artifact		100			10				2709
12	Penfluridol M I						100	35		1918
13	Penfluridol M II*								20	2240
02	Pimozide M I*			100						1730
01	Pimozide M II*			60				100		2750
14	Pipamperone		70							3040
15	(Androsterone)*		10	15	10		40			2581 (FID)
		189	191	223	233	235	245	287	297	
16	Bromperidol M I				100	98				1850
17	Bromperidol M II*								35	2335
18	Fluspirilene M*						15	7		2728
19	Haloperidol M I	100	35							1650
20	Haloperidol M II*	30	10			100				2150
21	Trifluperidol M I			100						1570
22	Trifluperidol M II*						5	35		2033
23	Fluorophenyloxobutanal									1490 (FID)
24	Bisfluorophenylbutyric acid									2228 (FID)

*Acetylated.

and may be useful to gas chromatographers without the latter facility and so they are given here.

The entire mass spectra are shown in Fig. 1 for the precise identification of the compounds. Formulae are proposed for probable structures of metabolites. It is possible that some of the metabolites were altered by the analytical procedures.

All investigations were carried out using the urine of man with the exception of bromperidol, fluanisone, fluspirilene, moperone and pimozide which were detected (in the absence of human samples) in the urine of rats. Benperidol, bromperidol, droperidol, fluspirilene, haloperidol, penfluridol, pimozide and trifluperidol are not detected in urine because they are almost completely excreted as their metabolites or they are not volatile under the applied GC conditions which are approved for toxicological analysis.

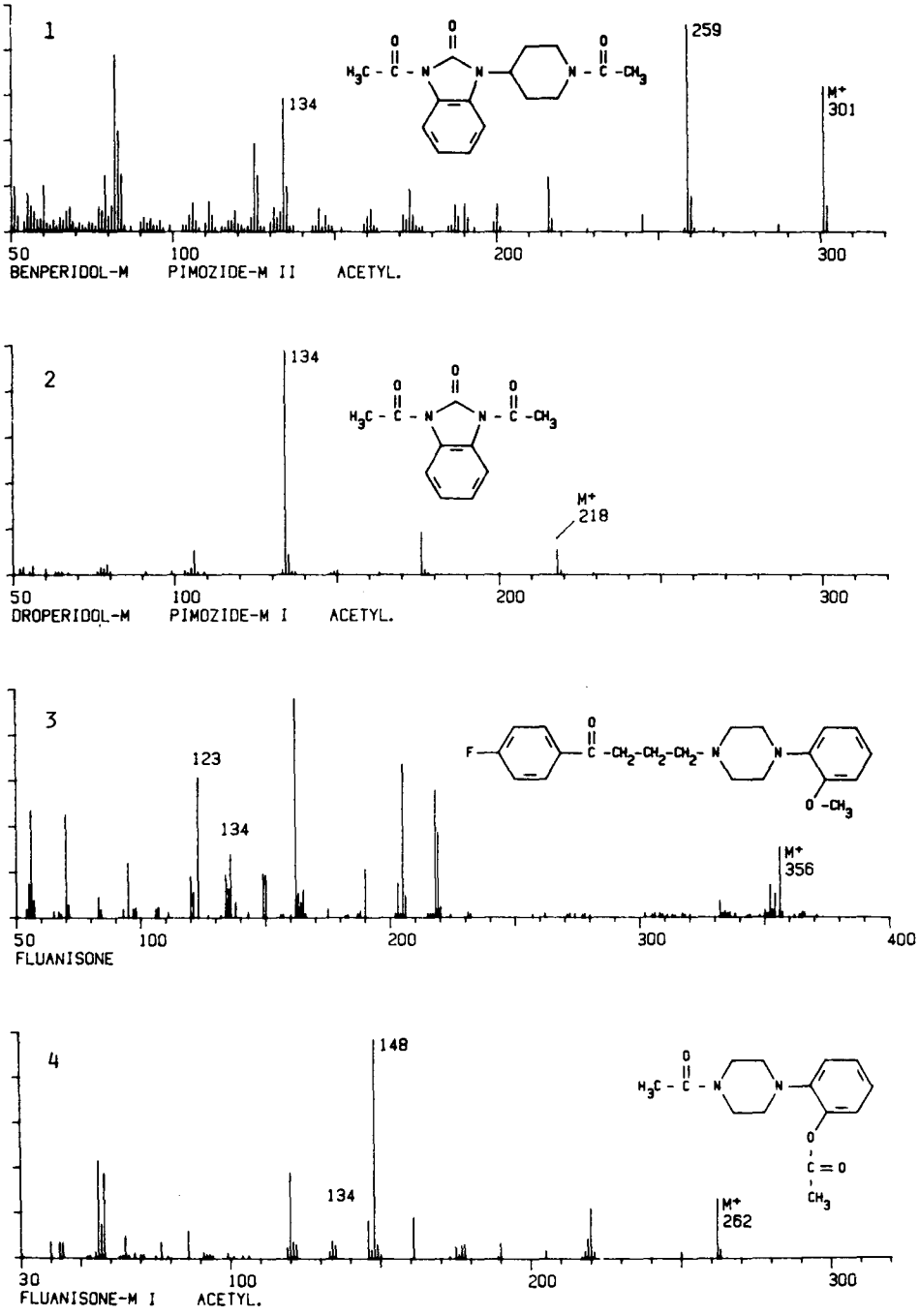
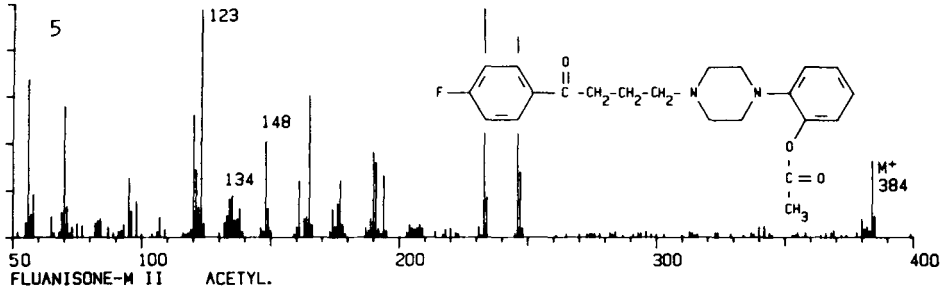
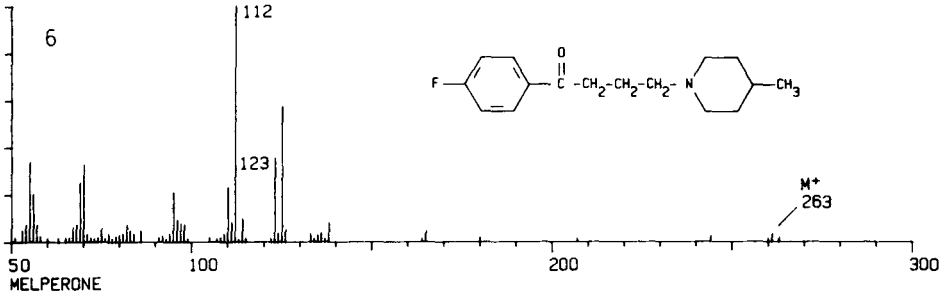
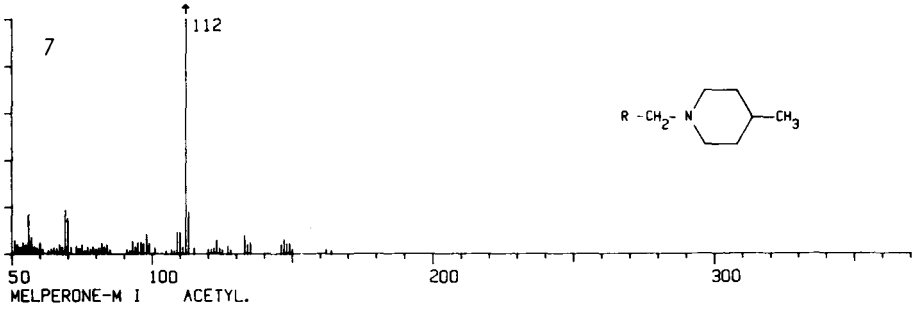

1
134
259
M+
301
50
100
200
300
BENPERIDOL-M
PIMOZIDE-M II
ACETYL.
2
134
M+
218
50
100
200
300
DROPERIDOL-M
PIMOZIDE-M I
ACETYL.
3
123
134
M+
356
50
100
200
300
400
FLUANISONE
4
148
134
M+
262
30
100
200
300
FLUANISONE-M I
ACETYL.

Fig. 1.







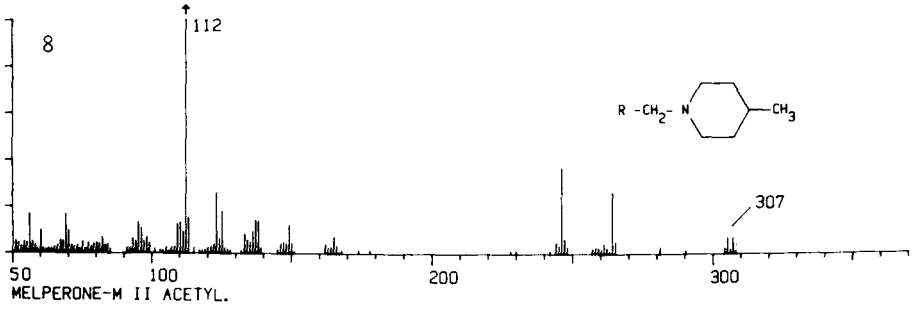


Fig. 1.

(Continued on p. 80)

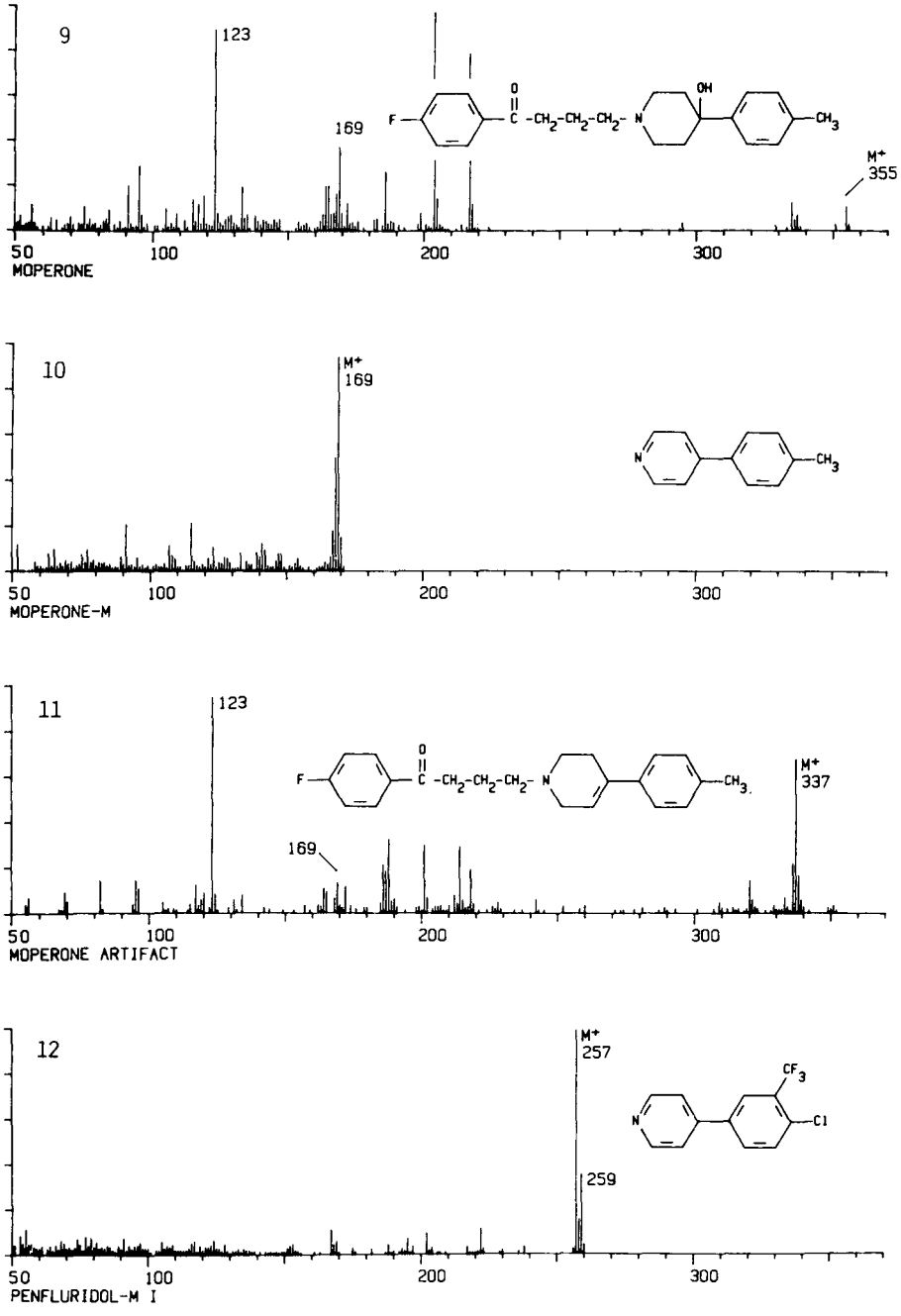
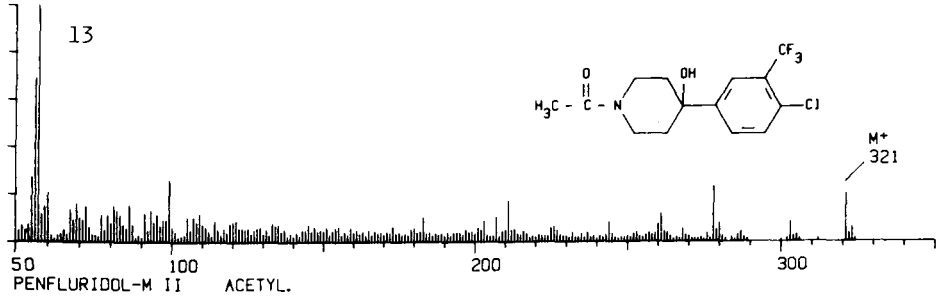
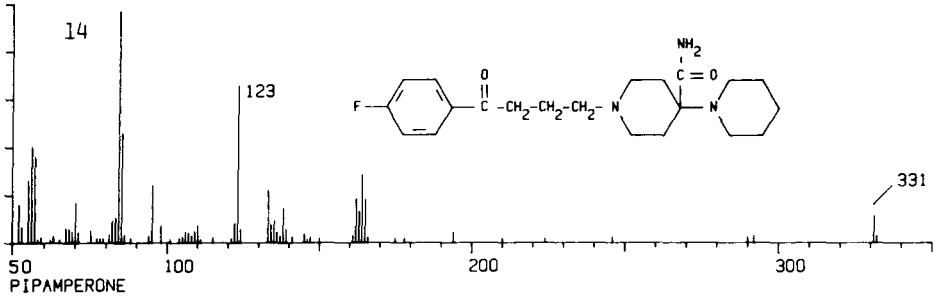
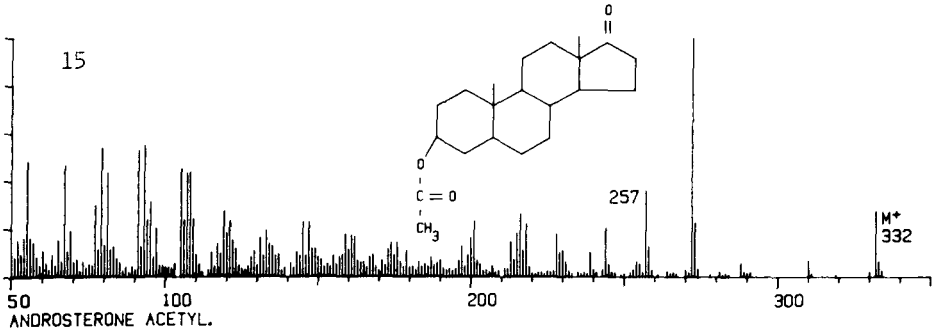

9
123
169
M+
355
50
100
200
300
MOPERONE
10
M+
169
50
100
200
300
MOPERONE-M
11
123
169
M+
337
50
100
200
300
MOPERONE ARTIFACT
12
M+
257
259
50
100
200
300
PENFLURIDOL-M I

Fig. 1.







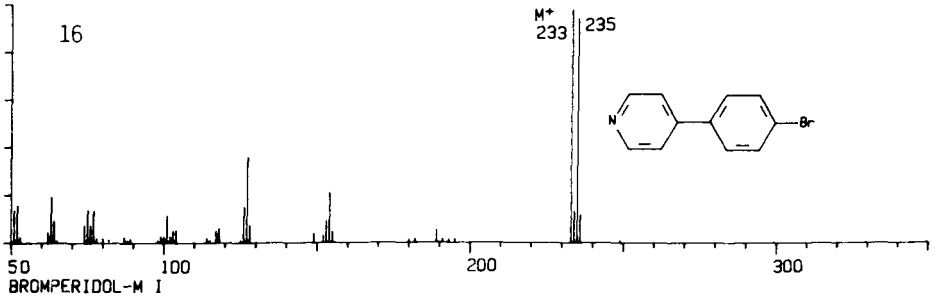


Fig. 1.

(Continued on p. 82)

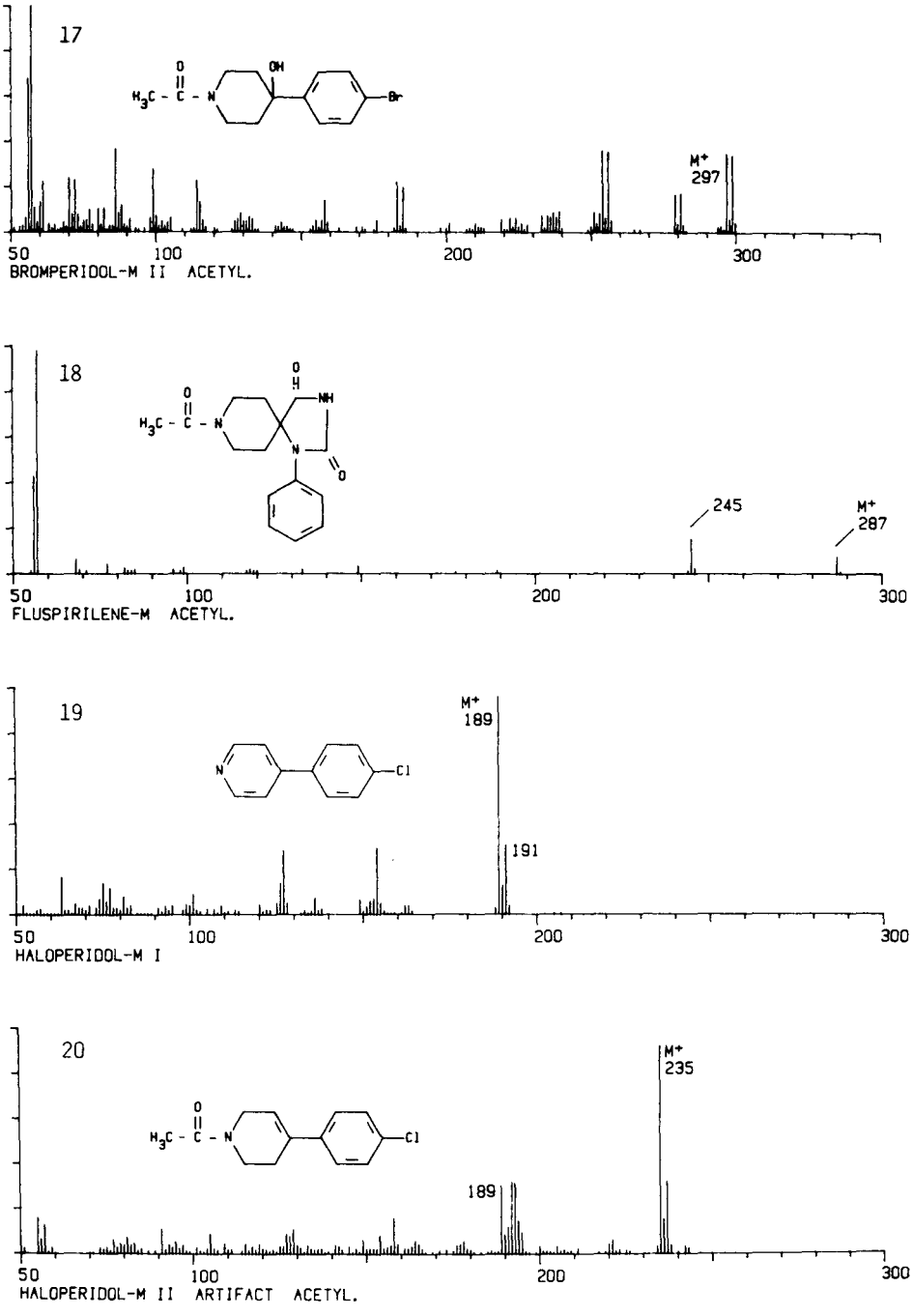

17
M+
297
50
100
200
300
BROMPERIDOL-M II ACETYL.
18
245
M+
287
50
100
200
300
FLUSPIRILENE-M ACETYL.
19
M+
189
191
50
100
200
300
HALOPERIDOL-M I
20
M+
235
189
50
100
200
300
HALOPERIDOL-M II ARTIFACT ACETYL.

Fig. 1.

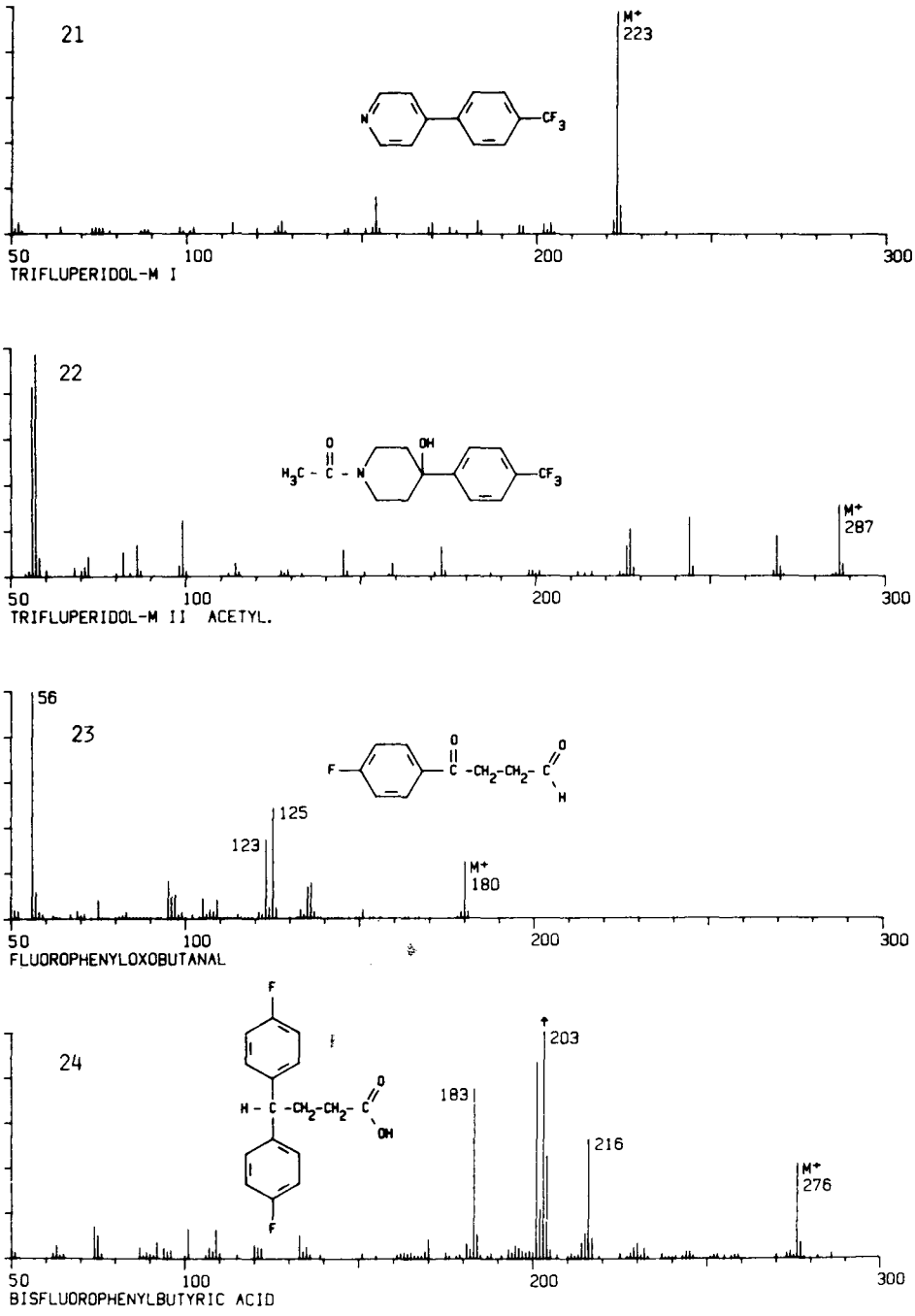


Fig. 1. Mass spectra of the compounds identified in urine after hydrolysis, extraction and acetylation.

Benperidol and droperidol each have a common metabolite with pimozide (mass spectra Nos. 1 and 2). Thus to determine if the patient has taken benperidol plus droperidol, or pimozide alone, it is necessary to analyse an acid extract. If benperidol and droperidol were taken, fluorophenyloxobutanal (mass spectrum No. 23) will be detected. If pimozide was taken, bisfluorophenylbutyric acid (mass spectrum No. 24) will be detected.

The moperone artifact (mass spectrum No. 11) is formed by the elimination of water during sample preparation because it is not present in the parent drug sample. The elimination of water was also observed for haloperidol M II (mass spectrum No. 20) but not for bromperidol M II, penfluridol M II or trifluperidol M II (mass spectra Nos. 17, 13, 22).

In our experience androsterone is the only endogenous physiological substance that appears in the mass fragmentogram. Because all compounds possibly indicated by the mass fragmentograms (e.g. the peaks at 134 and 148 in Fig. 3) can be precisely differentiated by comparison of the underlying mass spectra with those of standards (Fig. 1), interferences by other drugs are impossible.

To illustrate the method, a mass fragmentogram of a sample from a psychiatric patient administered a therapeutic dose of trifluperidol is shown in Fig. 2. The peak at *m/e* 223 indicates metabolite I (mass spectrum No. 21) and the peak at *m/e* 287 indicates the acetylated metabolite II of trifluperidol (mass spectrum No. 22).

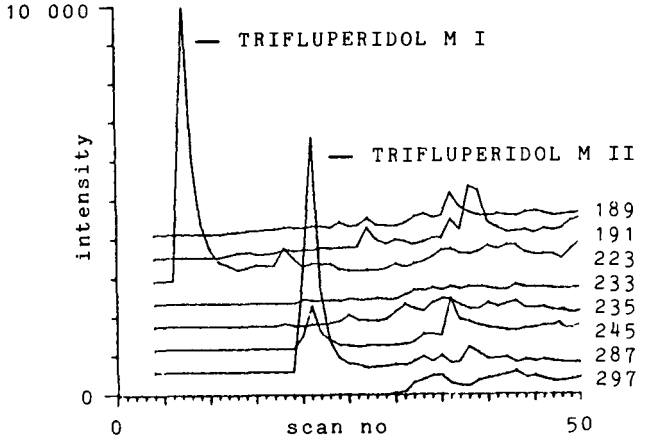


Fig. 2. Mass fragmentogram indicating metabolites of trifluperidol after a therapeutic dose.

Fig. 3 shows a mass fragmentogram of a sample from a child who had taken, accidentally, an unknown drug. The peaks at *m/e* 257, 259 indicate metabolite I (mass spectrum No. 12), the peak at *m/e* 321 the acetylated metabolite II of penfluridol (mass spectrum No. 13) and the second peak at *m/e* 257 indicates the acetylated physiological hormone androsterone (mass spectrum No. 15). The peaks at *m/e* 134 and 148 indicate unidentified compounds, which appeared in only this patient.

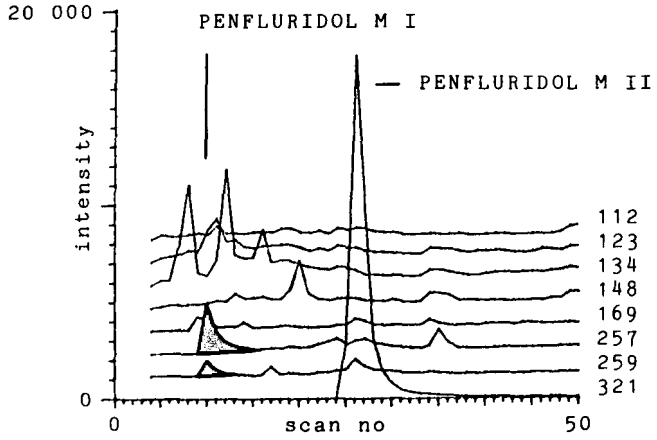


Fig. 3. Mass fragmentogram indicating metabolites of penfluridol in the urine of a child who had taken accidentally an unknown drug.

These examples show that the presented screening procedure allows a rapid and exact identification of butyrophenone and bisfluorophenyl neuroleptics and their predominant basic metabolites in urine.

ACKNOWLEDGEMENTS

We thank Cilag-Chemie, Delalande, Janssen and Tropon for substances and the DFG for sponsorship with instruments.

REFERENCES

1 H. Maurer and K. Pfleger, Naunyn-Schmiedebergs Arch. Pharmacol., 319 (Suppl.) (1982) R 6.
2 M.P. Quaglio, G. Sandri Cavicchi and M. Muraglia, Boll. Chim. Farm., 110 (1971) 385.
3 G. Bianchetti and P.L. Morselli, J. Chromatogr., 153 (1978) 203.
4 S.F. Cooper, J.M. Albert and R. Dugal, Int. Pharmacopsychiatry, 10 (1975) 78.
5 A. Forsman, E. Martensson, G. Nyberg and R. Öhman, Naunyn-Schmiedebergs Arch. Pharmacol., 286 (1974) 113.
6 F. Marcucci, L. Airoldi, E. Mussini and S. Garattini, J. Chromatogr., 59 (1971) 174.
7 I.A. Zingales, J. Chromatogr., 54 (1971) 15.
8 B.R. Clark, B.B. Tower and R.T. Rubin, Life Sci., 20 (1977) 319.
9 R. Hendriks, M. Michiels and J. Heykants, Preclinical Research Report of Janssen Pharmaceutica, Belgium, Serial number: R 4749/2, R 4584/2.
10 L.J.M. Michiels, J.J.P. Heykants, A.G. Knaeps and P.A.J. Janssen, Life Sci., 16 (1975) 937.
11 H. Maurer and K. Pfleger, J. Chromatogr., 222 (1981) 409.
12 H. Maurer, A. Weber and K. Pfleger, Z. Anal. Chem., 311 (1982) 414.

Journal of Chromatography, 272 (1983) 87—94
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1468

DETERMINATION OF THE ANTI-ISCHAEMIC DRUG BEPRIDIL IN HUMAN PLASMA USING GAS CHROMATOGRAPHY WITH NITROGEN-SENSITIVE DETECTION

JAN VINK* and HENK J.M. VAN HAL

Drug Metabolism R&D Labs, Organon International B.V., P.O. Box 20, 5340 BH Oss (The Netherlands)

and

JEAN-FRANÇOIS POGNAT and JEAN-LOUIS BOUQUET DES CHAUX

Centre Européen de Recherches Mauvernay, Route de Marsat, 63203 Riom (France)

(First received May 28th, 1982; revised manuscript received August 5th, 1982)

SUMMARY

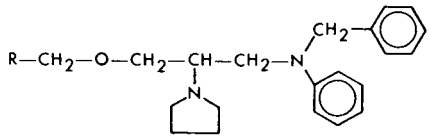
An assay has been developed to determine the anti-ischaemic drug bepridil (as its free base) in human plasma. The assay procedure comprises *n*-hexane extraction from basic plasma and gas chromatography using nitrogen-selective detection. An analogue of bepridil is used as internal standard. The accuracy and precision of the assay is determined by repeated analyses of drug-free plasma samples spiked with 5, 10, 20, 100, 400 and 1000 ng of bepridil per ml of plasma. The accuracy, defined as the relative difference between the mean bepridil concentration found and the true value, was 8% or better. The precision (relative standard deviation) was 13% at the 5 ng/ml level and 5% at the 1000 ng/ml level. The assay is suitable to monitor routinely bepridil plasma levels during clinical studies.

INTRODUCTION

In animal studies, bepridil (β-[(2-methylpropoxy)methyl]-N-phenyl-N-phenylmethyl-1-pyrrolidineethanamine; Fig. 1, 1) has been shown to possess anti-anginal [1—3] and anti-arrhythmic [4, 5] properties. In a recent clinical study [6], the efficacy of bepridil in the treatment of angina pectoris has been demonstrated.

To assess the pharmacokinetics of bepridil and the bioavailability of bepridil from different pharmaceutical formulations or to measure plasma levels of bepridil in clinical efficacy studies, assay methods have been developed for its

0378-4347/83/0000—0000/$03.00



Bepridil 1: R = $(CH_3)_2CH$

Internal standard 2: R = H

Fig. 1. Structure of bepridil and internal standard.

quantitative determination. In the present paper, the assay for the determination of bepridil in human plasma is reported. The assay is based on gas chromatography (GC) with nitrogen-selective detection using an analogue of bepridil as internal standard. Gas chromatography with nitrogen detection has been established as a reliable method for the quantitation of nitrogen-containing drugs in biological fluids using closely related compounds (in structure) as internal standards [7].

MATERIALS AND METHODS

Chemicals and glassware

The internal standard (Fig. 1, 2), the methyl-ether analogue of bepridil, was obtained from CERM-Reti, Riom, France.

The solvents *n*-hexane and methanol (Merck, Darmstadt, G.F.R.) were of Uvasol and analytical-reagent grade, respectively. Disodium hydrogen phosphate was purchased from Merck.

Solvent extraction of plasma aliquots was performed in 20-ml screw-capped disposable glass vials. The 10-ml conical glass tubes and 250-μl screw-capped conical glass vials (used for extract concentration and injection into the gas chromatograph, respectively) were rinsed three times with ethanol prior to use. In experiments to determine the recovery of bepridil from plasma during extraction and concentration, tritium-labelled bepridil was used. [^{3}H]Bepridil was obtained from the Organic Synthesis Group, Drug Metabolism R&D Labs, Organon International B.V., Oss, The Netherlands.

Gas chromatography

A Hewlett-Packard gas chromatograph Model 5710A, equipped with a 4 m × 2 mm I.D. glass column packed with 3% OV-17 on Gas-Chrom Q 80—100 mesh, was used. Chromatographic analyses were performed isothermally at a column temperature of 270°C. The temperature of the injector and N/P detector (Hewlett-Packard Model 18789A) was 300°C. Nitrogen was used as carrier gas at a flow-rate of 30 ml/min. The gas flow-rate for the N/P detector was 3 ml/min for hydrogen and 50 ml/min for air. The collector voltage was 15—20 V.

Assay procedure

The design of the clinical study, the administered dose and time of blood sampling was used to predict the bepridil plasma level which in turn was used

as the rationale to determine the volume of plasma to be processed. For expected bepridil levels in the range of 10—100 ng/ml, an aliquot of 1 ml of plasma was taken; for expected bepridil levels below 10 ng/ml generally more than 1 ml (maximum 3 ml) of plasma was processed; at expected bepridil levels above 100 ng/ml usually less than 1 ml of plasma was taken with a minimum manageable volume of 100 μl. The plasma aliquots were pipetted into 20-ml disposable glass vials. The internal standard was added to plasma as an aqueous solution containing 100 or 1000 ng/ml. The amount of internal standard added was approximately the same as the anticipated amount of bepridil in the plasma sample. The plasma was mixed thoroughly by vortexing and allowed to equilibrate for at least 30 min. The plasma pH was adjusted to approximately 8.9 by addition of a saturated Na_2HPO_4 solution. The volume of the Na_2HPO_4 buffer equalled the volume of plasma taken, with a minimum of 0.5 ml. The diluted, basic plasma was mixed thoroughly. The plasma sample was extracted with approximately 10 ml of *n*-hexane by vortexing, followed by centrifugation at 1500 *g*. The *n*-hexane extract was transferred to a 10-ml conical glass tube and the solvent evaporated under a gentle stream of nitrogen at 40°C. A second extraction of the water phase was carried out with approximately 8 ml of *n*-hexane. The organic layer was evaporated to dryness under nitrogen in the 10-ml tube containing the residue obtained after the first *n*-hexane extraction. The wall of the glass tube was rinsed thoroughly with 500 μl of methanol, which was evaporated under nitrogen at 40°C. The residue was taken up in 200 μl of methanol and transferred to a 250-μl conical glass tube. The extract was concentrated under a gentle stream of nitrogen at 40°C and taken up in 20—100 μl of methanol. An aliquot of this methanolic solution was injected into the gas chromatograph equipped with a nitrogen-sensitive detector.

Calibration

A series of drug-free plasma samples spiked with bepridil and its internal standard in different concentration ratios were prepared for calibration purposes. The series comprised plasma samples with a 1:1 concentration ratio of bepridil to internal standard and with concentration ratios around this unity ratio, e.g. 1:3, 1:2, 2:1 and 3:1. In each sample prepared for calibration the amount of bepridil and internal standard did not differ substantially from the amounts expected in the clinical samples. The samples prepared for calibration were processed in the same way as the clinical plasma samples received for analysis.

Quality control on the results of analysis

On each day of measurement, clinical plasma samples containing bepridil, samples prepared for calibration, drug-free plasma samples (blanks) and drug-free samples spiked with known amounts of bepridil were analyzed randomly. Analysis of drug-free plasma samples with and without bepridil added, ensured continuous quality control of the assay procedure. Clinical plasma samples in which the ratio of bepridil to internal standard eventually proved to be outside the upper or lower limit used in the series of calibration samples, were re-analyzed with adapted amounts of internal standard.

Automation and data handling

The laboratories involved in the development of an assay for bepridil had access to different facilities for automation and data handling. At Organon the following instruments were employed to facilitate the routine determination of bepridil plasma levels: a programmable microprocessor-controlled handpipette (MicroLab P from Hamilton, Bonaduz, Switzerland) was used to take aliquots of the plasma samples and to add the internal standard to plasma; a Hewlett-Packard 7672A automatic sampler was used for injection of plasma extracts into the GC column.

A Varian CDS 400 V laboratory data system equipped with a 983 K disc was used for data acquisition, subsequent data integration and temporary data storage. The integrated data were transmitted as peak heights found within a specified retention time window to a DEC PDP-11/RSTS computer network. Calibration functions and bepridil plasma levels were calculated using application programs written in BASIC.

At CERM-Reti, the gas chromatograph was equipped with a Hewlett-Packard 7672A Automatic Sampler. A Hewlett-Packard integrator type 3390A was used for signal integration. Further data handling was carried out with a Texas Instruments TI 51-III calculator using internally developed application programs.

Clinical study

To demonstrate the applicability of the assay method, plasma samples from a clinical study were analyzed. The dosage regimen in this study was such that steady-state levels were reached within two days. Bepridil monohydrochloride monohydrate was administered as an intravenous loading dose of 2 mg/kg given as an infusion over a period of 5 min. Four, 12 and 20 h after the infusion, oral doses of three tablets containing 100 mg of bepridil monohydrochloride monohydrate each were given; this was followed by oral doses of one 100-mg tablet at intervals of 8 h.

Blood samples were collected at regular intervals after the end of infusion and during oral drug administration just prior to each new dose. The blood was centrifuged and plasma samples were stored at −20°C until required for analysis.

RESULTS AND DISCUSSION

Recovery of bepridil and internal standard from plasma

In triplicate experiments with labelled bepridil at a plasma level of 100 ng/ml, 68.1% (S.D. 0.7%) of the amount of bepridil added to drug-free plasma appeared in the methanolic solution, from which aliquots were subjected to GC analysis. The observed "loss" of 32% of bepridil during the assay procedure included extraction as well as manipulation losses. However, this acceptable recovery of bepridil in the methanolic solution did not guarantee a similar recovery of the analogue used as internal standard. It was observed during the initial phase of the development of the assay that, using plasma samples from different sources spiked with known amounts of bepridil and internal standard, the peak height ratios of bepridil to internal standard fluctuated considerably. This phenomenon was attributed to differences between the plasma protein

binding of bepridil and its internal standard. Incubation of a bepridil-containing plasma sample with the internal standard for at least 30 min, dilution of plasma with the aqueous solution of the internal standard, addition of the concentrated Na_2HPO_4 buffer solution and the double *n*-hexane extraction were the measures taken to obtain reproducible peak height ratios of bepridil to internal standard.

Decomposition problems

Additional peaks not well separated from the peaks corresponding to bepridil and the internal standard appeared in the gas chromatograms when the GC column had been in use for a long time period. This was due to decomposition of bepridil and, to a lesser extent, of the internal standard. The decomposition problem was avoided by applying inert quartz wool to the injection side of the packed column and by regular changing of the quartz wool followed by careful conditioning of the column.

Selectivity

The selectivity of the assay is demonstrated in Fig. 2, which gives gas chromatograms of processed plasma samples from drug-free plasma, drug-free plasma spiked with a known amount of bepridil and human plasma from a clinical study. Interference from endogenous human plasma components was not observed at the retention times where bepridil and the internal standard eluted from the GC column. Interference from possible bepridil biotransformation products was unlikely judging from the gas chromatogram obtained after identical processing of a urine sample from a volunteer receiving chronic bepridil treatment. It is considered that the human metabolites of bepridil are either too polar to be extracted under the assay conditions used or can easily be separated from bepridil and its internal standard on the GC column.

Calibration curve

The calibration curve for the bepridil assay was calculated by linear or polynomial regression analysis. With the polynomial regression analysis using $y = ax^2 + bx + c$, where for example $a = 0.022$, $b = 0.692$, and $c = -0.021$, the deviation from linearity was, as expected, small.

Detection limit

From the signal and noise in the chromatograms obtained after processing a 3-ml plasma sample spiked with 3 ng of bepridil per ml (Fig. 2B), it was deduced that 1 ng of bepridil per ml of plasma could be detected with a signal-to-noise ratio of more than 10.

Accuracy and precision

The relative difference between the mean bepridil concentration found and the true value, and the relative standard deviation as measures for the accuracy and precision of the bepridil assay, respectively, were determined by analyzing drug-free plasma samples spiked with known amounts of bepridil. These spiked samples were analyzed concomitantly with plasma samples received from the clinical study over a period of one month. The accuracy and precision at the

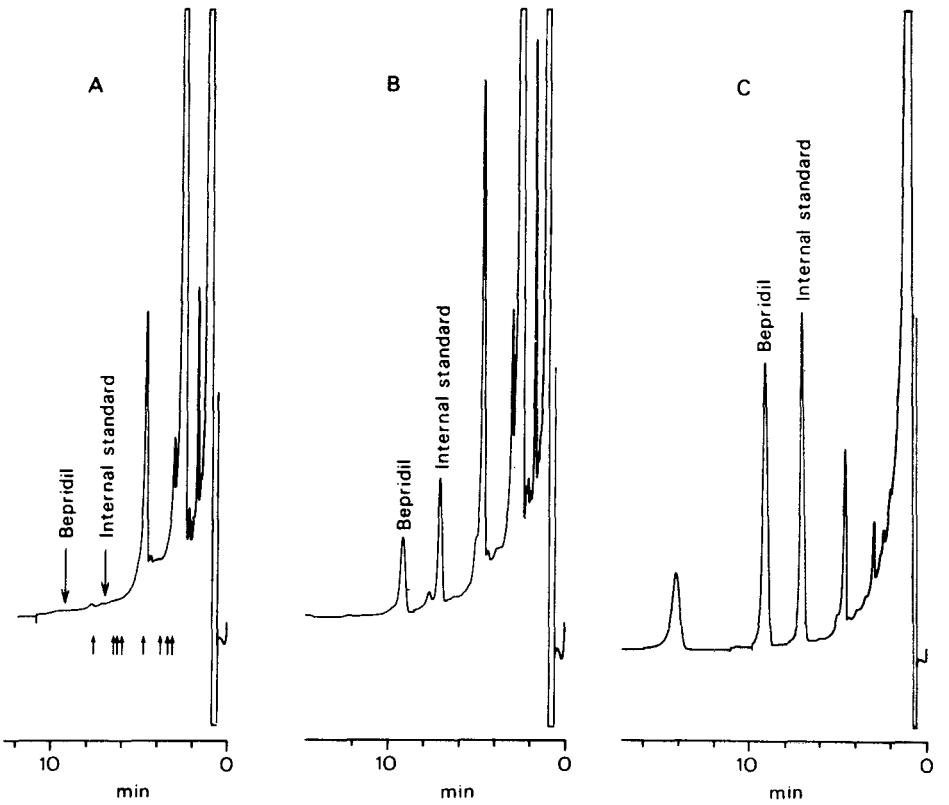


Fig. 2. Gas chromatograms of processed human plasma. (A) Drug-free human plasma. The expected positions of bepridil, the internal standard and metabolites extractable from human urine are indicated by arrows. The volume of plasma processed was 1 ml. (B) Spiked drug-free human plasma. Three nanograms of bepridil and 3 ng of internal standard per ml were added to 3 ml of blank plasma. (C) Clinical plasma sample. Fifty nanograms of the internal standard were added to 0.5 ml of the sample; the bepridil concentration was calculated to be 140 ng/ml.

5, 10, 20, 100, 400, 1000 ng/ml plasma levels are given in Table I.

The standard deviation as a function of the concentration can also be expressed in terms of an error model used for data point weighting in pharmacokinetic curve-fitting programs [8]. According to this error model, $s^2 = a(\bar{C})^b$, where s^2 is the square of standard deviation (variance), a and b are the appropriate coefficients and $\bar{C}$ is the mean concentration. A plot of the log (variance) versus the log (mean concentration) yielded a straight line ($r = 0.997$). The error model for the bepridil assay was $s^2 = 0.0186(\bar{C})^{1.731}$, which could be used to estimate the standard deviation at different plasma levels. For instance, at the 250 ng/ml level, the relative standard deviation as a measure of precision is expected to be 6.5%.

Routine application of the assay

The time course of plasma levels in one patient from the clinical study is shown in Fig. 3. The bepridil (free base) plasma level was 8 μg/ml 2 min

TABLE I

ASSAY CHARACTERISTICS DETERMINED BY ANALYSIS OF 1-ml DRUG-FREE PLASMA SAMPLES SPIKED WITH DIFFERENT AMOUNTS OF BEPRIDIL

Bepridil added (ng/ml)	No. of determinations	Mean value found (ng/ml)	Standard deviation (ng/ml)	Precision (%)	Accuracy (%)
1000	15	996	51	5	−1
400	20	392	24	6	−2
100	21	95.2	8.4	9	−5
20	16	20.2	1.7	8	+1
10	5	10.2	0.8	8	+2
5	5	4.6	0.6	13	−8

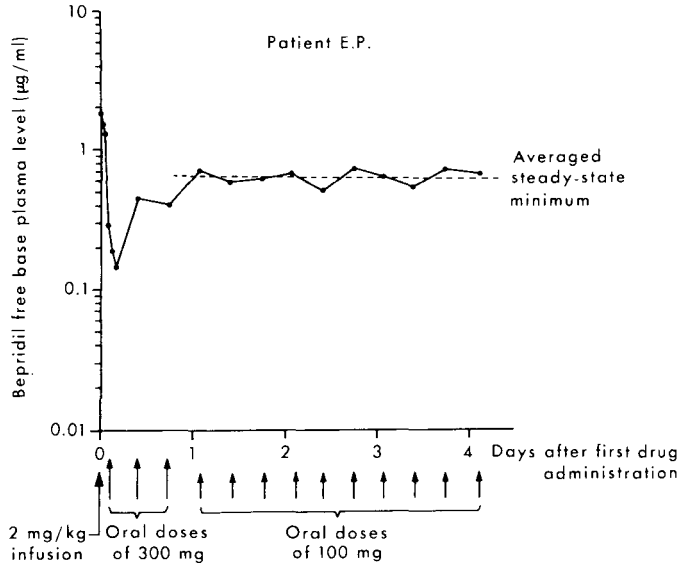


Fig. 3. Bepridil plasma levels vs. time in a clinical experiment undertaken by patients receiving an intravenous loading dose followed by oral drug administrations.

after the infusion finished; the plasma level declined to 140 ng/ml in the 4-h period preceding the first oral administration of 300 mg of bepridil monohydrochloride monohydrate, while the steady-state plateau with an average plasma level of 630 ng/ml was reached in approximately one day.

CONCLUSIONS

The GC assay method developed for the determination of bepridil in human plasma is sensitive, selective and applicable for routine measurement of plasma levels of bepridil in clinical studies. The use of GC with nitrogen-sensitive detection has produced an assay simply involving extraction of bepridil and the internal standard from basic plasma, followed by chromatography and detec-

tion without the need for purification of plasma extracts prior to chromatography. The selectivity for the determination of the unchanged drug with regard to possible metabolites and endogenous plasma components was guaranteed by the conditions chosen for extraction, GC separation and detection. The assay characteristics, expressed in terms of the precision and accuracy determined by analyzing spiked human plasma samples, allow bepridil plasma level measurement in pharmacokinetic and bioavailability studies.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. Paul Koppens and Mrs. Janny van de Logt who were involved in development of the bepridil assay.

The clinical study employing an intravenous loading dose followed by oral drug administration was supervised by Drs. Rowland and Krikler of the Hammersmith Hospital, London, Great Britain. Plasma and urine samples used to demonstrate the assay specificity originated from a bioequivalence study performed by Prof. K. Schnelle, IPHAR Institute for Clinical Pharmacology, 8012 Ottobrunn, G.F.R.

REFERENCES

1 M.T. Michelin, M. Cheucle and P. Duchene-Marullaz, Therapie, 32 (1977) 485.
2 D. Cosnier, P. Duchene-Marullaz, G. Rispat and G. Streichenberger, Arch. Int. Pharmacodyn. Ther., 225 (1977) 133.
3 P. Piris, M. Beaughard, D. Cosnier and C. Labrid, Arch. Int. Pharmacodyn. Ther., 235 (1978) 147.
4 C. Chassaing, N. Moins, J. Lavarenne and P. Duchene-Marullaz, J. Pharmacol., 8 (1977) 503.
5 M. Boucher and P. Duchene-Marullaz, Arch. Int. Pharmacodyn. Ther., 233 (1978) 65.
6 J.C. Canicave, J. Deu and F.X. Lesbre, Lyon Med., 243 (1980) 107.
7 A. Richens and V. Marks, Therapeutic Drug Monitoring, Churchill Livingstone, Edinburgh, 1981.
8 J.G. Wagner, Fundamentals of Clinical Pharmacokinetics, Drug Intelligence Publications, Hamilton, IL, 1975, p. 288.

Journal of Chromatography, 272 (1983) 95—102
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1469

GAS—LIQUID CHROMATOGRAPHIC EVALUATION OF FENQUIZONE IN BIOLOGICAL SAMPLES FOR PHARMACOKINETIC INVESTIGATIONS

A. MARZO*, G. QUADRO and E. TREFFNER

Medea Research, Scientific Division, Laboratory of Drug Metabolism and Pharmacokinetics, Via Pisacane 34/a, 20129 Milan (Italy)

(First received May 3rd, 1982; revised manuscript received August 3rd, 1982)

SUMMARY

Extractive alkylation was carried out on fenquizone, a sulphonamide diuretic, in order to devise a suitable method for its determination in pharmacokinetic and bioavailability studies. After extraction as a tetramethyl derivative, fenquizone was evaluated by gas—liquid chromatography with a ^{63}Ni electron-capture detector, which enables a limit of detection of 2 ng/ml of plasma or urine to be achieved. Linearity was verified in a range of 50— 10,000 pg for each injection with a fenquizone/internal standard ratio ranging from 4:1 to 1:4. Determination is very rapid, as one analysis only takes 5 min.

The preliminary results of the pharmacokinetic study performed in a volunteer human subject after a single oral administration of the drug are presented in this paper in terms of the plasma levels and the cumulative urinary excretion.

INTRODUCTION

Fenquizone (2-phenyl-6-sulphonamido-7-chloro-1,2,3,4-tetrahydro-4-quinazolinone) is a diuretic with a quinazoline structure like quinethazone and metolazone [1,2]. From the point of view of the structure—activity relationship, position 1 may be occupied by a CO- or an SO_2-group, shifting from quinazolinone to benzothiazine derivatives, such as chlorothiazide, hydrochlorothiazide, etc. [2].

The main interest of fenquizone lies in its activity at a single daily dose of 10—20 mg, which is quite low compared to other diuretics, and in its very good tolerability [3,4].

The pharmacokinetics of fenquizone were previously investigated in several animal species by detecting the ^{14}C-labelled drug in body fluids [5,6].

This paper reports an analytical method performed to achieve a sensitivity

0378-4347/83/0000—0000/$03.00

of 2 ng/ml which is required for human pharmacokinetic and bioavailability studies.

EXPERIMENTAL

Drugs and chemicals

Samples of fenquizone were supplied by Maggioni (Milan, Italy), and penfluridol by Studio Chimica (Milan, Italy).

All reagents were of analytical grade and were supplied by Merck (Darmstadt, G.F.R.).

Extractive alkylation procedure

Extractive alkylation was performed in a stoppered tube on 1 ml of human plasma or urine (2 ml may be used to increase sensitivity) to which 0.2 g of $NaHCO_3$ and 5 ml of methyl isobutyl ketone were successively added. The mixture was vigorously stirred and then centrifuged at 2400 *g* for 10 min. An aliquot of the organic layer was transferred to a second tube and alkalinized with 3 ml of 0.1 *N* sodium hydroxide and then shaken and centrifuged.

The organic layer was discarded and an aliquot of the aqueous layer was transferred to another test tube together with 5 ml of 0.5 *M* iodomethane in dichloromethane and 50 μl of 0.1 *M* tetrahexylammonium acid sulfate in dichloromethane. The test tube was stoppered and kept at 50°C under stirring for 20 min, after which it was cooled to room temperature and centrifuged. The organic layer, containing the tetramethyl derivative of fenquizone (Fig. 1), was dried over 500 mg of anhydrous Na_2SO_4, evaporated to dryness and finally dissolved in 100 μl of acetone. Penfluiridol was added as the internal standard (Fig. 2). This solution was ready for gas—liquid chromatographic (GLC) analysis and 1—3 μl were injected.

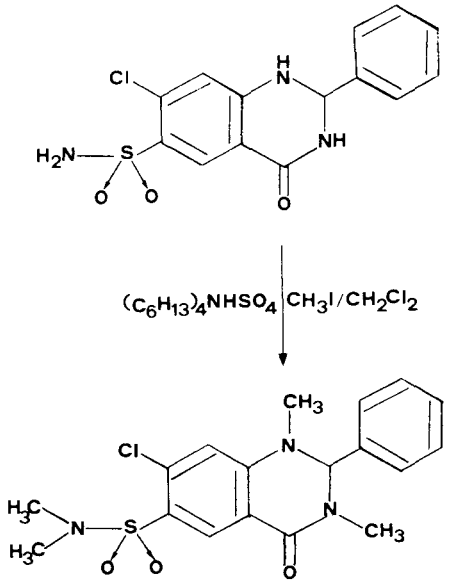
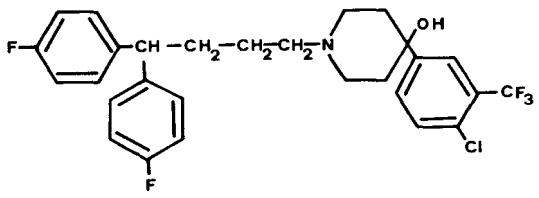


Fig. 1. N-Methylation of fenquizone.



Penfluridol

Fig. 2. Structure of penfluridol.

GLC analysis

A Perkin-Elmer Sigma 3B gas chromatograph equipped with an electron-capture detector (^{63}Ni) was employed. A silanized glass column (35 cm × 6 mm O.D. × 2 mm I.D.) was packed with 30% OV-101 on 80—100 mesh Chromosorb W AW. Oven, injection port and detector temperatures were maintained at 300°C, 320°C and 350°C, respectively. Nitrogen was used as carrier gas at a flow-rate of 60 ml/min.

The following retention times were measured: 3 min 40 sec for the fenquizone tetramethyl derivative, and 2 min 48 sec for the internal standard (I.S.) (Fig. 3). The relative retention volume of fenquizone was 1.31, assuming that of the I.S. to be 1.00.

The chemical identity of fenquizone was confirmed by GLC—mass spectrometry (Fig. 4) and the same was done to demonstrate that penfluridol was eluted unchanged.

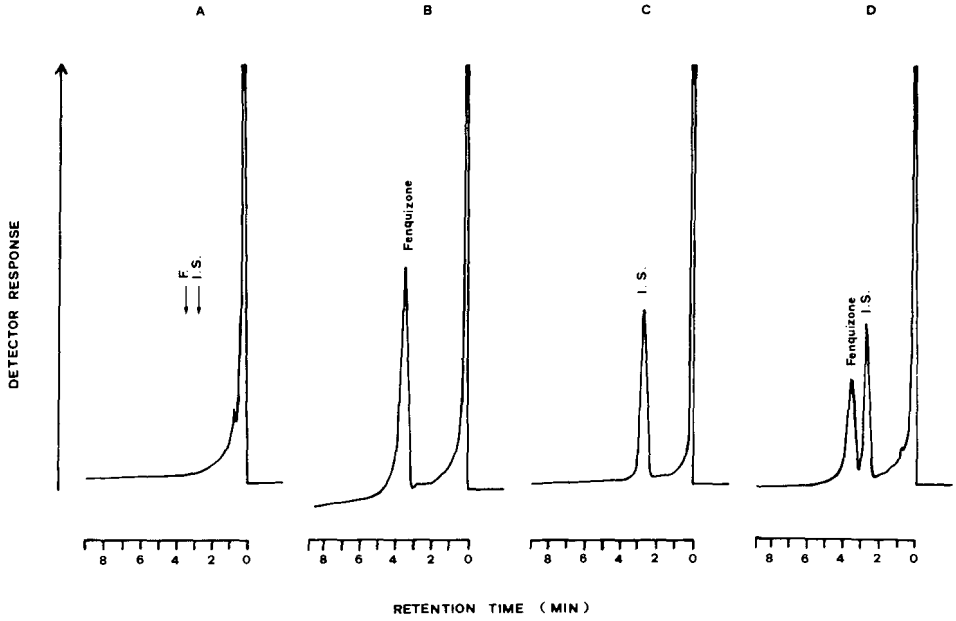


Fig. 3. Gas chromatograms of (A) a blank plasma sample in the absence of either fenquizone derivative or I.S., (B) fenquizone tetramethyl derivative, (C) penfluridol, and (D) fenquizone and I.S. after the described extraction procedure from plasma.

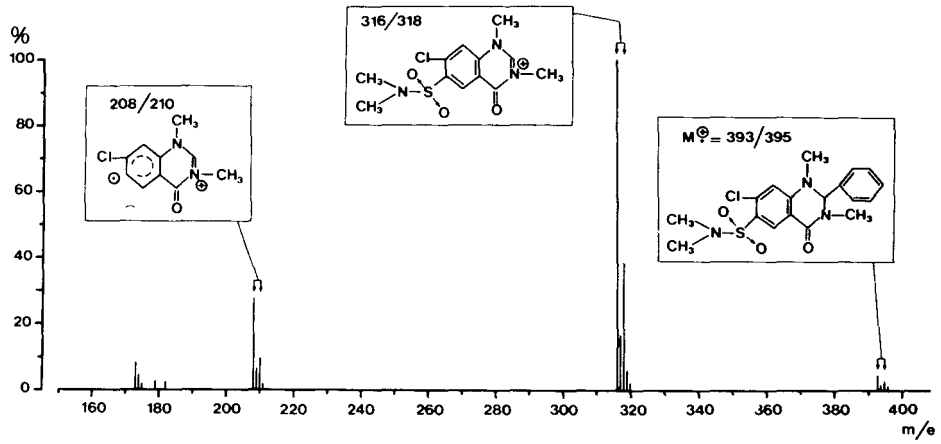


Fig. 4. Mass spectrum of the tetramethyl derivative of fenquizone, which confirms the chemical identity of the derivative used in GLC analysis.

Calibration curves, reproducibility and linearity of detector response

In order to evaluate the analytical reproducibility and the linearity of the detector response the following tests were performed:

(A) Nine different amounts of fenquizone (range 10–5000 pg) were injected into the gas chromatograph together with the I.S. at a fixed drug/I.S. ratio of 1:2. Each injection was performed twice.

(B) Fenquizone was mixed with the I.S. so as to obtain the five following drug/I.S. ratios: 1:0.25; 1:0.5; 1:1; 1:2; 1:4. Each injection was performed twice.

(C) A solution containing 250 pg of fenquizone and 500 pg of I.S. per μl was injected ten times into the gas chromatograph; the volume of each injection was 1 μl.

The recovery of the extraction was evaluated by adding nine different amounts of fenquizone, ranging from 2 to 1000 ng, to 1 ml of plasma and performing four tests at each concentration. A similar investigation was carried out on the urine in a range of concentration of the drug between 100 and 5000 ng/ml.

The concentration of fenquizone in plasma was evaluated from the fenquizone/I.S. area ratio of the peaks, which was corrected by the detector response factor and the recovery.

RESULTS AND DISCUSSION

Choice of the internal standard

Fig. 3 shows the gas chromatograms of a blank plasma sample without fenquizone, of fenquizone and of the internal standard. A series of diuretics with structures similar to that of fenquizone (furosemide, chlorothiazide, hydrochlorothiazide, chlorthalidone) were first tested as internal standards, but then discarded because their retention times were too short and there was interference between their peaks and the solvent tail. To improve resolution the

choice of a longer column and a lower temperature would have been necessary with a decrease in sensitivity and a loss of time as a consequence.

Penfluridol {1-[4,4-Bis(4-fluorophenyl)butyl]-4-bis(*p*-fluorophenyl)butyl-4-(4-chloro-α,α,α-trifluoro-*m*-tolyl)-4-piperiolinol} ($C_{28}H_{27}ClF_5NO$, M.W. = 523.99) is not structurally correlated with the thiazides (Fig. 2), but it is well evaluated in GLC by the electron-capture detector because of its six halogen atoms. Also it is quite stable because it eluted unchanged during GLC analysis, which was demonstrated by GLC—mass spectrometry, and it has a suitable retention time which allows clear separation from the fenquizone derivative and from the solvent peak (Fig. 3) to be achieved.

Overall recovery

The recovery of fenquizone extraction from plasma is depicted in Table I. By varying the amount of fenquizone added to 1 ml of plasma, the percentage recovery did not change. The mean value of the recovery, evaluated from the 36 tests, was 89.92 ± 7.10% (S.D.).

The linear relationship between fenquizone added to 1 ml of plasma (X) and fenquizone recovered (Y) was established by the least-squares method and was characterized by a linear regression coefficient of 0.9998. This linear correlation may be expressed by the function $Y = a + bX$, where $a = 0.019$ and $b = 0.9029$. The slope b, in percentage 90.29%, is very close to the mean recovery value directly calculated from the 36 tests performed.

The recovery of fenquizone from urine also gave good results with an average of 90%.

TABLE I

RECOVERY OF FENQUIZONE ADDED TO 1 ml OF PLASMA IN DIFFERENT AMOUNTS RANGING FROM 2 TO 1000 ng

Mean values, each obtained from four assays, are reported with their S.D.

Fenquizone added (ng)	Fenquizone found (ng)	Recovery* (%)
2	1.77 ± 0.24	88.75 ± 11.96
5	4.21 ± 0.44	84.25 ± 8.80
10	8.60 ± 0.65	86.00 ± 6.48
20	18.75 ± 1.63	93.75 ± 8.14
50	43.87 ± 2.72	87.75 ± 5.44
100	89.75 ± 6.60	89.75 ± 6.60
200	174.50 ± 9.98	87.25 ± 4.99
500	465.00 ± 27.39	93.00 ± 5.48
1000	897.50 ± 55.60	89.75 ± 5.56

*Mean recovery calculated from the 36 assays and covering the whole range is 88.92 ± 7.10%.

Reproducibility and linearity

Reproducibility was expressed as percentage S.D. in relation to the analytical data found in different assays. When the same solution containing 250 pg of fenquizone and 500 pg of I.S. per μl was injected ten times into the gas chromatograph, an S.D. of 2.2% could be determined. The S.D. reached 5.5%

by varying the fenquizone/I.S. ratio between 1:0.25 and 1:4 and rose to 6.3 when increasing fenquizone amounts from 10 to 5000 pg were injected at a fixed fenquizone/I.S. ratio. In both the latter cases the linearity of the response was well verified with an r value (correlation coefficient) very close to 1 (Table II).

Reproducibility in a complete analysis which includes all analytical manipulations can be evaluated from the recovery test (Table I).

An S.D. of 8.0% could be calculated as mean value from the 36 assays.

TABLE II

REPRODUCIBILITY AND LINEARITY OF DETECTOR RESPONSE IN FENQUIZONE ANALYSIS CARRIED OUT UNDER THREE DIFFERENT CONDITIONS

Condition A: varying the fenquizone injected at a fixed drug/I.S. ratio. Condition B: injecting a fixed amount of fenquizone and varying the drug/I.S. ratio. Condition C: fixing both parameters (ten assays).

Condition	Fenquizone injected (pg)	Drug/I.S. ratio	Detector response factor ± S.D.	S.D. (%)	Linear regression coefficient
A	From 10 to 5000, nine assays, each performed twice	1:2	0.441 ± 0.028	6.3	0.9994*
B	250	From 4 to 0.25, five different ratios, each performed twice	0.435 ± 0.024	5.5	0.9972**
C	250	1:2	0.446 ± 0.0097	2.2	

*Fenquizone injected versus fenquizone detected.
**Fenquizone/I.S. weight ratio versus area ratio.

Sensitivity

When tetramethyl fenquizone was injected into the gas chromatograph without any endogenous interference, small amounts of 5 or 10 pg gave a detectable response. But when fenquizone was added to a biological fluid and then extracted and analysed the lowest detectable concentration was 2 ng/ml with a reproducibility of 13.5% (S.D.). Even a concentration of 1 ng/ml could be determined, but in this case the S.D. rose to 20%.

Preliminary pharmacokinetic investigations

The extractive alkylation method just described, which had been already performed with other diuretics [7], also gave a satisfactory response with fenquizone. Indeed its high sensitivity (2 ng/ml) allows pharmacokinetic and bioavailability studies to be performed on human subjects. Fig. 5 shows plasma levels of fenquizone and Fig. 6 its cumulative urinary excretion in one healthy volunteer orally treated with 10 mg of the drug as its poassium salt, which corresponds to 8.937 mg of fenquizone as sulphonamide. Extractive alkylation may be carried out simultaneously on a series of samples and subsequent GLC analysis of each sample only takes about 5 min. Analysis is thus fairly rapid and inexpensive.

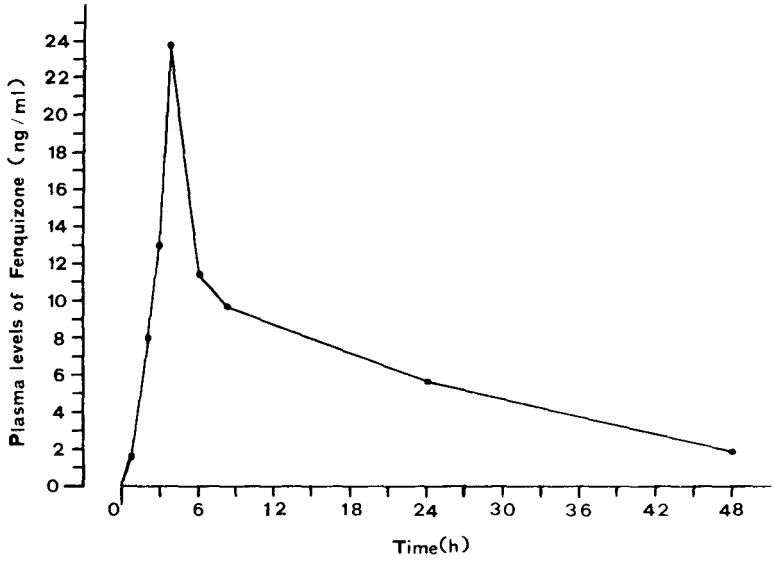


Fig. 5. Plasma levels of fenquizone determined in a human volunteer orally treated with 10 mg of the drug as its potassium salt.

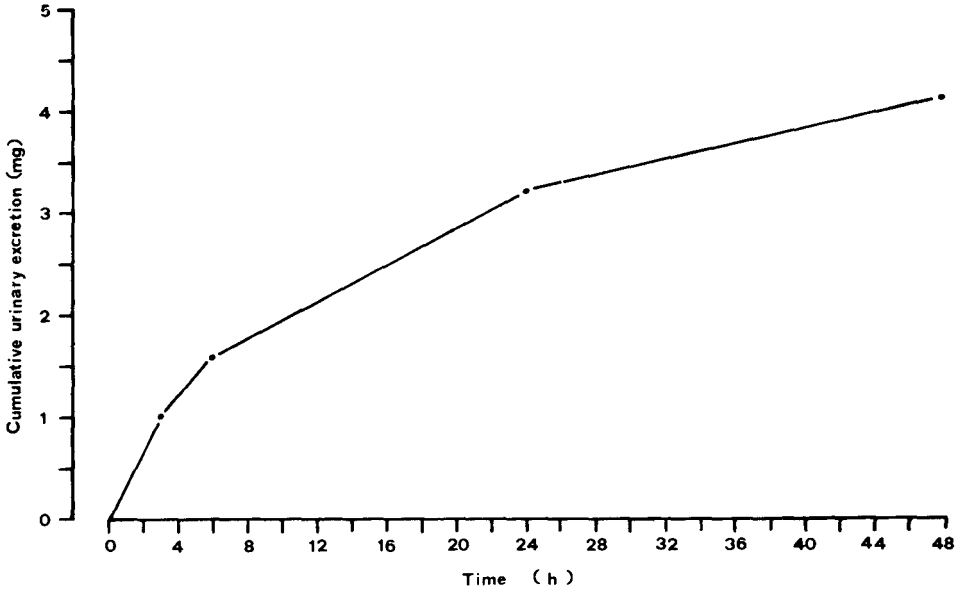


Fig. 6. Cumulative urinary excretion of fenquizone in the volunteer orally treated with 10 mg of the drug as its potassium salt.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Prof. G.C. Maggi and his team from the Bassini Hospital, Cardiology Division, Cinisello Balsamo (Milan),

who performed the drug administration and the related blood and urine sampling, and to Maggioni Farmaceutici S.p.A. for financial support.

REFERENCES

1 M.G. Biressi, G. Cantarelli, M. Carissimi, A. Cattaneo and F. Ravenna, Farmaco Ed. Sci., 24 (1969) 199.
2 O. Le Roy Salerni, Natural and Synthetic Organic Medicinal Compounds, C.V. Mosby Co., St. Louis, MO, 1976, p. 255.
3 C. Ferrando, J.M. Foy, C.N.F.W. Prott and J.R. Purvis, J. Pharm. Pharmacol., 33 (1981) 219.
4 C. Pasotti, R. Rossi and P. Gandolfi, Minerva Med., 72 (1981) 1.
5 D. Ceriani, A.M. Di Giulio and M. Panelli, Farmaco Ed. Sci., 5 (1976) 379.
6 G.F. Placidi, P. Fornaro and M. Guarnieri, Arzneim.-Forsch., 29 (1979) 1005.
7 M. Erwick and K. Gustavii, Anal. Chem., 46 (1974) 39.

Journal of Chromatography, 272 (1983) 103—110
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1470

GAS CHROMATOGRAPHIC DETERMINATION OF METOPROLOL IN HUMAN PLASMA

ANTOINE SIOUFI*, FREDERIQUE LEROUX and NADINE SANDRENAN

Ciba-Geigy, Centre de Recherche Biopharmaceutique, B.P. 308, 92506 Rueil-Malmaison Cedex (France)

(Received July 1st, 1982)

SUMMARY

A method for the determination of metoprolol at concentrations down to 10 ng/ml in human plasma is described. After addition of oxprenolol as internal standard, both compounds are extracted into diethyl ether—dichloromethane (4 :1, v/v) at basic pH, transferred into an acidic aqueous solution and back-extracted at basic pH into diethyl ether—dichloromethane. They are then derivatized with heptafluorobutyric anhydride. The derivatives are quantitatively determined by gas chromatography using a ^{63}Ni electron-capture detector. The linearity was demonstrated, and the technique was formally validated in the concentration range 10—500 ng/ml. The technique was applied in a study of the bioavailability of metoprolol after oral administration to man; mean plasma concentrations are reported.

INTRODUCTION

Various methods for the assay of metoprolol (Fig. 1, I) have been published. A gas chromatographic—mass spectrometric determination of metoprolol and its basic metabolites in plasma has been reported [1]. Several high-performance liquid chromatographic (HPLC) methods have been described [2—5], but they are all general methods for the assay of a series of β-adrenoreceptor blocking drugs. Gas chromatographic (GC) procedures [6—8] using electron-capture detection have also been described for the determination of metoprolol in plasma, but they suffer from certain disadvantages: they require 2 ml of plasma, and the internal standard is added just before injection [6,7]. The method recently described by Kinney [8] is sensitive, but needs a very high carrier-gas flow-rate (230 ml/min), which is not attainable with the gas chromatographs generally used.

This paper describes a GC assay procedure that permits the quantitative

0378-4347/83/0000—0000/$03.00

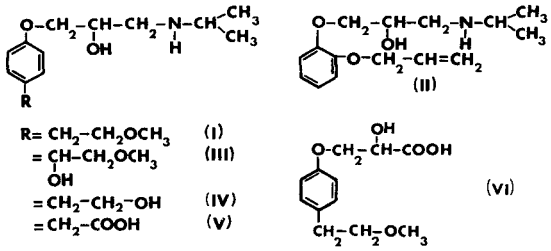


Fig. 1. Chemical structure of metoprolol (I), internal standard (oxprenolol, II), and main metoprolol metabolites (III, IV, V, VI).

determination of metoprolol in plasma down to 10 ng/ml. The technique makes use of an internal standard introduced into the plasma sample to correct for losses during extraction and derivatization. The extraction solvent used is the same as the one already described by Degen and Riess [9]. The specificity of the assay as regards the metabolites of metoprolol is demonstrated.

EXPERIMENTAL

Chemicals and reagents

Metoprolol and the internal standard oxprenolol (Fig. 1, II) were supplied by Ciba-Geigy (Basle, Switzerland).

Alkaline buffer (pH>13) was prepared by dissolving 30 g of tripotassium phosphate 3-hydrate (Merck 5102; E. Merck, Darmstadt, G.F.R.), or 38 g of tripotassium phosphate 7-hydrate (Merck 5103) and 16.8 g of potassium hydroxide (Merck 5033) in ultra-high-purity HPLC water (Alltech Assoc., Arlington Heights, IL, U.S.A.) and making the volume up to 100 ml. All reagents and solvents were of analytical grade: diethyl ether (Pestipur quality, S.D.S., Valdonne, France), hydrochloric acid (Merck 318), hexane (Pestipur quality, S.D.S.). Pyridine (Fluka 82702; Fluka, Buchs, Switzerland) was distilled at 115–116°C with potassium hydroxide pellets and stored over the same reagent. Heptafluorobutyric anhydride was purchased from Ventron (Ref. PCR, 1300-3; Ventron, Karlsruhe, G.F.R.); potassium dihydrogen phosphate (Merck 4873) was used as a saturated solution.

Calibration curves

Aliquots of 50 µl of six different aqueous solutions of metoprolol tartrate were added to 1 ml of plasma to produce reference samples in the range of concentrations 10–500 ng/ml of plasma.

The aqueous internal standard solution contained 1000 ng/ml of oxprenolol hydrochloride, and 100 µl of this solution were added to each plasma sample, resulting in a concentration of 100 ng/ml of oxprenolol in plasma.

Calibration solutions are prepared every ten days and stored at 4°C.

Equipment

All glassware was washed, dried at 100°C and immersed for 0.5 h in an ultrasonic bath, first in water and then in methanol. The dried glassware was then treated to prevent adsorption. It was immersed in a silanizing solution con-

taining hexamethyldisilazane, trimethylchlorosilane and pyridine (1% v/v each) for 15 min and rinsed with methanol. Silanization is unnecessary if quartz tubes are used instead of glass tubes.

A Hewlett-Packard (Model 5713 A) gas chromatograph equipped with a Hewlett-Packard electron-capture detector (Model 18713A) was used. The peak areas were calculated by a Hewlett-Packard computing integrator (Model 3388A) connected to the chromatograph. The column was operated at 200°C, the injector at 250°C and the detector at 300°C, with argon–methane (90:10) at a flow-rate of 60 ml/min. The glass column (2 m × 3 mm I.D.) was washed [10] and packed with 3% OV-1 on 80–100 mesh Gas-Chrom Q (Supelco 1-2096; Supelco, Bellefonte, PA, U.S.A.). The conditioning procedure has been described previously [10].

Extraction

One hundred microlitres of the internal standard solution were measured into a glass tube, to which 1 ml of plasma, 1 ml of alkaline buffer and 4 ml of diethyl ether–dichloromethane (4:1, v/v) were then added. The tube was shaken mechanically (Infors shaker) for 15 min at 300 rpm and centrifuged at 2500 *g* for 5 min. An aliquot of the organic phase was transferred to another tube, shaken with 2 ml of 0.1 *N* hydrochloric acid for 10 min at 300 rpm and briefly centrifuged. The organic phase was discarded, and 1 ml of the alkaline buffer and 4 ml of diethyl ether–dichloromethane (4:1, v/v) were added. The mixture was shaken for 10 min at 300 rpm. After centrifugation, the organic phase was removed and evaporated to dryness under a nitrogen stream at 40°C.

Derivatization and chromatography

To the dry residue were added 1 ml of 0.3% pyridine in hexane and 10 μl of heptafluorobutyric anhydride (conditioned in ampoules of 2 ml) with a glass pipette. The tube was stoppered and agitated on a Vortex mixer for 15 sec.

After 1 h at room temperature, 1 ml of a saturated aqueous solution of potassium dihydrogen phosphate was added, and the mixture was shaken for 15 min at 300 rpm, then centrifuged. The aqueous phase was frozen by immersing the tube in a methanol–dry-ice bath. An aliquot of the hexane phase was transferred to a 250-μl conical glass flask (Hewlett-Packard 5080-8779); 3 μl were injected into the gas chromatograph with a Hewlett-Packard automatic sampler (Model 7672A).

Bioavailability study in man

Twenty healthy subjects received 200 mg of metoprolol tartrate as two different formulations, given in three different dosage regimens (two 100-mg tablets every 24 h, one 100-mg tablet every 12 h, and one 200-mg sustained-release tablet every 24 h) for four days. Blood samples were collected on the first day at various times. The samples were centrifuged, and the plasma was removed and stored at −20°C until analysis.

RESULTS AND DISCUSSION

Reaction time

The duration of the derivatization reaction was varied from 15 min to 2 h. A maximum yield of derivative was reached after 1 h.

Linearity

Ln—ln straight lines were obtained for calibration curves in the concentration range of 10—500 ng/ml. At concentrations higher than 500 ng/ml, the lines began to deviate from linearity. The determinations could be extended beyond 500 ng/ml by taking less than 1 ml of plasma and diluting the plasma sample.

The method was formally validated over the concentration range 10—500 ng/ml (Table I).

The day-to-day reproducibility of the standard curves was shown in three consecutive experiments carried out on separate days. On each occasion, the

TABLE I

DAY-TO-DAY REPRODUCIBILITY OF CALIBRATION CURVES USED TO DETERMINE METOPROLOL IN PLASMA

Concentration added to plasma (ng/ml)	Peak area ratios				$100 \times \frac{E}{C}$ (%)
	Experimental (E)			Calculated from ln—ln regression line (C)	
	Day 1	Day 2	Day 3		
10	0.139			0.124	112.1
10		0.136		0.124	109.7
10			0.128	0.124	103.2
20	0.227			0.237	95.8
20		0.220		0.237	92.8
20			0.219	0.237	92.4
50	0.550			0.554	99.3
50		0.531		0.554	92.6
50			0.532	0.554	96.0
100	1.050			1.050	100.0
100		1.050		1.050	100.0
100			1.060	1.050	101.0
250	2.460			2.470	99.6
250		2.530		2.470	102.4
250			2.530	2.470	102.4
500	4.710			4.700	100.2
500		4.800		4.700	102.1
500			4.810	4.700	102.3
	Average ± C.V. (%)				100.2 ± 5.3

peak area ratio of metoprolol versus the internal standard plotted against six concentrations of metoprolol gave straight lines. A least-squares ln—ln regression line was generated from the eighteen data points of the three standard curves (Table I). It corresponds to the regression equation $\ln Y = 0.9280 \ln X$ (ng/ml) $- 4.2204$.

Reproducibility of calibration curves

A test of day-to-day reproducibility was made by expressing each data point as a percentage of the value read off the ln—ln line for the corresponding concentration (Table I). The distribution of these normalized (concentration-independent) data had an overall average (±C.V.) of 100.2 ± 5.3%, demonstrating a good reproduciblity between experiments.

The reproducibility of the calibration curves was tested over six weeks. Table II gives the slopes and intercepts on the y-axis obtained for twenty calibration curves. The results obtained show a good reproducibility; however, a variability between the curves is sometimes noted which is the reason why the calibration curve was generated daily.

TABLE II

REPRODUCIBILITY OF TWENTY CALIBRATION CURVES OVER SIX WEEKS USED TO DETERMINE METOPROLOL IN PLASMA

Determination No.	Slope (S)	Intercept on y-axis (i)	Correlation coefficient (R)
1	0.9142	−4.1114	0.9980
2	0.9353	−4.2406	0.9990
3	0.9134	−4.1725	0.9992
4	0.9103	−4.1683	0.9995
5	0.9139	−4.1515	0.9992
6	0.9285	−4.2215	0.9990
7	0.9405	−4.2805	0.9995
8	0.9465	−4.3302	0.9989
9	0.9216	−4.2418	0.9993
10	0.9154	−4.1666	0.9988
11	0.9208	−4.2112	0.9997
12	0.8949	−4.1057	0.9979
13	0.8422	−3.8882	0.9986
14	0.8885	−4.1210	0.9995
15	0.8838	−4.0393	0.9991
16	0.8750	−4.0020	0.9994
17	0.8830	−4.1282	0.9990
18	0.8844	−4.1623	0.9995
19	0.8839	−4.2037	0.9991
20	0.8488	−3.9561	0.9984
Mean	0.9022 ± 3.2%	−4.1451 ± 2.6%	

Precision and accuracy

Table III gives the results obtained when the described procedure was applied to spiked plasma samples. With a calibration curve generated on each

TABLE III

PRECISION AND RECOVERY OF THE DETERMINATION OF METOPROLOL IN SPIKED PLASMA SAMPLES

Amount added (ng/ml)	Mean amount found (ng/ml) (n=6)	Standard deviation	Recovery (mean, %)
10	10.4	±0.2	103.5
20	19.6	±0.3	97.8
50	49.6	±0.5	99.2
100	97.3	±0.8	97.3
250	256	±3	102.4
500	510	±16	101.9
			100.4 ± 2.6

day of analysis, the results demonstrate a good reproducibility of the assay down to concentrations of 10 ng/ml of plasma, which is taken as the quantitation limit of the method. Lower concentrations could still be detected.

Day-to-day validation

Over a period of six weeks, the method was validated on each working day by determining one concentration in duplicate (100 ng/ml). The assay was performed by two different analysts, and Table IV shows the day-to-day reproducibility of the method.

TABLE IV

PRECISION AND RECOVERY IN THE DAY-TO-DAY DETERMINATION OF METOPROLOL IN SPIKED PLASMA

Amount added (ng/ml)		Mean amount found (ng/ml) (n=20)	Standard deviation	Recovery (%)	C.V. (%)
100	First analyst	98.9	±19	98.9	6.5
100	Second analyst	100	±16	100	6

Plasma interference

Fig. 2 shows the chromatograms of an extract of human plasma and of the same extract spiked with 50 ng of metoprolol tartrate and 100 ng of internal standard. There is no interference from the normal components of the plasma extract.

Specificity

The four main metabolites of metoprolol, i.e. the basic metabolites

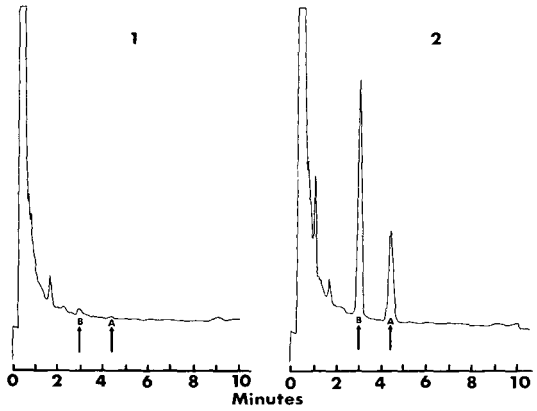


Fig. 2. Chromatograms of (1) human plasma blank (1 ml of plasma) and (2) the same plasma spiked with 50 ng/ml metoprolol tartrate (A) and 100 ng/ml internal standard (B).

α-hydroxymetoprolol and O-demethylmetoprolol (Fig. 1, III and IV), and the acidic metabolites H 117/04 and H 104/83 (Fig. 1, V and VI), were used to test the specificity. The two acidic metabolite derivatives were not detected after GC. O-Demethylmetoprolol was detected but it is reported [11] that this metabolite is only present in human urine. α-Hydroxymetoprolol was also detected; its retention time is longer than those of metoprolol and the internal standard.

Stability

Table V shows that no decrease in the metoprolol content (20 ng/ml and 250 ng/ml) occurred in plasma samples stored frozen for twelve months at −20°C.

TABLE V

STORAGE STABILITY OF METOPROLOL TARTRATE IN HUMAN PLASMA AFTER TWELVE MONTHS AT −20°C

Duration of storage at −20°C (months)	Amount of metoprolol tartrate added to plasma (ng/ml)	
	20	250
	Amount of metoprolol tartrate found (average of two assays) (ng/ml)	
0	22.3	259
1	21.5	262
2	20.3	239
3	21.5	261
6	19.6	262
9	18.6	242
12	19.9	272

Application

The technique was applied in a bioavailability study comparing commercial tablets of metoprolol with a new sustained-release formulation. Fig. 3 shows the average curves obtained from the plasma samples of twenty subjects given 200 mg of metoprolol tartrate in three dosage regimens.

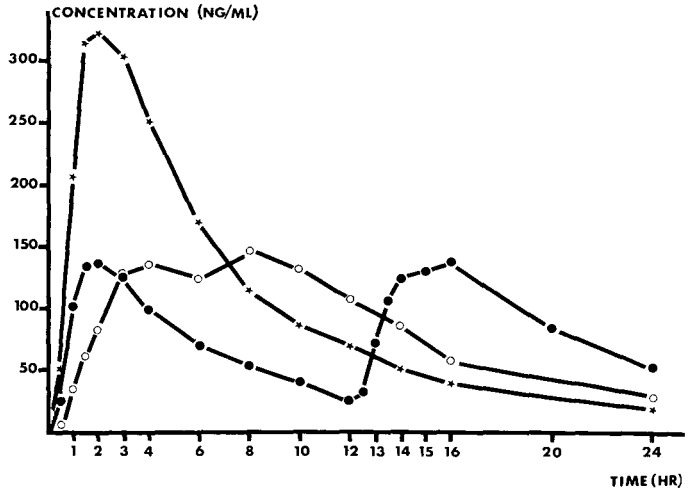


Fig. 3. Average plasma metoprolol concentrations obtained on the first day in twenty healthy subjects after administration of 200 mg of metoprolol tartrate given as: 100-mg metoprolol tablet every 12 h (●); 2 × 100-mg metoprolol tablet every 24 h (○); 200-mg sustained-release metoprolol tablet every 24 h (★).

CONCLUSION

The proposed technique permits the quantitative assay of metoprolol in human plasma at concentrations down to 10 ng/ml. It is specific, reproducible, and sufficiently sensitive for determinations of metoprolol in bioavailability studies.

REFERENCES

1 M. Ervik, K.J. Hoffmann and K. Kylberg-Hansen, Biomed. Mass Spectrom., 8 (1981) 322.
2 J. Kirschbaum and B. Patel, Abstr. Papers, Amer. Chem. Soc. 178th Meeting, Part 1, ANAL, 39 (1979).
3 M.A. Lefevre, J. Girault and J.B. Fourtillan, J. Liquid Chromatogr., 4 (1981) 483.
4 H. Winkler, Arch. Pharmacol. Suppl., 316 (1981) R59.
5 J. Hermansson and C. von Bahr, J. Chromatogr., 227 (1982) 113.
6 M. Ervik, Acta Pharmacol. Toxicol., 36 (Suppl. V) (1975) 136.
7 S. Zak, F. Honc and T.G. Gilleran, Anal. Lett., 13 (1980) 1359.
8 C.D. Kinney, J. Chromatogr., 225 (1981) 213.
9 P.H. Degen and W. Riess, J. Chromatogr., 121 (1976) 72.
10 A. Sioufi and F. Pommier, J. Chromatogr., 183 (1980) 33.
11 C.G. Regärdh, L. Ek and K.J. Hoffmann, J. Pharmacokin. Biopharm., 7 (1979) 471.

Journal of Chromatography, 272 (1983) 111—128
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1479

AN INVESTIGATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE ANALYSIS OF AMPHETAMINES

B.M. FARRELL and T.M. JEFFERIES*

University of Bath, School of Pharmacy and Pharmacology, Claverton Down, Bath, BA2 7AY (Great Britain)

(First received May 21st, 1982; revised manuscript received August 9th, 1982)

SUMMARY

Three pre-column derivatization reagents, namely *o*-phthalaldehyde, 4-chloro-7-nitrobenz-2,1,3-oxadiazole, sodium naphthaquinone-4-sulphonate and two ion-pair reagents, namely, naphthalene-2-sulphonate and sodium dodecylsulphate have been investigated for their suitability for the qualitative and quantitative analysis of urine and plasma samples containing amphetamines. The derivatization method employing sodium naphthaquinone-4-sulphonate was found to be selective and sufficiently sensitive for the routine determination of amphetamine and methylamphetamine in urine and plasma samples at the ng/ml level.

INTRODUCTION

The continual abuse of amphetamine for its central stimulant effects has led to thorough studies concerning their metabolism, distribution and excretion [1—4]. Many analytical procedures have been developed for qualitative and quantitative purposes especially in toxicology and forensic science. They include colour tests [5, 6], physicochemical methods [7, 8], UV spectrophotometry [9, 10], spectrofluorimetry [11, 12], microcrystallography [13], immunological methods [14], and all forms of chromatography: thin-layer chromatography (TLC) [15, 16], gas—liquid chromatography (GLC) [17, 18], GLC—mass spectrometry (MS) [19, 20] and high-performance liquid chromatography (HPLC) [21, 22]. The latter technique has not been widely applied to the analysis of amphetamine because its low specific extinction value (9.7 at 257 nm in water) renders the UV detector of limited value, and also amphetamine has very little natural fluorescence.

The purpose of this study was to overcome these detection difficulties and various techniques have been compared for their practical applicability for the monitoring, screening and analysis of amphetamines and related sym-

0378-4347/83/0000—0000/$03.00

pathomimetic amines having low UV absorptivities. One of the most important advantages of HPLC over GLC [23] is its ability to analyse aqueous samples, especially plasma and urine, with minimum sample preparation. Improvements in the detection of amphetamines would therefore permit rapid and selective methods to be devised having adequate sensitivity.

EXPERIMENTAL

Reagents and materials

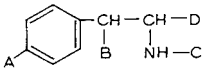
Acetonitrile, chloroform, cyclohexane, ethyl acetate, hexane, methanol, propan-2-ol and tetrahydrofuran of HPLC grade, and pentan-1-ol of laboratory reagent grade, were supplied by Fisons (Loughborough, Great Britain). Mercaptoethanol and methyl isobutyl ketone (MIBK) were from BDH (Poole, Great Britain). The water used for the preparation of aqueous mobile phases was double distilled from an all-glass still and deionised by passage through an Elgastat deioniser (Elga Products, Buckingham, Great Britain). The derivatizing reagent 4-chloro-7-nitrobenz-2,1,3-oxadiazole (NBD-Cl) was supplied by Phase Separations (Queensferry, Great Britain), *o*-phthalaldehyde (OPA) by BDH, and sodium β-naphthaquinone-4-sulphonate (NQS) by Kodak Eastman (Liverpool, Great Britain).

The following column packing materials were used: Hypersil-ODS (Shandon Southern, Runcorn, Great Britain), LiChrospher Si 100 and LiChrosorb Si 100 (Merck, Darmstadt, G.F.R.), Spherisorb ODS (Phase Separations) and Partisil (Whatman, Maidstone, Great Britain). Columns were packed using the upward slurry technique described by Bristow et al. [24], employing methanol—chloroform (20:80, v/v). The HPLC instrumentation used was assembled from commercially available components. Both constant flow-rate (Constametric III and Milton Roy Minipump, LDC, Stone, Great Britain, with pulse dampener) and constant pressure (Haskel Model 27502, Olin Energy Systems, Sunderland, Great Britain) pumps were used.

The structures of the amphetamines studied are given in Table I.

TABLE I

FORMULAE OF THE AMPHETAMINES AND RELATED COMPOUNDS USED



Compound	Source*	A	B	C	D
Phenethylamine	c	H	H	H	H
Amphetamine	a	H	H	H	CH_3
Methylamphetamine	a	H	H	CH_3	CH_3
Ephedrine	c	H	OH	CH_3	CH_3
Norephedrine	b	H	OH	H	CH_3
p-Hydroxyamphetamine	a	OH	H	H	CH_3
p-Hydroxynorephedrine	a	OH	OH	H	CH_3
p-Hydroxymethylamphetamine	a	OH	H	CH_3	CH_3

*a = Gift from Smith, Kline and French (Welwyn Garden City, Great Britain); b = May and Baker (Dagenham, Great Britain); c = BDH (Poole, Great Britain).

Whenever practical the temperatures of the mobile phase reservoir, the column and injector were controlled by immersion in a heated water bath. The output to the detector was recorded on a potentiometric chart recorder (Servoscribe IS RE 541.20, Smith Industries, London, Great Britain). The output was also monitored by a Perkin-Elmer (Beaconsfield, Great Britain) Sigma X Data Station. Samples were introduced on to the column by means of a loop valve (Rheodyne 7125, Jones Chromatography, Llanbradoach, Great Britain).

Two types of fluorimeter were used: Perkin-Elmer Fluorescence Spectrophotometer fitted with a xenon arc lamp and an LDC Fluoromonitor III, Model 1311, fitted with a standard low-pressure mercury and phosphor lamp, 370 nm excitation filter and 418—700 nm emission filter. The UV detectors were either a Cecil Model 2012 or a 212 variable-wavelength UV monitor using deuterium lamps (Cecil Instruments, Cambridge, Great Britain). Spectroscopic measurements were made using a Perkin-Elmer 550-S UV-VIS spectrophotometer.

Extraction

Samples of urine (20 ml) and plasma (10 ml) were made alkaline with 5 *N* sodium hydroxide to pH 11.4. The solutions were then introduced to columns (5-ml graduated pipettes) each containing about 1.5 g of clean XAD-2 resin (Amberlite, BDH). The columns were then washed with 10 ml water and the adsorbed solutes were eluted with 40 ml (for urine) or 20 ml (for plasma) of chloroform—isopropanol (3:1). Ethanolic hydrochloric acid (6 *N*, 100 μl) was added to the organic eluate which was then evaporated to dryness using a rotary evaporator (Buchi Rotavapor). The residue was dissolved in the appropriate solvent required for direct injection or derivatization.

The column for straight phase ion-pair HPLC was prepared using both methods described in detail by Crommen et al. [25]. The injection technique was preferred.

Ion-pair formation

Solutes for reversed-phase ion-pair formation with sodium dodecylsulphate (SDDS) were dissolved in mobile phase and chromatographed directly. The solutes for straight-phase ion-pair analysis were dissolved in aqueous stationary phase and then extracted into an equal volume of mobile phase and this was used for chromatography.

Derivatization

For UV detection [26]: Sample extracts were dissolved in 8% sodium hydrogen carbonate (1 ml) and an equal volume of 0.5% NQS solution was added. After heating in an oven at 70°C for 20 min, the aqueous solution was extracted (vortex mixed for 1 min) with an equal volume of chloroform, and the organic layer used for chromatography.

For fluorescence detection [27—29]: For reversed-phase: The OPA derivatizing reagent was prepared by dissolving OPA (200 mg) in methanol (2 ml) and undiluted mercaptoethanol (0.4 ml) was added. This solution was added to boric buffer (1 g boric acid in 38 ml water) adjusted to pH 10.4 with 4 *M*

potassium hydroxide. This reagent is stable for five days when protected from light and stored in a refrigerator. The biological sample extracts were taken up in ethanol (1 ml) and OPA derivatizing reagent (1 ml) was added. This solution was filtered prior to injection on to the column.

For straight-phase [30, 31]: Sample extracts were dissolved in 0.1 *M* sodium hydrogen carbonate (4 ml) and 1% NBD-Cl in MIBK (2 ml) was added. After shaking briefly the reaction vessel was heated at 80°C for 30 min. The upper (MIBK) layer was used for chromatography.

Definitions

The following expressions have been employed in order to assess various chromatographic parameters used in this study.

Resolution, R_s, of two adjacent solute bands is defined as being equal to the distance between the two band centres, divided by the average band width (W). Thus:

$$R_s = \frac{t_2 - t_1}{\frac{1}{2}(W_2 + W_1)} \tag{1}$$

where t is the elution time or chart distances of solutes 1 and 2. The selectivity factor, α, for two solutes with capacity ratios, κ_1 and κ_2 is given by:

$$\alpha = \frac{\kappa_2}{\kappa_1} \tag{2}$$

The functional group contribution towards retention may be defined as:

$$\tau = \log \alpha = \log \frac{\kappa_2}{\kappa_1} \tag{3}$$

where solutes 1 and 2 differ by a functional group and the reference solute 1 is taken as the parent compound.

RESULTS AND DISCUSSION

Reversed-phase ion-pair HPLC

The effect of changing the pairing-ion concentration in the mobile phase on retention (κ) was studied. The observed relationship (Fig. 1A) has been attributed to the retention mechanism in surfactant ion-pair HPLC [32] being a combination of ion-pair distribution and in situ ion exchange [33].

The initial sigmoidal effect can be attributed to an initial depletion of pairing-ion at low concentrations such that here retention is related to solute concentration and column load capacity. The influence of these side reactions is confirmed by the relationship between column efficiency and pairing-ion concentration (Fig. 1B) in which the plate height reaches a constant minimum at higher pairing-ion concentrations. This latter region produces reproducible κ values, whilst the maximum concentration is indicated by Fig. 1A suggesting a concentration range of approximately 2 to $12 \cdot 10^{-4}$ *M*. For the calibration

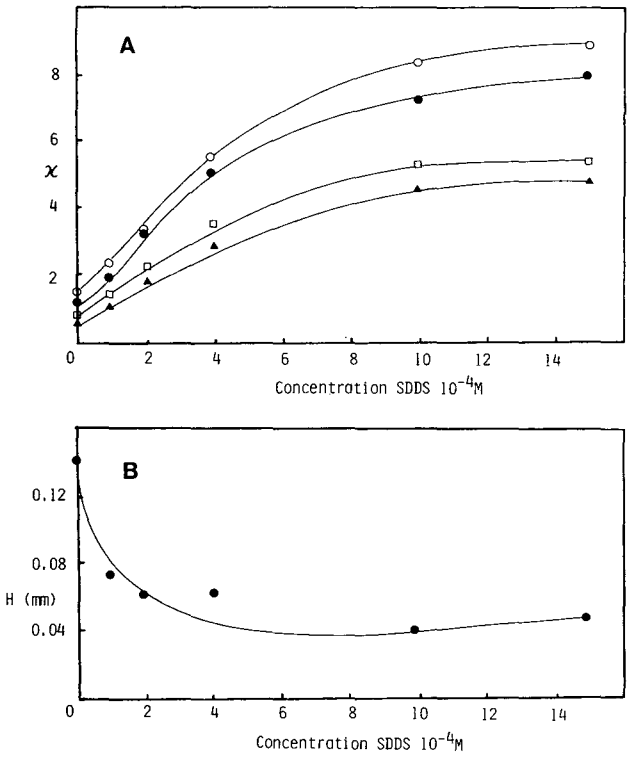


Fig. 1. Relationship between pairing-ion concentration and (A) the capacity ratio (κ) and (B) the plate height (H). Amphetamine (●), methylamphetamine (○), norephedrine (□) and ephedrine (▲). Conditions as in Table II for SDDS.

of amphetamine and hydroxyamphetamine, a pairing-ion concentration of $1 \cdot 10^{-3}$ M SDDS was used since this resulted in ideal capacity ratios and gave good column efficiencies ($h \approx 4$).

The estimations were made on the basis of peak height ratio using an internal standard and comparison with known standard solutions. The linearity of response was checked by a calibration curve of solutes dissolved in mobile phase in the concentration range 20—250 μg/ml. The correlation coefficient for amphetamine and hydroxyamphetamine using 5 points was 0.999 with coefficients of variation of the slopes of 0.8% and 1.8%, respectively. The peaks obtained (Fig. 2) were sharper than those in the other reversed-phase (OPA derivatization) method (see Fig. 7). There was some tailing but resolution was nevertheless 1.5 or greater, indicating complete separations. The limit of detection was approximately 5 μg/ml although this is lowered when samples of urine are processed (see Table III).

In general, metabolites of drug compounds are more polar than the parent compound since this facilitates urinary excretion by the kidney. In the reversed-phase mode, metabolites are generally eluted before the parent drug, which is an advantage when searching for metabolite peaks. The example in Fig. 2 is a good illustration of this point. Retention is due to the combined effects of ion-pair formation plus functional group contributions. The former

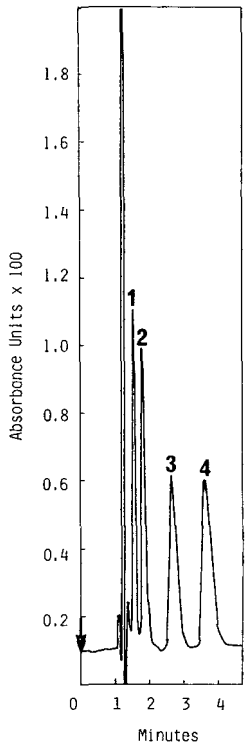


Fig. 2. Separation of amphetamine and three of its metabolites, dissolved in mobile phase. Conditions as in Table II, for SDDS. Peaks: 1 = *p*-hydroxynorephedrine; 2 = *p*-hydroxyamphetamine; 3 = norephedrine; 4 = amphetamine. Selectivity between adjacent peaks is 1.8, 2.1 and 1.6, respectively.

is a common factor, due to the protonated primary amino group in the four solutes. Differences in retention are due solely to differences in functional groups, which permit functional group contribution values, τ, to be calculated.

Aromatic hydroxylation of amphetamine and norephedrine produced falls in their κ values that may be expressed in terms of τ values as —0.52 and —0.51, respectively. The β-hydroxylation of amphetamine to norephedrine had less effect in reducing κ (τ = —0.21) probably due to steric hindrance of the hydroxyl group. Interestingly, the combined effect of aromatic hydroxylation plus β-hydroxylation as illustrated by the metabolism of amphetamine to *p*-hydroxynorephedrine, produced a fall in κ value equivalent to a τ value of —0.71, clearly demonstrating the additive effect of functional groups that do not interfere with one another. Functional group contribution values are relative and vary with solute type and reversed-phase material used. The percentage of carbon loading has a major influence on τ values, increasing the range of values obtained as carbon content is increased. The values obtained here are compatible with those of Riley et al. [34], who obtained —0.45 for the introduction of 4-OH into benzoic acid using a lower carbon-loaded material (Spherisorb-ODS).

A 24-h urine sample from a 22-year-old male subject was analysed using the developed assay. The urine was obtained 8 h after an unknown amount

of illegally obtained amphetamine sulphate had been taken. The pH of the urine was normal. Standard urine extracts containing 12.5, 25 and 50 μg/ml of amphetamine sulphate were used to compare peak height response to that of the clinical sample. The concentration of amphetamine in the urine was found to be 8.5 μg/ml corresponding to an original dose of 55 mg of amphetamine sulphate.

Straight-phase ion-pair HPLC

In this mode, highly UV-absorbing or fluorescing ions can be used as pairing-ions which permits extremely sensitive detection of non-UV-absorbing or non-fluorescing solutes. The ion-pair formed is lipophilic, increasing the solubility of the solute in the mobile phase.

Crommen et al. [25] published a method taking advantage of these factors for the resolution of amino acids, dipeptides and alkylamines. Using naphthalene-2-sulphonate (NS) as the highly UV-adsorbing pairing-ion contained in the stationary phase, they were able to separate and detect these compounds down to the 1-ng level. They compared their results with batch extraction data and showed that the retention of the cations, except the most hydrophobic, was due mainly to liquid—liquid distribution. The capacity ratio (κ) of the solute A^+ is given by:

$$\kappa_A = \frac{1}{E_{A,B}\,[B^-]} \cdot \frac{V_s}{V_m} \tag{4}$$

Where $E_{A,B}$ is the conditional extraction constant of the ion-pair, B^- is the pairing-ion and V_s/V_m is the phase volume ratio. In practice the capacity ratio can be varied to some extent by the concentration of the pairing-ion, by changing the nature of the pairing-ion, or by the regulation of $E_{A,B}$ by altering the composition of the organic mobile phase. The latter variable is the simplest method of varying κ values.

The choice of column packing material was based on surface area and pore size characteristics. In general, pore size is inversely proportional to surface area and for a support material to hold a maximum amount of stationary base, it should ideally have a large pore volume. LiChrospher Si 100 has spherical particles, 100 μm pore diameter and 1.2 ml/g pore volume.

Ion-pairs between naphthalene sulphonate and alkylamines have a high molar absorptivity (about 1400 at 254 nm). Naphthalene sulphonate is almost aprotic and can be used as a pairing-ion at a low pH (2.0) where distribution of many solutes, e.g. alkylamines, in non-charged form is negligible. This pairing-ion is very soluble in water and has a very low distribution to the organic mobile phase. The blank obtained with a mobile phase of chloroform and 1-pentanol depends on the amount of 1-pentanol present and the pH of the aqueous phase, both of which need to be determined before chromatographic conditions may be chosen. The curve showing the influence of pH (Fig. 3) indicates the narrow range providing minimal absorbance.

Solutions of NS in citrate buffer (disodium citrate and sulphuric acid, ionic strength 0.1) to be used as stationary phases were purified by repeated extraction with chloroform—pentanol (9:1) until the extract had a constant

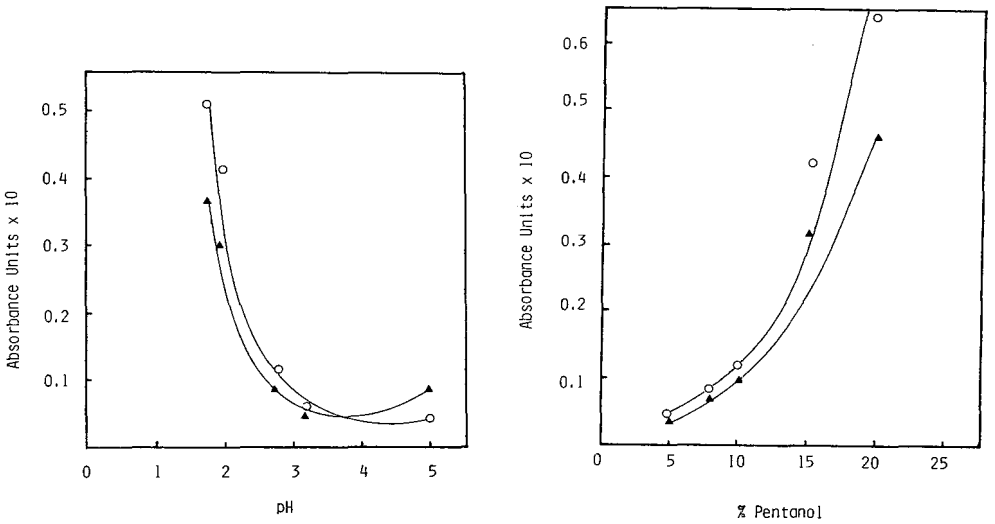


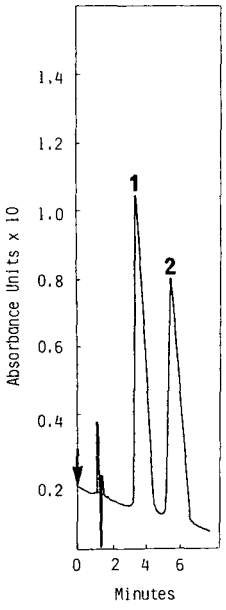
Fig. 3. Relationship between the absorbance of NS and the pH of buffer at 254 nm (▲) and 274 nm (○) at 10% pentanol and 0.1 *M* NS.

Fig. 4. Relationship between the absorbance of NS and the percentage of 1-pentanol in the mobile phase at 254 nm (▲) and 274 nm (○) at pH 3.0 and 0.1 *M* NS.

absorbance. The influence of 1-pentanol concentration on absorbance is shown in Fig. 4. From these graphs it was decided to use 10% 1-pentanol in the mobile phase and that the stationary phase should be buffered at pH 3.0.

The aqueous phase on the silica support material had a volume of approximately 1.0 ml/g of support, which is close to the specific pore volumes given by the manufacturer (1.2 ml/g). The complete filling of the pores is also illustrated by the fact that the fraction of the column volume occupied by the mobile phase after coating is 0.38—0.40, i.e. almost equal to the interstitial volume [25]. In order to ensure stable conditions during the recycling, the reservoir always contained 500 ml of mobile phase with a layer of stationary phase on the top. The mobile and stationary phase (on the column) was kept at a constant temperature (25°C) in a thermostatted water bath. This was necessary to maintain a constant solubility (and hence a constant background signal) of the NS in the mobile phase. The detector cell was kept at a higher temperature (35°C) than the mobile phase to ensure complete solubility of the stationary phase in it.

The log κ values for amphetamine and methylamphetamine were found to be directly proportional to the logarithm of the molar concentration of pentanol and a mobile phase containing 10% pentanol was chosen as it gave a selectivity factor of 2 and resolution of 1.56 for amphetamine and methylamphetamine (Fig. 5). With a stable system, there was very little background signal, sensitivity was high and the columns gave good symmetrical peaks without tailing, due to the silica surface being inactivated by the aqueous stationary phase. Unfortunately, a persistent lack of stability arose from the loss of aqueous stationary phase from the columns, causing an increase in



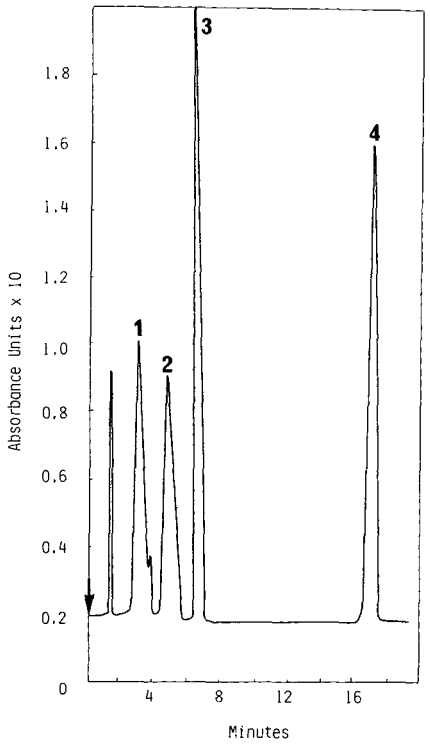


Fig. 5. Separation of methylamphetamine (1) and amphetamine (2) dissolved in mobile phase. Conditions as in Table II, for NS.

Fig. 6. Composite chromatogram of hydroxymethylamphetamine (1), phenylethylamine (2), hydroxyamphetamine (3) and hydroxynorephedrine (4) dissolved in mobile phase. Conditions as in Table II for NS.

background UV absorbance. Additionally, it was found that NS adhered to the quartz windows of the UV cell, eventually causing a full-scale deflection despite the precaution of increasing the temperature of the cell.

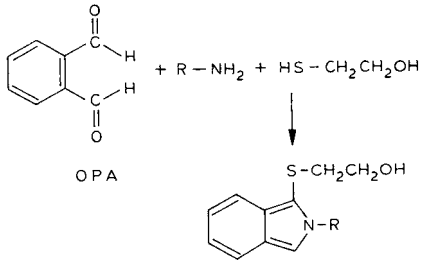
However, the results illustrated in Figs. 5 and 6 clearly indicate the excellent sensitivity of the method. Methylamphetamine and amphetamine were easily separated (α = 2) in 6 min, whilst the separation of three amphetamine metabolites and phenylethylamine gives some indication of the potential available. The elution order is clearly that expected in a straight-phase system, i.e. least polar compound eluted first. The addition of a β-hydroxy group produced a large increase in κ value, as shown by the retention of hydroxynorephedrine (Fig. 6, peak 4) compared with hydroxyamphetamine (Fig. 6, peak 3). Aromatic hydroxylation, as illustrated by hydroxyamphetamine compared to amphetamine gave a smaller increase in κ value. This difference in selectivity for β-hydroxy and aromatic hydroxy substituents is the opposite of that encountered in reversed-phase ion-pair HPLC. Secondary amines, i.e. methylamphetamine and hydroxymethylamphetamine (Fig. 6, peak 1), gave significantly lower κ values than their corresponding primary amines, i.e.

amphetamine and hydroxyamphetamine, the order of decrease being identical in both cases.

The method has been shown to be applicable to the amphetamines and potentially has both selectivity and sensitivity but further work is required to achieve column stability.

Reversed-phase HPLC with derivatization

The reaction of OPA in the presence of a strong reducing agent such as 2-mercaptoethanol was described by Roth [27] in 1971 for the fluorimetric detection of α-amino acids. Benson and Hare [28] found that: (a) it is five to ten times more sensitive than fluorescamine which is, in turn, more sensitive than ninhydrin used for UV detection of primary amines; (b) it has the advantage of being soluble and stable in aqueous buffers; (c) because of its aqueous solubility it is compatible with biological fluids and gives a stable baseline when mixed with an aqueous mobile phase; (d) OPA is considerably less expensive than fluorescamine and is therefore more economical for routine or automated use. Davies et al. [29] used it to detect nanogram quantities of biogenic amines. They compared their HPLC method to that of a GLC analysis and found it more sensitive. The fluorophore is produced as follows:



The excitation and emission maxima of OPA-amphetamine were found to be 345 and 445 nm, respectively. It also shows a UV-absorption maximum at 254 nm and spectrophotometric analysis showed that the absorbance is quantitative and sensitive at that wavelength. After studying the effect of varying methanol concentrations in the mobile phase, a composition of 73% was chosen as it gave a short overall analysis time (15 min) with resolution of 1.3 between peaks 1 and 2 and 2.6 between peaks 2 and 3 (Fig. 7). The stability of the derivatives was investigated by measuring the peak area ratios of three amphetamines injected every 30 min for 8 h. The results showed that maximum fluorescence occurs almost immediately and remains stable for at least 60 min. Thereafter the peak area decreases to a lower level where it is stable from 3.5 to 7 h. The calibration of amphetamine, hydroxyamphetamine and norephedrine produced a linear response with correlation coefficients of 0.997, 0.996 and 0.999, respectively, over the range 0.5—8 μg/ml. The percentage recovery of the drugs from biological fluids was high, ranging from 87% for hydroxyamphetamine to 98% for amphetamine.

Straight-phase HPLC with derivatization

UV detection. In 1922 Folin described a method for the determination of amino acid nitrogen which depended upon the combination of the amino

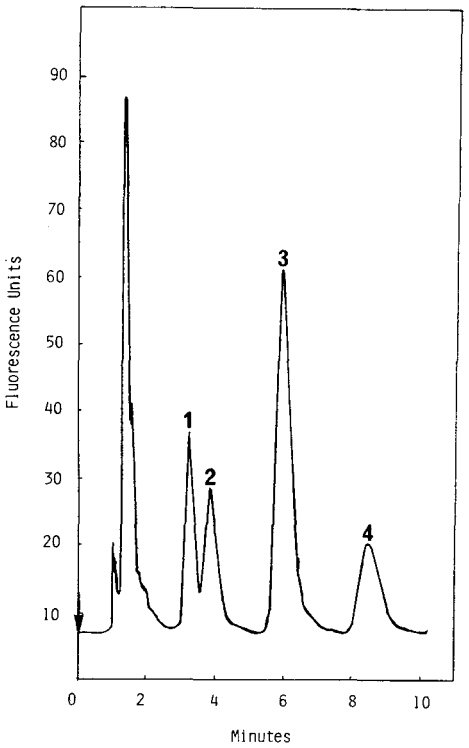
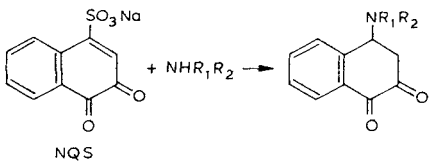


Fig. 7. Typical chromatogram of the OPA derivatives of norephedrine (1), hydroxyamphetamine (2), benzylamine (internal standard) (3) and amphetamine (4), prepared from underivatized standards added to urine. Conditions as in Table II. Unidentified peaks also occur in a urine blank. Suitable for plasma samples.

groups with 1,2-naphthaquinone-4-sulphonate (NQS) in alkaline solution to form highly coloured compounds which could be determined colorimetrically. Gürkan [35] applied the reaction to the investigation of sympathomimetic amines, including amphetamine. Absorbance maxima occurred in the visible region (447 nm) and in the UV region (245 nm). He found that sympathomimetic amines which contain a hydroxyl group, especially in the α-position, did not give a quantitative reaction. Also, after reduction with sodium borohydride, the coloured reaction products yielded an intense blue fluorescence. The fluorimetric analysis was more sensitive than UV detection but the fluorescent products were stable only for 10 min at room temperature. In 1978 Hashimoto et al. [16] used NQS for the quantitative analysis of phenylethylamine derivatives including amphetamine. The reaction products were isolated by TLC and subjected to elemental analysis, nuclear magnetic resonance (NMR), infra-red spectroscopy (IR) and MS. The results suggest that compounds have the general structure shown below.



NQS

Endo et al. [26] applied the method to the determination of amphetamine and methylamphetamine in urine by HPLC. They were able to detect 2 ng of drug at 280 nm, which was comparable to the sensitivity obtained by GLC. The mobile phase employed by us was similar to that used by Endo et al. and was a mixture of chloroform, ethyl acetate and hexane, saturated with water and one part of ethanol added after saturation. The water in the mobile phase "caps" the most active silanol groups on the silica packing material and the peaks produced are narrow and symmetrical. Retention was adjusted by varying the amount of ethyl acetate in the mobile phase. The separation of NQS-methylamphetamine from three of its metabolites and an internal standard under the chromatographic conditions listed in Table II is shown in Fig. 8.

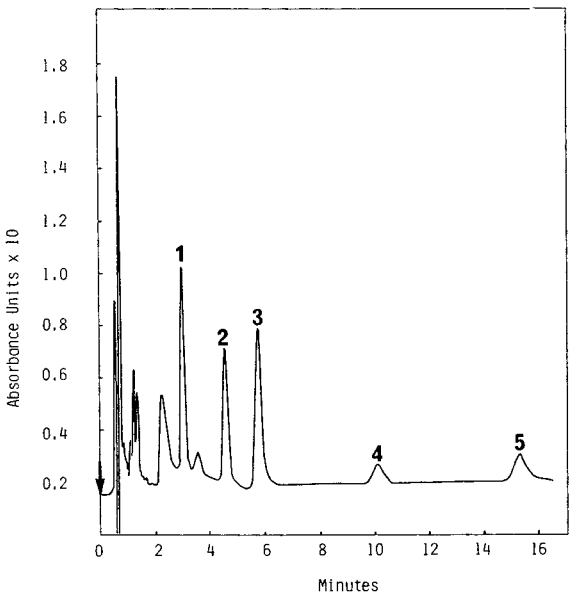


Fig. 8. Separation of the NQS derivatives of methylamphetamine (1), amphetamine (2), phenylethylamine (internal standard) (3), norephedrine (4) and hydroxyamphetamine (5) prepared from underivatized standards added to urine. Conditions as in Table II. Unidentified peaks also occur in a urine blank. Selectivity between adjacent drug peaks is 1.65, 1.31, 1.85 and 1.59. Suitable for plasma samples.

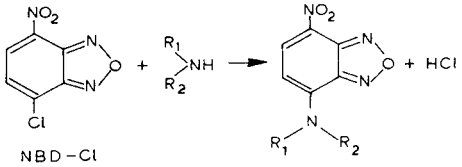
Hydroxyamphetamine and norephedrine were not included in quantitative analyses as they both contain a hydroxy group and according to Hashimoto et al. [16] do not react completely with NQS or form stable derivatives. The stability of other derivatives with time was investigated by injecting the samples into the chromatograph immediately after heating and extraction into chloroform and thereafter every 30 min for 8 h. The results using spiked plasma and urine extracts show that 30 min after removal of the reaction vessels from the oven, the derivatives had a stable maximum absorbance for at least 4 h. Injections were continued at 24-h intervals and the derivatives still showed good response after 60 h. The calibrations of NQS-methylamphetamine and NQS-amphetamine in water, plasma and urine produced linear

relationships between 0.5 and 5 μg/ml. All correlation coefficients were above 0.998. The limit of detection was 2 ng and confirmed that reported by Endo et al. [26]. The recovery of the drugs from biological fluids for methylamphetamine and amphetamine was 98 and 109% respectively (urine) and 88 and 95% respectively (plasma).

Fluorescence detection. Primary and secondary aliphatic amines react with NBD-Cl to produce intensely fluorescent derivatives. Although anilines, phenols and thiols also react with NBD-Cl, the derivatives produced have weak or no fluorescence, thus making the reaction more selective for amines. In 1968 Ghosh and Whitehouse [36] compared NBD-Cl with dansyl chloride and found that the former was more stable and soluble in aqueous solutions. Dansyl chloride and lutidine reagents are fluorescent and so produce significant blanks, whereas NBD-Cl is non-fluorescent. Another disadvantage of dansyl chloride and lutidine reagents is that they are non-specific and react with many naturally occurring amines in extracts of biological samples to produce interfering fluorophores.

Monforte et al. [37] used NBD-Cl for the analysis of amphetamines in blood and urine following TLC. MIBK was chosen as a solvent for NBD-Cl because the coupling reaction is faster in polar solvents such as alcohols or ketones but a solvent with a high boiling point was necessary since the reaction is carried out at 80°C (MIBK boiling point is 117°C) to reduce the reaction time for derivative formation.

Hopen et al. [38] chemically characterised the NBD-Cl derivative of amphetamine by NMR. They confirmed that the reaction with amphetamine proceeds as follows:



The production of hydrogen chloride requires the presence of a buffer to obtain a high product yield. The possibility of a hydrolysis reaction at the chlorine position of the reagent is prevented by the highly basic medium of the 0.1 *M* sodium bicarbonate buffer.

The excitation and emission maxima of NBD-amphetamine occur at 465 nm and 575 nm, respectively. Monforte et al. [37] showed that although the emission spectrum is not different from that of NBD-methylamphetamine, significant differences do exist in the excitation spectra and these may be used to distinguish between the primary and secondary amines. Chromatographic conditions for the separation and quantification of amphetamine and methylamphetamine are summarised in Table II. Using methylamine as an internal standard, the total elution time was 7 min. Resolution between the three solutes was 1.2 and 2.6 and selectivity was 5 and 3. The stability of the derivatives in biological fluid extracts with time was investigated over an 8-h period. Both were found to be extremely stable. Calibration of methyl-

TABLE II

SUMMARY OF CHROMATOGRAPHIC CONDITIONS EMPLOYED

	SDDS	NS	OPA	NQS	NBD-Cl
Internal standard	norephedrine 50 μg/ml	—	benzylamine 7.5 μg/ml	phenylethylamine 5 μg/ml	methylamine 10 μg/ml
Stationary phase	Partisil ODS-2 10 μm	LiChrospher Si 100, 10 μm, containing 0.1 *M* NS in citrate buffer *I* = 0.1, pH 3	Partisil ODS-2 10 μm	Partisil 5	Partisil 5
Column dimensions (mm)	250 × 5	150 × 5	250 × 5	150 × 5	200 × 5
Mobile phase	$1 \cdot 10^{-3}$ *M* SDDS in 0.01% H_2SO_4 (pH 2)—methanol (50:50)	10% pentan-1-ol in chloroform	0.2% EDTA in 73% methanol	chloroform—ethyl acetate—hexane—ethanol (25:35:50:1)	ethyl acetate—cyclohexane (40:60)
Injection volume (μl)	20	20	50	50	20
Detection wavelength (nm)	254	274	345 (ex) 445 (em)	248	465 (ex) 515 (em)
Temperature (°C)	20	25 35 (UV cell)	20	20	20
Flow-rate (ml/min)	1.0	1.0	1.8	2.5	2.0

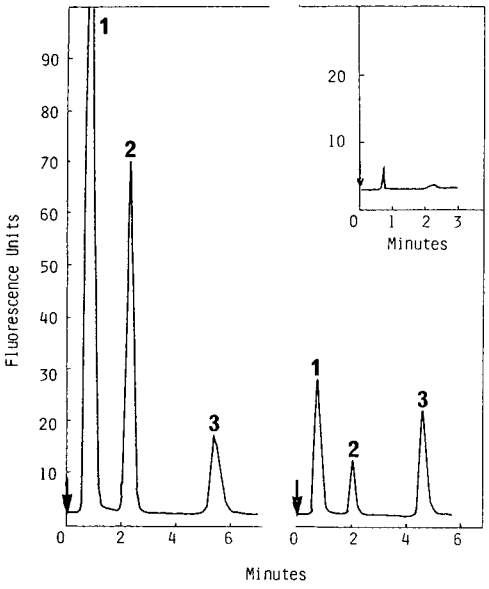


Fig. 9. Typical calibration chromatograms of the NBD derivatives of amphetamine (1), methylamphetamine (2) and methylamine (internal standard) (3). Conditions as in Table II. Inset: NBD blank. Suitable for urine and plasma samples.

amphetamine and amphetamine in water, plasma and urine over the range 0.5 to 5.0 μg/ml were found to be linear with correlation coefficients better than 0.996. Recovery of the derivatives from biological fluids was high. A typical chromatogram is shown in Fig. 9.

CONCLUSION

Both straight- and reversed-phase modes of HPLC have demonstrated their suitability for the resolution of methylamphetamine, amphetamine and their metabolites. Straight-phase columns are inherently highly efficient, elute the drug followed by the more polar metabolites, but require protection from irreversible adsorption that causes a fall in column efficiency. Reversed-phase columns can be highly efficient, elute the more polar metabolites before the drug, and are more tolerant towards the injection of complex biological solutions.

The starting point of this study was the reversed-phase ion-pair system employing SDDS, which clearly demonstrates the excellent chromatographic properties available and for which an improved detection system is required. Table III summarises some of the features of the HPLC systems studied to provide improved detection. The minimum detectable amount of drug for each method has been given for various injection solutions. For standard solutions the quantity represents the amount injected and expresses the sensitivity of the detection system. The levels recorded for urine and plasma samples depend upon the volumes of these materials available. For urine, 20-ml samples enable a concentration factor of ten times to be readily ob-

TABLE III

SUMMARY OF SOME OF THE FEATURES OF THE FIVE HPLC SYSTEMS STUDIED

Consideration	SDDS ion-pair	NS ion-pair	OPA derivatization	NQS derivatization	NBD-Cl derivatization
1. Sensitivity					
standards (ng)	100	20	30	2	5
Urine (20 ml; ng/ml)	250	not	60	4	25
Plasma (1 ml; ng/ml)	—	determined	600	20	125
2. Reliability	excellent	poor	good	good	poor
3. Application	primary and secondary amines non-specific	primary and secondary amines non-specific	primary amines only specific	primary and secondary amines specific	primary and secondary amines specific
4. Versatility	(a) chromatographic conditions easily changed	(a) chromatographic conditions not easily changed	(a) chromatographic conditions easily changed	(a) chromatographic conditions easily changed	(a) chromatographic conditions easily changed
	(b) UV detection	(b) UV detection	(b) UV and fluorimetric detection	(b) UV and fluorimetric detection	(b) fluorimetric detection
5. Speed of analysis	(a) preparation fast and simple	(a) preparation lengthy	(a) preparation relatively fast and simple	(a) preparation relatively simple	(a) preparation fairly lengthy
	(b) no heating, derivatization or extraction necessary	(b) extraction, but no derivatization or heating	(b) derivatization but no extraction or heating	(b) derivatization, heating and extraction	(b) derivatization, heating and extraction
	(c) chromatography 5 min	(c) chromatography 7 min	(c) chromatography 10 min	(c) chromatography 10 min	(c) chromatography 8 min
6. Stability	excellent	poor	derivatives stable for 1 h	derivatives stable for 4 h	derivatives stable for 7 h
7. Practical operation	very simple	complex	simple	simple	hazardous and prone to contamination

tainable using the XAD-2 resin extraction process. Plasma samples (1 ml) can, with care, be processed in the same way to obtain an injection solution of 1 ml, and for the NQS and NBD methods a concentration step is possible by evaporation of the final organic solvent (1 ml) and solvation of the residue in 250 μl solvent. For all the HPLC systems, sensitivities can be improved by increasing the volume injected, up to 100 μl, although the loss in resolution will depend upon the column efficiency.

After an oral dose of 10—15 mg amphetamine sulphate, peak plasma concentrations of 40—50 ng/ml are attained in 1—2 h, falling to about 2 ng/ml after 8—10 h [39]. This study has shown that only the method employing derivatization with NQS is able to provide the required sensitivity. It was found to be a simple, reliable and rapid method, suitable for the routine determination of amphetamine and methylamphetamine in plasma or urine. It is not suitable for hydroxylated metabolites, but these form a very minor percentage of the excreted forms. The reagent employed in the NBD method was found to accumulate in the injection valve and glassware, despite repeated and thorough rinsing. The reaction products were extremely darkly coloured and left a sticky residue upon evaporation. Although linear calibrations were obtained, the derivatization reaction was found to be unreliable for routine use. The OPA method was simple, reliable and rapid, but did not provide us with the sensitivity required. The straight-phase system employing NS had better sensitivity but we were unable to maintain column stability. Our studies on the methods of improving the detection of amphetamines and related compounds are continuing.

ACKNOWLEDGEMENT

The authors wish to acknowledge the valuable technical assistance of Mr. P. Reynolds.

REFERENCES

1 G.P. Cartoni and F. DeStefano, Ital. J. Biochem., 12 (1963) 296—309.
2 J. Caldwell, Drug Metab. Rev., 5 (1978) 219—280.
3 The Pharmaceutical Society of Great Britain, The Pharmaceutical Codex, The Pharmaceutical Press, London, 1979.
4 A. Wilson and H.O. Schild, Applied Pharmacology, Churchill, London, 1968.
5 M. Pesez and J. Bartos, Colourimetric and Fluorimetric Analysis of Organic Compounds and Drugs, Marcel Dekker, New York, 1974, pp. 150—151.
6 P.J. Cashman, J.D. Beedle and J.I. Thornton, J. Forensic Sci. Soc., 19 (1979) 137—141.
7 K.J. Schoen, J. Amer. Pharm. Assoc. Sci. Ed., 33 (1944) 116—118.
8 R.G. Martinek and E.S. Brady, Drug Stand., 22 (1954) 222—228.
9 J.E. Wallace, J.D. Biggs and S.L. Ladd, Anal. Chem., 40 (1968) 2207—2210.
10 H.M. Stevens, J. Forensic Sci. Soc., 13 (1973) 119—125.
11 F. Van Hoof and A. Heyndrickx, Anal. Chem., 46 (1974) 286—288.
12 J.T. Stewart and D.M. Lotti, J. Pharm. Sci., 60 (1971) 461—463.
13 C.C. Fulton, Modern Microcrystal Tests for Drugs, Wiley-Interscience, New York, 1969.
14 A.C. Moffat, in E.G.C. Clarke (Editor), Isolation and Identification of Drugs, The Pharmaceutical Press, London, 1974.

15 A.C. Moffat, J. Chromatogr., 110 (1975) 341—347.
16 Y. Hashimoto, M. Edno, K. Tominaga, S. Inuzuka and M. Moriyasu, Mikrochim. Acta, II (1978) 493—504.
17 A.H. Beckett and M. Rowland, J. Pharm. Pharmacol., 16 Suppl. (1964) 27T—31T.
18 A.H. Beckett, G.T. Tucker and A.C. Moffat, J. Pharm. Pharmacol., 19 (1967) 273—294.
19 C.R. Clarke, D.J. Teague, M.M. Wells and J.H. Ellis, Anal. Chem., 49 (1977) 912—915.
20 K. Kamei, M. Murata, K. Ishii, M. Nametaka and A. Momose, Chem. Pharm. Bull., 21 (1973) 1996—2003.
21 P.J. Cashman, J.I. Thornton and D.L. Shelman, J. Chromatogr. Sci., 11 (1973) 7—9.
22 W.A. Trinler, D.J. Reuland and T.B. Hiatt, J. Forensic Sci. Soc., 16 (1976) 133—138.
23 L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley-Interscience, New York, 1979.
24 P.A. Bristow, P.N. Brittain, C.M. Riley and B.F. Williamson, J. Chromatogr., 131 (1977) 57—64.
25 J. Crommen, B. Fransson and G. Schill, J. Chromatogr., 142 (1977) 283—297.
26 M. Endo, H. Imamichi, M. Moriyasu and Y. Hashimoto, J. Chromatogr., 196 (1980) 334—336.
27 M. Roth, Anal. Chem., 43 (1971) 880—882.
28 J.R. Benson and P.E. Hare, Proc. Nat. Acad. Sci. U.S., 72 (1975) 619—622.
29 T.P. Davis, C.W. Gehrke, C.W. Gehrke, Jr., T.D. Cunningham, K.C. Kuo, K.O. Gerhardt, H.D. Johnson and C.H. Williams, J. Chromatogr., 162 (1979) 293—310.
30 Technical Bulletin, Fluorotags, Regis Chemical Company, IL, U.S.A.
31 J.F. Lawrence and R.W. Frei, Chemical Derivatization in Liquid Chromatography, Elsevier, Amsterdam, Oxford, New York, 1976.
32 E. Tomlinson, T.M. Jefferies and C.M. Riley, J. Chromatogr., 159 (1978) 315—358.
33 Cs. Horváth, W. Melander, I. Molnár and P. Molnár, Anal. Chem., 49 (1977) 2259—2305.
34 C.M. Riley, E. Tomlinson and T.M. Jefferies, J. Chromatogr., 185 (1979) 197—224.
35 T. Gürkan, Microchim. Acta, I (1976) 165—171.
36 P.B. Ghosh and M.W. Whitehouse, Biochem. J., 108 (1968) 155—156.
37 J. Monforte, R.J. Bath and T. Sunshine, Clin. Chem., 18 (1972) 1329—1333.
38 T.J. Hopen, R.C. Briner, H.G. Sadler and R.L. Smith, J. Forensic Sci., 21 (1976) 842—850.
39 A.H. Beckett, in H. Steinberg (Editor), Scientific Basis of Drug Dependence, Churchill, London, 1969, pp. 129—148.

Journal of Chromatography, 272 (1983) 129—136
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1473

DETERMINATION OF ISOSORBIDE DINITRATE AND ITS MONONITRATE METABOLITES IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY—THERMAL ENERGY ANALYSIS

J. MADDOCK*, P.A. LEWIS, A. WOODWARD, P.R. MASSEY and S. KENNEDY

Analytical Division, Simbec Research Limited, Merthyr Tydfil, Mid Glamorgan CF48 4DR (Great Britain)

(First received April 21st, 1982; revised manuscript received August 11th, 1982)

SUMMARY

An accurate and sensitive method for the simultaneous determination of isosorbide dinitrate and its 2- and 5-mononitrates in human plasma has been developed. Following extraction of 3.0 ml of plasma with 12.0 ml of dichloromethane—ethyl acetate (1:1) the extract is subjected to high-performance liquid chromatography employing a Zorbax NH_2 column. The eluent stream is introduced into a thermal energy analyser, employing chemiluminescence as a specific means of detection. The minimum quantifiable level of the compound in plasma is 200 pg/ml allowing the quantitation of isosorbide dinitrate in human plasma following single oral administration. Nitroglycerin is employed as internal standard.

INTRODUCTION

Isosorbide dinitrate (ISDN) is widely used for the prophylactic treatment of angina pectoris and for the treatment of refractory congestive heart failure. Following the single oral administration of therapeutic doses of ISDN, plasma levels in the sub-nanogram to low-nanogram per ml range are encountered for the parent compound and a number of gas—liquid chromatographic (GLC) procedures [1—3] have been developed which are sensitive to these levels, using electron-capture detection. Although these methods are suitable for the parent compound, high background responses interfere with the analysis of the metabolites despite the use of elaborate clean-up procedures. A capillary column GLC—electron-capture procedure has been reported [4] which does not suffer this interference, but in our hands the sensitivity for ISDN was

0378-4347/83/0000—0000/$03.00

inadequate and the claimed detection limits of 0.5 ng/ml can rarely be attained in routine use.

High-performance liquid chromatography (HPLC) with thermal energy analysis (TEA) detection has been employed [5] to determine another polynitric ester, nitroglycerin in dog plasma using normal-phase silica columns and gradient elution.

The thermal energy analyser uses the chemiluminescence of nitrogen dioxide radicals [6] produced by pyrolysis of the organic nitrates as the basis of detection.

A preliminary method for the determination of ISDN and its metabolites using HPLC–TEA with gradient elution has been reported [7]. This method employs Preptube extraction cartridges (Thermo Electron Corp., Waltham, MA, U.S.A.) for sample preparation which is time-consuming and costly and gave variable recoveries in our hands.

This present paper describes an HPLC–TEA procedure for ISDN and its 2- and 5-mononitrates which is highly sensitive and reproducible without the necessity for elaborate clean-up procedures and redistillation of reagents. This method, using a polar bonded phase and isocratic elution is thus eminently suitable for routine use.

The developed procedure has been used for the analysis of plasma ISDN and mononitrate concentrations in pharmacokinetic studies with adult healthy subjects of ISDN sublingual tablets, sustained-release tablets and capsule formulations and creams.

EXPERIMENTAL

Reagents and materials

All reagents used were of analytical grade and aqueous based reagents were prepared using double glass distilled water. *n*-Hexane and methanol were of far-UV S grade (Rathburn Chemicals, Walkerburn, Great Britain) and the ethyl acetate used was Nanograde (Mallinckrodt, St. Louis, MO, U.S.A.). All glassware was treated with Surfasil [Pierce and Warriner (UK), Chester, Great Britain] and allowed to dry prior to use. Standard solutions of ISDN were prepared freshly each day at a concentration of 10 mg per 100 ml in *n*-hexane, whereas the isosorbide 2-mononitrate (I-2-MN) and isosorbide 5-mononitrate (I—5-MN) were prepared in similar concentrations in dichloromethane–ethyl acetate (1:1). The internal standard, nitroglycerin, was prepared at a concentration of 10 mg per 100 ml in *n*-hexane. Working standard solutions were then prepared at a concentration of 0.1 mg per 100 ml for each component, diluting in the appropriate solvent used for the stock standard solutions.

Pure, authentic samples of ISDN, I-2-MN, I-5-MN and nitroglycerin were kindly donated by Professor A.H. Beckett, Department of Pharmacy, Chelsea College, London, Great Britain.

Collection of blood samples

Blood samples (10 ml) were taken into glass collection tubes (pretreated with Surfasil) containing 500 units of lithium heparin and 25 μl of 0.002 *M* silver nitrate solution. Following centrifugation the plasma was withdrawn and

stored in glass tubes at −20°C.

Extraction procedure

Aliquots (3.0 ml) of plasma samples were pipetted into 15.0-ml glass extraction tubes fitted with screw caps provided with PTFE liners. Nitroglycerin internal standard solution (30.0 μl, 30 ng) was added to all tubes giving an internal standard concentration of 10 ng/ml plasma. Then 12.0 ml of dichloromethane—ethyl acetate (1:1) were added, the tubes capped tightly and shaken mechanically for 5 min at 250 cycles/min. Following centrifugation at 550 *g* for 5 min at 4°C the organic phases were transferred to 20-ml glass vials using pasteur pipettes. The organic phases were evaporated to approximately 20-μl volumes under a gentle stream of nitrogen at room temperature. The vials were stored frozen at −20°C until just prior to chromatography.

Chromatography

Chromatography was performed on a 25 cm × 0.04 cm I.D. stainless-steel column, prepacked with Zorbax NH_2 polar bonded phase material (10 μm particle size) [DuPont Instruments (UK), Hitchin, Great Britain]. The mobile phase consisted of 5.0% methanol in *n*-hexane maintained at a flow-rate of 5.0 ml/min using an LDC Constametric IIC pump (Milton Roy Corporation, FL, U.S.A.). The eluate stream was directly coupled to a thermal energy analyser, Model 502A (Thermo Electron Corp.) with a furnace temperature of 575°C, argon flow-rate of 15 ml/min, oxygen flow-rate of 25 ml/min, with a slush bath of methanol and solid carbon dioxide pellets maintained at −77°C.

The sample extracts were introduced into the chromatograph using a Rheodyne 100-μl loop injector valve (Rheodyne, CA, U.S.A.).

RESULTS AND DISCUSSION

Calibration

Calibration samples were prepared using pooled control (drug-free) plasma. Aliquots (3.0 ml) were spiked by the addition of ISDN, I-2-MN, and I-5-MN working standard solutions to produce concentration ranges of 0.5—20.0 ng/ml, 1.0—40.0 ng/ml and 5.0—80.0 ng/ml, respectively.

Standard calibration lines were constructed from plotting the peak height ratios of the compound and its metabolites versus internal standard against the concentrations in the calibration standards. Linear regression analysis showed that the calibrations were linear over the concentration ranges: ISDN: $Y = 0.093X + 0.017$; I-2-MN: $Y = 0.0576X + 0.028$; and I-5-MN: $Y = 0.3523X + 0.0674$.

Table I shows typical values of the peak height ratios obtained on different occasions over a period of several weeks indicating good precision for each compound.

Further, calibrations over the standard range assayed in triplicate on the same day and on two further days consecutively, gave coefficients of variation (C.V.) for the slopes after linear regression analysis which again indicates good reproducibility (Table II).

The correlation coefficients of the linear regression analysis were found to be

TABLE I

PRECISION OF REPLICATE CALIBRATIONS OF THE HPLC—TEA ASSAY FOR ISDN AND ITS MONONITRATE METABOLITES IN PLASMA

Each value is the mean of five replicates.

Isosorbide dinitrate			Isosorbide-2-mononitrate			Isosorbide-5-mononitrate		
Value (ng/ml)	Mean peak height ratio (± S.D.)	Coefficient of variation (%)	Value (ng/ml)	Mean peak height ratio (± S.D.)	Coefficient of variation (%)	Value (ng/ml)	Mean peak height ratio (± S.D.)	Coefficient of variation (%)
0.5	0.03 (0.0049)	16.35	1.0	0.07 (0.015)	21.6	2.5	0.11 (0.015)	13.6
1.0	0.08 (0.009)	11.8	2.5	0.16 (0.197)	12.3	5.0	0.22 (0.02)	9.09
2.5	0.20 (0.019)	9.7	5.0	0.37 (0.035)	9.6	10.0	0.44 (0.08)	18.2
5.0	0.37 (0.026)	7.04	10.0	0.60 (0.044)	7.4	15.0	0.59 (0.07)	12.2
10.0	0.68 (0.036)	5.29	20.0	1.15 (0.08)	7.7	20.0	0.88 (0.18)	11.5
20.0	1.41 (0.075)	5.35	30.0	1.84 (0.11)	6.2	40.0	1.57 (0.2)	9.4
40.0	2.75 (0.132)	4.8	40.0	2.54 (0.13)	5.3	60.0	2.12 (0.16)	5.5

TABLE II

REPRODUCIBILITY OF CALIBRATION CURVES

Compound	C.V. (%)			
	Day 1	Day 2	Day 3	Mean
ISDN	2.93	0.17	1.33	1.48
I-2-MN	1.84	3.26	2.08	2.39
I-5-MN	13.86	0	4.02	5.96

0.999, 0.9986 and 0.9985 for ISDN, I-2-MN and I-5-MN, respectively.

Reproducibility

The reproducibility of the analysis for each compound was determined by assaying control spiked plasma samples with each batch, the data for which are found in Table III. This shows excellent reproducibility with coefficients of variation of 9.97, 13.45 and 9.9% overall for ISDN, I-2-MN and I-5-MN, respectively.

Recovery

Recovery of the compound and its metabolites was calculated over the

TABLE III

RECOVERY OF ISDN AND ITS MONONITRATE METABOLITES FROM SPIKED PLASMA CONTROL SAMPLES ASSAYED SINGLY OVER A PERIOD OF FOURTEEN DAYS

ISDN (ng/ml)		I-2-MN (ng/ml)		I-5-MN (ng/ml)	
Spiked value	Assayed value	Spiked value	Assayed value	Spiked value	Assayed value
2.0	2.2	10.0	11.0	10.0	11.2
6.0	5.7	20.0	18.5	30.0	30.5
2.7	3.5	12.0	13.4	10.0	9.0
20.0	19.8	2.5	2.5	47.0	43.0
2.5	2.7	13.3	11.8	60.0	58.0
10.0	8.9	60.0	62.5	10.0	9.5
20.0	19.4	6.7	5.5	40.0	39.0
13.3	14.0	13.3	14.5	6.0	8.0
6.7	6.5	33.0	32.0	10.0	9.0
20.0	20.5	6.0	6.5	60.0	58.0
3.3	3.2	3.0	4.5	15.0	15.0
3.0	3.0	10.0	9.0	30.0	32.0
7.0	7.5	60.0	62.0	70.0	68.1
2.5	2.2	2.5	2.5	10.0	10.0
1.7	1.5	10.0	12.0	20.0	22.0
5.0	5.0	25.0	28.5	40.0	37.5
30.0	32.0	60.0	63.0	10.0	9.5
2.5	2.8	3.3	3.0	20.0	19.0
5.0	4.6	10.0	10.5	50.0	53.0
7.0	6.5	13.3	12.5	12.0	13.0
20.0	19.0	20.0	20.0	66.0	63.5

calibration range by comparing the peak height ratios for non-extracted standards to those taken through the complete analytical procedure, including extraction. After correcting for the recovery of the internal standard (64.5%) the mean overall recoveries for the drug and its 2- and 5-mononitrate metabolites were found to be 98.2, 78.3 and 78.0%, respectively, comparing very favourably with other published data.

Chromatography

Typical chromatograms of a blank plasma sample and of a plasma sample from a subject in receipt of a single oral dose of 40 mg sustained release ISDN formulation are shown in Fig. 1. Under the conditions described in the Experimental section, the retention times of ISDN, nitroglycerin internal standard, I-2-MN and I-5-MN are 3.3 min, 5.0 min, 5.8 min and 8.4 min, respectively.

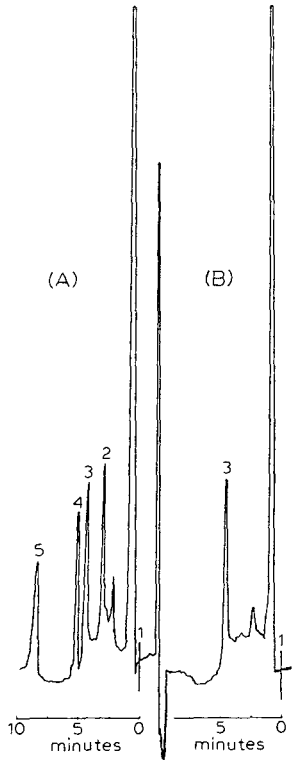


Fig. 1. (A) Chromatogram of human plasma from a subject in receipt of 40 mg ISDN. Peaks: 1 = injection point; 2 = isosorbide dinitrate; 3 = nitroglycerin internal standard; 4 = isosorbide-2-mononitrate and 5 = isosorbide-5-mononitrate. (B) Chromatogram of a control (drug-free) plasma extract.

Detection limits

The observed detection limits, based on the minimum peak to give a signal-to-noise ratio of 2:1 over baseline was 0.25 ng/ml for ISDN, 0.5 ng/ml for I-2-MN and 1.0 ng/ml for I-5-MN. The detection limits did vary from day to day depending on a number of factors: fluctuation in slush bath temperature,

fluctuation in detector vacuum, gradual contamination of the detector filter and the photomultiplier cooling temperature.

Fluctuations in slush bath temperature and photomultiplier temperature can easily be regulated since these parameters are continuously monitored. However, changes in detector vacuum and contamination of the detector filter can cause gradual loss of sensitivity. In order to control these variables a standard solution is injected daily to monitor sensitivity and when significant losses are found the detector is taken down and cleaned.

The values quoted above represent the best possible limits when conditions were optimal and more typically the detection limits on a routine basis were 0.5 ng/ml for ISDN, 0.8 ng/ml for I-2-MN and 1.2 ng/ml for I-5-MN. This is confirmed by calculating the minimum quantitation levels (MQL) by computer program based on the 80% confidence limits of a value being differentiated from zero. From the data presented in Table I, the MQL values for ISDN, I-2-MN and I-5-MN were found to be 0.56 ng/ml, 0.86 ng/ml and 1.66 ng/ml, respectively.

Human studies

The HPLC—TEA procedure has been employed in the study of ISDN pharmacokinetics with a number of different dosage forms. Fig. 2 illustrates two dose—response curves for ISDN in plasma, (A) mean plasma concentrations from twelve adult healthy male subjects in receipt of a 40-mg sustained release ISDN tablet showing mean peak concentrations of plasma ISDN at 6 h at 4.5 ng/ml with mean concentrations of 1.15 ng/ml persisting at 12 h post dosing, and (B) for comparison the mean plasma concentrations from the same twelve subjects following a 5-mg sublingual tablet administration with a peak ISDN concentration of 18.2 ng/ml 20 min following dosing.

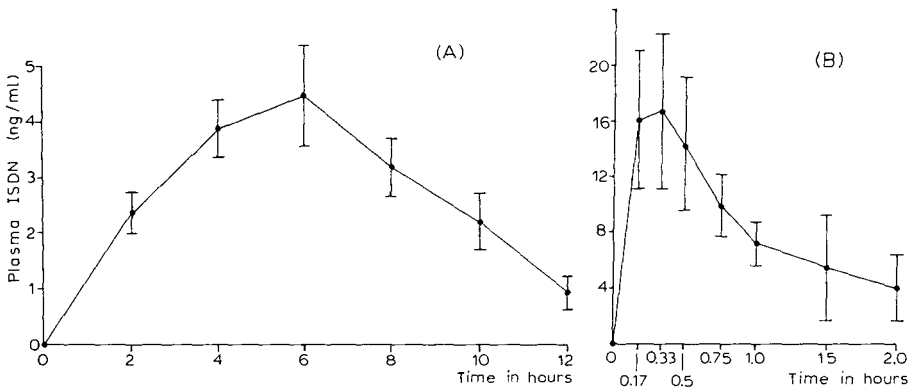


Fig. 2. (A) Mean plasma ISDN concentrations in twelve subjects following a single oral dose of a 40-mg ISDN sustained release tablet. (B) Mean plasma ISDN concentrations in the same twelve subjects following a single dose of a 5-mg sublingual ISDN tablet.

CONCLUSION

An accurate, sensitive and rapid procedure for determining ISDN and its mononitrate metabolites was developed using HPLC with TEA detection.

The assay is eminently suitable for the analysis of large numbers of samples, each chromatography run taking only 10 min and the procedure involves a simple extraction step with the minimum of reagent preparation, e.g. redistillation of solvents, etc. In our hands, the HPLC—TEA system is used manually. However, the system could be fully automated if some method could be devised to flush the cold trap of mobile phase periodically. In addition to ISDN the method allows for the sensitive analysis of its mononitrate metabolites during the same chromatographic run, and in this respect this reported procedure is superior to published methods employing GLC with electron-capture detection.

REFERENCES

1 K.H. Gobbeler, Pharm. Ztg., 27 (1971) 961.
2 M.T. Rosseel and M.G. Bogaert, J. Pharm. Sci., 62 (1973) 754.
3 H. Laufen, F. Scharpf and G. Bartsch, J. Chromatogr., 146 (1978) 457.
4 M.T. Rosseel and M.G. Bogaert, J. Pharm. Sci., 68 (1979) 659.
5 R.J. Spanggord and R.G. Keck, J. Pharm. Sci., 69 (1980) 444.
6 I.S. Krull, T.Y. Fan, M. Wolf, R. Ross and D.H. Fine, in G. Hawk (Editor), Biological and Biomedical Applications of Liquid Chromatography, Marcel Dekker, New York, 1978, pp. 443—474.
7 A. Silvergleid, W. Yu and B. Morriseau, Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Pittsburgh, PA, 1980.

Journal of Chromatography, 272 (1983) 137—148
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1483

DISTRIBUTION OF CHLOROQUINE AND ITS METABOLITE DESETHYL-CHLOROQUINE IN HUMAN BLOOD CELLS AND ITS IMPLICATION FOR THE QUANTITATIVE DETERMINATION OF THESE COMPOUNDS IN SERUM AND PLASMA

YNGVE BERGQVIST* and BRITTA DOMEIJ-NYBERG

Department of Clinical Chemistry and Department of Rheumatology, Falun Central Hospital, S-791 82 Falun (Sweden)

(First received February 22nd, 1982; revised manuscript received August 24th, 1982)

SUMMARY

The amount of chloroquine and desethyl-chloroquine was determined in samples of total blood and in blood cell fractions from three normal subjects after one oral dose of 1000 mg of chloroquine diphosphate. About 70—85% of the total whole blood content of choroquine and its metabolite desethyl-chloroquine were recovered in blood cells isolated from whole blood, indicating that these compounds have a high cell/plasma concentration ratio. They were mainly present in thrombocytes and granulocytes.

A study of 40 patients taking chloroquine regularly as a treatment for rheumatoid arthritis showed significantly higher concentrations of chloroquine and desethyl-chloroquine in serum than in plasma. The concentration of chloroquine was about two times higher in serum than in plasma and for desethyl-chloroquine the concentration was about four times higher in serum than in plasma. These differences may be explained by a release of chloroquine and desethyl-chloroquine from thrombocytes during the coagulation of blood. The practical implication of the results is that the samples taken for chloroquine determination must be clearly identified as serum or plasma.

INTRODUCTION

In the measurement of drug concentration, binding of drugs to the cellular components of human blood has hitherto received little attention. Ehrnebo et al. [1] reported that 45—51% of the amount of pentazocine in whole blood is bound to blood cells. For chlorthalidone the amount in whole blood is ten times larger [2] than in plasma. At a given plasma concentration of tricyclic antidepressants, a six-fold interindividual variation in the erythrocyte levels was reported [3]. For digoxin the amount in erythrocytes is 2—6 times greater

0378-4347/83/0000—0000/$03.00

than in plasma [4], and the amount of carbamazepine in erythrocytes is 11—23% of the amount in plasma [5].

It has been shown that the time elapsed between the collection and centrifugation of blood significantly effects the plasma concentrations of gentamicin [6] and furosemide [7].

In evaluating the significance of drug concentration in blood for routine therapeutic drug monitoring it is therefore important to know whether the analytical determinations were carried out on whole blood, plasma or serum.

Some basic drugs such as chloroquine (CQ) [8] are accumulated in the organelles in thrombocytes. Thrombocytes have been shown to strongly bind another basic drug, imipramine [9].

Despite the widespread use of CQ in the prevention of malaria and in the treatment of rheumatoid arthritis, relatively little is known about its distribution in blood cells.

The aim of the present study was to measure the distribution of CQ and its metabolite desethyl-chloroquine (CQM) in blood cells, i.e. erythrocytes, granulocytes and thrombocytes, both in vivo and in vitro, and thereby evaluate some aspects of the sample handling before the assay of CQ and CQM.

EXPERIMENTAL

Blood samples and sample handling

Studies of the in vivo blood cell binding were performed on three healthy volunteers aged 35—46 years (two male and one female). They were given one oral dose of 1000 mg of chloroquine diphosphate. After 7 h, samples of antecubital venous blood were withdrawn into tubes containing EDTA.

Furthermore, EDTA-stabilized venous blood from two of these persons was used for experiments of the in vitro uptake of CQ and CQM in blood cells (about 7—8 months after the oral dose of 1000 mg of chloroquine diphosphate).

Studies on the concentration difference of CQ and CQM between plasma and serum were made on venous blood samples from 40 patients undergoing continuous CQ therapy for rheumatoid arthritis. They were between 41 and 65 years old and had been taking one oral dose of 0.16—0.25 g of chloroquine diphosphate daily for at least two months. The samples were withdrawn in the morning before the daily dose.

Studies on the effect of the time elapsed between the collection and centrifugation of blood samples were made on venous blood from six of these patients. Five samples each of heparin- and EDTA-stabilized whole blood were kept standing undisturbed at room temperature (23°C) for 0.5, 2, 4, 6 and 24 h before the plasma was separated by centrifugation. The plasma was then kept frozen (—65° C) until assayed.

The effect of variation of the centrifugal force was studied on the venous blood samples from three of the above six patients. Seven samples of heparin-stabilized whole blood from each patient were centrifuged 1—3 h after the collection at different centrifugal force for 10 min at room temperature (23° C) in a Laborfuge II centrifuge, (Heraeus Christ, Osterode, G.F.R.).

The sample tubes used for serum and EDTA-plasma came from Vacutainer (Becton-Dickinson, Rutherford, NJ, U.S.A.) and those used for heparin-plasma came from Venoject (Terumo, Tokyo, Japan).

Chemicals and reagents

Chloroquine, desethyl-chloroquine and the internal standard 6,8-dichloro-4-(1-methyl-4-diethylamino-butylamino)-quinoline, used in the liquid chromatographic determinations, were kindly donated by Sterling-Winthrop (Skärholmen, Sweden).

Lymphoprep^{R} was supplied from Nyegaard & Co. (Oslo, Norway) and Dextran 70, 6% in 154 mmol/l sodium chloride, came from Pharmacia Fine Chemicals (Uppsala, Sweden). All other reagents were analytical grade from Merck (Darmstadt, G.F.R.). The aqueous solutions were prepared using high-purity water obtained from a Milli-Q deionized water system (Millipore, Bedford, MA, U.S.A.). Polypropylene tubes from Sarstedt (Malmö, Sweden) and siliconized glass tubes were used in the cell separations.

Liquid chromatography assay

The analyses of CQ and CQM in whole blood, serum, plasma and various cell suspensions were assayed using a high-performance liquid chromatographic (HPLC) method with fluorescence detection. Concentrations down to 0.5 nmol/l (0.15 ng/ml) can be determined with a relative standard deviation of 12%. A description of this method has previously been published by our laboratory [10].

This method requires an extraction step. Unfortunately, the recovery of CQ and CQM from aqueous solutions in the concentration range of 200—500 nmol/l was only about 70—80%. Therefore, for analysis of cell suspension an equal amount of EDTA-plasma (from a plasma pool free from CQ or CQM) was added before the extraction. The result was an almost 90% recovery of CQ and CQM from aqueous solutions of 200 nmol/l, as seen in Fig. 1.

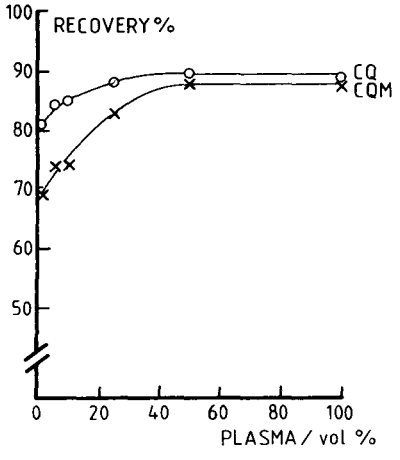


Fig. 1. Effect of different amounts of plasma in 154 mmol/l NaCl in phosphate buffer pH 7.0—7.4, on recovery of 200 nmol/l chloroquine (CQ) and desethyl-chloroquine (CQM) extracted as base with ethylene dichloride.

Isolation of blood cells

The isolation scheme is outlined in Fig. 2. All reagent solutions used contain 154 nmol/l NaCl in a phosphate buffer pH 7.0--7.4. This buffer was mixed with an equal amount of CQ- and CQM-free plasma (plasma-buffer) and was used as a suspension medium for cell counting and HPLC analysis.

On the basis of Bφyum's method for isolation of lymphocytes [11] and granulocytes, a technique was developed for the isolation of thrombocytes, granulocytes and erythrocytes. The aim was to obtain as pure cell fractions as possible with a reasonable recovery and not too many steps. The lymphocytes are in principle very interesting in experiments with blood cell uptake of drugs. However, the maximal recovery of lymphocytes was only about 20%. This was due to high losses of lymphocytes during the washing steps which were necessary to reduce the thrombocyte contamination.

Thrombocytes. A centrifugation isolation technique was employed. Eighteen millilitres of freshly drawn EDTA-blood were centrifuged at 100 *g*, for 15 min at 4°C. The plasma, containing the thrombocytes, was pipetted off into siliconized glass tubes using polypropylene pipettes. Since the plasma could not be completely removed without disturbing the buffy coat, about 10--15% of the plasma volume was left to avoid contamination with leucocytes. The

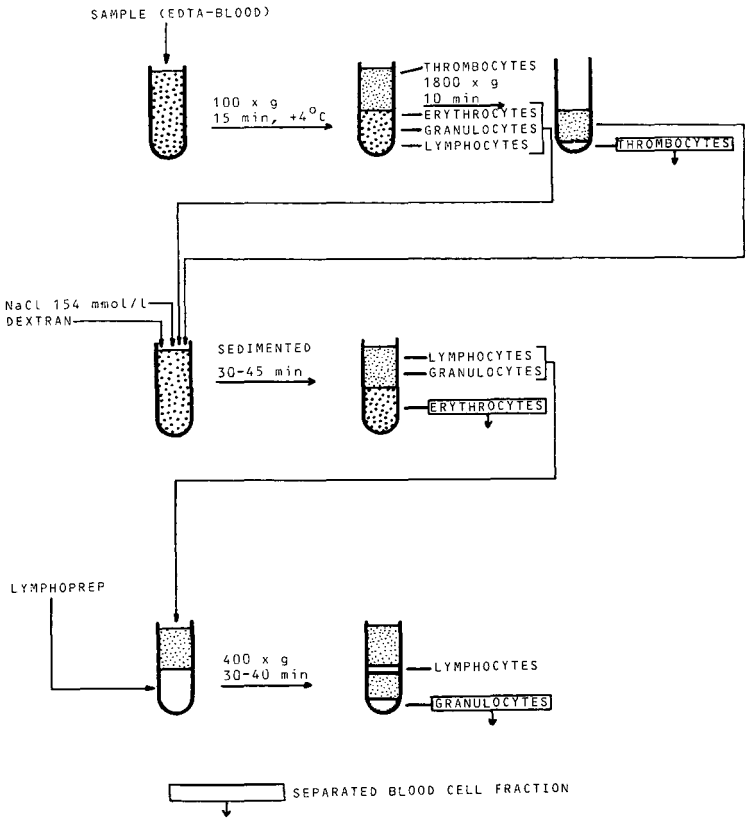


Fig. 2. Isolation of blood cells.

thrombocytes were centrifuged into a pellet at 1800 *g* for 10 min, leaving plasma which was free from thrombocytes above the pellet. This plasma was added to and mixed with the contents of the original blood tube for further separation of other blood cells. The pellets were resuspended in 5 ml of phosphate buffer containing 0.03 mol/l EDTA and 0.1 ml of plasma which was free from CQ and CQM. Contaminating erythrocytes and leucocytes were removed by centrifugation (100 *g*, 15 min) leaving a pure thrombocyte fraction. After centrifugation the thrombocytes were suspended in 6 ml of plasma-buffer.

Granulocytes. Whole blood, from which the main fraction of thrombocytes was removed by the above centrifugation technique, was prepared essentially as described by Bφyum [11]. Ten millilitres of blood were mixed with 4 ml of phosphate buffer, and then 2 ml of 6% Dextran 70. When the erythrocytes had sedimented (1 *g*, 30—45 min) the supernatant was pipetted off and carefully layered onto 8 ml of Lymphoprep. The tube was then centrifuged (400 *g*, 30--40 min). The supernatant was sucked up, leaving the granulocytes in the last 0.5—1.0 ml. To this fraction were admixed 5 ml of phosphate buffer containing 0.03 mol/l EDTA and 0.1 ml of plasma which was free from CQ and CQM. By centrifugation at 100 *g* for 10 min a granulocyte pellet was produced. Most of the contaminating lymphocytes and thrombocytes were left in the supernatant, which was removed. The washed granulocytes were suspended in 6 ml of plasma-buffer. The preparations contained 80—90% granulocytes, the contaminating cells being erythrocytes and lymphocytes.

Erythrocytes. The erythrocytes from the Dextran sedimentation step in the granulocyte separation above were centrifuged at 1000 *g* for 10 min and the supernatant and buffy coat were removed. The erythrocytes were washed three times with 7 ml of 154 mmol/l NaCl in phosphate buffer pH 7.0—7.4 and packed by centrifugation at 2000 *g*, 10 min. The erythrocyte fraction was almost free from other cells.

Cell counting

From the suspensions of the different blood cells, serial dilutions in three steps were made with the plasma-buffer. These diluted cell suspensions were counted in an automated blood-counting apparatus, Hemalog-8 (Technicon, Tarrytown, NY, U.S.A.), which is designed to count simultaneously erythrocytes, leucocytes and thrombocytes in blood samples. The purity of the blood cell fractions was also evaluated microscopically by manual counting in a cell chamber.

Analysis of CQ and CQM in cell fractions

The amounts of CQ and CQM in the different fractions were determined after adding 2 ml of 1 mol/l NaOH and freezing at −65° C to achieve cell lysis. The completeness of the cell lysis was controlled microscopically. The lysed cell suspensions were then extracted exactly as a plasma sample in the HPLC method [10]. Chromatograms from a granulocyte and a thrombocyte fraction are presented in Fig. 3 with a chromatogram of plasma standards for comparison. Fig. 3 also shows an unidentified metabolite present in the

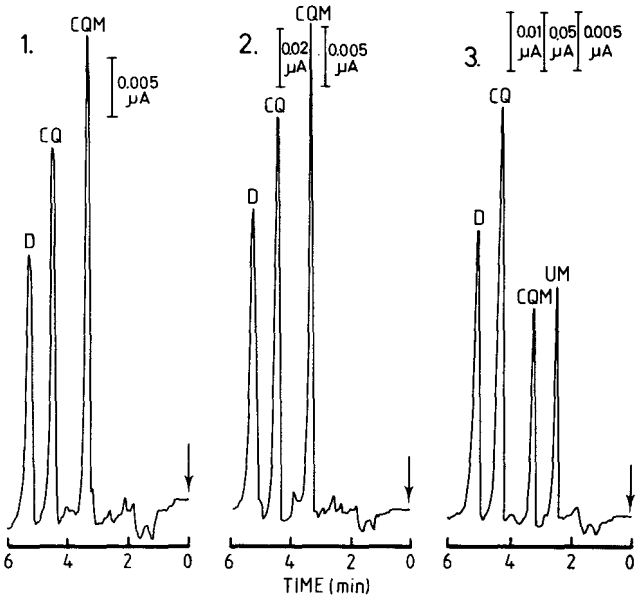


Fig. 3. Representative chromatograms from blood cell fractions and plasma standard of chloroquine (CQ) and desethyl-chloroquine (CQM). D = internal standard; UM = unidentified metabolite. (1) Plasma standard: 50 nmol/l CQ and CQM. (2) Granulocyte fraction: 130 nmol CQ per 10^9 cells and 27 nmol CQM per 10^9 cells from subject 1. (3) Thrombocyte fraction: 1.8 nmol CQ per 10^9 cells and 1.6 nmol CQM per 10^9 cells from subject 3.

thrombocyte fraction. The same metabolite is probably also present in plasma and urine [10].

In vitro uptake of CQ and CQM by blood cells

CQ and CQM, corresponding to about 1500—2000 nmol/l, were added to 25 ml of EDTA-stabilized whole blood from haematologically normal individuals. After incubation for 60 min at 37°C with mixing every 10 min, the blood cells were separated and the amount of CQ and CQM in the different blood cell fractions was determined as described above.

RESULTS

The concentration difference between plasma and serum

Fig. 4 shows the difference between serum and plasma concentrations of CQ and CQM in samples from 40 rheumatoid patients. The concentration of CQ in serum was two times higher, and the concentration of CQM in serum was four times higher than in plasma. The higher concentrations of CQ and CQM in serum might be explained as being due to the release of CQ and CQM from leucocytes and thrombocytes during the clotting process.

Sample handling

Fig. 5 shows that plasma should be separated from whole blood within 1—2 h to avoid enhanced plasma levels. In order to centrifuge the blood cells containing CQ and CQM, the centrifugal force must be above 1000 *g*, as seen in Fig. 6.

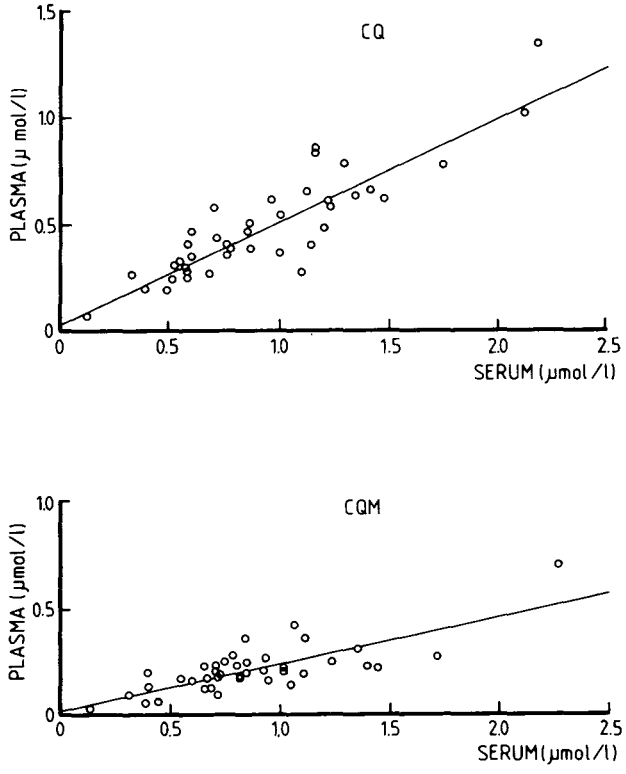


Fig. 4. Comparison between serum (X) and plasma (Y) from 40 patients on chloroquine therapy for chloroquine (CQ) and desethyl-chloroquine (CQM). Top graph: $Y = 0.50X + 0.024$, $r = 0.89$, $n = 40$. Bottom graph: $Y = 0.22X + 0.023$, $r = 0.76$, $n = 40$.

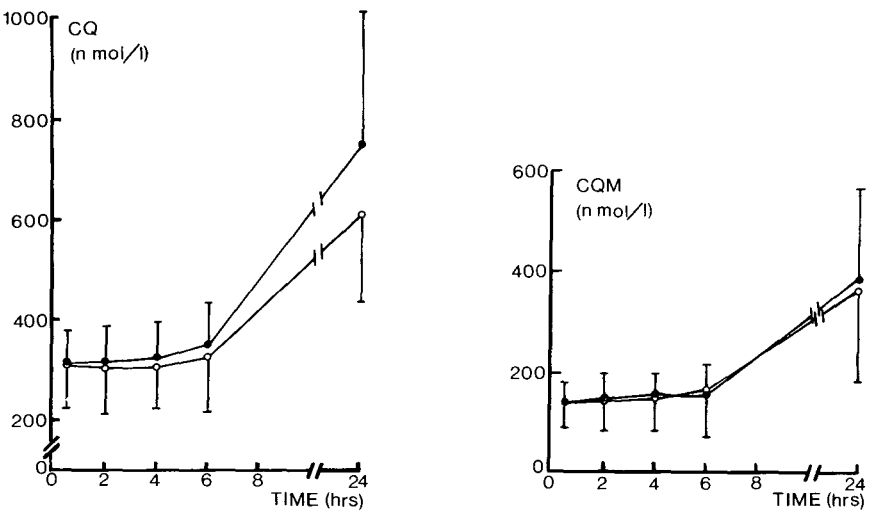


Fig. 5. The effect of storage of whole blood at room temperature (23°C) before centrifugation on the concentration of CQ (upper) and CQM (lower) in plasma. The length of the bar corresponds to the standard deviation. (•) Heparin-plasma; (○) EDTA-plasma.

TABLE I

IN VIVO BLOOD CELL, PLASMA, AND WHOLE BLOOD CONCENTRATIONS OF CHLOROQUINE (CQ) AND DESETHYL-CHLOROQUINE (CQM) (A) FROM THREE HEALTHY SUBJECTS GIVEN AN ORAL DOSE OF 1000 mg OF CHLOROQUINE PHOSPHATE AND (B) AFTER INCUBATION OF WHOLE BLOOD IN VITRO WITH 1500—2000 nmol/l CQ AND CQM FOR 60 min

Samples were taken 7 h after ingestion for the in vivo measurements. Subjects 1 and 2 are the same in vivo and in vitro.

	Subjects														
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
	No. of cells in whole blood ($\times 10^9$ cells/l)			CQ						CQM					
				nmol/10^9 cells			nmol/l whole blood*			nmol/10^9 cells			nmol/l whole blood*		
(A) In vivo															
Blood cells															
Granulocytes	4.5	3.3	3.6	130	221	105	585	729	378	27	24	17	122	79	61
(Lymphocytes	2.7	1.8	2.7)	—	—	—	—	—	—	—	—	—	—	—	—
Thrombocytes	259	254	205	3.9	2.4	1.8	1010	610	387	2.6	1.7	1.6	673	432	328
Erythrocytes	4100	5000	5100	0.101	0.040	0.030	414	200	153	0.046	0.016	0.014	189	80	71
Plasma**,***				—	—	—	547	246	149	—	—	—	224	57	62
Whole blood***				—	—	—	2880	2050	1350	—	—	—	1100	707	507
Recovery+				—	—	—	2560 (89%)	1790 (87%)	1070 (79%)	—	—	—	1210 (110%)	650 (92%)	520 (103%)
(B) In vitro															
Blood cells															
Granulocytes	2.9	3.2	5.9	233	120	71	676	384	419	75	29	27	218	93	159
(Lymphocytes	4.7	2.0	5.1)	—	—	—	—	—	—	—	—	—	—	—	—
Thrombocytes	251	250	385	1.7	1.5	1.2	440	375	462	4.7	3.0	2.7	1180	750	1040
Erythrocytes	4300	5400	4900	0.053	0.044	0.030	207	238	162	0.065	0.046	0.044	280	225	216
Plasma**,***				—	—	—	268	241	95	—	—	—	318	233	173
Whole blood***				—	—	—	1870	1440	1460	—	—	—	2080	1570	1770
Recovery§				—	—	—	1590 (85%)	1240 (86%)	1140 (78%)	—	—	—	2000 (96%)	1300 (83%)	1590 (90%)

*The numbers in these columns represent the contribution of the fractions to the total whole blood concentration.
**The concentration of CQ and CQM in plasma is corrected for the hematocrit.
***Plasma and whole blood concentration of CQ and CQM were analysed by the method described in ref. 10.
§The recovery in whole blood is calculated by adding the concentration contribution of the different blood cells to that of plasma. This value is compared with the total concentration in whole blood.

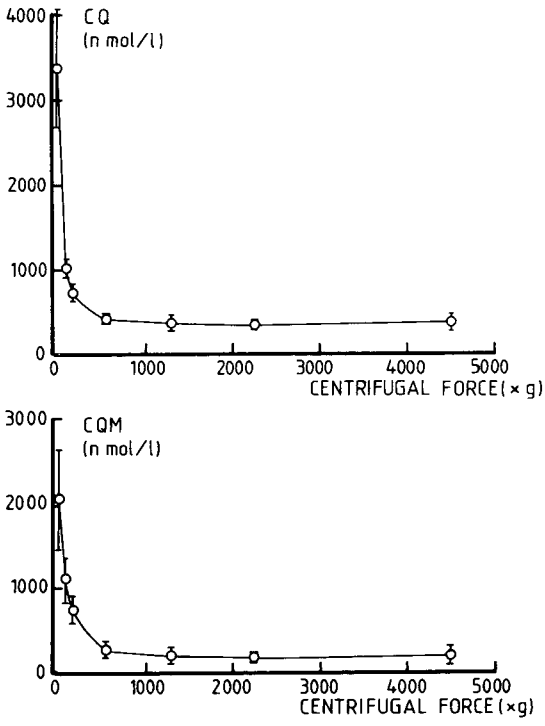


Fig. 6. The effect of variation of the centrifugal force (*g*) on CQ (upper) and CQM (lower) concentration in plasma. Centrifugation time 10 min. The length of the bar corresponds to the standard deviation.

Recovery of the cell fractions

The recovery of the different cell types from whole blood with the presented separation technique was for thrombocytes 64 ± 14%, for granulocytes 47 ± 8%, and for erythrocytes 73 ± 5%, calculated from seven experiments.

In vivo uptake of CQ and CQM by blood cells

Four different dilutions were made from the blood cell fractions of erythrocytes, granulocytes and thrombocytes. Cell counts and CQ and CQM analyses were made on these dilutions and plotted as shown, for granulocytes and thrombocytes from one subject, in Fig. 7. This demonstrates the good positive linear correlation between the cell counts for thrombocytes and granulocytes and the HPLC determinations in blood cells, and confirms that the measuring ranges are suitable for determination of CQ and CQM in blood cells.

The slope of the regression lines provides the CQ and CQM amount per 10^9 cells. This, together with the count of erythrocytes and thrombocytes in the original whole blood sample, gives the contribution to the total blood CQ and CQM concentrations from these cells. In a similar manner, the granulocyte contribution is calculated from the total leucocyte count and from the differential counting.

Table I shows the amounts and concentrations of CQ and CQM in different blood cells from the three subjects who had taken one oral CQ dose. The large

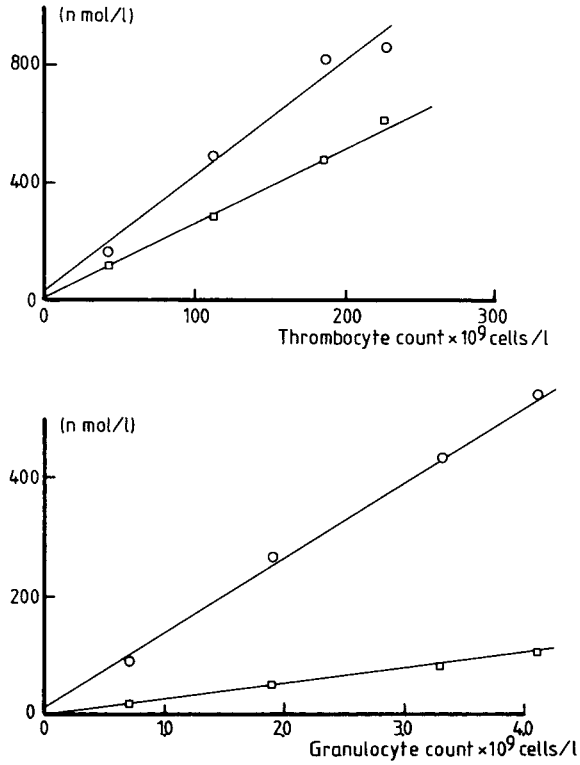


Fig. 7. Relationship between thrombocyte and granulocyte cell counts and chloroquine (CQ) and desethyl-chloroquine (CQM) concentration. Top panel: for CQ (○), $Y = 3.92 \cdot 10^{-9} X + 35$, $r = 0.986$; for CQM (□), $Y = 2.60 \cdot 10^{-9} X + 6$, $r = 0.998$. Bottom panel: for CQ (○), $Y = 130 \cdot 10^{-9} X + 9$, $r = 0.999$; for CQM (□), $Y = 27 \cdot 10^{-9} X - 1.8$, $r = 0.999$.

amount of CQ and CQM in granulocytes and thrombocytes is especially noteworthy.

Of the total concentration in whole blood, 84 ± 5% of CQ and 96 ± 9% of CQM were recovered in the different fractions in vivo as well as in vitro (Table I). However, there are many steps in the cell separation procedure. The error of cell counts and differential counts is 5—10% and the error in the chromatographic analysis is about 5%. The systematic recovery deficit could be explained by the assumption that the lymphocytes contain CQ and CQM.

In vitro uptake of CQ and CQM by blood cells

The uptake in vitro of CQ and CQM in blood cells was about the same as in vivo. The results are also presented in Table I.

DISCUSSION

In this study we have found large amounts of CQ and CQM in thrombocytes and granulocytes in vivo as well as in vitro. In other investigations [12] it has been shown that erythrocytes from monkeys infected with *Plasmodium*

falciparum bind CQ to a high degree. In uninfected erythrocytes such high binding could not be seen. It is a common practice that the erythrocyte cell concentration of drugs is calculated from analysis on serum and whole blood [13]. If the blood/plasma distribution ratio exceeds 1.0, the drug is considered to be distributed in the erythrocytes [14]. As we have shown, other blood cells should also be taken into consideration.

A very important question in clinical practice is which biological material is the most suitable for the analysis of drugs. Concerning the determination of CQ and its metabolite CQM we may conclude that heparin- or EDTA-plasma are the most suitable biological materials, if they are depleted of thrombocytes and granulocytes within 1—2 h after the sampling by centrifugation at high speed (>1000 *g*, 15 min).

As reported in a previous paper [15], we found no change in CQ and CQM values when the plasma samples were stored for up to eleven months at −20° C. Repeated freezing and thawing, five times, of a plasma pool sample from patients undergoing treatment with CQ did not result in any change of CQ and CQM concentration.

In a recent investigation [16] it was found that the concentration of the antiarrhythmic drug quinidine was higher in serum than in plasma. Quinidine and CQ are both 4-aminoquinolines and therefore could have similar binding properties to the thrombocytes and granulocytes. The quinidine results could be explained in the same way as the CQ and CQM serum/plasma discrepancy.

The uptake of CQ and CQM by blood cells, demonstrated in this investigation, could be important for the effect of CQ on the inflamed tissue in rheumatoid arthritis. The effect of CQ is not clearly understood, but an accumulation of CQ has been shown in the lysosomes of many different cells [17], including fibroblasts [18, 19], from many different tissues. Both granulocytes and thrombocytes possess a large amount of lysosomes [20, 21]. It has also been shown that CQ has a stabilizing effect on the lysosome membrane [22], inhibits the transformation of lymphocytes [23], and reduces the activity of several lysosomal enzymes [18, 24]. The transportation of CQ with the granulocytes to the inflamed tissue would improve the possibility for a higher concentration locally, thereby making an enhanced action of CQ possible.

In conclusion, the study has shown the importance of sample handling and choice of material for analysis of drugs in blood. The technique used for estimation of the amount of CQ and CQM in blood cells could easily be adapted for other drugs provided suitable analytical methods are available.

ACKNOWLEDGEMENTS

We are very grateful to Dr. Lars Funding and Dr. Ejvind Lyders-Hansen for valuable discussions on the manuscript and their kind interest in this work. We are also very grateful to Solveig Eckerbom and Christer Hed for stimulating collaboration and skilful technical assistance.

This work was supported by the Gustav V Research Foundation, Swedish National Association against Rheumatoid Diseases, and the Ester Asberg-Lindbergs Foundation.

REFERENCES

1 M. Ehrnebo, S. Agurell, L.O. Boreus, E. Gordon and U. Lönroth, Clin. Pharmacol. Ther., 16 (1974) 424—429.
2 P. Collste, M. Garle, M.D. Rawlins and F. Sjökvist, Eur. J. Clin. Pharmacol., 9(1976) 319—325.
3 M. Linnoila, F. Dorrity and K. Jobson, Amer. J. Psychiat., 135 (1978) 557—561.
4 R. Gorodisscher, S. Jusko and S.J. Yaffe, Clin. Pharmacol. Ther., 19 (1976) 256--263.
5 S. Pynnönen and T. Yrjänä, Int. J. Clin. Pharmacol., 15 (1977) 222—226.
6 M.G. Lee, M.-L. Chen, S.-M. Huang and W.L. Chiou, Biopharm. Drug Dispos., 2 (1981) 89--97.
7 M.G. Lee, M.-L. Chen and W.L. Chiou, Res. Commun. Chem. Pathol. Pharmacol., 34 (1981) 17—28.
8 M. Da Prada and A. Pletscher, Eur. J. Pharmacol., 32 (1975) 179—185.
9 S.Z. Langer, M.S. Briley, R. Raisman, J.F. Henry and P.L. Morsellei, Arch. Pharmacol., 313 (1980) 189—194.
10 Y. Bergqvist and M. Frisk-Holmberg, J. Chromatogr., 221 (1980) 119—127.
11 A. Bøyum, Scand. J. Immunol., 5 (Suppl. 5) (1976) 9—15.
12 C.D. Fitch, Science, 169 (1970) 289—290.
13 J.S. van Stone, J. Lab. Clin. Med., 88 (1976) 87—90.
14 K.P. Maguire, G.D. Burrows, R.T. Norman and B.A. Scoggins, Clin. Chem., 26 (1980) 1624—1625.
15 Y. Bergqvist and S. Eckerbom, J. Chromatogr., 226 (1981) 91—97.
16 S.O. Daylily, W.J. Poznanski and F.M. Smith, Clin. Biochem., 13 (1980) 297—300.
17 N.J. Zvaifler, Arthritis Rheum., 7 (1964) 760—761.
18 M. Wibo and B. Poole, J. Cell Biol., 63 (1974) 430—440.
19 C. de Duve, T. de Barsy, B. Poole, A. Trouet, P. Tulkens and F. van Hoff, Biochem. Pharmacol., 15 (1974) 2495—2531.
20 G.J. White and J.M. Gerrard, in G. Weissman (Editor), The Cell Biology of Inflammation, (Elsevier), Elsevier/North-Holland Biomedical Press, Amsterdam, 1980, p. 83.
21 M. Baggiolini, in G. Weissman (Editor), The Cell Biology of Inflammation, Elsevier/ North-Holland Biomedical Press, Amsterdam, 1980, p. 169.
22 G. Weissman, N. Engl. J. Med., 273 (1965) 1143—1149.
23 D.G. Trist and M. Weatherall, J. Pharm. Pharmacol., 33 (1981) 434—438.
24 J. Block, A.-A. Mulder-Stapel, L.-A. Ginsel and W. Daems, Cell Tissue Res., 218 (1981) 227—251.

Journal of Chromatography, 272 (1983) 149—155
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1489

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF DILTIAZEM AND ITS METABOLITE IN PLASMA

CHACKO VERGHESE, MARK S. SMITH, LIN AANONSEN, EDWARD L.C. PRITCHETT and DAVID G. SHAND*

Divisions of Clinical Pharmacology and Cardiology, Departments of Medicine and Pharmacology, Duke University Medical Center, Durham, NC 27710 (U.S.A.)

(First received July 12th, 1982; revised manuscript received August 26th, 1982)

SUMMARY

A rapid, selective and reproducible high-performance liquid chromatographic method for the analysis in plasma of the calcium channel blocking agent, diltiazem, and one of its metabolites, deacetyldiltiazem is described. The method involves extraction with the methyl *tert.*-butyl ether of the drugs and the internal standard (verapamil), back-extraction into sulphuric acid and reversed-phase chromatography with UV detection. Over a concentration range of 10—1000 ng/ml the average coefficient of variation for diltiazem was 5.4% and for deacetyldiltiazem was 8.3%.

INTRODUCTION

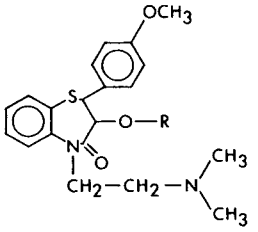
Diltiazem (Fig. 1) is a newer member of the calcium channel blocking agents which is currently undergoing intensive investigation. To date some pharmacokinetic data have been obtained [1—3] using the gas chromatographic method of Rovei et al. [4]. This method which uses a nitrogen detector is quite time consuming as it involves several extraction steps and requires silylation for the quantitation of the parent drug and its metabolite, deacetyldiltiazem. We have therefore developed a simpler high-performance liquid chromatographic (HPLC) method for the measurement of diltiazem and deacetyldiltiazem in plasma that is suitable for the analysis of clinical samples.

EXPERIMENTAL

Reagents and materials

Diltiazem, *d*-3-acetoxy-*cis*-2,3-dihydro-5-[2-(dimethylamino)ethyl]-2-(*p*-methoxyphenyl-1,5-benzothiazepin-4(5H)-one, hydrochloride and its deacetyl metabolite (II) were supplied by Marion Laboratories (Kansas City, MO,

0378-4347/83/0000—0000/$03.00



R = $COCH_3$ (I)

R = H (II)

Fig. 1. Structural formulae of diltiazem (I) and deacetyldiltiazem (II).

U.S.A.). Verapamil, α-[3-[[2-(3,4-dimethoxyphenyl)ethyl]-methylamino]propyl]-3,4-dimethoxy-(1-methylethyl)-benzeneacetonitrile, hydrochloride, was used as an internal standard and supplied by Searle Labs. (Chicago, IL, U.S.A.). All analytical standards were of pharmaceutical grade (> 99% purity) and stock solutions in methanol were stored at −20°C. Ammonium dihydrogen phosphate and triethylamine were purchased from Aldrich (Milwaukee, WI, U.S.A.). Methanol and methyl *tert.*-butyl ether were of HPLC grade and obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). All other reagents were of analytical grade.

Sample preparation

Plasma (1 ml) is placed in a 15-ml capacity culture tube fitted with a PTFE-lined screw cap and the internal standard, 50 μl of verapamil (750 ng per 50 μl) is added. The samples are extracted with 5 ml of methyl *tert.*-butyl ether by shaking for 10 min on a Labquake Shaker at 80—100 strokes/min. Following centrifugation at 1000 *g* for 5 min, the tubes are immersed in a dry ice—acetone mixture and the upper organic phase transferred to a glass tube with an elongated cone bottom of approximately 100-μl volume; then 80 μl of 0.05 mol/l sulphuric acid are added and the mixture agitated in a Vortex mixer for 45 sec. After brief centrifugation, a 50-μl sample of the aqueous phase is injected onto the column.

Chromatography

The HPLC solvent delivery system is a Constametric III pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.). The analytical column is a prepacked (25 cm × 4.6 mm I.D.) stainless-steel column containing Zorbax™ CN (6 μm) polar bonded-phase packing used in the reversed-phase mode (DuPont, Wilmington, DE, U.S.A.). A six-port rotary valve injector (Model 7125, Rheodyne, Berkeley, CA, U.S.A.) with a 50-μl sample loop is used for sample injection. A cyano guard column cartridge (Brownlee Labs., Santa Clara, CA, U.S.A.) is used between the injector and the analytical column which effectively minimizes the accumulation of particulate matter on the analytical column. The mobile phase is a mixture of methanol—0.05 *M* ammonium dihydrogen phosphate—triethylamine (45:55:0.25, v/v), the pH being adjusted to 5.0 with 1.0 *M* phosphoric acid. The solvent flow-rate is 1.5 ml/min with a column inlet pressure of 103.4 bars. The eluate is monitored continuously for absorbance at 237 nm using a variable-wavelength Spectro-Monitor III (Laboratory Data Control) and the detector output is displayed on a Linear Instruments Model 858 dual-pen recorder (Irvine, CA, U.S.A.).

Calibration

Calibration curves were constructed by transferring the respective stock solutions of diltiazem and deacetyldiltiazem in methanol (10—1000 μl) to culture tubes in amounts to give final concentrations of 10, 25, 50, 100, 250, 500, 800 and 1000 ng/ml. These standards were evaporated to dryness under nitrogen at 37°C and the residue dissolved in 1.0 ml plasma before extraction and assay. The amounts of the drug and metabolite in the unknown samples were obtained through weighted linear regression analysis of the standards. The sample peaks were measured and analyzed using peak height ratios (peak height of diltiazem or the deacetyl metabolite divided by the peak height of the internal standard) using linear regression employing the reciprocal of the concentration ($1/c$) as the weighting factor [5]. A 100-fold change in concentration is used in our calibration curve due to a wide variation in plasma levels depending on the route of administration and the dosage given. To quantitate the lower concentrations with better relative accuracy it was necessary to employ $1/c$ as the weighting factor rather than unit weighting which is generally used in linear regression analysis. As an alternative to weighted linear regression, two standard curves could be used, one covering the lower range (10—500 ng/ml), and another covering the higher range (500—1000 ng/ml) of concentrations. The advantage in choosing the former method lies in its convenience, and the simplicity of having one equation to define the entire concentration range. The coefficient of variation (C.V.) was used to determine the day-to-day reproducibility of the method and was calculated using normalized peak height ratios (the peak height ratio divided by the concentration of the standard).

Application of the method

This method of analysis has been employed by us in the study of diltiazem kinetics in patients with paroxysmal supraventricular tachycardia. On separate days, patients received a 20-mg infusion of diltiazem, and a 60- or 90-mg oral dose. Samples of venous blood were drawn at various intervals after each dose for 12 h. The plasma was separated and stored at —70°C for later analysis.

RESULTS AND DISCUSSION

The present HPLC assay involves an extraction into methyl *tert.*-butyl ether, followed by back-extraction into 0.05 mol/l sulphuric acid and reversed-phase chromatography using a Zorbax CN column and UV detection. The back-extraction step into 0.05 mol/l sulphuric acid greatly decreased the chromatographic interference from plasma constituents. Initial studies showed that altering the pH of the plasma samples was unnecessary for good recovery. The absolute and relative recoveries for diltiazem and its metabolite are shown in Table I. Compared with diethyl ether, the use of methyl *tert.*-butyl ether gave much cleaner chromatograms of blank plasma samples and back-extraction into 0.05 mol/l sulphuric acid gave increased sensitivity compared with other acids such as acetic, hydrochloric and phosphoric acid. The normality of the sulphuric acid was very critical for the resolution of the peaks of interest. Below 0.05 mol/l sulphuric acid the resolution was lost, and above 0.05 mol/l sulphuric acid the column life deteriorated.

TABLE I

ANALYTICAL RECOVERIES AND EXTRACTION PRECISION

Each value represents the mean of five determinations.

Drug	Concentration (ng/ml)	Recovery* (%)			
		Relative	C.V. (%)	Absolute	C.V. (%)
Diltiazem	50	100	7.8	95	13.4
	500	100	4.2	88	11.3
Deacetyldiltiazem	50	99	9.6	93	15
	500	98	3.5	92	11.5

*The relative recovery was determined by comparing peak heights of the compounds after an extraction out of 1 ml plasma to an identical extraction out of water. To determine absolute recovery the drug was evaporated in a test tube and was extracted in the same manner as the plasma and water extraction. This extraction was compared to the plasma extraction to determine the absolute recovery. Verapamil (750 ng) was added to all tubes just prior to the back-extraction into 0.05 mol/l sulphuric acid.

The Zorbax CN column was chosen after comparison with reversed-phase (C_{18}) and adsorption (silica gel) columns. Of the microparticulate columns tested the Zorbax CN column exhibited a greater separation selectivity. The relative retention times of deacetyldiltiazem, diltiazem, and the internal standard were 2.50, 3.35 and 4.0, respectively. Fig. 2 illustrates a typical chromatogram for control human plasma and a sample from a patient who received an oral dose of diltiazem. There were no interfering peaks detected in control human plasma or clinical samples at the retention times corresponding to diltiazem, its metabolite and the internal standard. However, we found — as did Rovei et al. [4] — that an interfering, endogenous peak was detected from samples that were assayed after having been frozen for a period of 3—4 weeks. The response of the UV detector to the amount of diltiazem and deacetyldiltiazem added to human plasma was linear over the 100-fold range in drug

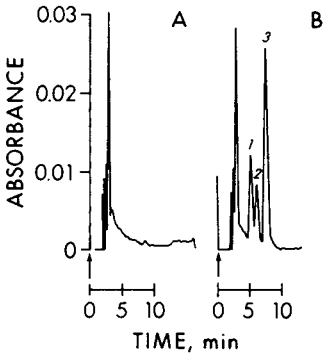


Fig. 2. Chromatograms of (A) control plasma and (B) plasma from a patient who had received diltiazem orally, showing peaks for deacetyldiltiazem (1), diltiazem (2) and the internal standard, verapamil (3) at concentrations of 13, 15 and 600 ng/ml, respectively. Mobile phase flow-rate was 1.5 ml/min and detector output displayed at a recorder chart speed of 12 cm/h; a.u.f.s. 0.01.

concentrations used (10—100 ng/ml). Correlation coefficients of 0.999 were obtained from the five calibration curves for both drug and metabolite.

The combination of low detector noise following injections of plasma extracts, high extraction efficiencies, and the high molar absorptivities at 237 nm in 0.05 mol/l sulphuric acid of diltiazem and its metabolite permit their determination in human plasma at low concentrations. The accuracy and within-run precision of the present method were determined by assaying plasma samples containing added diltiazem and deacetyldiltiazem (Table II).

TABLE II

WITHIN-RUN PRECISION AND ACCURACY

Each value represents the mean of eight determinations.

Drug	Concentration (ng/ml)		C.V. (%)
	Added	Found (±S.D.)	
Diltiazem	10	9.8 ± 1.25	12.8
	25	25.8 ± 2.85	11.1
	50	51.2 ± 3.18	6.2
	100	98.5 ± 4.31	4.3
	250	243.4 ± 4.48	1.8
	500	498.3 ± 25.19	5.1
	800	814.0 ± 38.27	4.7
	1000	989.0 ± 34.29	3.5
Deacetyldiltiazem	10	10.4 ± 0.521	5.0
	25	26.0 ± 2.32	8.9
	50	51.8 ± 4.76	9.2
	100	99.0 ± 6.35	6.4
	250	257.0 ± 16.38	6.3
	500	486.8 ± 28.60	5.8
	800	789.5 ± 39.18	4.9
	1000	1054.0 ± 58.24	5.5

The limit of quantitation is 10 ng/ml for diltiazem and deacetyldiltiazem defined as minimum signal-to-noise ratio of 4 and a C.V. of 15% or less. The lower limit of detection however, was less than 5 ng/ml. These concentrations generally yielded C.V. values greater than 15%. Therefore, 10 ng/ml is taken as a lower practical limit of quantitation. The reproducibility of the daily standard curves over a period of one month had an average C.V. of 5.4% for diltiazem and 8.3% for deacetyldiltiazem over the linearity range of the assay (Table III).

Several drugs which are often concurrently administered to cardiac patients were also examined for their possible interference with the quantification of diltiazem and its metabolite using this assay procedure (Table IV). None of the drugs tested interfered in the assay.

The application of the method in a patient who received an infusion of diltiazem is illustrated in Fig. 3. Diltiazem concentrations rose to a peak

TABLE III

STANDARD CURVE REPRODUCIBILITY OVER A PERIOD OF ONE MONTH

Drug	Standard curve No.	*X* of NPHR*	Standard deviation	C.V. (%)
Diltiazem	1	0.0042	0.0002	4.7
	2	0.0078	0.0003	3.7
	3	0.0049	0.0003	6.2
	4	0.0075	0.0003	3.7
	5	0.0042	0.0004	8.4
	6	0.0044	0.0003	5.9
			Mean =	5.4
Deacetyldiltiazem	1	0.0097	0.0011	11
	2	0.0115	0.0009	7.9
	3	0.0075	0.0006	7.7
	4	0.0113	0.0007	6.2
	5	0.0116	0.0013	12
	6	0.0118	0.0006	4.8
			Mean =	8.3

*NPHR = Peak-to-height ratio divided by concentration; C.V. = standard deviation divided by NPHR.

TABLE IV

RELATIVE RETENTION (*K'*) OF DILTIAZEM, ITS MAJOR METABOLITE, OTHER CARDIOVASCULAR DRUGS AND DRUGS OFTEN ADMINISTERED CONCURRENTLY WITH DILTIAZEM

Drug	*K'*
Diltiazem	2.50
Deacetyldiltiazem	3.25
Verapamil	4.00
Disopyramide	1.25
Lidocaine	0.88
Procainamide	0.50
N-Acetylprocainamide	0.75
Quinidine	2.95
Pindolol	1.25
Timolol	1.00
Metoprolol	0.75
Propranolol	2.00
Atenolol	0.50
Cimetidine	0.50
Diazepam	1.50
Clonazepam	1.45
Lorazepam	1.00
Nitrazepam	1.00
Oxazepam	1.40

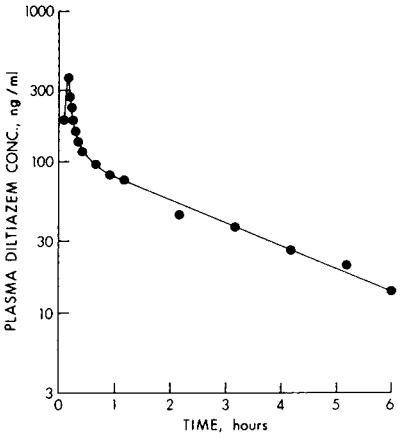


Fig. 3. Semilogarithmic plot of plasma diltiazem concentrations during and after a 10-min infusion of 20 mg of the drug.

at the end of the infusion and then declined biexponentially with a terminal half-life of 2 h consistent with the literature [2]. Concentrations of deacetyldiltiazem (not shown) rose early to a peak of 30 ng/ml and were undetectable by 30 min.

The present HPLC method described for the analysis of diltiazem and its deacetyl metabolite is accurate and reproducible within the apparent range of effective concentrations in plasma and is rapid enough that one person can analyze 15—20 samples daily in addition to the calibration curve samples. The present method is currently used in this laboratory for monitoring plasma levels during oral, intravenous, and steady state pharmacokinetic studies.

ACKNOWLEDGEMENTS

This work was supported in part by grants RR-30, HL24920 5-T32-HL-07101, and LM 03373 from the National Institutes of Health and a grant-in-aid from Marion Labs. Dr. Shand is a Burroughs Welcome Scholar in Clinical Pharmacology.

REFERENCES

1 P.L. Morselli, V. Rovei, M. Mitchard, A. Durand, R. Gomeni and J. Larrbaad, in R.J. Bing (Editor), New Drug Therapy with a Calcium Antagonist, Diltiazem, Excerpta Medica, Amsterdam, 1978, p. 152.
2 Y. Oyama, in R.J. Bing (Editor), New Drug Therapy with a Calcium Antagonist, Diltiazem, Excerpta Medica, Amsterdam, 1978, p. 169.
3 R.F. Zelis and E.L. McKinney, Amer. J. Cardiol., 49 (1982) 529.
4 V. Rovei, M. Mitchard and P. Morselli, J. Chromatogr., 138 (1977) 391.
5 N.R. Draper and H. Smith, Applied Regression Analysis, Wiley, New York, 1966.

Journal of Chromatography, 272 (1983) 157—165
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1484

DETERMINATION OF FLUPHENAZINE IN PLASMA BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

C.M. DAVIS* and D.C. FENIMORE*

Texas Research Institute of Mental Sciences, 1300 Moursund Avenue, Houston, TX 77030 (U.S.A.)

(First received April 13th, 1982; revised manuscript received August 26th, 1982)

SUMMARY

Determination of fluphenazine in blood plasma by in situ fluorescent detection after separation by high-performance thin-layer chromatography is described. Enhancement of fluorescent emission of the drug is accomplished by exposure to UV light in the presence of paraffin oil which permits a limit of detectability of approximately 0.1 ng/ml in blood plasma. Thirty samples or more can be processed in a 7-h period with excellent precision (less than 3% relative standard deviation at 2.5 ng/ml). Investigation of extraction procedures, chromatographic conditions, photodevelopment, and fluorescent detection are described.

INTRODUCTION

Fluphenazine (FPZ) is among the most potent of the phenothiazine drugs used in the treatment of schizophrenia. Esterification of the hydroxyl group in the piperazine side chain with heptanoic or decanoic acid yields dosage forms for intramuscular injection which release the drug slowly over a period of 1 to 4 weeks thus simplifying maintenance of therapeutic levels in the blood and central nervous system. The concentration of FPZ circulating under these conditions is quite low and has been shown by radiolabeled experiments to be less than 1 ng/ml of blood plasma over most of the dosage period [1]. Determination of drug blood levels at such low concentrations under the constraints of limited sample volume, minimal assay time, and acceptable cost of analysis, pose serious problems particularly where large patient populations are involved in therapeutic drug monitoring programs.

Procedures for the determination of FPZ reported in recent years are based primarily on gas chromatography with electron-capture detection [2, 3] or

*Present address: Clarke Analytical Systems, P.O. Box 744, Sierra Madre, CA 91024, U.S.A.

0378-4347/83/0000—0000/$03.00

with nitrogen specific detection [4, 5]. The sensitivity of these methods are, for the most part, inadequate for blood level monitoring under therapeutic regimens with perhaps the exception of the procedure described by Dekirmenjian et al. [5] which employs the acetylated derivative of FPZ with a reported subnanogram limit of detectability. A radioimmunoassay reported by Wiles and Franklin [6] achieved very high sensitivity but cross-reacted significantly with some of the metabolic products of FPZ, as well as, with other phenothiazine drugs.

This report describes a sensitive and selective procedure involving in situ fluorescent detection of FPZ after separation by high-performance thin-layer chromatography (HPTLC). The improvements in TLC methodology and materials which have resulted in HPTLC permit sensitive, selective, and rapid assays [7, 8] that may be applied economically to large numbers of samples. The limit of detectability for FPZ by the present procedure is approximately 0.1 ng/ml of plasma using a 4-ml sample. Thirty to sixty patient samples and calibration standards (depending on HPTLC plate size) can be processed in less than 6 h with a precision of better than 3% relative standard deviation.

EXPERIMENTAL

Materials and apparatus

Hydrochloride salts of FPZ and trifluopromazine (TFP) were obtained from E.R. Squibb & Sons (New Brunswick, NJ, U.S.A.) and the Upjohn Co. (Kalamazoo, MI, U.S.A.), respectively. Toluene, heptane, isoamyl alcohol, and acetone (Fisher Scientific, Pittsburg, PA, U.S.A.) were distilled in an all-glass system and stored in glass bottles with PTFE-sleeved glass stoppers. Hydrochloric acid, ammonium hydroxide, sodium carbonate and paraffin oil were also purchased from Fisher Scientific. Absolute ethanol (U.S. Industrial Chemical Co., Tuscola, IL, U.S.A.) was used as received. HPTLC plates (Silica Gel 60, Cat. No. 5633, E. Merck, Darmstadt, G.F.R.) were pre-cleaned by overnight development in absolute ethanol in the presence of vapor from concentrated ammonium hydroxide.

Either a Zeiss KM-3 (Carl Zeiss, Oberkochen, G.F.R.) or a Shimadzu CS-910 (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.) scanning densitometer equipped with a mercury lamp was used for fluorodensitometric determination, and samples were applied to the HPTLC plates with a Contact Spotter, Model 1010 (Clarke Analytical Systems, Sierra Madre, CA, U.S.A.). All glassware was silylated by a vapor phase method [9].

Stock standards

A standard containing 250 ng/ml and 300 ng/ml FPZ and TFP respectively was prepared in heptane containing 25% ethanol and 0.05% *n*-dodecane. The amount of FPZ and TFP in 40 μl of this standard was equivalent to the central point (2.5 ng/ml) of the calibration curve produced by extracts of plasma standards and as such served as an external standard to monitor the assay procedure. A standard containing 120 ng/ml TFP was prepared in heptane to introduce 12 ng of the internal standard to the plasma before extraction. An

additional standard containing 10 μg/ml of FPZ in heptane was prepared for the standard curve. All weights of FPZ and TFP represent the free form of the drug.

Standard curve

Standard curves were prepared by evaporating 125 μl of the 10 μg/ml FPZ standard under nitrogen at 40°C in a 250-ml volumetric flask. The drug was redissolved in 100 μl of 0.05 *N* hydrochloric acid, diluted to volume with drug-free plasma, and mixed for approximately 30 min. Additional dilutions were obtained by transferring 50-ml and 10-ml aliquots of the 5 ng/ml plasma standard to 100-ml volumetric flasks and adding drug-free plasma to volume. These standards were then transferred in 4-ml amounts to 30-ml silylated screw-top test tubes fitted with PTFE-lined tops and frozen at −70°C for further use.

Procedure

The following solutions were pipetted into 30-ml silylated screw-top test tubes containing 4 ml of patient plasma or plasma standard: 100 μl of internal standard solution, 0.6 ml of saturated sodium carbonate solution, and 20 ml distilled heptane containing 0.50% isoamyl alcohol. The tubes were capped with PTFE-lined closures, mixed for 45 min on a tube rocker (Lab-Industries, Berkeley, CA, U.S.A.) and centrifuged at 1800 *g* for 30 min. The aqueous phase was then frozen by immersing each tube momentarily in dry ice—acetone, and the organic phase was decanted into another silylated screw-top test tube containing 2 ml of 0.05 *N* hydrochloric acid. The tubes were then shaken for 15 min, centrifuged at 1500 *g* for 5 min, and the organic layer was removed by aspiration and discarded. The samples were then back-extracted by the addition of 0.3 ml saturated sodium carbonate solution and 4.5 ml of distilled pentane followed by shaking for 15 min and centrifugation at 1500 *g*. The aqueous phase was again frozen and the organic layers decanted into Reacti-vials (Pierce, Rockford, IL, U.S.A.). The pentane was removed by evaporation under nitrogen at 40°C, and the residue was redissolved in 50 μl heptane—ethanol—*n*-dodecane (75:25:0.05). Each extracted solution was then deposited in the separate indentations of the Contact Spotter as was a 40-μl volume of the spotting standard. After evaporation at the "low" temperature setting of the Contact Spotter, the residues were transferred to a 10 × 10 cm pre-washed HPTLC plate, 8 mm from the edge of the plate.

The plates were developed in a pre-equilibrated linear development chamber (Camag, Applied Analytical Industries, Wilmington, NC, U.S.A.) containing toluene—acetone (60:40). This solvent moved the FPZ and internal standard to an R_F of 0.1, and interfering plasma components moved with the solvent front. A second, pre-equilibrated development in toluene—acetone—ammonium hydroxide (60:40:2) was carried out to a distance 4 cm from the origin and moved the FPZ and internal standard to R_F values of 0.2 and 0.5, respectively. Chromatographic separation occurred in an air-conditioned laboratory at approximately 60% relative humidity and 23°C.

After evaporation of solvent the plate was placed in 5% paraffin oil (Saybolt viscosity 125/135) in toluene for 15 min. The toluene was evaporated

from the adsorbent layer, and the plate was exposed to UV light (Blak-Ray B100A, Ultraviolet Products, San Gabriel, CA, U.S.A.) for 15 to 20 min. The photodeveloped plate was then scanned in the fluorescent mode at 254-nm excitation wavelength with a 400-nm cut-off filter.

RESULTS AND DISCUSSION

The phenothiazine drugs can be detected on TLC plates by a variety of reagent sprays [10] or by UV absorption with or without preliminary reagent treatment [11, 12]. The sensitivity achieved with these methods, however, is not sufficiently sensitive to permit reliable quantitation of FPZ at the plasma concentration associated with therapeutic dosage. Fluphenazine, like many of the phenothiazine drugs, is fluorescent under appropriate conditions, a quality that would appear to offer some advantage with respect to sensitivity and selectivity of detection. Unfortunately, the fluorescent yield of FPZ adsorbed on silica gel is not adequate for determination when chromatographed plasma extracts are examined by conventional fluorescent scanning techniques.

In attempts to enhance the fluorescence of FPZ, the adsorbent layer of the HPTLC plate was exposed to paraffin oil, a treatment which had been shown to increase fluorescent yield when applied to other classes of compounds [13]. This recourse was not successful in itself, but it was observed that when the paraffin oil-treated plate was exposed to UV light, small amounts of FPZ became visible after 3 or 4 min and reached maximum emitted intensity after 15—20 min. If the plate was exposed for periods longer than 30 min, background fluorescence increased thus diminishing the overall sensitivity. Photodevelopment in the absence of paraffin oil did not enhance fluorescence.

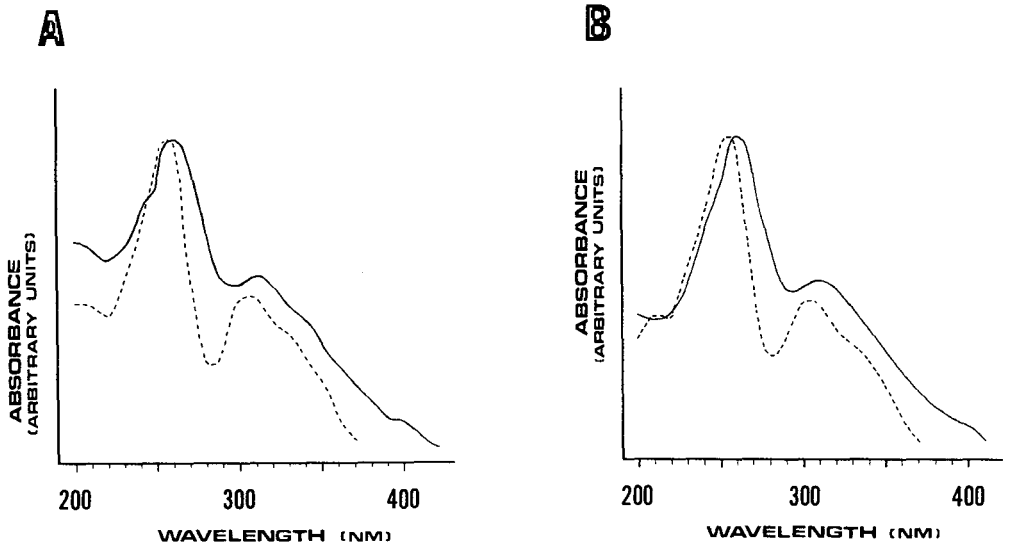


Fig. 1. (A) UV spectrum of fluphenazine (FPZ) before (— — —) and after (——) photodevelopment procedure. (B) UV spectrum of triflupromazine (TFP), internal standard, before (— — —) and after (——) photodevelopment procedure.

The fluorescent products of both FPZ and the internal standard, TFP, formed in this manner were stable on the HPTLC plates for periods as long as 6 months. Although no attempts were made to characterize these products, their UV spectra were different from the parent compounds (Fig. 1). The fluorescent product of FPZ was examined on an Aminco Bowman Spectrofluorometer equipped with a xenon lamp and was found to have major excitation wavelengths at 285 and 355 nm with emission maximum at 510 nm. In order to utilize one of these excitation maxima, it was originally elected to employ a xenon lamp attachment with the Shimadzu CS-910 densitometer; but sensitivity and stability were difficult to maintain with this configuration. Consequently, the less expensive mercury lamp attachment was evaluated at the various mercury emission lines, and it was found that maximum excitation occurred at the 253.7-nm line resulting in a 17-fold increase in response over the best fluorescent emission obtained with the xenon lamp.

The extraction of drug and internal standard from plasma followed a fairly straightforward scheme of liquid—liquid partitioning with back-extractions at appropriate conditions of acidity and basicity. It was found that certain precautions had to be taken, however, to exclude fluorescent contamination from reagents. The sodium carbonate used to prepare the saturated pH 11.6 solution contained an unacceptably large amount of fluorescent material and the 0.05 *N* hydrochloric acid showed trace contamination. These contaminants were removed by multiple extractions with 30% volumes of distilled heptane. Once cleaned, the solutions were held in glass bottles sealed with PTFE-sleeved glass stoppers. When contamination was suspected, it was readily checked by processing a water blank through the entire procedure.

Plasma extracts contained fluorescent substances that emitted at the same wavelength range as FPZ, but most of this interference was removed by careful selection of pH and by the use of non-polar extraction solvents. Fig. 2 demonstrates the extraction of FPZ into pentane from plasma adjusted to specific pH values. The addition of 0.6 ml saturated sodium carbonate solution to 4 ml plasma as described in the procedure resulted in a pH of 10 which was sufficient to affect maximum partitioning without co-extracting interfering substances.

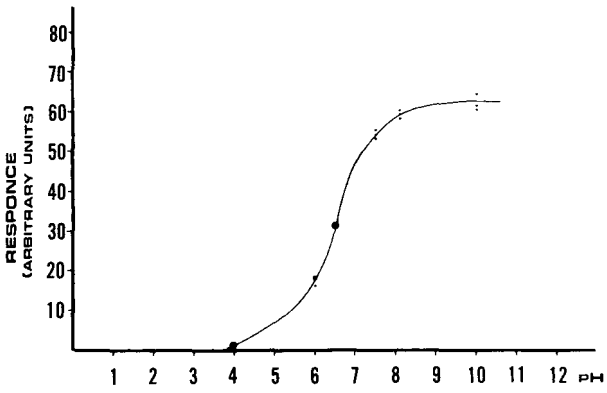


Fig. 2. Effect of pH on the partitioning of fluphenazine (FPZ) out of plasma. Spectrophotometric measurements were carried out on the FPZ which extracted into pentane.

A number of organic solvents were evaluated with respect to extraction efficiency. The more polar solvents such as diethyl ether and ethyl acetate extracted interfering substances while non-polar solvents such as pentane or heptane provided clean extracts with fairly good recovery. The addition of a small amount of isoamyl alcohol (0.50%) to the heptane resulted in a lower coefficient of variation when the same plasma sample was extracted a number of times. Larger amounts of isoamyl alcohol added to the heptane increased the extraction of interfering substances from the plasma. The complete extraction procedure as described resulted in approximately 75% recovery of drug, however, about 95% of the drug was recovered during the back extraction steps suggesting the greatest loss occurred in the initial extraction of plasma.

TABLE I

R_F VALUES OF 32 BASIC DRUGS SCREENED FOR INTERFERENCE

The solvent system used was toluene—acetone—ammonia (60:40:1).

Drugs	R_F	Relative in situ fluorescent yield
Mesoridazine	0.00	6
Protriptyline	0.06	7
Desipramine	0.08	0
Thiothixine	0.09	14
Acetophenazine	0.09	60
Carphenazine	0.11	70
Nortriptyline	0.14	0
Methdilazine	0.15	0
Perphenazine	0.15	2
Butaperazine	0.19	64
Fluphenazine	0.20	40
Prochlorperazine	0.23	3
Trifluoperazine	0.23	47
Clozapine	0.27	0
Nicotine	0.28	0
Promazine	0.30	1
Caffeine	0.32	0
Imipramine	0.35	1
Chlordiazepoxide	0.35	10
Ethopropazine	0.36	1
Thioridazine	0.37	16
Doxepin	0.38	2
Promethazine	0.41	1
Chlorpromazine	0.43	7
Loxapine	0.43	0
Haloperidol	0.43	0
Amitriptyline	0.45	0
Flurazapam	0.47	0
Triflupromazine	0.47	48
Chlorprothixine	0.57	100
Methotrimeprazine	0.64	1
Diazepam	0.73	0

Samples were mixed with extraction solvent by gently rocking in order to avoid excessive emulsification. Centrifugation at 1800 *g* for 30 min yielded a clean separation without a pronounced emulsion layer between the two phases. This emulsion layer must be minimized to maintain good recovery.

After the extracts were taken to dryness, they were redissolved in 50 μl of a solvent mixture containing 0.05% *n*-dodecane. The 25 nl of *n*-dodecane which remained with the extract after evaporation on the Contact Spotter were necessary to insure complete sample transfer to the HPTLC plate during the sample application process. Simultaneous transfers of 15 extracts were placed 6 cm apart on each of two opposing edges of a 10 × 10 cm plate for separation of 30 samples with each chromatographic development. The Camag linear development chamber applied developing solvent to these opposing edges, and sample separation occurred as the solvent fronts moved towards

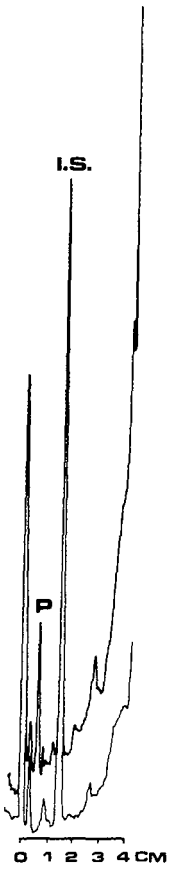


Fig. 3. Fluorescent scan of a patient on Prolixin (fluphenazine) therapy at a concentration of 0.6 ng/ml. Lower trace is blank plasma containing the internal standard. Peaks: P = FPZ; I.S. = TFP, internal standard.

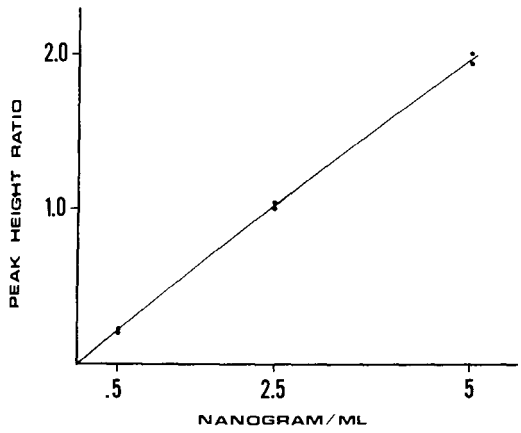


Fig. 4. Standard curve of the peak height ratio of FPZ/I.S. extracted from plasma. Each concentration was assayed in duplicate.

one another at the center of the plate, thus chromatographing the 30 samples in a single 7-min period. Pre-equilibration of the HPTLC plate for 2 min with vapors of the developing solvent was necessary to reduce "edge effect" and provide more uniform R_F values among sample lanes. The relative standard deviation obtained from the repeated analysis (n = 12) of the 2.5 ng/ml plasma standard using a linear development chamber (2.8%) was approximately half the relative standard deviation found with the conventional chromatography tank (5%).

Table I shows the R_F values and relative fluorescent responses for 32 drugs screened for possible interference. The R_F values presented here are slightly lower than those obtained by the dual development system, because the drugs were separated by only the final solvent system. Since most of these basic drugs have very little, if any, fluorescent emission at an excitation wavelength of 253.7 nm, they do not contribute to interferences in the detection process. A chromatographic tracing of a plasma extract after development in both solvent systems is shown in Fig. 3 and represents 0.6 ng/ml of FPZ from a patient on Prolixin therapy.

TABLE II

PLASMA FLUPHENAZINE LEVELS

Patient No.	Days of FPZ treatment	Dosage of FPZ*	Interval** (days)	Plasma FPZ (ng/ml)
I	1	a	1	1.06
	20	a	1	0.72 ± 0.02
	45	a	4	0.60 ± 0.05
	49	a	1	0.70 ± 0.05
	87	a	5	0.45 ± 0.05
	104	a	9	0.55
	118	a	10	0.53 ± 0.03
	125	a	3	0.25 ± 0.05
	133	a	9	0.35 ± 0.05
	140	a	2	0.35 ± 0.05
II	1	a	1	0.38 ± 0.08
	14	a	1	0.23 ± 0.08
	28	a	1	0.35 ± 0.05
	38	a	8	0.32
III	1	c	0.5	0.56
	7	a	4	ND***
	14	a	10	ND
	31	a	14	0.23
IV	1	c	0.5	1.35 ± 0.05
	18	a and	3	
		b	0.5	2.5
V	1	b	0.5	0.65
	7	a	4	0.30
VI	1	c	0.5	1.40 ± 0.05

*a = Fluphenazine decanoate, 25 mg every 2 weeks; b = fluophenazine·HCl, 5 mg three times per day; c = fluphenazine·HCl, 10 mg three times per day.
**Interval between dosage and blood sampling.
***ND = not detected.

The capability of HPTLC for accepting many samples simultaneously greatly simplifies calibration, for little additional effort is required to include several standards with any particular determination. Standard curves, such as that illustrated in Fig. 4, prepared from duplicate plasma extracts over three concentrations were run concurrently with each group of patient samples. This practice effectively excluded those errors which may arise from small variations in chromatographic conditions in processing each discrete HPTLC plate. Analysis of plasma containing 0.5 ng/ml or 2.5 ng/ml of FPZ resulted in a relative standard deviation of 8.1% and 2.8%, respectively (n = 12).

The applicability of this method to the monitoring of patient blood levels during therapy with long-acting dosage forms of FPZ is shown in Table II. The values obtained were generally consistent with the amount and dosage form of the drug over extended periods of observation.

REFERENCES

1 S.H. Curry, R. Whelpton, P.J. DeSchepper, S. Vranckx and A.A. Schiff, Lancet, i (1978) 1217—1218.
2 L. Rivera-Calimlim and A. Siracusa, Commun. Psychopharmacol., 1 (1977) 233—242.
3 N.-E. Larsen and J. Naestoft, Med. Lab. Technol., 30 (1973) 129—132.
4 M. Franklin, D.H. Wiles and D.J. Harvey, Clin. Chem., 24 (1978) 41—44.
5 H. Dekirmenjian, J.I. Javaid, B. Duslak and J.M. Davis, J. Chromatogr., 160 (1978) 291—296.
6 D.H. Wiles and M. Franklin, Brit. J. Clin. Pharmacol., 5 (1978) 265—268.
7 W. Bertsch, S. Hara, R.E. Kaiser and A. Zlatkis, Instrumental HPTLC, Proceedings of the First International Symposium on Instrumentalized High Performance Thin-Layer Chromatography (HPTLC), Bad Dürkheim, West Germany, Dr. Alfred Hüthig Verlag, New York, 1980.
8 D.C. Fenimore and C.M. Davis, Anal. Chem., 53 (1981) 252A—266A.
9 D.C. Fenimore, C.M. Davis, J.H. Whitford and C.A. Harrington, Anal. Chem., 48 (1976) 2289—2290.
10 J.G. Kirchner, in A. Weissberger (Editor), Thin-Layer Chromatography, Techniques of Chemistry, John Wiley & Sons, New York, 2nd ed., 1978.
11 D.C. Fenimore, C.J. Meyer, C.M. Davis, F. Hsu and A. Zlatkis, J. Chromatogr., 142 (1977) 399—409.
12 D.C. Fenimore, C.M. Davis and C.J. Meyer, Clin. Chem., 24 (1978) 1386—1392.
13 R.S. Day, R.E. De Salamanca and L. Eales, Clin. Chim. Acta, 89 (1978) 25—33.

Journal of Chromatography, 272 (1983) 166—169
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1447

Note

Simple gas chromatographic analysis of 3-methylthiopropionate in human urine

HIROSHI KAJI* and KAZUO SAITO

Department of Environmental Medicine, Graduate School of Environmental Science, Hokkaido University, Nishi-5, Kita-10, Kita-ku, Sapporo 060 (Japan)

NARIYOSHI SAITO and MASAYA HISAMURA

The First Department of Medicine, Hokkaido University School of Medicine, Nishi-7, Kita-15, Kita-ku, Sapporo 060 (Japan)

and

MAKOTO ISHIMOTO and HIROYUKI KONDO

Department of Chemical Microbiology, Faculty of Pharmaceutical Sciences, Hokkaido University, Nishi-6, Kita-12, Kita-ku, Sapporo 060 (Japan)

(First received February 15th, 1982; revised manuscript received July 21st, 1982)

In our earlier studies with healthy volunteers, it was demonstrated that exhalation of dimethyl sulfide and urinary excretion of α-keto-γ-methiolbutyrate increased markedly following administration of D-methionine, and that, by contrast, these excretions were quite trivial following administration of L-methionine [1—3]. In order to study this metabolic difference between the optical isomers of methionine and to verify the precursors of dimethyl sulfide, it is essential to determine 3-methylthioproprionate as one of the intermediates of D-methionine metabolism.

In the present communication, a simple and sensitive gas chromatographic quantitation of urinary 3-methylthiopropionate without derivatization is presented.

0378-4347/83/0000—0000/$03.00

MATERIALS AND METHODS

Synthesis and identification of 3-methylthiopropionate

3-Methylthiopropionate was chemically synthesized from 3-methylthiopropionaldehyde (methional), which was obtained from Sigma (St. Louis, MO, U.S.A.), by a modification of the synthesis of 3-thiophenecarboxylic acid as described by Campaigne and LeSuer [4]. The identity of 3-methylthiopropionate was verified by element analysis, nuclear magnetic resonance spectroscopy, and mass spectrometry. Since the sodium salt of 3-methylthiopropionate was difficult to crystallize and the potassium salt was highly deliquescent, the lithium salt was used as the standard in the present investigation.

Extraction, preconcentration, and quantitation of 3-methylthiopropionate in standard aqueous solution and in urine

A 20-ml standard solution of the lithium salt of 3-methylthiopropionate or 20- to 150-ml urine samples were acidified by hydrochloric acid to pH 1.0 and extracted three times with chloroform. The pooled chloroform phases were evaporated to dryness and dissolved in 500 μl of methanol. Three microliters of the final methanol solution of 3-methylthiopropionate were directly injected into the injection port of the gas chromatograph.

Gas chromatography

A gas chromatograph equipped with a flame photometric detector (FPD) and a hydrogen flame ionization detector (FID) (Model GC-7AGPrFFP, Shimadzu, Kyoto, Japan) was used for the present investigation. The glass column (2.1 m × 3 mm I.D.) was packed with 10% SP-1200/1% H_3PO_4 on Chromosorb W AW 80--100 mesh (Wako, Osaka, Japan). The column temperature was initially isothermal at 140°C for 32 min, then increased to 195°C at a rate of 16°C/min with a hold at 195°C. The injection port temperature was 200°C. The FPD with a 394-μm filter was operated at 750 V. Nitrogen was the carrier gas at a flow-rate of 50 ml/min. The recorder responses were calculated by a Chromatopac C-RIA (Shimadzu).

RESULTS AND DISCUSSION

For the separation of 3-methylthiopropionate by gas chromatography, the operating conditions were determined as described in the Methods. The retention time of 3-methylthiopropionate was 19.36 min (Fig. 1). Reproducibility of analyses and linearity of the calibration curve were then studied by triplication at three different concentrations (Table I). The linear range for amount of 3-methylthiopropionate injected was 1 ng to 14.4 μg. The sensitivity of the FPD was about ten times higher than that of the FID and the detection limit was 1 ng. The recovery of 3-methylthiopropionate from urine was compared with that from distilled water solution at three different concentrations: 108% at 10 nmol/ml, 105% at 100 nmol/ml and 96% at 1 μmol/ml. Since the

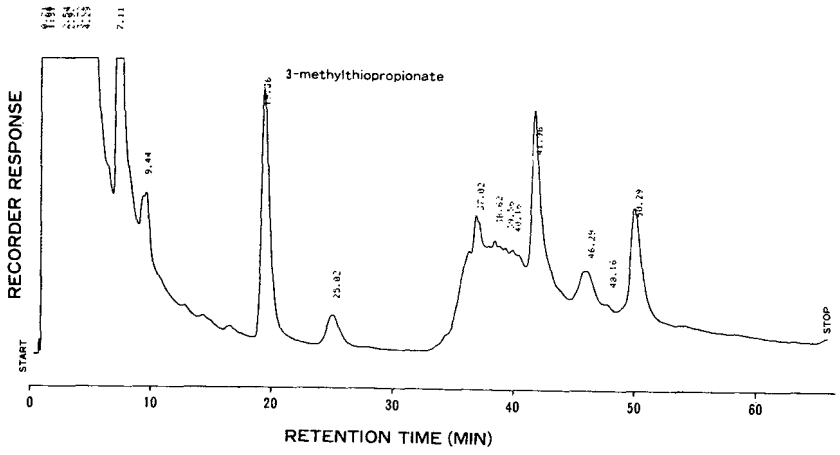


Fig. 1. Gas chromatographic analysis and flame photometric detection of 3-methylthiopropionate in a 2-h urine sample obtained from a 43-year-old healthy male following ingestion of 2 g of D-methionine. Retention time of 3-methylthiopropionate: 19.36 min.

TABLE I

REPRODUCIBILITY* OF THE DETERMINATION OF 3-METHYLTHIOPROPIONATE IN METHANOL SOLUTION BY GAS CHROMATOGRAPHY

Dose injected	Sensitivity and attenuation	Recorder response	Mean	S.D.
3.36 μg	$10^3 \times 32$	1322	1345	43.3
		1308		
		1406		
336 ng	10 × 8	2224	2179	127.4
		2005		
		2307		
33.6 ng	10 × 1	101	92	7.3
		83		
		92		

*$y = 4.9555 \cdot x^{0.42797}$ ($r = 0.9998$), where y = dose injected and x = recorder response.

creatinine and/or 3-methylthiopropionate concentration in urine varies over a wide range, it is difficult to specify in advance the sample volume of urine for measuring this acid. The detection limit of 3-methylthiopropionate was 1 ng per 3 μl final methanol solution. Because of its specificity and higher sensitivity, flame photometric detection was more useful.

In most studies on human methionine metabolism, methionine concentration has been directly measured in blood or urine [5—10]. In addition, there are other approaches using the quantitative analysis of several metabolites of methionine [11—13]. In our earlier studies, marked increases in exhalation of dimethyl sulfide and urinary excretion of α-keto-γ-methiolbutyrate following administration of the D-isomer of methionine were confirmed in

healthy volunteers [1—3]. Recently, the transamination pathway of L-methionine metabolism has been given attention and has been re-investigated in vitro in rat and monkey liver homogenate and in rat liver mitochondrial systems [14—17].

The simple gas chromatographic determination of 3-methylthiopropionate in urine presented here would seem to play an important role in the studies on the metabolism of methionine isomers.

REFERENCES

1 H. Kaji, M. Hisamura, N. Saito, H. Sakai, T. Aikawa, T. Kondo, H. Ide and M. Murao, Clin. Chim. Acta, 93 (1979) 377.
2 H. Kaji, N. Saito, M. Murao, M. Ishimoto, H. Kondo, S. Gasa and K. Saito, J. Chromatogr., 221 (1980) 145.
3 H. Kaji, M. Hisamura, N. Saito and M. Murao, Res. Commun. Chem. Pathol. Pharmacol., 32 (1981) 515.
4 E. Campaigne and W.M. LeSuer, in C.C. Price (Editor), Organic Synthesis, Vol. 33, John Wiley, New York, 1953, p. 94.
5 L.W. Kinsell, H.A. Harper, H.C. Barton, M.E. Hutchin and J.R. Hess, J. Clin. Invest., 27 (1948) 677.
6 L.W. Kinsell, H.A. Harper, G.K. Giese, S. Margen, D.P. McCallie and J.R. Hess, J. Clin. Invest., 28 (1949) 1439.
7 T.L. Perry, D.F. Hardwick, G.H. Dixon, C.L. Dolman and S. Hansen, Pediatrics, 36 (1965) 236.
8 W.H. Peters, H. Lubs, M. Knoke and M. Zschiesche, Acta Biol. Med. Germ., 36 (1977) 1435.
9 K.J. Printen, M.C. Brummel, E.S. Cho and L.D. Stegink, Amer. J. Clin. Nutr., 32 (1979) 1200.
10 L.D. Stegink, L.J. Filer, Jr. and G.L. Baker, J. Nutr., 112 (1982) 597.
11 C. Hooft, J. Timmermans, J. Snoeck, I. Antener, W. Oyaert and C. van den Hende, Amer. Paediat., 205 (1965) 73.
12 S. Chen, L. Zieve and V. Mahadevan, J. Lab. Clin. Med., 75 (1970) 628.
13 D. Glaubitt and K.E. Hampel, Proc. First World Congr. Nucl. Med., (1974) 516.
14 R.D. Steele and N.J. Benevenga, J. Biol. Chem., 253 (1978) 7844.
15 R.D. Steele and N.J. Benevenga, J. Biol. Chem., 254 (1979) 8885.
16 J.L. Dixon and N.J. Benevenga, Biochem. Biophys. Res. Commun., 97 (1980) 939.
17 G. Livesey and P. Lund, Biochem. Soc. Transaction, 8 (1980) 540.

Journal of Chromatography, 272 (1983) 170—175
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1492

Note

Analysis of corticosterone in the serum of mice and rats using high-performance liquid chromatography

KOJI SHIMIZU, SAKAE AMAGAYA and YUKIO OGIHARA*

Faculty of Pharmaceutical Sciences, Nagoya City University, 3-1, Tanabe-dori, Mizuho-ku, Nagoya 467 (Japan)

(First received June 22nd, 1982; revised manuscript received August 31st, 1982)

Corticosterone is an endogenous glucocorticoid which is secreted mainly in mice and rats. While a number of workers have published assays for endogenous glucocorticoids in rats and humans by means of radioimmunoassay [1, 2], quantitative analysis by chromatographic methods is very rare [3, 4]. Also, the determination of corticosterone in the serum of mice has not been reported previously. The purpose of the present work is the application of high-performance liquid chromatography (HPLC) in the quantitative analysis of serum corticosterone in a reproducible, accurate and rapid manner and comparison of corticosterone levels between mice and rats to develop an assay for corticosterone in mouse serum as a preliminary experiment in investigating the biochemical response to the administration of drugs. This paper describes the identification and the determination of minimum amounts of corticosterone in the serum of mice and rats by HPLC and gas chromatography combined with mass spectrometry (GC—MS).

EXPERIMENTAL

Standards and reagents

Corticosterone and dexamethasone, the internal standard, were obtained from Sigma (St. Louis, MO, U.S.A.). Acetonitrile, methylene chloride, sodium hydroxide, sulfuric acid, pyridine and N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from Nakarai Chemical Co. (Kyoto, Japan).

Apparatus

A gas chromatograph combined with a mass spectrometer (GCMS9020DF,

0378-4347/83/0000—0000/$03.00

SCAP1123; Shimadzu) was used. The glass column (1 m × 2 mm I.D.) packed with 3% OV-1 on Gas-Chrom Q (80—100 mesh) was used. The column temperature was 290°C, the injection port and detector temperature 330°C. The carrier gas was helium (30 ml/min). The conditions of the mass spectrometer were: electron energy 30 eV; ion source electron impact; temperature of ion source 250°C; current 60 μA; acceleration voltage 3.5 kV; gain 50; scan speed 8. Samples were derivatized to give the 21-mono-trimethylsilyl (TMS) derivative by BSTFA—pyridine (1:1) for 30 min at room temperature; 1 μl was injected.

For HPLC a Shimadzu Model 4A chromatograph with a Shimadzu Model SPD-2A UV detector was employed. A stainless-steel column (25 cm × 4 mm I.D.), packed with reversed-phase Fine SIL C_{18}-5 (5 μm; JASCO, Tokyo, Japan), was used. The mobile phase was acetonitrile—0.03% sulfuric acid solution (36:64). The column temperature was 45°C, the flow-rate was 1.2 ml/min, detection wavelength was 240 nm and the sensitivity was 0.005 a.u.f.s. Peak area was measured using a Shimadzu C-R1A computing integrator.

Animals

Male Wistar rats weighing 180—200 g and male ddY mice weighing 20—25 g were used. They were kept in an air-conditioned room (24°C) under a light—dark cycle (light phase, 06:00—18:00 hours) for seven days. They were given commercial diet (CLEA, Tokyo, Japan) and water ad libitum, unless otherwise specified. To avoid stress-induced release of corticosterone, they were calmed by daily handling once a day in the morning for seven days. Rats were decapitated every 2 h between 10:00 and 08:00 hours to investigate the rhythm of corticosterone secretion over 24 h.

Extraction

Blood samples were collected and allowed to stand for 1 h at room temperature. After centrifugation at 7400 *g* for 10 min, 200 μl of serum were transferred to a 10-ml separating funnel and internal standard solution of dexamethasone (5 μl = 50 ng) was added. Then 0.05 ml of 0.25 *M* sodium hydroxide and 4 ml of methylene chloride were added. The mixture was shaken by hand for a minute. The organic layer was washed with water, transferred to a 5-ml flask and evaporated in vacuo at 30°C. The residue was dissolved in 100 μl of methanol and 60 μl were injected into the HPLC column.

Calibration graph

Corticosterone at concentrations varying from 5 to 30 μg per 100 ml and internal standard at a fixed concentration of 10 μg per 100 ml were dissolved in 3% albumin solution and a calibration graph was obtained using the procedure described above.

RESULTS AND DISCUSSION

Typical chromatograms obtained from mouse and rat serum spiked with dexamethasone (internal standard, 10 μg per 100 ml) are shown in Fig. 1. The retention times of corticosterone and dexamethasone are 18 min and 9

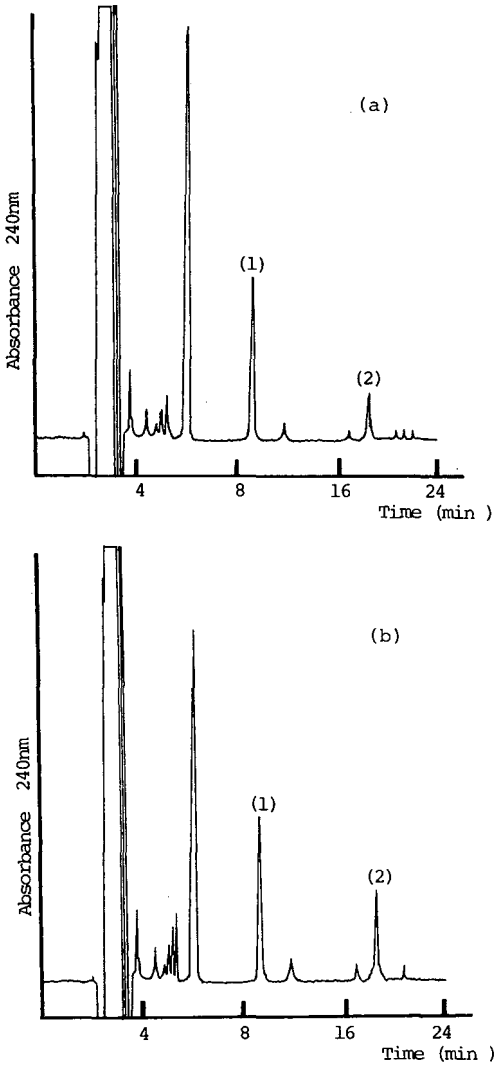


Fig. 1. High-performance liquid chromatograms of (a) mouse serum and (b) rat serum spiked with 10 μg per 100 ml internal standard. Peaks: 1 = dexamethasone (internal standard); 2 = corticosterone.

min, respectively. The patterns of the chromatograms are very similar. The identity of the separated mouse corticosterone was confirmed by GC—MS. Mild trimethylsilylation using BSTFA—pyridine (1:1) for 30 min at room temperature gave the 21-mono-TMS derivative selectively. To obtain the di-TMS derivative, it was necessary to stand for 1 h at 60° C. The GC peak was analyzed by mass spectrometry and selected ion monitoring of parent ions at *m/e* 418, 403, 315, 269, 251 and 227 for 21-mono-TMS corticosterone. A mass spectrum and chromatograms are shown in Fig. 2. This chromatogram corresponds well with that of corticosterone standard. From these results, the peak separated by HPLC was recognized as pure corticosterone.

A calibration graph for serum corticosterone obtained by plotting the ratio

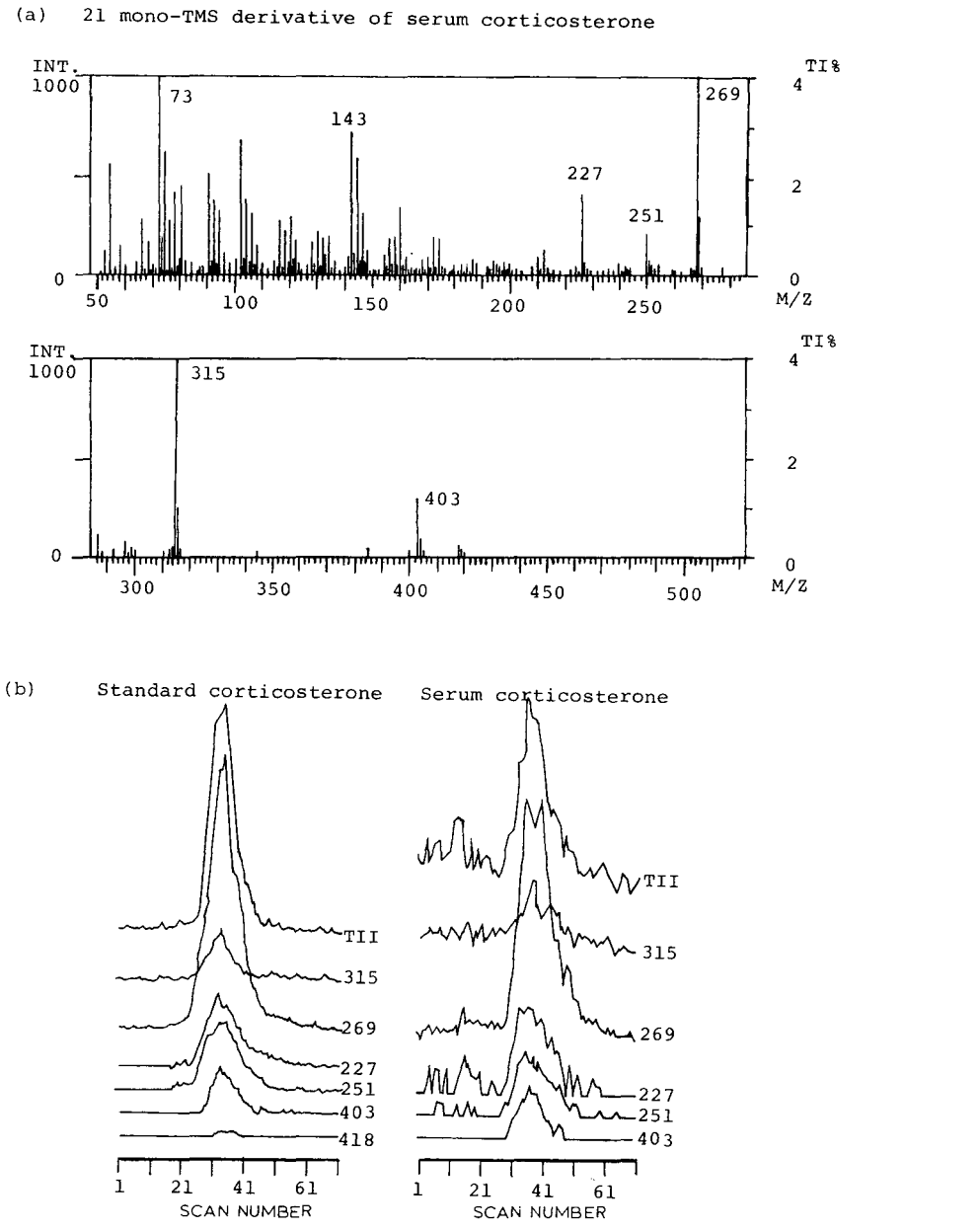


Fig. 2. Mass spectrum (a) and mass chromatogram (b) of 21-mono-TMS derivative of mouse corticosterone separated by HPLC.

of the peak area of corticosterone to that of internal standard against the concentration of corticosterone in mouse and rat serum showed linearity in the range 1—60 ng (8.3—500 ng/ml serum). The minimum measurable level was 1 ng (8.3 ng/ml serum).

Fig. 3 shows the circadian rhythm of serum corticosterone in rats. The concentration of serum corticosterone between 02:00 and 14:00 hours exhibits a

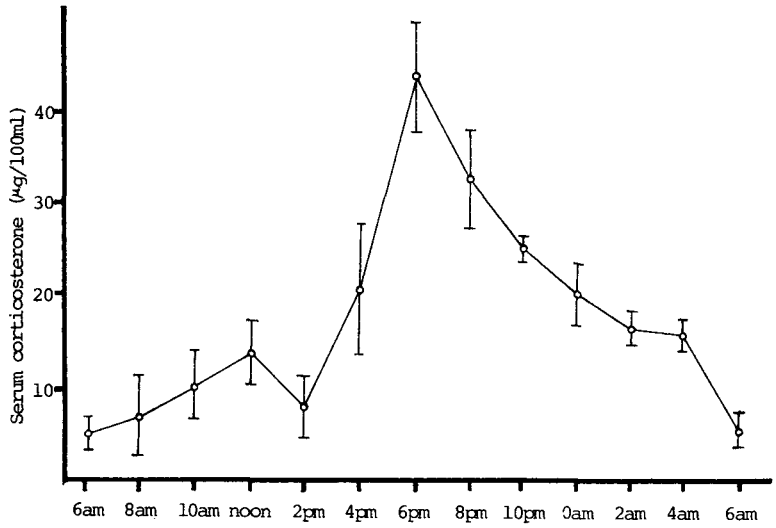


Fig. 3. Circadian rhythm of serum corticosterone in rats. Each point represents the mean of six rats and vertical bars indicate the S.E.

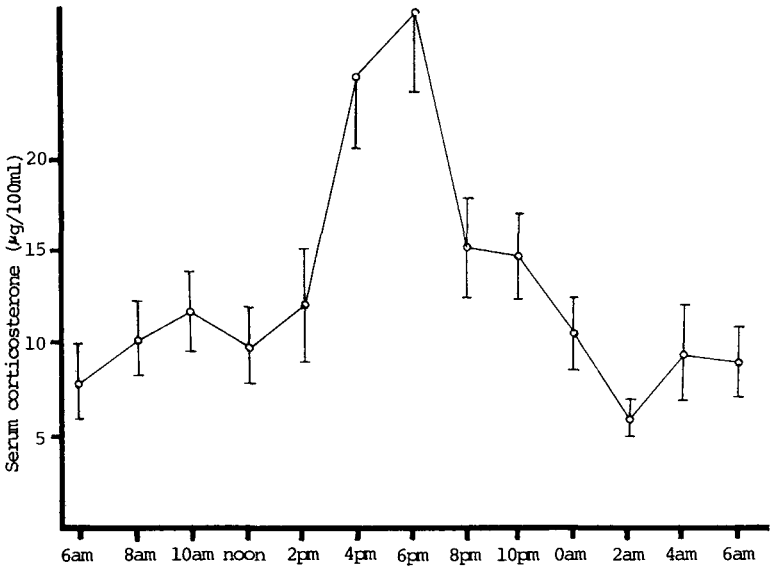


Fig. 4. Circadian rhythm of serum corticosterone in mice. Each point represents the mean of six mice and vertical bars indicate the S.E.

low level of around 5 μg per 100 ml; the level then increased from 15:00 hours and reached a maximum, 44 μg per 100 ml, at 18:00 hours. After that, the level decreased gradually until morning.

Fig. 4 shows the circadian rhythm of serum corticosterone in mice. The pattern of the rhythm is very similar to that of rats. The minimum level is 5 μg per 100 ml, which is equal to that of rats, and the maximum level is 15 μg per 100 ml which is lower than that of rats.

From these results, it is seen that the serum corticosterone levels and the circadian rhythm of the two animals are very similar, and mice can therefore be used for the assay of serum corticosterone instead of rats. The analysis of serum corticosterone by HPLC was reproducible, the most outstanding point in the method being the rapidity and accuracy in analysis of serum corticosterone above 1 ng (8.3 ng/ml serum) in comparison with radioimmunoassay.

REFERENCES

1 C.J. Migeon, J. Clin. Endocrinol., 16 (1956) 622.
2 D.T. Krieger, Endocrinology, 95 (1977) 1195.
3 S. Ohnishi, Hormon To Rinsho, 24 (1976) 303.
4 N.R. Scott, J. Chakraborty and V. Marks, Anal. Biochem., 108 (1980) 266.

Journal of Chromatography, 272 (1983) 176—180
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1481

Note

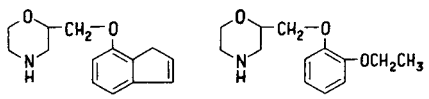
Determination of indeloxazine, a new antidepressant agent, in human plasma by gas—liquid chromatography with electron-capture detection

AILEEN G. HAYES and TSUN CHANG*

Warner-Lambert/Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, MI 48160 (U.S.A.)

(First received June 16th, 1982; revised manuscript received August 16th, 1982)

Indeloxazine hydrochloride, (±)2-[(1H-inden-7-yloxy)methyl]morpholine hydrochloride (Fig. 1), is a new potential antidepressant drug structurally related to viloxazine. In various animal models indeloxazine was shown to have a novel psychopharmacological profile different from that of tricyclic antidepressants or viloxazine [1]. As part of the clinical pharmacokinetics and bioavailability studies, a sensitive and specific electron-capture gas—liquid chromatographic assay was developed for the determination of indeloxazine in biological fluids at nanogram levels.



INDELOXAZINE VILOXAZINE

Fig. 1. Chemical structures of indeloxazine and viloxazine.

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 5730A gas chromatograph equipped with a 15 mCi ^{63}Ni electron-capture detector, a glass-lined heated injector, and a Model 7672A automatic sampler was used for all analyses. The instrument was fitted with a silanized coiled glass column (1.2 m × 2 mm I.D.) packed with pretested 3% OV-225 on 100—120 mesh Gas-Chrom Q. Argon—methane (95:5) with a preset head pressure of 0.275 MPa and a flow-rate of 50 ml/min was used as the carrier gas. The injection port, column oven, and detector were maintained at 250°C, 200°C, and 300°C, respectively.

0378-4347/83/0000—0000/$03.00

An Autolab IV integrator (Spectra-Physics, Mountain View, CA, U.S.A.) was interfaced with the electrometer and the automatic sampler for unattended operations.

Chemicals

Indeloxazine hydrochloride was obtained from Yamanouchi Pharmaceutical Co. (Tokyo, Japan). The internal standard, viloxazine hydrochloride, was supplied by ICI (Macclesfield, Great Britain); toluene, distilled in glass, from Matheson, Coleman, and Bell (Cincinnati, OH, U.S.A.); heptafluorobutyric anhydride in sealed glass ampoules from Pierce Chemical (Rockford, IL, U.S.A.); pretested 3% OV-225 on 100—120 mesh Gas-Chrom Q from Applied Science Labs. (State College, PA, U.S.A.); argon–methane (95:5) from Matheson Gas Products (East Rutherford, IL, U.S.A.). All other chemicals were of analytical grade.

Reagents

Stock solutions of indeloxazine or viloxazine (internal standard) were prepared by dissolving the appropriate amount of drug in 0.1 *N* hydrochloric acid to make a 1 mg/ml solution as the free base. Aliquots of these solutions were diluted separately with 0.1 *N* hydrochloric acid to yield 200 ng/ml and 50 ng/ml working standards of indeloxazine and 500 ng/ml of the internal standard.

Buffer solution, pH 9.5, was prepared by combining 250 ml saturated ammonium chloride and 105 ml concentrated ammonium hydroxide.

Sample preparation

Aliquots of plasma (0.2—0.5 ml), 0.2 ml of the internal standard (100 ng), 2 ml of ammonium chloride—ammonium hydroxide buffer, and 5 ml of toluene were measured into a set of 16-ml glass-stoppered tubes. The tubes were shaken on a mechanical shaker for 10 min, and centrifuged to separate the phases. A 4-ml aliquot of the toluene layer was back-extracted with 3.5 ml of 0.1 *N* hydrochloric acid in a duplicate set of tubes for 10 min. Following centrifugation 3 ml of the aqueous phase were transferred into another set of tubes containing 0.3 ml 1 *N* sodium hydroxide and 1 ml of ammonium chloride—ammonium hydroxide buffer, and the mixture was extracted with 3.5 ml of toluene as above. Toluene solution (3 ml) was mixed with 50 μl of heptafluorobutyric anhydride in a 13 × 100 mm glass-stoppered tube. The lower one-third of the tube was heated in a 75°C dry heating block for 30 min.

The tube was cooled to room temperature and excess reagent was removed by shaking with 1 ml of water for 1 min, followed by 1 ml of 5% ammonium hydroxide for another minute. After centrifugation, the organic phase was removed and evaporated to dryness at 55°C under nitrogen. The residue was redissolved in 1 ml of toluene (containing 1.5% isoamyl alcohol). The sample was transferred into a 1-ml glass sample vial and capped with a PTFE-faced rubber septum aluminum seal using a hand crimper. The prepared samples were then loaded into the automatic sampler ready for injection (3 μl).

Calibration and precision

Six calibration standards containing 12.5, 25, 50, 100, 150, and 200 ng of indeloxazine in 0.5 ml of blank human plasma were processed daily with each set of unknowns. Calibration curves were constructed by plotting the peak area ratios of indeloxazine to the internal standard versus the amount of indeloxazine in each sample. To determine the precision of the assay procedure, plasma standards were analyzed on three separate days to yield 10—12 replicate values for each of the six concentrations.

Quality control and stability studies

Quality control samples at concentrations of 300 ng/ml, 150 ng/ml, and 75 ng/ml were prepared in pooled blank human plasma, and 0.8-ml portions were stored frozen in disposable glass vials. Samples of 0.5 ml plasma at each concentration were analyzed daily to gauge the reliability of each day's analyses and the stability of indeloxazine in frozen plasma.

RESULTS AND DISCUSSION

Method evaluation

A sensitive and specific gas—liquid chromatographic assay has been developed for indeloxazine from 0.5 ml or less of plasma. The procedure is based on the electron-capture properties of the heptafluorobutyryl derivative which permits detection of low levels of indeloxazine in plasma. Viloxazine serves as an excellent internal standard owing to its structural similarity to indeloxazine and the ease of formation of the heptafluorobutyryl derivative [2]. Fig. 2 shows typical chromatograms obtained from extracts of blank human plasma containing indeloxazine and internal standard, blank human plasma, and a post-dose plasma sample from a human volunteer. The back-extraction

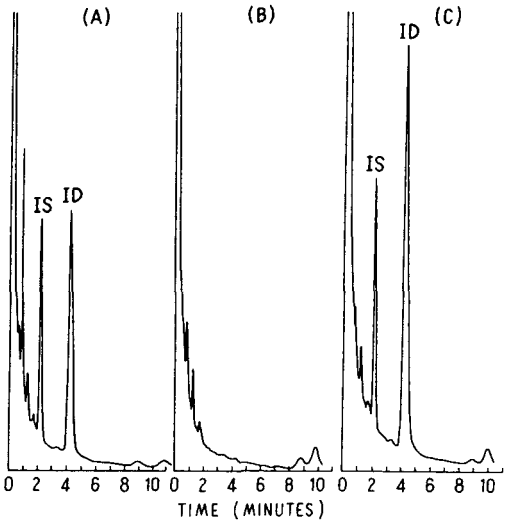


Fig. 2. Chromatograms of an extract of (A) blank human plasma containing indeloxazine (ID) and internal standard (IS); (B) blank human plasma; (C) post-dose plasma sample from a human volunteer.

step was necessary to produce chromatographic tracings free of endogenous interferences. The derivatization reaction was essentially complete within 15 min, but the samples were routinely heated for 30 min to ensure completeness of reaction. The heptafluorobutyryl derivatives appeared to be stable in the toluene solution for several days.

The relationship between the peak area ratio (indeloxazine/internal standard) and the amount of indeloxazine (12.5—200 ng) in 0.5 ml of plasma was linear over the concentration range studied (r = 0.999). The slopes of the calibration curves constructed over a 3-month period showed a relative standard deviation (R.S.D.) of 3.21%.

Precision and accuracy of the assay procedure were determined at six different concentrations ranging from 12.5—200 ng (Table I); the relative standard deviation (R.S.D.) varied from 2.55—6.05%. System reproducibility based on the peak area ratios of six sequential injections of standards had a mean R.S.D. of 1.3%.

TABLE I

PRECISION AND ACCURACY OF THE PROCEDURE APPLIED TO SPIKED HUMAN PLASMA SAMPLES (n = 9)

Amount drug added (ng)	Amount drug found (ng)	Relative standard deviation (%)
12.5	12.40	6.05
25.0	25.27	4.59
50.0	50.02	4.52
100.0	100.23	2.55
150.0	150.17	3.24
200.0	197.56	2.81

The limit of detection was approximately 5 ng/ml using 0.5-ml aliquots of plasma. Extraction recovery from plasma averaged 95%.

Quality control samples containing 300 ng/ml, 150 ng/ml, and 75 ng/ml of indeloxazine were frozen and assayed daily over a 3-month period. The mean values obtained were 302.8 ng/ml, 150.4 ng/ml, and 77.2 ng/ml with a R.S.D. of 2.69%, 3.00%, and 3.84%, respectively, indicating excellent drug stability in frozen plasma.

Human plasma levels

The described method has been successfully applied to clinical bioavailability studies after a single 100-mg oral dose of capsules and enteric coated tablets. The resulting plasma concentration versus time profiles for a representative volunteer are illustrated in Fig. 3. The peak plasma concentrations of 303 ng/ml and 326 ng/ml were reached in 2 h and 4 h, respectively, for the capsule and enteric coated tablet. The apparent disposition half-life was 3.15 h for the capsule and 3.58 h for the enteric coated tablet.

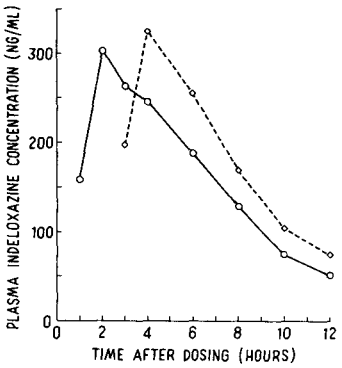


Fig. 3. Plasma indeloxazine concentrations in a normal volunteer following a single 100-mg oral dose of capsule (○——○) or enteric coated tablet (◇ — — — ◇).

The method as presented is suitable for pharmacokinetic and bioavailability studies following a single therapeutic dose.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. J.R. Goulet, Clinical Research Department, for the clinical specimens obtained during Phase 1 evaluation, and to Dr. G.J. Yakatan for critical review of the manuscript.

REFERENCES

1 S. Tachikawa, M. Harada, and H. Maeno, Arch. Int. Pharmacodyn., 238 (1979) 81.
2 D.E. Case, J. Pharm. Pharmacol., 25 (1973) 800.

Journal of Chromatography, 272 (1983) 181—186
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1487

Note

Determination of valproic acid in human serum by gas—liquid chromatography on OV-17 using nitrogen-specific detection

NEIL J. COOK and DAVID A. JOWETT*,*

Toxicology Laboratory, Department of Laboratory Services, Wellington Hospital, Private Bag, Wellington (New Zealand)

(First received April 19th, 1982; revised manuscript received August 27th, 1982)

The determination of valproic acid (di-*n*-propylacetic acid) in serum or plasma is an important part of the clinical management of epileptic patients receiving that drug. Although high-performance liquid chromatographic (HPLC) [1—4], enzyme immunoassay [5, 6], isotachophoresis [7] and direct insertion mass spectrometry [8] methods have been reported, gas—liquid chromatographic (GLC) methods have been the most extensively used.

Due to the volatility of valproic acid, concentration by evaporation is avoided in several GLC methods which employ extraction into a small amount of organic solvent, followed by chromatography without derivatization [9—32]. However, these methods require specific and frequently unstable stationary phases (e.g. FFAP, SP-1000), and large sample volumes — a significant disadvantage since this drug is commonly used with pediatric patients. Procedures involving derivatization decrease the volatility of valproic acid and allow the use of more stable stationary phases. The GLC of valproic acid as its methyl [33—37], butyl [38], propyl [39] and phenacyl [40, 41] esters have been reported.

GLC using the stationary phase OV-17, coupled with nitrogen-specific detection, is extensively employed in the analysis of several other antiepileptic drugs [42]. We investigated the determination of valproic acid as its *p*-nitrophenacyl ester since Gupta et al. [40] previously noted that this nitrogen-containing derivative showed good chromatographic properties on OV-17. This paper reports a sensitive, simple and precise method for the determination of valproic acid in human serum employing derivatization with α-bromo-*p*-nitroacetophenone followed by chromatography on OV-17 using a gas chromatograph equipped with a nitrogen—phosphorus specific detector.

*Present address: Cawthron Institute, P.O. Box 175, Nelson, New Zealand.

0378-4347/83/0000—0000/$03.00

EXPERIMENTAL

Materials

The internal standard, cyclohexane carboxylic acid, and triethylamine (99%) were supplied by Aldrich (Milwaukee, WI, U.S.A.). α-Bromo-*p*-nitroacetophenone was obtained from Pfaltz and Bauer (Stanford, CT, U.S.A.), *n*-hexane 98% (rein) from Merck (Darmstadt, G.F.R.) and sodium valproate from Reckitt and Colman (Avondale, New Zealand). All materials were used without further purification.

Extraction and derivatization

To 100 μl of internal standard solution (0.02% cyclohexane carboxylic acid in 0.5 *M* ammonium hydroxide, prepared fresh weekly and stored at 4°C) in a 12-ml glass-stoppered conical tube were added 100 μl of serum and 100 μl of 1 *M* hydrochloric acid. The mixture was vortex-mixed and then extracted with 4 ml *n*-hexane by vigorous mixing on a platform shaker for 10 min. The organic layer was transferred to a 75 × 12 mm glass tube and 20 μl of α-bromo-*p*-nitroacetophenone solution (0.5% in acetonitrile prepared monthly and stored at 4°C) and 20 μl of triethylamine were added. This was vortex-mixed, placed in a heating block (65°C), allowed to react for 15 min and then taken to dryness under a stream of air. The residue was taken up in 50 μl of *n*-hexane and a 2-μl aliquot was injected into the gas chromatograph.

Chromatography

A Hewlett-Packard 5840A gas chromatograph, equipped with a nitrogen—phosphorus detector, was used. The 2 m × 2 mm glass column was silanized before packing with 2% OV-17 on Chromosorb W HP, 100—120 mesh. The operating conditions were: injection port, 280°C; column, 230°C; and detector 280°C. The flow-rate of the carrier gas (nitrogen) was 24 ml/min.

Quantitation

The concentration of valproic acid in serum samples was calculated with the aid of standards prepared by adding sodium valproate to blank serum to give concentrations of 10—150 μg/ml valproic acid. These were analysed by the described procedure and calibration curves were prepared by plotting the ratio of the peak area of valproic acid to that of the internal standard against the known concentrations of valproic acid. Analyses were also carried out using 50-μl and 20-μl aliquots of the standards (using 100-μl aliquots of a 2-fold and 5-fold dilution of the internal standard solution, respectively).

RESULTS

A typical chromatogram of an extract of drug-free serum and that of serum containing valproic acid (75 μg/ml) are shown in Fig. 1. The retention time of the *p*-nitrophenacyl ester of valproic acid is 3.6 min, while that of the ester of cyclohexane carboxylic acid is 6.0 min. *n*-Hexane was used to reconstitute the final residue since the use of more polar solvents, such as methanol, led to the appearance of extraneous peaks in the chromatogram.

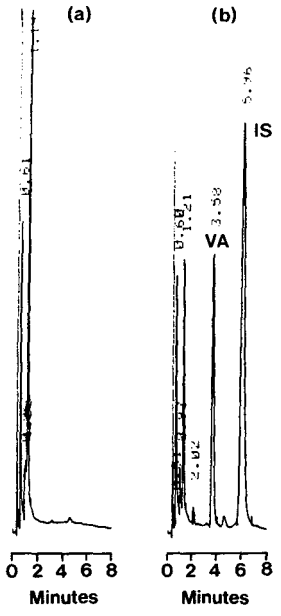


Fig. 1. Elution profiles of (a) an extract of drug-free serum and (b) an extract of serum containing 75 μg/ml valproic acid (VA) and internal standard (IS).

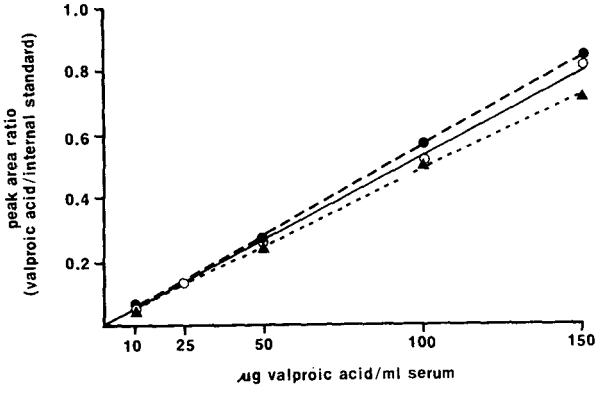


Fig. 2. Calibration graphs for analyses using 100-μl (○), 50-μl (▲) and 20-μl (●) aliquots of serum containing valproic acid.

When samples containing 10—15 μg/ml valproic acid were analysed using 100-, 50- and 20-μl aliquots the resulting calibration curves were linear with correlation coefficients of 0.9994, 0.9991 and 0.9998, respectively (Fig. 2). Samples containing valproic acid concentrations of 0.5—10 μg/ml similarly gave a linear calibration curve (correlation coefficient of 0.9998) when 100-μl aliquots were assayed.

The intra-batch and inter-batch coefficients of variation (C.V.), determined using the paired-sample technique [43], were 3.1% (n = 18 pairs, range 26.1—148.9 μg/ml) and 3.5% (n = 37 pairs, range 23.8—153.9 μg/ml), respectively.

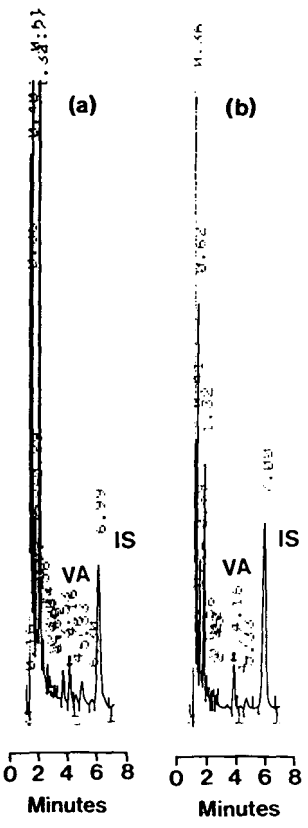


Fig. 3. Elution profiles of (a) serum spiked to 2 μg/ml and (b) patient serum containing 2 μg/ml valproic acid (VA, arrowed). The internal standard (IS) added was a 12.5-fold dilution of the solution used in the standard assay. Oven temperature was 225°C.

The repeated analysis of a single sample over a 6-month period gave an inter-batch C.V. of 3.7% (n = 50, mean = 78.1 μg/ml, S.D. = 2.87). The repeated analysis of a single sample spiked to 2 μg/ml, using 100 μl serum and 100 μl 12.5-fold diluted internal solution, gave an intra-batch C.V. of 6.3% (n = 10, mean = 1.87 μg/ml) (Fig. 3).

Mean absolute recoveries, determined by adding an increment of valproic acid after hexane extraction, were 72.1, 79.5, 73.5 and 73.5% at serum valproic acid concentrations of 2, 40, 80 and 120 μg/ml, respectively (n = 5 in each case).

The following drugs were added to blank sera to 50 mg/l and the sera were analysed as for valproic acid: amitriptyline, beclamide, benzhexol, benztropine, betazole, chloral hydrate, chlorpromazine, danthron, diazepam, doxapram, ethosuximide, fluphenazine, haloperidol, methylprednisolone, naproxen, nitrazepam, nortriptyline, oxazepam, pericyazine, phenobarbitone, phenytoin, poloxamer, primidone, prochlorperazine, prothiaden, thioridazine, triazolam and trifluoperazine. None interfered with valproic acid quantitation. Haemolysed, icteric and lipaemic samples similarly did not interfere with quantitation.

Serum which had been in prolonged contact with the red, grey or green stoppers of Vacutainer brand (Becton Dickinson, Rutherford, NJ, U.S.A.) blood collection tubes manufactured before 1980 gave an extra peak eluting between valproic acid and the internal standard. This is possibly the plasticizer tributoxyethylphosphate which has been eliminated from the most recent formulation of the stoppers. The extra peak was not seen when Venoject brand (Terumo Medical, Elkton, MD, U.S.A.) blood collection tubes were used.

DISCUSSION

The formation of the *p*-nitrophenacyl ester of valproic acid permitted the analysis of small samples of serum containing concentrations of valproic acid as low as 2.0 μg/ml. At this concentration each 2-μl injection represented the equivalent of 8 ng of valproic acid. This sensitivity was achieved because (i) the ester is less volatile than the parent drug and thus could be concentrated by removal of the extracting solvent and (ii) the nitro-group on the ester permitted the use of a nitrogen-sensitive detector. An important additional factor was the use of *n*-hexane as the extracting solvent and as the final reconstituting solvent since the resulting chromatograms contained far fewer extraneous peaks than when more polar solvents were used. The recovery of valproic acid from serum using *n*-hexane was similar to that obtained by Gupta et al. [40] using pentane.

A significant advantage of the method described over many other methods is that the commonly used stationary phase, OV-17, is employed. This phase is more stable than polar phases such as FFAP and SP-1000 which are commonly used in the analysis of fatty acids. It is likely that the method presented here could be readily extended to the analysis of other fatty acids.

REFERENCES

1 R. Farinotti, M.C. Pfaff and G. Mahuzier, Ann. Biol. Clin., 36 (1978) 347.
2 R.N. Gupta, P.M. Keane and M.L. Gupta, Clin. Chem., 25 (1979) 1984.
3 R. Alric, M. Cociglio, J.P. Blayac and R. Puech, J. Chromatogr., 224 (1981) 289.
4 H. Cisse, R. Farinotti, S. Kirkiacharian and A. Dauphin, J. Chromatogr., 225 (1981) 509.
5 A.A. Elyas, V.D. Goldberg, N. Ratnaraj and P.T. Lascelles, Ann. Clin. Biochem., 17 (1980) 307.
6 S.L. Braun, A. Tausch, W. Vogt, K. Jakob and M. Knedel, Clin. Chem., 27 (1981) 169.
7 F. Mikkers, Th. Verheggen, F. Everaerts, J. Hulsman and C. Meijers, J. Chromatogr., 182 (1980) 496.
8 G.M. Schier, I.E. Gan, B. Halpern and J. Korth, Clin. Chem., 26 (1980) 147.
9 L.J. Dusci and L.P. Hackett, J. Chromatogr., 132 (1977) 145.
10 A.J. Fellenberg and A.C. Pollard, Clin. Chim. Acta, 81 (1977) 203.
11 M.H. Wood, D.C. Sampson and W.J. Hensley, Clin. Chim. Acta, 77 (1977) 343.
12 H.-U. Schulz and P.A. Toseland, Ann. Clin. Biochem., 14 (1977) 240.
13 J. Balkon, J. Anal. Toxicol., 2 (1978) 207.
14 D.J. Berry and L.A. Clarke, J. Chromatogr., 156 (1978) 301.
15 C. Jakobs, M. Bojasch and F. Hanefeld, J. Chromatogr., 146 (1978) 494.
16 R.H. Levy, L. Martis and A.A. Lai, Anal. Lett., B11 (1978) 257.
17 J.-C. Libeer, S. Scharpé, P. Schepens and R. Verkerk, J. Chromatogr., 160 (1978) 285.

18 A.E. Hershey, J.R. Patton and K.H. Dudley, Ther. Drug Monit., 1 (1979) 217.
19 G.A. Peyton, S.C. Harris and J.E. Wallace, J. Anal. Toxicol., 3 (1979) 108.
20 B. Pileire, J. Chromatogr., 162 (1979) 446.
21 D.J. Freeman and W. Rawal, Clin. Chem., 26 (1980) 674.
22 J.H. Goudie, K. Reed, G.J. Ayers and D. Burnett, Clin. Chem., 26 (1980) 1929.
23 N. Grgurinovich and J.O. Miners, J. Chromatogr., 182 (1980) 237.
24 W.R. Külpmann, J. Clin. Chem. Clin. Biochem., 18 (1980) 339.
25 A. Kumps and Y. Mardens, Clin. Chem., 26 (1980) 1759.
26 M. Puukka, R. Puukka and M. Reunanem, J. Clin. Chem. Clin. Biochem., 18 (1980) 497.
27 A. Sioufi, D. Colussi and F. Marfil, J. Chromatogr., 182 (1980) 241.
28 R.O. Fullinfaw and J.J. Marty, Clin. Chem., 27 (1981) 1776.
29 K.A. Odusote and A.L. Sherwin, Ther. Drug Monit., 3 (1981) 103.
30 M.I.A. Peña, J. Chromatogr., 225 (1981) 459.
31 C. Manfredi and L. Zinterhofer, Clin. Chem., 28 (1982) 246.
32 A. Wu, M.L. Pearson, S.K. Mertens, D.D. Bretl and G.S. Wolffe, Clin. Chem., 28 (1982) 544.
33 O. Gyllenhaal and A. Albinsson, J. Chromatogr., 161 (1978) 343.
34 N.L. Tupper, E.B. Solow and C.P. Kenfield, J. Anal. Toxicol., 2 (1978) 203.
35 S. Willox and S.E. Foote, J. Chromatogr., 151 (1978) 67.
36 R. Varma and A.Y. Hoshino, Neurosci. Lett., 11 (1979) 353.
37 B.A. Calendrillo and G. Reynoso, J. Anal. Toxicol., 4 (1980) 272.
38 A. Hulshoff and H. Roseboom, Clin. Chim. Acta, 93 (1979) 9.
39 Y. Morita, T.I. Ruo, M.L. Lee and A.J. Atkinson, Jr., Ther. Drug Monit., 3 (1981) 193.
40 R.N. Gupta, F. Eng and M.L. Gupta, Clin. Chem., 25 (1979) 1303.
41 S.C. Chan, Clin. Chem., 26 (1980) 1528.
42 C.L. Least, Jr., G.F. Johnson and H.M. Solomon, Clin. Chem., 23 (1977) 593.
43 N.D.H. Balazs and T.D. Geary, The Clinical Biochemist, Reviews, (1980) 51.

Journal of Chromatography, 272 (1983) 187—192
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1490

Note

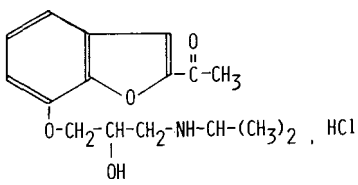
Gas chromatographic determination of the glucuronide of 2-(1-hydroxy-ethyl)-7-(2-hydroxy-3-isopropyl-aminopropoxy)-benzofuran, a metabolite of befunolol, in human urine

K. KAWAHARA* and T. OFUJI

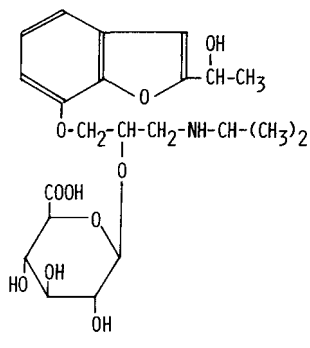
Research Laboratories, Kakenyaku-kako Co. Ltd., 14 Minami-kawaramachi, Shinomiya, Yamashinaku, Kyoto 607 (Japan)

(First received June 8th, 1982; revised manuscript received September 1st, 1982)

Befunolol (Fig. 1) is a β-adrenergic receptor blocking agent [1]. 2-(1-Hydroxy-ethyl)-7-(2-hydroxy-3-isopropyl-aminopropoxy)-benzofuran (MI) and its glucuronide (MIG, see Fig. 1) have been known as the metabolites of befunolol excreted in animal and human urine [2, 3]. MIG in human urine after oral administration of befunolol has been determined by enzymatic hydrolysis of glucuronide followed by gas chromatography (GC) and gas chromatographic—mass spectrometric (GC—MS) analysis of the released aglycone (MI) [4, 5]. Direct GC methods have been reported for the quantitative determination of glucuronides of thiamphenicol, chloramphenicol [6], oxazepam and lorazepam [7] after methyl-trimethylsilylation, and of trimetozine [8] after permethylation.



befunolol hydrochloride



MIG

Fig. 1. Chemical structures of befunolol and MIG.

0378-4347/83/0000—0000/$03.00

In this paper, we describe a GC method for the quantitative determination of MIG after trimethylsilylation. The conditions for pretreatment of urine and for trimethylsilylation of MIG are investigated. The method established is applied to a comparison of the excretion rate of MIG with those of befunolol and MI (unconjugated).

EXPERIMENTAL

Reagents and materials

Ethyl acetate, diethyl ether, methanol (for pesticide residue analysis) and acetic acid (analytical grade) were obtained from Wako (Osaka, Japan). N,O-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and N-(trimethylsilyl)imidazole (SIM) were specially prepared reagents for GC analysis (Nakarai Chemical, Kyoto, Japan). 6-Bromo-2-naphthyl-β-D-glucuronide used as an internal standard (IS) was obtained from Sigma (St. Louis, MO, U.S.A.), and *n*-tetracontane used as an external standard (ES) was from Applied Science Labs. (State College, PA, U.S.A.). IS and ES were used as aqueous solution (40 μg/ml) and chloroform solution (22 μg per 50 μl), respectively.

Amberlite XAD-2 resin (Rohm & Haas, Philadelphia, PA, U.S.A.) was purified as described in the previous paper [8]. QAE-Sephadex A-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) was swollen in distilled water for three days and washed with distilled water.

Authentic MIG was isolated from the urine of dog dosed with befunolol by the method described in the previous paper [3].

Drug administration to volunteers

Two healthy male adults each received an oral dose of a 20-mg befunolol capsule after a 16-h fast. Urine specimens were collected just before and at 1, 2, 3, 4, 5, and 6 h after administration.

Sample preparation

A 2-ml portion of urine was placed on a QAE-Sephadex A-25 column (3 cm × 5 mm I.D.) and eluted with 7 ml of distilled water. The eluate was washed twice with 10 ml of ethyl acetate. The trace of ethyl acetate remaining in the aqueous layer was removed by washing twice with 10 ml of diethyl ether. After complete removal of diethyl ether by evaporation under reduced pressure at room temperature for 10 min, a 1-ml portion of IS solution was added to the aqueous layer and the mixture was placed on an Amberlite XAD-2 column (5 cm × 10 mm I.D.). The column was first washed with 20 ml of distilled water and subsequently eluted with 20 ml of methanol containing 1.5% acetic acid. The latter eluate was collected and evaporated to dryness in a flask under reduced pressure at 40°C. The residue was trimethylsilylated by reaction with 40 μl of SIM and 30 μl of BSTFA for 20 min at room temperature. A 1-μl portion of the reaction mixture was injected into the gas chromatograph.

GC conditions

An Hitachi 073 gas chromatograph equipped with a flame ionization

detector was used. A U-shaped glass column (0.5 m × 3 mm I.D.) was packed with 1.5% OV-17 on Chromosorb W AW DMCS, 80—100 mesh (Shimadzu, Kyoto, Japan). The column temperature was 230°C, the injection port and detector temperature 260°C; the carrier gas (nitrogen) flow-rate was 70 ml/min.

RESULTS AND DISCUSSION

Pretreatment of urine specimens

The endogenous urinary components were removed as far as possible to avoid interference with subsequent trimethylsilylation and GC separation of MIG and IS. Acidic components were removed by passing through a QAE-Sephadex A-25 column. Recovery of MIG from the column eluted with distilled water was 97.8 ± 0.03% (mean ± S.E.), which was determined by its UV absorbance at 244 nm. The neutral components were removed from the eluate of a QAE-Sephadex A-25 column by washing with ethyl acetate and then diethyl ether, while MIG remained in the aqueous eluate. A trace of organic solvent remaining in the eluate was completely removed by evaporation under reduced pressure, since it interferes with adsorption of glucuronides on an Amberlite XAD-2 column. IS should be added to the aqueous layer at this point, because it is strongly adsorbed on a QAE-Sephadex A-25 column and easily extracted into the organic layer.

The recoveries of MIG and IS from an Amberlite XAD-2 column were examined using methanol containing 0–1.5% acetic acid as an elution solvent. The recovery of MIG was 85.2%, but that of IS was less than 5% when they were eluted with methanol. An acetic acid concentration above 1% in the elution solvent gave almost constant recoveries of MIG (97.5%) and IS (91.7%). From these results, the procedure for the clean-up of urine was settled as described in the Experimental section.

Trimethylsilylation of MIG and IS

The time and temperature dependencies of trimethylsilylation were investigated with varying reaction times between 2 and 60 min and reaction temperatures of 15, 25 and 35°C. *n*-Tetracontane was used as an external standard (ES) to evaluate the effect of these conditions on the yields of MIG and IS derivatives. The peak area ratios of MIG and IS derivatives against ES indicate that trimethylsilylation of MIG and IS was complete within 10 min. These derivatives were stable for at least 60 min at a reaction temperature of 25°C, and the yields of MIG and IS derivatives were independent of temperature (reaction time 20 min). When MIG was trimethylsilylated with SIM alone, a minor secondary peak of MIG was sometimes observed on the chromatogram, which, however, was reduced by the addition of BSTFA.

GC separation

Fig. 2 shows chromatograms of human urine before and after the oral dose of 20 mg of befunolol as a capsule. The TMS derivatives of MIG and IS are clearly separated on the OV-17 column with retention times of 10.9 min and 6.3 min, respectively.

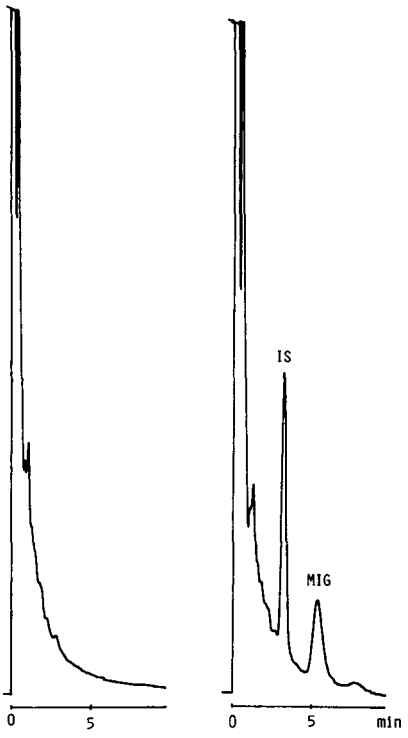


Fig. 2. Chromatograms of urine extracts. Left: normal urine. Right: urine from volunteer A who received a single oral dose of 20 mg of befunolol; MIG = 151 ng (5.3 μg/ml at 6 h after administration)

Calibration graph

The calibration graph of MIG against IS was drawn using 2 ml of control urine spiked with several known amounts of MIG. The calibration graph constructed with peak area ratios of MIG to IS is linear in the range 5—100 μg per 2 ml with a correlation coefficient of 0.998.

The accuracy and precision of the present method are shown in Table I. The average recovery of MIG from spiked urine was 101.1 ± 1.2% and the minimum detectable concentration of MIG was 2.5 μg/ml when 2 ml of human urine were used.

TABLE I

ACCURACY AND PRECISION OF THE ASSAY APPLIED TO SPIKED HUMAN URINE

Amount of MIG added to urine (μg per 2 ml)	Amount of MIG found (μg per 2 ml) (mean of seven experiments)	Precision (coefficient of variation, %)
5	4.99	9.7
10	10.28	9.0
25	24.02	3.3
50	50.83	4.5
100	105.43	4.8

Urinary excretion of MIG

The present method was applied to the determination of MIG in the urine of volunteers receiving 20 mg of befunolol by the oral route. The semi-logarithmic plot for the excretion rate of MIG is shown in Fig. 3. The apparent excretion rate constants of MIG in volunteers A and B calculated from the declining slopes were 0.101 h^{-1} and 0.295 h^{-1}, respectively. On the other hand, befunolol (unconjugated) and MI (unconjugated) were determined by the GC method [4] (minor modification), and the excretion rate constants of befunolol (unconjugated) and MI (unconjugated) in volunteers A and B were 0.401 h^{-1} and 0.464 h^{-1}, and 0.364 h^{-1} and 0.428 h^{-1}, respectively. Comparison of these results shows that the excretion rate constant of MIG is the smallest of all.

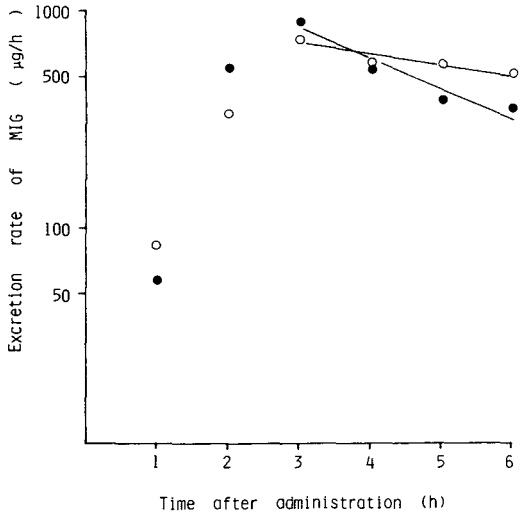


Fig. 3. Urinary excretion of MIG in volunteers after a single oral dose of 20 mg of befunolol. (○) Volunteer A; (●) volunteer B.

CONCLUSION

The present method has advantages over the previous methods in that it avoids the error accompanying enzymatic hydrolysis employed in the GC method, and that it does not need the preparation of monodeutero-MIG (glucuronide of monodeutero-MI) as internal standard and the complicated calculations required in the GC—MS method. The present method is highly sensitive, specific and reproducible, and can be used for the determination of MIG in human urine.

ACKNOWLEDGEMENTS

The authors thank Professor T. Uno and Dr. T. Nakagawa of the Faculty of Pharmaceutical Sciences, Kyoto University, for many suggestions during this work.

REFERENCES

1 F. Takenaka, T. Ishihara, Y. Maruyama, S. Masumoto, H. Inoue, M. Okumura, and H. Honma, Folia Pharmacol. Japon., 70 (1974) 385.
2 H. Kitagawa, Y. Sakai, Y. Matsumura, H. Kojima, T. Matsuda, M. Tohno and T. Ofuji, Pharmacometrics, 18 (1979) 889.
3 M. Tohno, K. Kimura, M. Nagahara, Y. Sakai, T. Ofuji and T. Nadai, Yakugaku Zasshi, 99 (1979) 944.
4 H. Funaki and T. Matsuda, J. Kyoto Prefect. Univ. Med., 84 (1975) 793.
5 M. Tohno, Y. Matsumura, T. Ofuji, A. Tatematsu, M. Suzuki, H. Harada and T. Nadai, Mass Spectrosc., 26 (1978) 343.
6 T. Nakagawa, M. Masada and T. Uno, J. Chromatogr., 111 (1975) 355.
7 F. Marcucci, R. Bianchi, L. Airoldi, M. Salmona, R. Fanelli, C. Chiabrando, A. Frigerio, E. Mussini and S. Garattini, J. Chromatogr., 107 (1975) 285.
8 K. Kawahara and T. Ofuji, J. Chromatogr., 231 (1982) 333.

Journal of Chromatography, 272 (1983) 193—199
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1467

Note

Dosage du méthoxy-5 psoralène dans le plasma par chromatographie en phase liquide et detection spectrofluorimétrique

P. PROGNON, G. SIMON et G. MAHUZIER*

Laboratoire de Chimie Analytique, Centre d'Études Pharmaceutiques, Université Paris Sud, rue Jean Baptiste Clément, 92220 Chatenay-Malabry (France)

(Reçu le 8 avril 1982; manuscrit modifié reçu le 4 août 1982)

Le méthoxy-5 psoralène (5 MOP, I) est une furocoumarine qui vient d'être introduite récemment dans le traitement du psoriasis.

Deux heures après son absorption orale, une irradiation de la lésion par des rayonnements UVA (320—400 nm) entraîne après plusieurs séances la disparition des symptômes cliniques. Cette association, appelée puvathérapie [1], est depuis longtemps pratiquée avec son homologue le méthoxy-8 psoralène (8 MOP, II) [2]. Avec ce dernier produit, l'apparition d'effets secondaires pour certaines posologies et la nécessité de déterminer sa pharmacocinétique ont nécessité la mise au point de méthodes faisant appel à la chromatographie en phase gazeuse [3—6], à son couplage avec la spectrométrie de masse [7, 8], ou à la chromatographie liquide. Dans ce cas, la détection met à profit l'absorbance dans l'ultraviolet des psoralènes [9—11].

De telles techniques peuvent selon toute vraisemblance être appliquées au dosage du 5 MOP. Cependant celui-ci présente une fluorescence dix fois plus importante que le 8 MOP qui peut être exploitée pour obtenir une meilleure sensibilité et surtout une meilleure spécificité de détection.

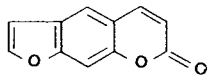
La méthode proposée met en oeuvre une chromatographie liquide avec une détection spectrofluorimétrique. L'étalon interne est le psoralène (III). Elle permet ainsi d'atteindre une limite de 5 ng de 5 MOP par ml de plasma, alors qu'en spectrophotométrie d'absorption la limite inférieure se situe aux alentours de 15 ng/ml.

MATÉRIEL ET MÉTHODE

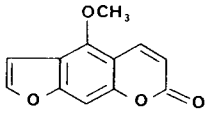
Appareillage

Micropipettes automatiques réglables type SMI, tubes à extraction en verre

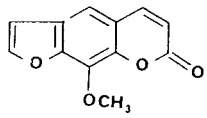
0378-4347/83/0000—0000/$03.00



PSORALÈNE (III)



5 MOP (I)



8 MOP (II)

pyrex de 10 ml, tubes à hémolyse, agitateur type Vortex, évaporateur à sec Biobloc type 92617, spectrofluorimètre Perkin Elmer MPF 3L.

Le système chromatographique comprend: une pompe Chromatem 330 (Touzart et Matignon, Paris, France), un injecteur à boucle (20 μl) Rheodyne 7125, une colonne en acier inox (30 cm × 4.6 mm I.D.) remplie de Spherosil C_{18}, 5 μm (Waters Assoc.) selon la méthode de Coq et al. [12], un spectrofluorimètre Schoeffel type FS 970 équipé d'un monochromateur d'entrée à 330 nm et d'un filtre de sortie (440 nm), un enregistreur multicanaux Kipp en Zonen type BD 40 réglé avec un déroulement de papier de 5 mm/min.

Réactifs

Méthanol, éthanol, acétate d'éthyle Normapur (Prolabo, France), acide sulfurique, benzène "pour fluorimétrie" (Merck), Psoralène (Laboratoires Promedica, France), méthoxy-5 psoralène (Laboratoire GOUPIL, France), bisulfate de quinine (Prolabo, France).

Étalons

Solution de psoralène à 5 mg/l dans l'éthanol (solution A); solution de méthoxy-5 psoralène à 1 mg/l dans l'acétate d'éthyle (solution B).

Méthode d'extraction

1 ml de sérum ou de plasma est additionné de 100 μl de solution A (étalon interne), agité au Vortex 30 sec, extrait par 2 ml de benzène après agitation mécanique de 15 min. L'ensemble est centrifugé pendant 10 min à 3000 rpm. La phase organique est prélevée, transférée dans des tubes à hémolyse puis évaporée à sec. L'extrait sec est repris par 50 μl de méthanol et 20 μl sont injectés dans le chromatographe.

Chromatographie en phase liquide

La phase mobile est constituée d'un mélange isocratique méthanol—eau (70:30, v/v). Le débit est maintenu à 1.5 ml/min. Les concentrations sont calculées à partir des rapports des hauteurs de pic par rapport à une courbe d'étalonnage.

Cet étalonnage est réalisé par extraction de sérum surchargé par des quan-

tités croissantes de 5 MOP (10—500 ng/ml) et par une même quantité d'étalon interne (500 ng/ml).

RÉSULTATS

La Fig. 1A représente un chromatogramme obtenu à partir du sérum d'un sujet non traité, la Fig. 1B représente celui obtenu chez le même sujet deux heures après l'absorption d'un gélule renfermant 45 mg de 5 MOP. Comme on le constate, les constituants normaux du plasma ne peuvent interférer sur le dosage du fait de la spécificité de la détection spectrofluorimétrique. La puvathérapie étant en principe réservée à des sujets n'ayant pas d'autres médications, il n'a pas été besoin de rechercher les interférences médicamenteuses.

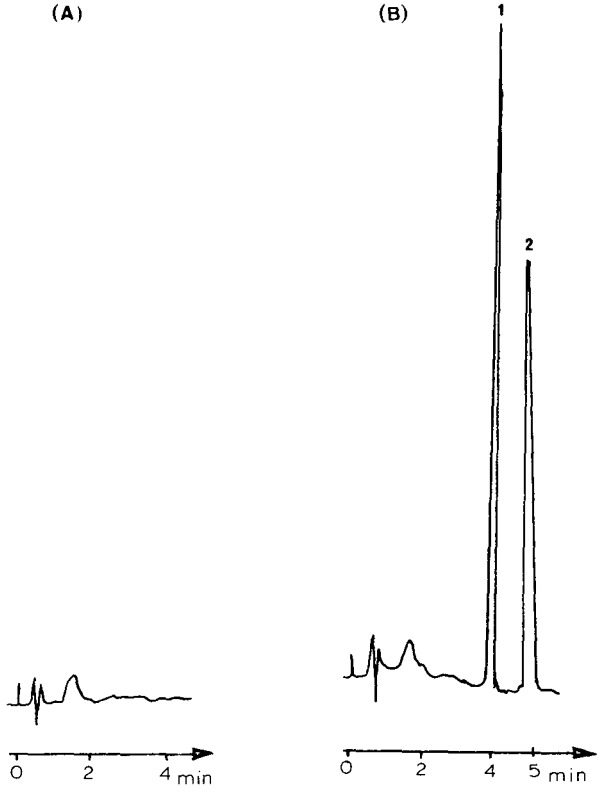


Fig. 1. (A) Chromatogramme d'un sérum de malade non traité; (B) chromatogramme d'un sérum de malade, 3 h après l'administration d'une gélule dosée à 45 mg de 5 MOP. (1) psoralène: temps de rétention 4 min, (2) 5 MOP: temps de rétention 5 min.

Les temps de rétention du psoralène et du 5 MOP sont respectivement de 4 et 5 min dans les conditions décrites. Les caractéristiques de la méthode ont été étudiées:

Le rendement d'extraction du 5 MOP est de 95 ± 1.60%, la reproductibilité déterminée par l'analyse de dix échantillons surchargés donne des coefficients

de variations de 6.10% pour 25 ng/ml, 4.80% pour 100 ng/ml et 4% pour 500 ng/ml.

La linéarité de la méthode pour des concentrations de 0—500 ng/ml (0—0.0231 nmol/ml) est satisfaisante ($Y = 0.039\ X + 0.022$; $r = 0.999$). La limite de sensibilité est de 4 ng/ml soit 0.007 nmol injectées dans un volume de 20 μl.

DISCUSSION

Les propriétés de fluorescence présentées par le 5 MOP et le psoralène ont été mises à profit pour leur détection en chromatographie liquide. Au préalable, il a été nécessaire d'envisager l'influence, sur l'intensité de cette fluorescence, des différents facteurs pouvant intervenir lors de la séparation: nature de la phase mobile, variation du pH et de la force ionique.

Des essais préliminaires ont montré que le pH du milieu entre 2.5 et 7.5 ainsi que la force ionique entre 0.02 et 0.5 *M* ne modifient pas cette fluorescence. Par contre, celle-ci est influencée par la composition du mélange binaire eau—méthanol, classiquement utilisé en chromatographie liquide en phase inverse, qui intervient sur la longueur d'onde d'émission et sur l'intensité de cette fluorescence.

On constate en effet (Tableau I) que si la position des maxima d'excitation varie peu en fonction de la composition du solvant; les maxima d'émission subissent un effet bathochrome marqué avec l'augmentation de polarité du mélange.

TABLEAU I

INFLUENCE DU SOLVANT SUR LA POSITION DES LONGUEURS D'ONDES MAXIMA D'EXCITATION ET D'EMISSION DU 5 MOP

Solvant	λ_{exc}^{max}	λ_{em}^{max}
Méthanol	329	480
Méthanol—eau (70:30)	330	485
Méthanol—eau (5:95)	328	492

Ceci traduit l'augmentation de l'interaction dipole—dipole entre l'état excité légèrement polarisé de la molécule et le solvant qui caractérise une émission de fluorescence $\pi^* \rightarrow \pi$.

Les variations d'intensité de fluorescence du 5 MOP et du psoralène en fonction de la teneur en méthanol de la phase mobile ont été ensuite étudiées en déterminant les rendements quantiques relatifs de ces deux molécules selon la méthode proposée par Parker et Rees [13].

Cette notion présente l'avantage de définir précisément l'intensité de fluorescence d'une molécule dans des conditions de concentration et de solvant déterminée en la comparant à celle d'une substance dont les caractéristiques de fluorescence sont bien établies.

Les rendements quantiques présentés dans le Tableau II ont été calculés par rapport au bisulfate de quinine 10^{-6} *M* en solution sulfurique 0.1 *N* en se plaçant à une longueur d'excitation de 330 nm. Le rendement quantique du psoralène est maximum dans l'eau, alors que celui du 5 MOP l'est dans le méthanol. Un mélange méthanol—eau (50:50) permet cependant d'atteindre une bonne sensibilité.

TABLEAU II

RENDEMENT QUANTIQUE RELATIF DU 5 MOP ET DU PSORALÈLE EN FONCTION DE LA TENEUR EN MÉTHANOL DE LA PHASE MOBILE

	Méthanol (%)			
	0	50	70	100
5 MOP	$3.2 \cdot 10^{-3}$	$5.8 \cdot 10^{-3}$	$8.3 \cdot 10^{-3}$	$1.2 \cdot 10^{-2}$
Psoralène	$1.5 \cdot 10^{-2}$	$1.2 \cdot 10^{-2}$	$1.0 \cdot 10^{-2}$	$6.5 \cdot 10^{-3}$

À partir de ces résultats, des essais rapportés dans le Tableau III ont été effectués pour préciser la composition et le débit de la phase mobile permettant d'obtenir une bonne séparation.

Il apparaît que, d'une part, le pH apparent est sans influence sur la résolution des deux molécules, d'autre part que l'augmentation de la teneur en méthanol ainsi que l'augmentation du débit diminuent les k'. Les valeurs de 50, 60 et 70% en méthanol semblent acceptables, mais une valeur de 70% a été cependant choisie car dans ces conditions le rendement quantique du 5 MOP est plus élevé ($8.3 \cdot 10^{-3}$) et l'on obtient ainsi une meilleure sensibilité de détection spectrofluorimétrique.

Un exemple d'application de cette technique est donné sur la Fig. 2 où sont reportés les taux sériques déterminés chez un même malade ayant ingéré à jeun en l'absence de toute autre thérapeutique, par voie orale, 45 mg de 5 MOP sous forme de gélule et la même dose sous forme de solution hydroalcoolique [eau—éthanol (50:50) à 90°]. Comme pour le 8 MOP, les posologies habituelles sont de 0.60 mg/kg. Les équations des concentrations sanguines, au cours du temps, sont également reportées ainsi que les constantes d'absorption, d'élimination et les temps de demie-vie qui indiquent un modèle de type monocompartimental pour les deux formes.

CONCLUSION

La méthode proposée est simple, rapide et reproductible. Son originalité repose sur l'exploitation des propriétés de fluorescence du 5 MOP qui lui confère une sensibilité et une spécificité applicable aux dosages de cette molécule dans les milieux biologiques lors des études pharmacocinétiques et de métabolisme ou lors de la détermination des susceptibilités individuelles.

TABLEAU III

VARIATION DES FACTEURS DE CAPACITÉ k' DU 5 MOP ET DU PSORALÈNE EN FONCTION DE LA COMPOSITION DE LA PHASE MOBILE, DU DÉBIT ET DU pH APPARENT

	pH apparent 5; débit 1.5 ml/min Eau—méthanol (v/v)				pH apparent 5; eau—méthanol (30:70, v/v) Débit (ml/min)				Débit 1.5 ml/min; eau—méthanol (30:70, v/v) pH apparent			
	50:50	40:60	30:70	20:80	0.5	1	1.5	2	2.5	4	5	7.5
k' 5 MOP	5	3.7	2.8	2	3.5	3	2.8	2.6	2.8	2.83	2.8	2.81
k' PSO	3.6	2.5	1.9	0.95	2.7	2	1.9	1.8	1.9	1.9	1.9	1.92

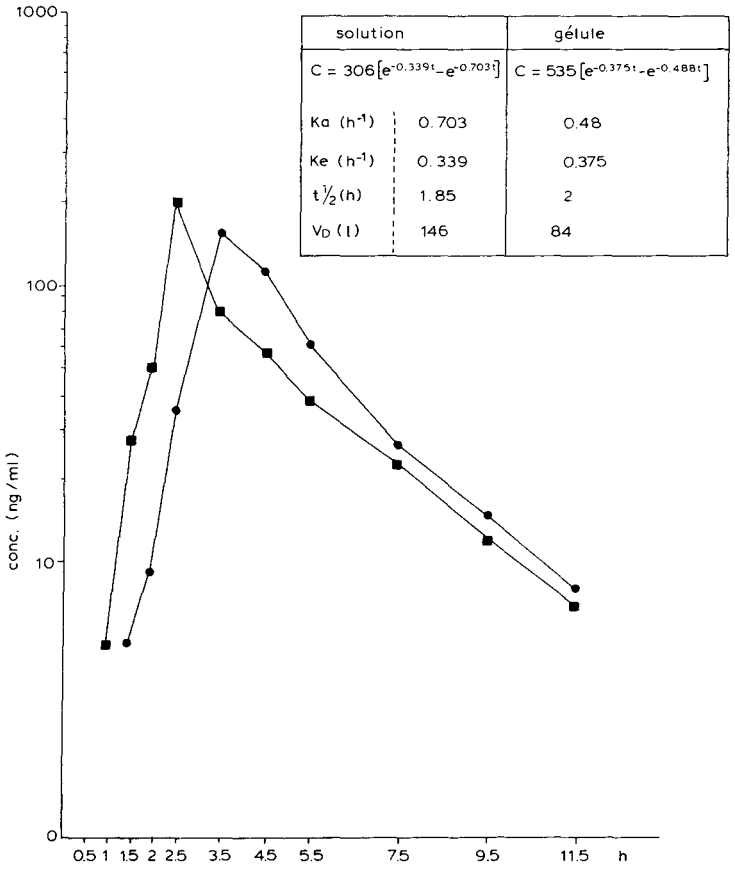


Fig. 2. Taux sériques de 5 MOP chez un même malade ayant ingéré deux formes orales de 5 MOP également dosées: 45 mg en une seule prise sous forme de solution (▪——▪) ou gélule (●——●).

BIBLIOGRAPHIE

1 T.F. Anderson et J.J. Voorhees, Ann. Rev. Pharmacol. Toxicol., 20 (1980) 235.
2 K. Wolff, T.D. Fitzpatrick, J.A. Parrish, F. Gschnait, B. Gilchrest, H. Honigsmann, M.A. Pathack et L. Tannenbaum, Arch. Dermatol., 112 (1976) 943.
3 J. Gazith et H. Schaefer, Biochem. Med., 18 (1977) 102.
4 H. Ehrsson, S. Eksborg, I. Wallin, N. Kallberg et G. Swanbeck, J. Chromatogr., 140 (1977) 157.
5 J. Schmid et F.W. Koss, J. Chromatogr., 146 (1978) 498.
6 J. Gazith, W. Schalla et H. Schaefer, Arch. Dermatol. Res., 263 (1978) 215.
7 C.N. Hensby, Clin. Exp. Dermatol., 3 (1978) 355.
8 J. Schmid, A. Prox, H. Zipp et F.N. Koss, Biom. Mass Spectrom., 7 (1980) 560.
9 C.V. Puglisi, J.A.F. de Silva et J.C. Meyer, Anal. Lett., 10 (1977) 39.
10 J. Kreuter et T. Higuchi, J. Pharm. Sci., 68 (1979) 451.
11 B. Ljungeren, D.M. Carter, J. Albert et T. Reid, J. Invest. Dermatol., 74 (1980) 59.
12 B. Coq, G. Gonnet et J.L. Rocca, J. Chromatogr., 106 (1975) 249.
13 C.A. Parker et W.T. Rees, Analyst (London), 85 (1960) 587.

Journal of Chromatography, 272 (1983) 200—204
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1471

Note

Determination of the biological response modifier MVE-2 (AD-022) in biological fluids by high-performance liquid chromatography

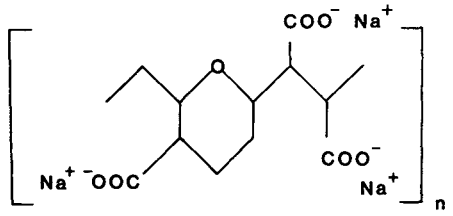
MICHAEL G. ROSENBLUM*, EVAN M. HERSH and TI LI LOO

Department of Clinical Immunology and Biological Therapy and The Department of Developmental Therapeutics, The University of Texas, M.D. Anderson Hospital and Tumor Institute at Houston, Houston, TX 77030 (U.S.A.)

(First received March 8th, 1982; revised manuscript received June 18th, 1982)

Maleic vinyl ether (MVE) copolymers are polyanionic macromolecules with broad molecular weights ranging from 1000 to 1,000,000 daltons. They have been shown to possess antitumor activity against several murine tumors [1—6] as well as antiviral activity against vaccina virus, herpes simplex virus, and influenza type A virus [7]. The putative mechanism of antitumor action of these agents is through the stimulation of the reticuloendothelial system and enhancement of endogenous interferon production [8—12].

Five MVE fractions having a discrete molecular weight range (from 12,500 to 52,600 daltons) were synthesized in 1974 and designated MVE-1 through MVE-5 [13]. Among these, MVE-2 (AD-022, Fig. 1), with an average molecular



MALEIC ANHYDRIDE - DIVINYL ETHER COPOLYMER
(AD-022, MVE-2)

Fig. 1. Structure of MVE-2.

weight of 15,500 daltons, was found to have antitumor and antiviral activity in murine model systems [14, 15] and is currently undergoing phase I

0378-4347/83/0000—0000/$03.00

clinical trials. In concert with these trials, we have developed a high-performance liquid chromatographic (HPLC) method for the analysis of MVE-2, as well as all of the other MVE polymers, in biological fluids in preparation for pharmacokinetic and metabolism studies of this agent in man.

MATERIALS AND METHODS

All materials purchased from regular commercial suppliers were of reagent grade or higher. High-purity water and distilled-in-glass methanol were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All solvents were filtered and vacuum-degassed immediately before use. ^{14}C-Labeled MVE-2 (0.47 μCi/mg) was a generous gift from Adria Laboratories (Columbus, OH, U.S.A.).

Chromatography

All analyses were performed with a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph consisting of a Model 710B sample processor, two Model M6000A pumps, a Model 720 system controller, a data module, and a Model 450 variable-wavelength UV detector. An analytical reversed-phase (5 μm particle size, silica matrix) short alkyl chain (C2,3) column (200 × 4.6 mm I.D.) from Custom L.C. (Houston, TX, U.S.A.) was used for all analyses. Solvent A consisted of 0.02 *M* Na_2HPO_4 adjusted to pH 4.0 with H_3PO_4. Solvent B consisted of 0.02 *M* Na_2HPO_4 (pH 4.0) in 80% methanol. The flow-rate was 2 ml/min, and a nonlinear gradient (curve 7) was generated from 0% B to 100% B over 25 min. The column eluate was monitored for UV absorbance at 220 nm.

Preparation of biological fluids

Plasma. Specimens (4 ml) containing MVE-2 were placed in 10-ml plastic centrifuge tubes and admixed rapidly with a 400-μl aliquot of 0.2 *N* sodium hydroxide and 4 ml of hot water (ca. 85°C). The samples were allowed to stand for 5 min and applied to mini-columns constructed from 1-ml blue plastic pipette tips (Eppendorf). Washed macroporous anion-exchange resin (AGMP-1, 100—200 mesh, from Bio-Rad Labs., Richmond, CA, U.S.A.) was added to a bed size of 2.0 × 0.5 cm. A small plug of glass wool prevented loss of the resin. After the samples were loaded, the columns were washed with 3 ml of water previously adjusted to pH 11 with sodium hydroxide. The columns were washed with 4 ml of distilled water, 2 ml of 1 *M* formic acid, and 3 ml of 8.5 *M* formic acid. MVE-2 was eluted from the column with 2.5 ml of 14 *M* formic acid. The 14 *M* acid wash was collected in plastic test tubes and reapplied to mini-columns containing washed Bio-Rex 70 cation-exchange resin (100—200 mesh, bed size 2.0 × 0.5 cm). The samples were washed through with 0.5 ml of distilled water. The column eluates were collected in plastic centrifuge tubes, a 50-μl aliquot of 0.1 *N* sodium hydroxide was added and the samples were lyophilized to dryness. The samples were reconstituted with 250 μl of buffer A and chromatographed as described above.

Urine. Urine specimens (4 ml) were similarly prepared without the addition of hot water. Samples were lyophilized, reconstituted and chromatographed as above.

Radiochemical techniques

The HPLC column eluates were collected at 30-sec intervals directly in glass scintillation vials after injection of radiolabeled MVE-2. Aquosol scintillant (12 ml, New England Nuclear, Boston, MA, U.S.A.) was added to each vial. The vials were counted in a Packard Tri-Carb liquid scintillation spectrometer Model 2650 to determine ^{14}C activity.

RESULTS AND DISCUSSION

The MVE-2 polymer is composed of macromolecules with a variety of molecular weights ranging from a few thousand to several hundred thousand daltons. However, the majority of molecular weights center at approximately 15,500 daltons. Nevertheless, as shown in Fig. 2 (top), this polymeric mixture can be chromatographed and eluted as a discrete peak (retention time 14.5 min) using a short alkyl chain reversed-phase column and the elution buffers and gradients described. The curved line in Fig. 2 shows the gradient profile. Injection of an equal amount of MVE-4 (M.W. 34,000 daltons) in this

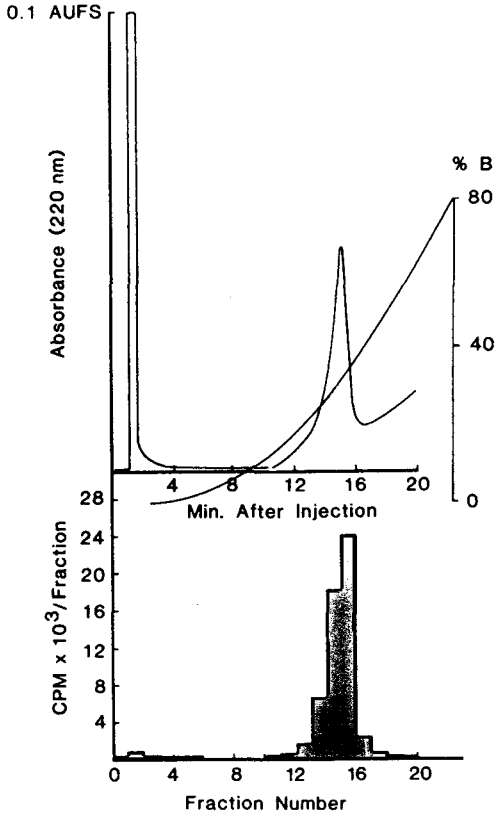


Fig. 2. HPLC analysis of MVE-2. Top panel shows the UV profile (220 nm) of the column eluate after an injection of unlabeled MVE-2 (100 μg total injected). Bottom panel shows the radioactive profile of the column eluate after injection of ^{14}C-labeled MVE-2. Recovery of injected radioactivity was 98 ± 4%.

chromatographic system resulted in an identical peak at 14.5 min, suggesting that this system may be useful for analysis of the entire MVE polymeric series. The chromatographic separation used in this analysis may consist of size exclusion, ion-exchange, normal-phase and reversed-phase partition modes. The relative contribution of each mode is unknown.

Co-injection with ^{14}C-labeled MVE-2, fractionation, and scintillation counting of the column eluate showed that most (96%) of the injected counts were in the MVE-2 peak (Fig. 2, bottom panel). The radioactivity in fractions 2—4 may represent unreacted ^{14}C-labeled maleic acid. Total recovery of injected radioactivity was 98 ± 4%.

A method for the extraction of MVE-2 from plasma and urine was developed using ion-exchange chromatography (see Materials and methods). This method was utilized in conjunction with the HPLC analysis of MVE-2. Standard curves for MVE-2 were constructed by the addition of various amounts of MVE-2 standard to plasma and urine aliquots. The lowest limits of detection for MVE-2 by this method were approximately 10 μg and 20 μg per ml plasma and urine, respectively. The recovery rate of MVE-2 from plasma was 73—85%, while the recovery rate from urine was 40—65%. For plasma, standard curves were linear ($Y = 0.35X + 3.07$, $r^2 = 0.99$) over the concentration range tested. Standard curves for MVE-2 in urine were also linear ($Y = 0.16X + 0.91$, $r^2 = 1$) over the concentration range tested. Intra-assay variability of triplicate samples averaged ± 8% while inter-assay variability was ±10%. Incubation of MVE-2 with plasma and urine at 37°C for 24 h produced no evidence of drug degradation.

Analysis of both a plasma blank and a patient plasma sample drawn 5 min after MVE-2 administration (Fig. 3, 650 mg/m^2 dose) showed that the method described for MVE-2 analysis is sensitive enough for monitoring patient samples after drug administration. Studies are ongoing to determine the plasma pharmacokinetics and urinary excretion of MVE-2 in man. This method may

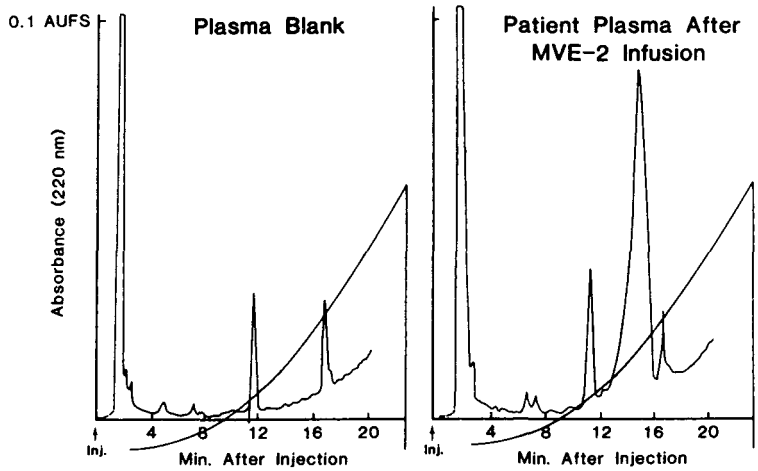


Fig. 3. Chromatographic profile of patient plasma prior to and after infusion of MVE-2. The concentration of MVE-2 in this patient sample withdrawn 5 min after the end of MVE-2 infusion was 100 μg/ml.

also be applicable to clinical pharmacology studies of other MVE polymers.

ACKNOWLEDGEMENTS

This work was supported by Grant CA-29769 from the National Cancer Institute, Grant CA-05831, Biomedical Research Support Grant RR5511-18, and by a grant from Adria Laboratories Inc.

REFERENCES

1 S.J. Mohr, M.A. Chirigos, F.S. Fuhrman and J.W. Pryor, Cancer Res., 35 (1975) 3750.
2 P.S. Morahan, J.A. Munson, L.G. Baird, A.M. Kaplan and W. Regelson, Cancer Res., 34 (1974) 506.
3 J.W. Pearson, M.A. Chirigos, S.D. Chaparas and N.A. Sher, J. Natl. Cancer Inst., 52 (1974) 463.
4 J. Sandberg and A. Goldin, Cancer Chemother. Rep., 55 (1971) 233.
5 R.M. Schultz, J.D. Papamatheukis, J. Luetzeler, P. Ruiz and M.A. Chirigos, Cancer Res., 37 (1977) 358.
6 R.M. Schultz, W.A. Woods, S.J. Mohr and M.A. Chirigos, Cancer Res., 36 (1976) 1641.
7 P.S. Morahan, W. Regelson and A.E. Munson, Antimicrob. Ag. Chemother., 2 (1972) 16.
8 N.A. Pavlidis, R.M. Schultz, M.A. Chirigos and J. Luetzeler, Cancer Treat. Rep., 62 (1978) 1817.
9 W. Regelson and A.E. Munson, Ann. N.Y. Acad. Sci., 173 (1970) 831.
10 T.C. Merigan, Nature (London), 214 (1967) 416.
11 T.C. Merigan and W. Regelson, N. Engl. J. Med., 277 (1967) 1284.
12 K. Kapila, C. Smith and A.A. Rubin, J. Reticuloendothel. Soc., 9 (1971) 447.
13 D.S. Breslow, U.S. Patient Office Pat. No. 3,794,622, patented Feb. 26, 1974.
14 W. Braun, W. Regelson, Y. Yajima and M. Ishizuka, Proc. Soc. Exp. Med., 133 (1970) 171.
15 P.S. Morahan, D.W. Barnes and A.E. Munson, Cancer Treat. Rep., 62 (1978) 1797.

Journal of Chromatography, 272 (1983) 205—209
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1476

Note

Rapid determination of metoprolol and α-hydroxymetoprolol in human plasma and urine by high-performance liquid chromatography

M.S. LENNARD* and J.H. SILAS

University Department of Therapeutics, Royal Hallamshire Hospital, Glossop Road, Sheffield S10 2JF (Great Britain)

(First received July 12th, 1982; revised manuscript received August 11th, 1982)

Several methods are available for the analysis of the β-adrenoceptor antagonist, metoprolol, alone or together with its metabolites, in biological fluids. Although sensitive, gas—liquid chromatographic procedures involve a derivatisation step [1—3] and, in the case of gas chromatography—mass spectrometry [4] require expensive equipment not readily available in most laboratories. High-performance liquid chromatography (HPLC) overcomes these disadvantages, but published HPLC assays for metoprolol are associated with either a time-consuming extraction procedure [5, 6] or lengthy chromatographic retention times [7, 8].

This paper describes HPLC methods for the analysis of metoprolol in plasma and for the drug and its α-hydroxylated metabolite in urine. Both procedures are sensitive and selective and significantly more rapid than other HPLC assays.

Recent work in our laboratory, using debrisoquine as a probe for defective drug oxidation in man [9], has indicated that the metabolism of metoprolol exhibits genetic polymorphism [10]. To illustrate the application of the present assays the plasma and urine profiles of metoprolol and α-hydroxymetoprolol in a poor and in an extensive metaboliser of debrisoquine are presented.

MATERIALS AND METHODS

Chemicals and drugs

Metoprolol tartrate and α-hydroxymetoprolol *p*-hydroxybenzoate were gifts from Astra Pharmaceuticals (St. Albans, Great Britain) and Hässle (Mölndal, Sweden), respectively. The internal standard, 1-(4-butyramido-2-butyrylphenoxy)-2-hydroxy-3-isopropylaminopropane hydrochloride, was donated by May and Baker (Dagenham, Great Britain). HPLC-grade acetonitrile and

0378-4347/83/0000—0000/$03.00

methanol and glass-distilled dichloromethane were purchased from Rathburn Chemicals (Walkerburn, Great Britain). All other chemicals were of analytical grade.

HPLC instrumentation and conditions

The chromatograph consisted of a Model 6000A pump (Waters Assoc., Northwich, Great Britain), a Model 7125 Rheodyne injector (0.5-ml loop) (HPLC Technology, Macclesfield, Great Britain) a Model 970FS Schoeffel fluorimetric detector (Kratos, Manchester, Great Britain). The stainless-steel columns (10 cm × 5 mm I.D.) used were packed with either Hypersil 5-ODS (method I) or Spherisorb Phenyl (method II) reversed-phase materials (both 5-μm particle size) (HPLC Technology).

In method I, the mobile phase consisted of water—acetonitrile (80:20) containing 1% triethylamine and adjusted to pH 3 with orthophosphoric acid. Water—methanol (55:45) containing 5 m*M* sodium heptanesulphonate and 0.1% (w/v) acetic acid was the mobile phase in method II.

Chromatography was performed isocratically at a flow-rate of 2 ml/min and at ambient temperature. The detector excitation wavelength was set at 222 nm and a 320-nm emission filter was used.

Extraction procedures

Method I: metoprolol in plasma. Plasma (1.0 ml), internal standard (400 ng) and sodium hydroxide (100 μl, 0.1 *M*) were gently shaken with dichloromethane (2 ml) for 10 min. After centrifugation (900 *g*, 2 min) and removal of the upper aqueous layer the organic extract was transferred to a 10-ml conical tube and evaporated to dryness on a Buchler Vortex Evaporator (Baird and Tatlock, Romford, Great Britain). The residue was reconstituted in mobile phase (100 μl) and an aliquot (30—100 μl) was injected into the chromatograph.

Method II: metoprolol and α-hydroxymetoprolol in urine. Urine (1.0 ml), internal standard (20 μg) and sodium carbonate (250 μl, 0.5 *M*) were mixed and then extracted with dichloromethane in an identical manner to that described for metoprolol in plasma.

RESULTS

Under the chromatographic conditions of both methods I and II, metoprolol, α-hydroxymetoprolol and internal standard gave rapidly eluting, fully resolved and essentially symmetrical peaks (Fig. 1). In method I the retention times for metoprolol and internal standard were 2.3 and 5.9 min, respectively. Retention times for metoprolol, α-hydroxymetoprolol and internal standard in method II were 5.1, 3.0 and 7.7 min, respectively.

Although no endogenous compounds were found to co-elute with any of the drug peaks, a small, slowly eluting peak (retention time 11.2 min) was observed in chromatograms from several of the plasma extracts. By careful timing of injections, interference from this material could be easily avoided.

Samples from patients taking a variety of cardiovascular drugs were run through the assays. Of these drugs, only presumed metabolites of hydralazine

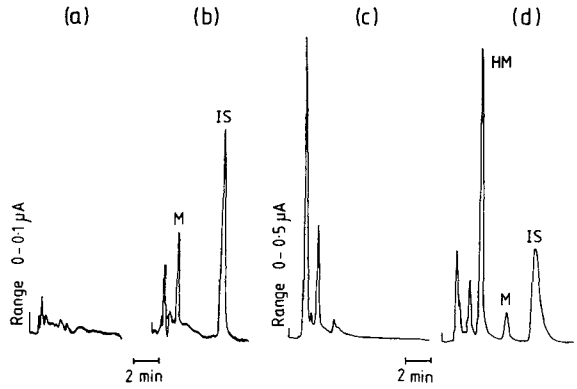


Fig. 1. Chromatograms of extracts of plasma and urine taken from a subject before and after oral administration of 200 mg metoprolol tartrate. Method I: (a) pre-dose plasma, (b) post-dose plasma containing 60 ng base per ml of metoprolol (M), and 400 ng per ml of internal standard (IS). Method II: (c) pre-dose urine, (d) post-dose urine containing 10.8 µg base per ml of α-hydroxymetoprolol (HM), 0.95 µg base per ml of metoprolol (M), and 20 µg per ml of internal standard (IS).

caused interference.

In addition, the following drugs, when injected directly did not interfere with either assay: sotalol, nadolol, propranolol, timolol, metoprolol, oxprenolol, lignocaine, disopyramide, mexiletine, warfarin, canrenone, nifedipine, isosorbide dinitrate, frusemide, hydralazine and three of its metabolites: 3-hydroxymethyltriazolophthalazine, methyltriazolophthalazine and phthalazinone.

Calibration curves for metoprolol and α-hydroxymetoprolol in both methods I (range 10—400 ng base per ml) and II (range 0.5—40 µg base per ml) were linear and passed through the origin ($r^2 > 0.99$). Standards prepared by spiking control plasma or urine with known amounts of drug and metabolite were included in each analytical run. Intra-assay coefficients of variation are shown in Table I. The lowest measurable concentration of metoprolol in plasma was about 5 ng/ml and of metoprolol and α-hydroxymetoprolol in urine about 0.2 µg/ml.

TABLE I

INTRA-ASSAY COEFFICIENTS OF VARIATION (%) FOR THE PLASMA AND URINE ANALYSIS OF METOPROLOL AND α-HYDROXYMETOPROLOL (n = 6)

Compound	Plasma (method I)		Urine (method II)	
	50 ng/ml	200 ng/ml	1 µg/ml	20 µg/ml
Metoprolol	4.8	2.1	2.3	4.7
α-Hydroxymetoprolol	—	—	4.8	5.1

DISCUSSION

Enhancement of chromatographic peak shape and therefore resolution, of weak bases like metoprolol on reversed-phase HPLC is most often accomplished by the addition to the mobile phase of an ion-pair reagent of negative charge, e.g. heptanesulphonate. Another approach, namely the use of alkylamines as modifiers, can also give extremely good resolution thereby leading to rapid analysis times. For example, a baseline separation of four weakly basic local anaesthetics in less than 3 min has been obtained on reversed-phase HPLC by the addition of 1% triethylamine to the mobile phase [11]. There is some doubt as to whether the modifier acts by blocking residual silanol groups on the stationary phase, or through an ion-pairing mechanism [11, 12].

The rapid, sensitive and selective HPLC method for the plasma analysis of metoprolol described in this paper utilises triethylamine as a modifier. Because of the single extraction step and short chromatographic analysis times, as many as forty samples can be processed in one day.

Owing to interferences from endogenous compounds, difficulties were encountered in extending the plasma assay to the measurement of metoprolol and α-hydroxymetoprolol in urine. After evaluating various stationary and mobile phases, good resolution was obtained by substituting a phenyl for an octadecylsilane column and using a methanol—water mobile phase containing heptanesulphonate. The same internal standard was used and the

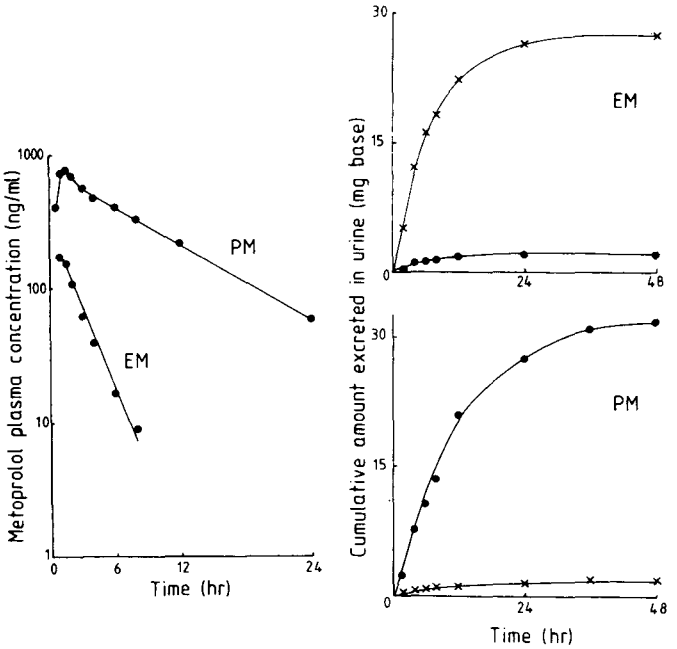


Fig. 2. Plasma concentrations of metoprolol and cumulative urinary excretion of metoprolol (•) and α-hydroxymetoprolol (×) in a poor (PM) and an extensive metaboliser (EM) of debrisoquine following a single oral dose of 200 mg metoprolol tartrate.

extraction procedure required only minor alteration. This method offers the advantage over that described by Pautler and Jusko [8] of having a much shorter analysis time (9 min instead of 28 min) making it better suited to routine use.

Large differences in the plasma and urine kinetics of metoprolol and α-hydroxymetoprolol were observed between the extensive and the poor metaboliser of debrisoquine (Fig. 2). The urine assay possessed sufficient sensitivity to detect the low drug and metabolite concentrations seen in the extensive and in the poor metaboliser, respectively.

ACKNOWLEDGEMENT

We are grateful to Dr. G.T. Tucker for valuable discussions of the manuscript.

REFERENCES

1 M. Ervik, Acta Pharmacol. Toxicol., 36 , Suppl. V (1975) 136—144.
2 C.P. Quarterman, M.J. Kendall and D.B. Jack, J. Chromatogr., 183 (1980) 92—98.
3 C.D. Kinney, J. Chromatogr., 225 (1981) 213—218.
4 M. Ervik, K.-J. Hoffmann and K. Kylberg-Hanssen, Biomed. Mass Spectrom., 8 (1981) 322—326.
5 M.A. Lefebre, J. Girault and J.B. Fourtillan, J. Liquid Chromatogr., 4 (1981) 483—500.
6 H. Winkler, W. Ried and B. Lemmer, J. Chromatogr., 228 (1982) 223—234.
7 M.T. Rosseel, F.M. Belpaire, I. Bekaert and M.G. Bogaert, J. Pharm. Sci., 71 (1982) 114—115.
8 D.B. Pautler and W.J. Jusko, J. Chromatogr., 228 (1982) 215—222.
9 D.A.P. Evans, A. Mahgoub, T.P. Sloan, J.R. Idle and R.L. Smith, J. Med. Genet., 17 (1980) 102—105.
10 M.S. Lennard, J.H. Silas, S. Freestone and J. Trevethick, Brit. J. Clin. Pharmacol., 14 (1982) 301—303.
11 B.A. Bidlingmeyer, J. Chromatogr. Sci., 18 (1980) 525—539.
12 N.H.C. Cooke and K. Olsen, J. Chromatogr. Sci., 18 (1980) 512—524.

Journal of Chromatography, 272 (1983) 210—215
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1480

Note

Reversed-phase high-performance liquid chromatographic assay for zomepirac in urine

JOSEPH M. GRINDEL*, JOANNA F. HILLS and NED L. RENZI

Department of Drug Metabolism, McNeil Pharmaceutical, Spring House, PA 19477 (U.S.A.)

(First received May 13th, 1982; revised manuscript received August 11th, 1982)

Zomepirac sodium, sodium 5-(4-chlorobenzoyl)-1,4-dimethyl-1H-pyrrole-2-acetate dihydrate, is an orally active, non-narcotic analgesic agent [1—3]. Prior publications from these laboratories have detailed the pharmacokinetics and disposition of zomepirac in man [4, 5] and animals [5, 6]. These studies utilized a normal-phase high-performance liquid chromatographic (HPLC) assay for zomepirac in plasma [7].

Recently, a reversed-phase HPLC assay for zomepirac in plasma and serum was published by Welch et al. [8]. However, neither of these assays are directly applicable for analysis of zomepirac in urine since they both utilize a diethyl ether extraction step at neutral pH prior to the acidic ether extraction. Such a prewash step is effective at removing interference peaks when the drug is highly bound to plasma protein, but extracts large amounts of drug from urine where the drug is not bound to protein.

Since urinary excretion accounts for 5% of the dose as zomepirac and 80% as zomepirac glucuronide in man, a rapid, sensitive and reproducible assay in urine was required for these chemicals. Thus, a reversed-phase HPLC assay was developed for zomepirac and hydrolyzed zomepirac conjugates in urine with the goal of minimal sample manipulation, adequate sensitivity and wide dynamic range.

MATERIALS AND METHODS

Reagents

Zomepirac sodium and the internal standard, 5-(4-chlorobenzoyl)-1,4,α-trimethyl-1H-pyrrole-2-acetic acid, were supplied by McNeil Pharmaceutical (Spring House, PA, U.S.A.). Methanol and water were distilled in glass grade from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Hexane and isoprop-

0378-4347/83/0000—0000/$03.00

anol were reagent grade from Mallinckrodt (St. Louis, MO, U.S.A.). Sodium acetate (J.T. Baker, Phillipsburg, NJ, U.S.A.) and glacial acetic acid (Mallinckrodt) were used to make 0.01 *M* acetate buffer (pH 4.0).

Chromatography

A Waters Assoc. (Milford, MA, U.S.A.) Model 6000A solvent delivery system was utilized with a U6K injector and a Model 440 UV detector (254 nm). Separation of components was effected with a 25 cm × 4.6 mm I.D. Whatman (Clifton, NJ, U.S.A.) Partisil-10 ODS (10 μm) column with a 6.5 cm × 4.1 mm I.D. Bondapak C_{18}/Corasil (Waters Assoc.) pre-column. The mobile phase was a methanol—0.01 *M* acetate buffer (pH 4.0) (45:55, v/v) solution run at 2.0 ml/min.

Procedure

Zomepirac. Urine (0.5 ml) is added to a 15-ml glass screw-top centrifuge tube with PTFE-cap liners. To the urine is added 0.5 ml of a methanol solution (240 μg/ml) of the internal standard. The sample is vortexed, allowed to stand for 15 min, centrifuged and aliquots (20 μl) of the supernatant are injected directly into the chromatograph.

Zomepirac conjugates. Urine (0.5 ml) is placed in a 15-ml glass screw-top centrifuge tube with PTFE-cap liners, after which 25 μl of 6 *N* sodium hydroxide solution are added. The solution is vortexed and allowed to stand for 1 h. Then 27 μl of 6 *N* hydrochloric acid solution are added, the solution is vortexed, and the internal standard solution (0.5 ml) is added. The remainder of the procedure is as described for zomepirac.

Calibration

Daily standards are prepared over the range of 4—1000 μg/ml. Zomepirac sodium (6.0 mg) is dissolved in 50 ml of methanol, appropriate aliquots are pipetted into centrifuge tubes, evaporated to dryness and reconstituted with 0.5 ml of water. Sample treatment then begins as previously described. Seeded quality control urine samples were prepared by adding appropriate aliquots of the methanol solution of zomepirac to glass bottles, evaporating to dryness and reconstituting with 100 ml of human urine. Concentrations of 800, 100 and 10 μg/ml were prepared.

Assay validation

Validation of the HPLC assay was performed by comparison of results with those obtained by thin-layer radiochromatographic techniques. Two pooled urine samples were prepared from samples obtained from a male subject who had received a single 200-mg oral dose of [^{14}C] zomepirac sodium (100 μCi/dose).

Each pooled urine sample was assayed in quadruplicate by the HPLC assay as described and by the thin-layer chromatographic (TLC) assay as follows: 1 ml of urine is made basic with 25 μl of 6 *N* sodium hydroxide and incubated overnight at 37°C (hydrolysis step is omitted when assaying for unconjugated zomepirac). The sample was then neutralized with 27 μl of 6 *N* hydrochloric acid, and 50 μl of each sample were applied directly to a 20 cm × 5 cm silica

gel GF TLC plate (Analtech, Newark, DE, U.S.A.). Unlabelled zomepirac (50 μg) was co-chromatographed as a standard. The plates were developed in a solvent system of hexane—isopropanol—glacial acetic acid (90:9:1, v/v/v). The mean R_F value for zomepirac was 0.35. The plates were analyzed by removing a 2-cm silica gel zone at the R_F of zomepirac, transferring it to a liquid scintillation vial containing 0.5 ml of water, adding 10 ml of Biofluor (New England Nuclear, Boston, MA, U.S.A.) and assaying for total radioactivity by liquid scintillation spectrometry. Assay controls were two seeded urine samples at 6 and 100 μg/ml, respectively, assayed in duplicate on each analysis day.

Statistical approaches

Calibration curve data were fitted by least squares linear regression analysis of zomepirac/internal standard peak height ratio (weighted by 1/variance of peak height ratio) vs. zomepirac concentration. Results of the assay validation were compared by paired t-test with significance set at $p < 0.05$.

RESULTS AND DISCUSSION

Chromatography

Separation of zomepirac and the internal standard from the background components of urine was effected using a C_{18} reversed-phase column (10 μm particle size) and methanol—acetate buffer mobile phase. As can be seen in Fig. 1, there were no extraneous peaks observed in a chromatogram of blank human urine at the retention times of zomepirac (4.1 min) and the internal standard (6.2 min). Also, it is apparent that baseline resolution was achieved

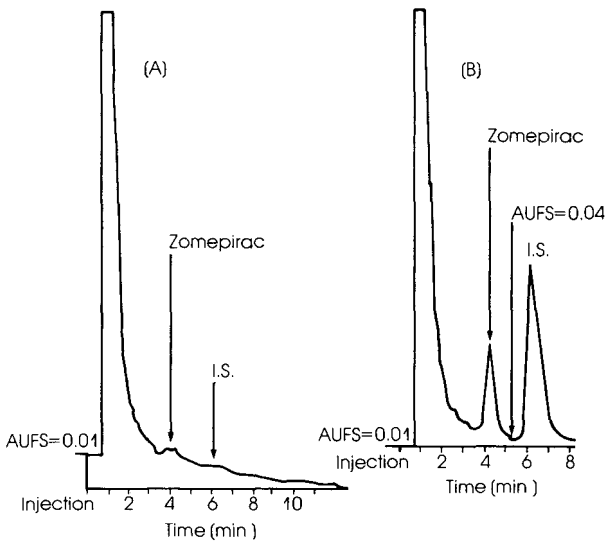


Fig. 1. Analysis of human urine by HPLC. Chromatograms of (A) blank human urine; and (B) human urine containing zomepirac (20 μg/ml) and the internal standard (I.S., 240 μg/ml).

for all components of interest as seen in the chromatogram of urine containing zomepirac and the internal standard. Minor alterations in the composition of the mobile phase will substantially affect the retention times of both zomepirac and the internal standard. Therefore, care must be taken to prepare the mobile phase in the same manner every day. The base hydrolysis step has no detectable effect on the chromatography.

Calibration and sensitivity

Calibration of the assay was performed daily using standards prepared over the range of 4.0—1000 μg/ml. Accuracy of the daily analyses was ensured by analyzing duplicate aliquots (0.5 ml) of the seeded urine pools. The daily calibration curves were linear and passed through the origin using 1/variance of the peak height ratio as the weighting factor. The regression line was described by the parameters: slope = 0.0065 ± 0.00003; intercept = 0.0036 ± 0.00064; $r^2 = 0.9995$.

Table I presents data on the reproducibility and accuracy of the assay.

TABLE I

REPRODUCIBILITY AND ACCURACY OF THE HPLC ASSAY FOR ZOMEPIRAC IN URINE

The average coefficient of variation of the curve of concentration vs. normalized peak height ratio at 4—1000 μg/ml (1 per day, 22 days, 6 points per range) is 3.38 ± 0.86%

	Zomepirac concentration (μg/ml)			
	4.0	10	100	1000
Precision				
C.V. (%)				
Intra-day (n = 6)	5.1	2.1	1.6	4.6
Inter-day (n = 22)	5.3	3.4	3.3	2.5
Bias				
Deviation of mean from amount spiked (%)				
Intra-day (n = 6)	−1.25	−0.20	+3.00	−6.2
Inter-day (n = 22)	−1.25	−0.23	+1.60	−7.1

Mean inter- and intra-day variability was less than 10% and accuracy was within 10%. Accurate quantitation was performed down to 4 μg/ml. Sensitivity could be enhanced by decreasing the amount of internal standard added and constructing a more limited calibration curve. Under these conditions the assay is capable of accurately quantitating as little as 0.2 μg/ml. Preliminary work has demonstrated that detection at 313 nm in addition to detection at 254 nm provides adequate sensitivity with less background interference. Also, the assay published by Welch et al. [8], has demonstrated the utility of detection at 320 nm.

Validation

The accuracy of the assay was also ascertained by comparison of assay results with a thin-layer radiochromatographic assay. Pooled urine samples were prepared from specimens obtained in a clinical metabolic study. Assays for zomepirac and hydrolyzed zomepirac conjugate (the acylglucuronide) were performed using the reversed-phase HPLC assay and the TLC assay [9]. The accuracy and reproducibility of the TLC assay for zomepirac are demonstrated in Table II. The TLC assay for zomepirac caused little or no hydrolysis

TABLE II

VALIDATION OF THE TLC ANALYSIS OF ZOMEPIRAC IN URINE

Seeded zomepirac concentration (μg/ml)	*n*	Concentration found (μg/ml) (mean ± S.D.)	Recovery (%) (mean ± S.D.)
6.0	6	6.16 ± 0.34	102.6 ± 5.7
100	8	96.3 ± 5.6	96.3 ± 5.6

of the glucuronide conjugate unless preceded by the base hydrolysis step. A comparison of assay results by the HPLC and TLC assays is presented in Table III. As can be seen, the analysis for zomepirac agrees reasonably well between the two assays although the HPLC assay results were statistically significantly ($p < 0.05$) higher. The assays for zomepirac plus zomepirac glucuronide are in excellent agreement and no statistically significant differences exist. The HPLC assay has subsequently been used to assay well over 2000 human and monkey urine samples.

TABLE III

RECOVERY OF ZOMEPIRAC IN 0—24 h HUMAN URINE ANALYZED BY HPLC AND TLC

Each sample was assayed in quadruplicate by both assay procedures.

Pooled urine sample	Zomepirac (μg/ml) (mean ± S.D.)		Zomepirac + conjugates (μg/ml) (mean ± S.D.)	
	HPLC*	TLC	HPLC	TLC
A	26.7 ± 2.3	19.5 ± 1.3	367 ± 12	353 ± 51
B	16.9 ± 1.1	13.6 ± 1.4	152 ± 14	151 ± 13

*Value different from TLC assay ($p < 0.05$).

CONCLUSION

A rapid, reproducible and accurate HPLC assay for zomepirac in urine has

been described. Addition of a base hydrolysis step allows for quantitation of zomepirac glucuronide, the major urinary metabolite.

REFERENCES

1 S.A. Cooper, J. Clin. Pharmacol., 20 (1980) 230.
2 W.M. Baird and D. Turek, J. Clin. Pharmacol., 20 (1980) 243.
3 S.L. Wallenstein, A. Rogers, R.F. Kaiko, G. Heidrich, III, and R.W. Houde, J. Clin. Pharmacol., 20 (1980) 250.
4 R.K. Nayak, K.T. Ng, S. Gottlieb and J. Plostnieks, Clin. Pharmacol. Ther., 27 (1980) 392.
5 J.M. Grindel, P.J. O'Neill, K.A. Yorgey, M.H. Schwartz, L.A. McKown, B.H. Migdalof and W.N. Wu, Drug Metab. Disp., 8 (1980) 343.
6 W.N. Wu, L.E. Weaner, J. Kalbron, P.J. O'Neill and J.M. Grindel, Drug Metab. Disp., 8 (1980) 349.
7 K.T. Ng and T. Snyderman, J. Chromatogr., 178 (1979) 241.
8 C.L. Welch, T.M. Annesley, H.S. Luthra and T.P. Moyer, Clin. Chem., 28 (1982) 481.
9 P.J. O'Neill, personal communication.

Journal of Chromatography, 272 (1983) 216—220
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1488

Note

Analysis of plasma physostigmine concentrations by liquid chromatography

ROBIN WHELPTON

Department of Pharmacology and Therapeutics, The London Hospital Medical College, Turner Street, London E1 2AD (Great Britain)

(First received June 24th, 1982; revised manuscript received August 26th, 1982)

Physostigmine, an alkaloid from the Calabar bean, is a potent inhibitor of cholinesterase. Unlike other carbamate anticholinesterases, such as neostigmine and pyridostigmine which are quaternary ammonium compounds, physostigmine is a tertiary amine and is rapidly absorbed after oral or subcutaneous administration. It readily penetrates the central nervous system. It may be used to treat poisoning by anticholinergic compounds (e.g. atropine or tricyclic antidepressants) and certain neurological disorders or to investigate central cholinergic mechanisms.

Low doses (typically 0.5—2 mg) coupled with rapid metabolism mean that a plasma assay must be capable of measuring nanogram or even sub-nanogram amounts. The aim of the present investigation was to measure plasma concentrations after a single subcutaneous injection of 1 mg physostigmine salicylate (equivalent to 0.67 mg of the base). If this amount were distributed instantaneously through total body water then the plasma concentration would be about 15 ng ml^{-1} in a 70-kg individual (i.e. 9.5 $\mu g\ kg^{-1}$). A combination of an absorptive phase following subcutaneous injection, an apparent volume of distribution greater than total body water (which is likely, considering the lipophilic nature of the compound) and rapid metabolism will produce plasma concentrations considerably less than this. The existing enzymatic method [1] with a sensitivity of ca. 7 ng ml^{-1} in blood was considered unsuitable. A liquid chromatographic assay has been applied to measuring physostigmine in cat brains after intravenous injection of 270 $\mu g\ kg^{-1}$ [2]. The sensitivity was 100 ng g^{-1} of tissue. Other analytical methods have only been applied to assaying pharmaceutical preparations [3—5].

0378-4347/83/0000—0000/$03.00

EXPERIMENTAL

Reagents and stock solutions

All reagents were of analytical grade apart from the methanol used for preparing the eluent, which was HPLC grade (Fisons Scientific Apparatus, Loughborough, Great Britain). Stock solutions of physostigmine and neostigmine bromide (Sigma, Poole, Great Britain) were prepared at 1 mg ml^{-1} in methanol and water, respectively. Eseroline and rubreserine were synthesized as described by Ellis [6].

Plasma samples

A male volunteer, aged 34 years and weighing 72 kg, was injected subcutaneously with 1 ml physostigmine salicylate solution B.P. (equivalent to 0.67 mg base). Venous blood (10 ml) was withdrawn into heparinised tubes and neostigmine bromide solution (1 mg ml^{-1}, 10 μl) added. The blood was mixed and centrifuged at 4°C to separate the plasma, after which it was stored at 4°C until assay later in the day. Samples were taken before and at 15, 30, 60, 90 and 120 min after injection. The protocol was approved by the Tower Hamlets District Ethics Committee.

Extraction procedure

Plasma (3 ml) and ammonium hydroxide solution (1 *M*, 1 ml) were pipetted into a screw-cap tube. Diethyl ether (5 ml) was added and the capped tube shaken mechanically for 20 min. After centrifugation the ether layer (4 ml) was transferred to a pointed tube and evaporated at 40°C under a gentle stream of nitrogen. The residue was dissolved in methanol (60 μl) and 50 μl injected into the chromatograph.

Standard solutions were prepared at 20, 10, 5, 2, 1, 0.5 and 0 ng ml^{-1} in plasma containing neostigmine bromide (10 μg ml^{-1}) and taken through the extraction procedure along with the unknown samples.

Chromatographic system

The stainless-steel column, 250 × 4.5 mm I.D., was slurry packed with 5-μm silica particles (Spherisorb, Phase Separations, Queensferry, Great Britain) in methanol. The eluent was methanol—1 *M* ammonium nitrate buffer, pH 8.6 (9:1) and degassed to remove dissolved oxygen before use. The flow-rate was maintained at 1 ml min^{-1} using a Laboratory Data Control Constametric pump. Samples were introduced via a Rheodyne valve fitted with a 50-μl loop. Detection was by either a fixed-wavelength (254 nm) UV detector or a Bioanalytical Systems electrochemical detector. The Type 8A glassy-carbon cell was operated at a potential of 0.8 V relative to the silver—silver chloride electrode (SSCE).

RESULTS AND DISCUSSION

Physostigmine is hydrolysed, enzymatically or in alkali, to the phenol eseroline which, in the presence of air, is rapidly oxidised to the orthoquinone, rubreserine. Under the conditions described the retention volumes were: rubreserine, 4.1 ml; physostigmine, 5.6 ml and eseroline, 6.1 ml. Eseroline was

not completely resolved from physostigmine but the retention times were sufficiently different for the two not to be confused. Furthermore, the current—voltage curves were so different that the compounds could be distinguished by changing the oxidation potential (Fig. 1). Eseroline was more readily oxidised than physostigmine, having a half-wave potential of 0.21 V relative to the SSCE compared with 0.70 V relative to the SSCE for physostigmine. Rubreserine showed no signs of electrochemical oxidation up to a potential of 1 V.

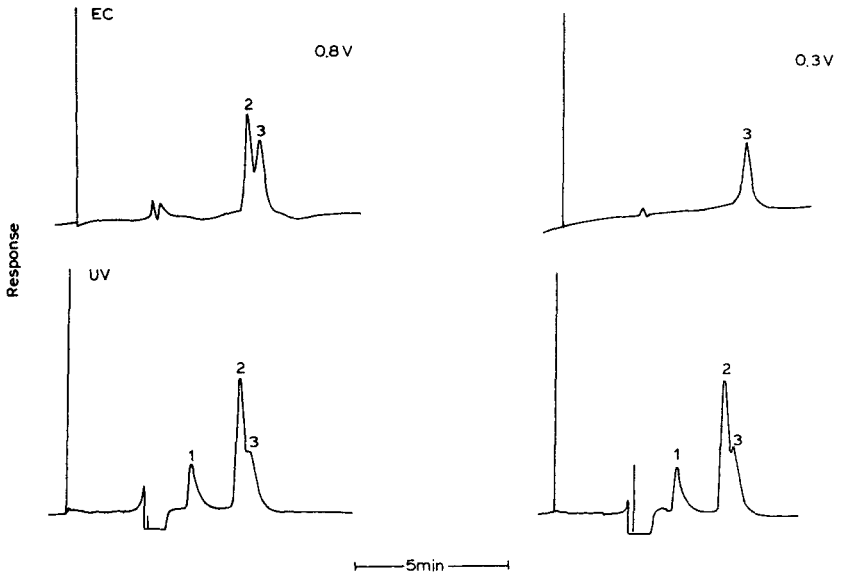


Fig. 1. Chromatograms showing simultaneous recording of UV (254 nm) and electrochemical responses to illustrate the effect of oxidation potential on the responses to reference compounds. Left: electrochemical detector at 0.8 V; right: electrochemical detector at 0.3 V. Compounds (50 ng of each): (1) rubreserine, (2) physostigmine and (3) eseroline.

Using eluent containing ammonium nitrate buffer, pH 8.6, physostigmine was resolved from an electro-active co-extractant which has been present in all the plasma samples tested to date. At pH 9.0, the retention volumes of physostigmine was reduced and the compound was no longer resolved from the contaminant. UV detection was unsuitable for plasma extracts because of a second contaminant which chromatographed at almost the same retention time as physostigmine and absorbed light at 254 nm. Fortunately, this compound was devoid of electro-activity at 0.8 V and it was for this reason that electrochemical detection was chosen.

Physostigmine contains two basic nitrogen atoms with pK_a values of 1.8 and 7.9 [7]. Consequently, solvent extraction is from alkaline aqueous solutions. If the pH of the aqueous medium is high, too much physostigmine may be hydrolysed during the extraction procedure. One millilitre of 1 *M* ammonium hydroxide in 3 ml of plasma gave pH 10 (approximately two units greater than the higher pK_a value). Assuming hydrolysis in dilute solution to

be pseudo-first order, an estimate of the decomposition during extraction can be made using the data of Christenson [8]. The decomposition at 25°C and pH 10 is approximately 5%, whereas at pH 11 it is 38%/h and by pH 12, less than 5% of the original concentration would be present after 1 h. Extractions were completed in less than 1 h, and standard plasma solutions extracted at the same time as the unknown samples to minimise the effects of decomposition. Once extracted, the residues can be stored overnight at 4°C without noticeable losses.

Neostigmine was added to the samples to prevent enzymatic hydrolysis before extraction. Non-enzymatic hydrolysis was not considered important for the few hours that plasma samples were stored at 4°C as at 25°C and pH 7.8 (the lowest value for which data were available) the rate of decomposition is $< 0.1\%/h$.

Precision and sensitivity

Intra-assay coefficients of variation, determined by assaying six samples containing 10 or 1 ng ml^{-1}, were 6.3% and 7.3%, respectively. Recovery did not appear to be concentration dependent: the mean value was 93%, after correction for aliquot losses, at both concentrations.

The sensitivity of the method was judged to be in the order of 0.5 ng ml^{-1}, using 3-ml plasma samples. At this concentration peaks were (typically) 3—5 mm high and distinguishable from the background. The calibration line between 0.5 and 20 ng ml^{-1} was linear (e.g. $r = 0.9999$, $n = 5$) with a slightly negative, but insignificant, intercept (e.g. —0.0231 ± 0.0146 cm). From this it was concluded that adsorptive or other non-exponential losses, either in the extraction or chromatography were absent or unimportant over the range of concentrations studied.

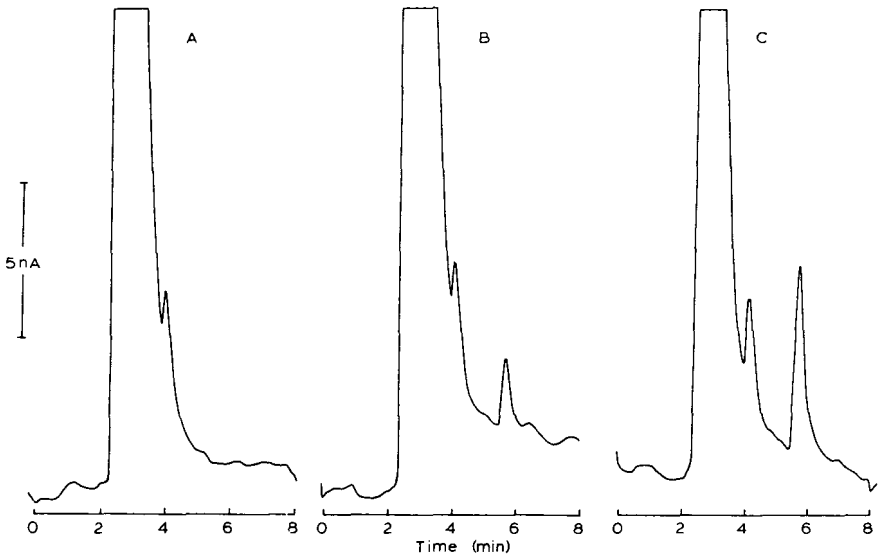


Fig. 2. Chromatograms of physostigmine. (A) Pre-dose plasma; (B) plasma collected 15 min after 1 mg physostigmine salicylate was administered subcutaneously; (C) plasma spiked with physostigmine at 10 ng ml^{-1}. Electrochemical detection at 0.8 V.

Plasma concentrations

Physostigmine concentrations in the samples from the volunteer were 3.6, 1.3 and 0.5 ng ml^{-1} at 15, 30 and 60 min respectively after the dose. By 90 min the concentration had fallen below the limit of detection. Plasma collected before the dose was free of interfering peaks at the retention volume of physostigmine (Fig. 2). The absence of a rising phase probably reflects partly the speed with which a subcutaneous dose is absorbed, and partly the difficulty of ensuring that the injection is purely subcutaneous. The rate of decline from plasma suggests an elimination half-time in the order of 15—20 min. This is in keeping with the idea that a subcutaneous dose is largely destroyed in about 2 h [9].

CONCLUSION

The described method is selective and sensitive enough for monitoring physostigmine concentrations after single doses in the therapeutic range.

ACKNOWLEDGEMENTS

I should like to thank The London Hospital Blood Bank for kindly giving me samples of plasma for construction of the calibration curves, and Mr. Charles Glanville for taking the blood samples.

REFERENCES

1 W.A. Groff, R.I. Ellin and R.L. Skalsky, J. Pharm. Sci., 66 (1977) 389.
2 D.J. de Wildt, A.J. Porsius and H.H. van Rooy, J. Chromatogr., 225 (1981) 381.
3 A.R. Rogers and G. Smith, J. Chromatogr., 87 (1973) 125.
4 A.R. Rogers and G. Smith, Pharm. J., 211 (1973) 353.
5 M. Kneczke, J. Chromatogr., 198 (1980) 529.
6 S. Ellis, J. Pharmacol. Exp. Ther., 79 (1943) 364.
7 Martindale, The Extra Pharmacopoeia, The Pharmaceutical Press, London, 27th ed., 1977, xxvii.
8 I. Christenson, Acta. Pharm. Suecica, 6 (1969) 287.
9 P. Taylor, in A.G. Gilman, L.S. Goodman and A. Gilman (Editors), The Pharmacological Basis of Therapeutics, Macmillan, New York, 6th ed., 1980, p. 108.

Journal of Chromatography, 272 (1983) 221—225
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1478

Note

High-performance liquid chromatographic determination of 4-aminopyridine and 3,4-diaminopyridine in rat cerebrospinal fluid and serum

D. LAMIABLE* and H. MILLART

Laboratoire de Pharmacologie Médicale Faculté de Médecine, 51 rue Cognacq-Jay, 51095 Reims Cedex (France)

(First received April 8th, 1982; revised manuscript received August 11th, 1982)

As far as we know, only 4-aminopyridine (4-AP) has been used in clinical practice for treatment of human neuromuscular diseases [1—4], but its usefulness has been limited by its central nervous system stimulant effect [5]. 3,4-Diaminopyridine (3,4-DAP) has been shown to be six to ten times more potent than 4-AP in increasing evolked transmitter release at the neuromuscular junction in vitro and two times less convulsant and toxic than 4-AP after acute intravenous injection in mice.

The existing high-performance liquid chromatographic method was developed for determining 4-AP in stomach contents of horses and was not applicable for biological fluids [6]. This paper describes a rapid, sensitive and selective assay developed to compare the ability of 4-AP and 3,4-DAP to cross the blood—brain barrier.

EXPERIMENTAL

Materials

4-AP and 3,4-DAP were purchased from Aldrich-Europe (Beerse, Belgium). Acetonitrile and methanol were of analytical grade. Potassium dihydrogen phosphate buffer (0.05 *M*), containing trimethylammonium chloride (0.02 *M*), was prepared in freshly glass-bidistilled water and adjusted to pH 7.4. Stocks solutions of 4-AP and 3,4-DAP were prepared at the concentrations of 100 μg/ml and 150 μg/ml, respectively, in methanol. Standard solutions of 4-AP and 3,4-DAP were prepared in methanol at the concentrations of 10 μg/ml and 15 μg/ml, respectively.

0378-4347/83/0000—0000/$03.00

Sample preparation

To 100 μl of serum were added 200 μl of methanol. The mixture was stirred for 30 sec on a Vortex Genie Mixer (Scientific Industries, Bohemia, NY, U.S.A.), centrifuged for 5 min at 2600 *g* and a 10μl portion of the supernatant was injected into the chromatograph. The cerebrospinal fluid (CSF) samples were shaken, centrifuged at 2600 *g* for 5 min and a 10-μl portion of the supernatant was injected directly into the chromatograph.

Calibration

4-Aminopyridine. Serum calibration curves were constructed by adding 0.25, 0.5, 1, and 2 μg of 4-AP to 100-μl serum samples via the 200 μl of methanol used for the deproteinization (2, 5, 10, 20 mg/l 4-AP). CSF calibration curves were constructed by dilution of the standard solution in water (0.5, 1, 3, and 5 mg/l 4-AP).

3,4-Diaminopyridine. Serum calibration curves were constructed in the same way as for 4-AP, but the final concentrations were 3.75, 7.5, 15, and 30 mg of 3,4-DAP per l of serum. For the CSF samples, the final dilutions were 0.25, 0.75, 1.5, and 3 mg of 3,4-DAP per l of water.

The peak heights were plotted against the concentration of the standards to give the calibration curves. Equations of the computed regression lines and correlation coefficients were calculated.

Chromatography

A liquid chromatograph (Varian Model 5000 liquid chromatograph) equipped with a variable-wavelength detector (Varichrom, Varian) was used in a reversed-phase system with MicroPak C_{18} as the stationary phase (300 × 4 mm I.D.; particle size 10 μm) (MCH 10, Varian) and acetonitrile–phosphate buffer (0.05 *M*) + tetramethylammonium chloride (0.02 *M*) (60:40) as the mobile phase. The volume of sample injected was 10 μl (Valco Valve). The effluent was monitored at 260 nm for 4-AP and 290 nm for 3,4-DAP with a sensitivity of 0.02 or 0.01 absorbance unit full scale (a.u.f.s.). The mobile phase flow-rate was 2 ml/min and the chart speed 0.25 cm/min.

RESULTS AND DISCUSSION

Typical chromatograms are shown in Fig. 1. The retention times of 3,4-DAP and 4-AP were 175 sec and 216 sec, respectively.

Extraction efficiency

Known amounts of each component were added to serum free from drug. All samples were extracted as previously described. The results were compared with those obtained from the injection of equivalent concentrations of pure drugs in water. The results for extraction efficiency are summarised in Table I.

Precision

The within-run precision was established by spiking serum and CSF with 3,4-

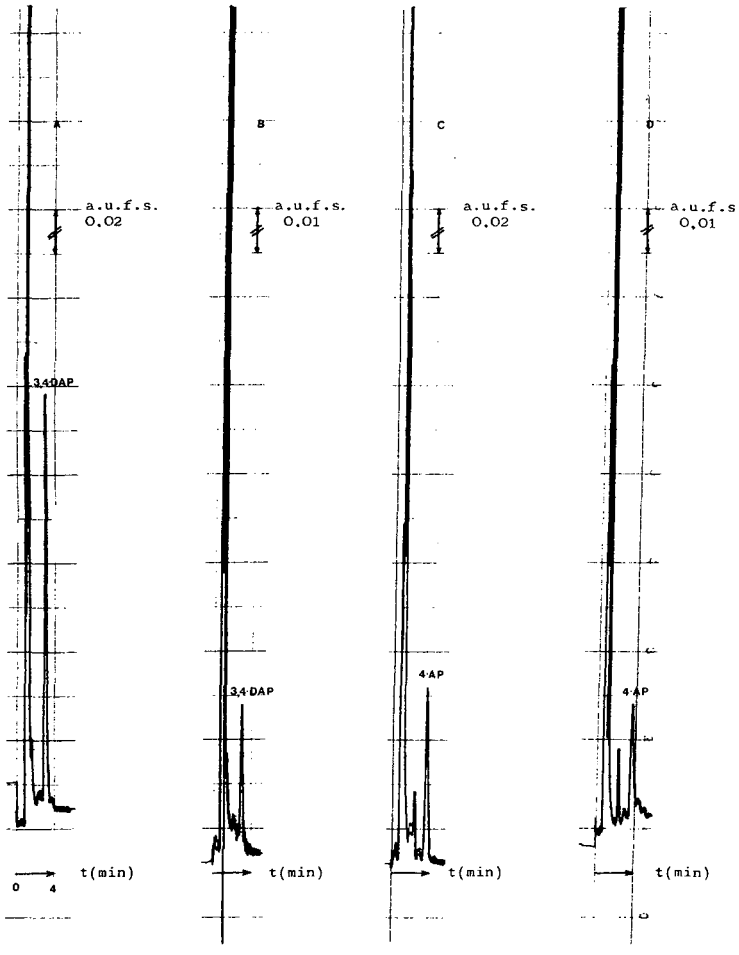


Fig. 1. Chromatograms obtained from rat serum and CSF samples after intravenous injection of 4-AP (7 mg/kg) or 3,4-DAP (16 mg/kg). (A) serum sample containing 20 mg/l 3,4-DPA, (B) CSF sample containing 1.3 mg/l 3,4-DAP, (C) serum sample containing 6 mg/l 4-AP and (D) CSF sample containing 1.6 mg/l 4-AP.

TABLE I

EXTRACTION EFFICIENCY OF 3,4-DAP AND 4-AP IN SERUM

Compound	Concentration (mg/l)	*n*	Mean ± S.D. (%)
3,4-DAP	15	5	99.8 ± 3
4-AP	5	5	97.9 ± 2

DAP and 4-AP in a concentration corresponding, approximately, to the middle range to be encountered after a single intravenous dose of 16 mg/kg 3,4-DAP or 7 mg/kg 4-AP. The results are summarised in Table II. The higher levels of 3-4 DAP and 4-AP in serum explain the better precision for serum than for

TABLE II

PRECISION OF THE METHOD (WITHIN-RUN)

Compound analyzed	Biological fluid	Concentration (mg/l)	*n*	C.V. (%)
3,4-DAP	CSF	1.5	8	5.07
	serum	15	10	2.31
4-AP	CSF	1	9	5.46
	serum	5	10	3.05

CSF. The use of an internal standard (3,4-DAP for the determination of 4-AP and 4-AP for the determination of 3,4-DAP) was not found to improve the precision of the assay, and has, therefore, been omitted.

Linearity

A linear relationship was observed between the peak height (Y, mm) and the amount of 3,4-DAP added to serum (X, mg/l) ($Y = 6.892\,X - 2.913$; $r = 0.999$; concentration range 3.75—30 mg/l; a.u.f.s. = 0.02) and 3,4-DAP added to water ($Y = 38.623\,X + 1.132$; $r = 0.995$; concentration range 0.25—3 mg/l; a.u.f.s. = 0.01). The same was observed with amount of 4-AP added to serum ($Y = 9.655\,X + 2.719$; $r = 0.998$; concentration range 2—20 mg/l; a.u.f.s. = 0.02) and to water ($Y = 25.263\,X + 2.829$; $r = 0.998$; concentration range 0.5—5 mg/l, a.u.f.s. = 0.01).

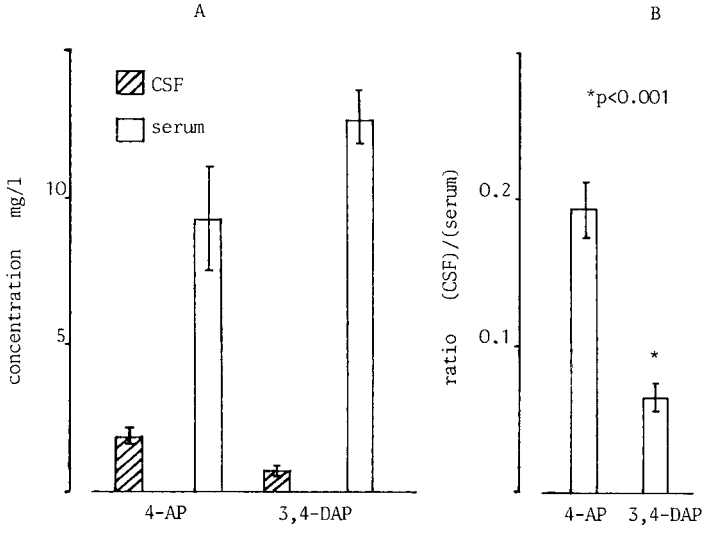


Fig. 2. (A) Mean concentrations in the CSF and serum for 4-AP (7 mg/kg, $n = 7$) and 3,4-DAP (16 mg/kg, $n = 8$) 5 min after intravenous injection. (B) Mean ratios of the concentrations (CSF/serum) with significant difference between 4-AP and 3,4-DAP.

Detection limit

No interfering peaks were present in pre-dose plasma or CSF with the same retention time as 3,4-DAP or 4-AP. The lower limits of detection in serum measured at a detector sensitivity of 0.02 a.u.f.s. and allowing a signal-to-noise ratio of two are 100 μg/l for 3,4-DAP and 50 μg/l for 4-AP.

Concentration of the drugs in rat CSF and serum

The concentrations of the drugs were measured in the CSF and in the serum of anesthetized rats (urethane) 5 min after intravenous injection of 4-AP (7 mg/kg) or 3,4-DAP (16 mg/kg). The mean concentration of 3,4-DAP in the CSF was lower than that of 4-AP. The ratios of the concentrations found in the CSF to those found in the serum were significantly higher with 4-AP than with 3,4-DAP (Fig. 2) [7].

CONCLUSION

In conclusion, 3,4-DAP does not cross the blood–brain barrier as easily as 4-AP and this can account for its lower central nervous system stimulant action and toxicity in vivo.

REFERENCES

1 H. Lundh, O. Nilsson and I. Rosen, J. Neurol. Neurosurg. Psychiat., 40 (1977) 1109–1112.
2 S. Agoston, T. van Weerden, P. Westra and A. Broekert, Brit. J. Anaesth., 50 (1978) 383–385.
3 H. Lundh, O. Nilsson and I. Rosen, J. Neurol. Neurosurg. Psychiat., 42 (1979) 171–175.
4 N.M.F. Murray and J. Newson-Davis, Neurology, 31 (1981) 265–271.
5 D.A. Spyker, C. Lynch, J. Shabanowitz and J.A. Sinn, Clin. Toxicol., 16 (1980) 487–497.
6 A.C. Ray, J.N. Dwyer, G.W. Fambro and J.C. Reagor, Amer. J. Vet. Res., 39 (1978) 329–333.
7 M. Lemeignan, H. Millart, N. Letteron, D. Lamiable, J. Josso, H. Choisy and P. Lechat, in S. Thesleff and W.C. Bouwmann (Editors), Advances in the Biosciences, Vol. 35, Pergamon Press, Oxford, 1982, p. 222.

Vol. 272, No. 1

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MEETINGS

FIFTH INTERNATIONAL SYMPOSIUM ON CAPILLARY CHROMATOGRAPHY, 1983

Because of the great interest and enthusiasm shown for the biennial Hindelang Capillary Chromatography Symposia, this series will be continued. The "Fifth International Symposium on Capillary Chromatography, 1983" will be held at Palazzo dei Congressa in Riva del Garda, Italy, from April 26–28, 1983. Both format and atmosphere will be as similar as possible to the successful Hindelang meetings.

The scientific program will consist of invited papers by experts and submitted papers presented in poster sessions in order to achieve intensive discussion. Scholarships for young scientists presenting invited papers are available, proposals are welcome. The program will cover basic and practical aspects of capillary gas and liquid chromatography with emphasis on: columns and their evaluation; quantitation (trace analysis, headspace, etc.); instrumentation (sampling, detection, ancillary techniques); and applications (petrochemistry, environment, biomedicine, food and drug analysis).

A book of the symposium proceedings will be available at the registration desk. The papers will be published in the *Journal of Chromatography* after the usual reviewing.

The Organizing and Scientific Committee will consist of: W. Bertsch (U.S.A.); C. Cramers (The Netherlands); M. Lee (U.S.A.); J. Rijks (The Netherlands); P. Sandra (Belgium); and G. Schomburg (G.F.R.). Local arrangements will be made by S. Trestianu (Italy).

Authors intending to submit papers for the symposium will be required to adhere to the following schedule: abstracts of 500–700 words should be submitted before December 15, 1982; and manuscripts ready for direct photo reproduction should be sent before March 1, 1983, to: Dr. P. Sandra, Laboratory of Organic Chemistry, University of Ghent, Krijgslaan 281 (S4), B-9000 Ghent, Belgium.

Registration fees for delegates from outside Italy will be US $ 150.00; Italian delegates, 160,000 lires; and students, 50% reduction of the above fees.

Accommodation will be available in a limited number of reasonably priced hotels within walking distance from the conference hall. Details regarding hotels and the social program for delegates and their guests will be completed at a later date.

For application forms and more detailed information concerning the symposium and the instrument exhibition contact Dr. P. Sandra at the above address.

INTERNATIONAL SYMPOSIUM ON CHROMATOGRAPHY AND MASS SPECTROMETRY IN NUTRITION SCIENCE AND FOOD SAFETY

Encouraged by the positive comments and appreciation of previous participants and in an effort to interpret their suggestions, the Italian Group for Mass Spectrometry in Biochemistry and Medicine and the Nestlê Research Department of the Nestlê Products Technical Assistance Co. Ltd. are organizing

an International Symposium on Chromatography and Mass Spectrometry in Nutrition Science and Food Safety to be held in Montreux, Switzerland, at the Hotel Eden au Lac on June 20–23, 1983. The symposium will consist of lectures by prominent invited speakers, contributed papers, and discussions.

The symposium will illustrate and discuss all the latest aspects of chromatography, mass spectrometry, and chromatography–mass spectrometry. The areas of application will be limited to the fields of nutrition science and food safety. In particular, the preferred topics will include: food science, flavours and aromas, biochemistry of nutrition in humans and animals, disease in relation to nutrition, food safety, and improvements on the methodology of chromatography and mass spectrometry in nutrition science and food safety.

For further information contact: Secretariat, International Symposium on Chromatography and Mass Spectrometry in Nutrition Science and Food Safety, c/o Italian Group for Mass Spectrometry in Biochemistry and Medicine, Via Eritrea 62, 20157 Milan, Italy. Tel. (02) 35 54 546. Telex: 331268 Negri I.

NEW FEATURE IN TrAC

INTERFACE

"*Interface*" is a new, monthly feature in *TrAC, Trends In Analytical Chemistry,* on computers and related subjects. *Interface* articles are designed to promote an awareness among non-specialists about advances in computer applications, chemometrics and information systems. They are written by experts in a readable, jargon-free manner to enable the users, developers and managers of analytical systems to take practical advantage of these advances.

At a time of limited financial resources, it is a particularly important function of *TrAC* to highlight ways in which available resources can be used optimally. *Interface* will provide information in this area.

Note for editors: further information can be obtained from: Peter T. Shepherd, Trends in Analytical Chemistry, Elsevier, 68 Hills Road, Cambridge CB2 1LA, U.K. (Tel: 0223 - 315961).

CALENDAR OF FORTHCOMING MEETINGS

March 7–12, 1983 Atlantic City, NJ, U.S.A.	**1983 Pittsburgh Conference and Exhibition on Analytical Chemistry and Applied Spectroscopy** Contact: 1983 Pittsburgh Conference, 437 Donald Road, Dept. FP, Pittsburg PA 15235, U.S.A.
March 15–18, 1983 Rome, Italy	**EURO FOOD CHEM II** Contact: Societa Italiana di Scienca dell'Alimentazione, Viale Monte Oppio, 24, I-00124 Roma, Italy.
April 5–8, 1983 Cardiff, Great Britain	**International Symposium in Electroanalysis in Biomedical, Environmental and Industrial Sciences** Contact: Short Courses Section, University of Wales Institute of Science and Technology (UWIST), Cardiff CF1 3NU, Wales, Great Britain.
April 17–23, 1983 Limassol, Cyprus	**1st Cyprus Conference on New Methods in Drug Research** Contact: Conference Secretariat, 1st Cyprus Conference on New Methods in Drug Research, P.O. Box 121, Oxford, Great Britain.
April 26–28, 1983 Riva del Garda, Italy	**5th International Symposium on Capillary Chromatography** Contact: Dr. P. Sandra, Laboratory of Organic Chemistry, University of Ghent, Krijgslaan 281 (S4), B-9000 Ghent, Belgium

May 2–6, 1983 Baden-Baden, G.F.R.	**VIIth International Symposium on Column Liquid Chromatography** Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, Varrentrapstrasse 40–42, D-6000 Frankfurt (Main) 90, G.F.R.
May 15–17, 1983 Indianapolis, IN, U.S.A.	**1983 Symposium on LCEC and Voltammetry** Contact: LCEC Symposium, P.O. Box 2206, West Lafayette, IN 47096, U.S.A.
May 30–June 3, 1983 Melbourne, Australia	**International Conference on Chromatographic Detectors** Contact: The Secretary, International Conference on Chromatographic Detectors, University of Melbourne, Parkville, Victoria 3052, Australia. (Further details published in Vol. 216.)
June 1–3, 1983 Budapest, Hungary	**The Budapest Chromatography Conference** Contact: Dr. Tibor Devenyi, Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary, or Dr. Haleem J. Issaq, NCI-Frederick Cancer Research Facility, P.O. Box B, Frederick, MD 21701, U.S.A.
June 5–10, 1983 Cologne, G.F.R.	**29th Congress of the International Union of Pure and Applied Chemistry (IUPAC)** Contact: Dr. M. Williams, Executive Secretary, IUPAC, Bank Court Chambers, 2–3 Pound Way, Cowley Centre, Oxford OX4 3YF, Great Britain.
June 7–10, 1983 Brussels, Belgium	**1st International Symposium on Drug Analysis** Contact: Ms. C. Van Kerchove, Secretary, Société Belge des Sciences Pharmaceutiques/Belgisch Genootschap voor Pharmaceutische Wetenschappen, rue Archimedesstraat 11, B-1040 Brussels, Belgium. Tel.: (02) 733 98 20, ext. 33.
June 13–17, 1983 Annapolis, MD, U.S.A.	**5th International Symposium on Affinity Chromatography and Biological Recognition** Contact: Fifth International Symposium Secretariat, 9650 Rockville Pike, Bethesda, MD 20814, U.S.A.
June 20–23, 1983 Montreux, Switzerland	**International Symposium on Chromatography and Mass Spectrometry in Nutrition Science and Food Safety** Contact: Secretariat, International Symposium on Chromatography and Mass Spectrometry in Nutrition Science and Food Safety, c/o Italian Group for Mass Spectrometry in Biochemistry and Medicine, Via Eritrea 62, 20157 Milan, Italy. Tel: (02) 35 54 546; Telex: 331268 Negri I.
June 26–July 1, 1983 Amsterdam, The Netherlands	**23rd Colloquium Spectroscopium Internationale** Contact: Conference Secretariat 23 CSI, c/o Organisatie Bureau Amsterdam BV, Europaplein, 1078 GZ Amsterdam, The Netherlands. Tel.: (020) 44 08 07; Telex: 13499 raico nl.
June 27–July 1, 1983 Gatlinburg, TN, U.S.A.	**3rd Symposium on Separation Science and Technology for Energy Applications** Contact: A.P. Malinauskas, Oak Ridge National Laboratory, P.O. Box X, Oak Ridge, TN 37830, U.S.A. (Further details published in Vol. 245, No. 1.)
July 17–23, 1983 Edinburgh, Scotland, Great Britain	**SAC '83, International Conference and Exhibition on Analytical Chemistry** Contact: The Royal Society of Chemistry, Analytical Division, Burlington House, London W1V 0BN, Great Britain. Tel.: 01-734-9971. (Further details published in Vol. 214, No. 2.)

Aug. 28–Sept. 2, 1983 Amsterdam, The Netherlands	**9th International Symposium on Microchemical Techniques** Contact: Symposium Secretariat, c/o Municipal Congress Bureau, Oudezijds Achterburgwal 199, 1012 DK Amsterdam, The Netherlands. Tel: (020) 552 3459.
Aug. 29–Sept. 2, 1983 Bratislava, Czechoslovakia	**4th Danube Symposium on Chromatography and 7th International Symposium "Advances and Application of Chromatography in Industry"** Contact: Dr. Ján Remen, The Analytical Section of the Czechoslovak Scientific and Technical Society, Slovnaft, 823 00 Bratislava, Czechoslovakia. (Further details published in Vol. 235, No. 1.)
Sept. 5–9, 1983 Montreux, Switzerland	**43rd International Congress on Pharmaceutical Sciences** Contact: Mr. L.G. Felix-Faure, Administrative Director, International Pharmaceutical Federation, 11 Alexanderstraat, 2514 JL The Hague, The Netherlands.
Nov. 10–16, 1983 Düsseldorf, G.F.R.	**9th International Congress and Exhibition for Instrumentation and Automation (INTERKAMA 83)** Contact: INTERKAMA 83, Düsseldorfer Messegesellschaft mbH, NOWEA, Postfach 32 02 03, D-4000 Düsseldorf 30, G.F.R.
Dec. 7–10, 1983 Singapore, Singapore	**Chem Asia '83 Conference** Contact: Singapore Exhibition Services, Ltd., 601 Cathay Building, Singapore 0922, Singapore.
May 20–25, 1984 New York, NY, U.S.A.	**8th International Symposium on HPLC** Contact: Professor Cs. Horváth, Mason Laboratory, Yale University, P.O. Box 2159, Yale Station, New Haven, CT 06520, U.S.A.
Oct. 1–5, 1984 Nürnberg, G.F.R.	**15th International Symposium on Chromatography** Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, Varrentrappstrasse 40–42, D-6000 Frankfurt (Main), G.F.R.
Nov. 22–24, 1984 Barcelona, Spain	**3rd International Congress on Analytical Techniques on Environmental Chemistry** Contact: 3rd International Congress on Analytical Techniques on Environmental Chemistry/EXPOQUIMIA, Av. Reina Ma. Christina, Palacio No. 1, Barcelona 4, Spain. Tel.: 223.31.01; telex: 50458 FOIMB-E.
Nov. 22–24, 1984 Barcelona, Spain	**14th Annual Symposium on Analytical Chemistry of Pollutants** Contact: 3rd International Congress on Analytical Techniques on Environmental Chemistry/EXPOQUIMIA, Av. Reina Ma. Christina, Palacio No. 1, Barcelona 4, Spain. Tel.: 223.31.01; telex: 50458 FOIMB-E.

NEW BOOKS

Advances in thin layer chromatography: clinical and environmental applications, edited by J.C. Touchstone, Wiley, Chichester, New York, 1982, *ca.* 544 pp., price *ca.* US$ 66.50, £ 37.00, ISBN 0-471-09936-8.

Biological/biomedical applications of liquid chromatography IV, edited by G.L. Hawk, Marcel Dekker, New York, Basel, 1982, XVII + 367 pp., price SFr. 156.00, ISBN 0-8247-1842-0.

Literature guide to the GLC of body fluids, by A.V. Signeur, IFI/Plenum, New York, Washington, London, 1982, IX + 385 pp., price US $ 85.00, ISBN 0-306-65203-X.

Electrophoresis – A survey of techniques and applications, Part B, Applications, edited by Z. Deyl, Elsevier, Amsterdam, Oxford, New York, 1983, XIV + 462 pp., price Dfl. 225.00, US$ 104.75, ISBN 0-444-42114-9.

Gel electrophoresis of nucleic acids – A practical approach, edited by D. Rickwood and B.D. Hames, IRL Press, Oxford, Washington, DC, 1982, XV + 242 pp., price £8.50, US$18.00, ISBN 0-904-14724-X.

Mass spectrometry for chemists and biochemists, by M.E. Rose and R.A.W. Johnstone, Cambridge University Press, Cambridge, 1982, XVI + 307 pp., price £ 27.50 (hardbound), £ 9.95 (paperback), ISBN 0-521-23729-7 (hardbound), 0-521-28148-9 (paperback).

Radioimmunoassay of gut regulatory peptides, edited by S.R. Bloom and R.G. Long, Praeger, Eastbourne, New York, 1982, IX + 194 pp., price US$ 24.95, ISBN 0-03-062116-X.

A textbook of pharmaceutical analysis, by K.A. Connors, Wiley, Chichester, New York, **3rd ed.,** 1982, *ca.* 608 pp., price *ca.* US$56.55, £ 31.45, ISBN 0-471-09034-4.

Progress in pesticide biochemistry, Vol. 2, edited by D.H. Houston and T.R. Roberts, Wiley, Chichester, New York, 1982, X + 226 pp., price £22.50, ISBN 0-471-10118-4.

Proceedings of the International Symposium on Quantum Biology and Quantum Pharmacology, Palm Coast, FL, March 5–7, 1981, edited by P.-O. Lowdin, Wiley, Chichester, 1982, 498 pp., price US$ 66.50, £ 39.00, ISBN 0-471-86671-7.

Cumulative author, subject and technique indexes for Vols. 1–100 of Analytica Chimica Acta, *Anal. Chim. Acta,* Vol. 137, Elsevier, Amsterdam, New York, 634 pp., price Dfl. 264.00, US$ 105.60 (incl. postage).

Plasma and cellular modulatory proteins, edited by D.H. Bing and R.A. Rosenbaum, Center for Blood Research, Boston, MA, 1981, XII + 240 pp.

Structure of complexes between biopolymers and low molecular weight molecules, by W. Bartmann and G. Snatzke, Wiley-Heyden, Chichester, 1982, *ca.* 220 pp., price *ca.* US$ 56.00, £ 26.00, ISBN 0-85501-720-1.

PITTSBURGH CONFERENCE TRAVEL PACKAGE

The Conference

The 1983 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy will be held in Atlantic City, NJ, U.S.A., March 7-12 1983. It is the world's largest meeting for analytical chemists (20,000 attended in 1982) and the Exposition presents the most comprehensive exhibit of analytical instrumentation to be found anywhere.

The Travel Package

A low-price "all-in" package tour to the Pittsburgh Conference has been organized in collaboration with Select International Travel, Utrecht, The Netherlands, a company experienced in setting up professional travel packages for professional people.
The package includes:

- direct flights from Amsterdam, London and Frankfurt to New York (JFK)
- 2 nights first class accommodation in New York City
- transfer by special luxury coach to Atlantic City, NJ
- 6 nights first class accommodation in Atlantic City, NJ
- porterage, taxes and hotel service charges
- airport assistance
- assistance with advance registration

Note:

Although the basic package is based on group flight departure, individual extension of your stay is possible at extra cost. Other departure points can also be arranged.
Ask for details.

Interested?

For full details and a reservation form, contact:
Pittsburgh Conference Travel Package,
TrAC,
Elsevier,
68 Hills Road,
Cambridge, United Kingdom, CB2 1LA,
Tel: (0223) 31 59 61, Telex: 81623

Prices

From:	Amsterdam	Dfl.	2515.00
	London	£	573.00
	Frankfurt	DM	2412.00

Prices are based on double room occupancy. Single room supplement: Dfl. 880.00, £ 186.00, DM 800.00.

TrAc and TrAC Travel

Deadline for reservations: January 15, 1983.

7256

Affordable ways to bring analytical chemists together

PUBLICATION SCHEDULE FOR 1983

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

MONTH	D 1982	J	
Journal of Chromatography	252 253/1 253/2	254 255 256/1	The publication schedule for further issues will be published later.
Chromatographic Reviews			
Biomedical Applications		272/1	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 244, No. 2, pp. 401–404. A free reprint can be obtained by application to the publisher.)

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

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

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

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

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the lay-out of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints of Full-length papers, Short communications and Notes will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

News. News releases of new products and developments, and information leaflets of meetings should be addressed to: The Editor of the News Section, Journal of Chromatography/Journal of Chromatography, Biomedical Applications, Elsevier Scientific Publishing Company, P.O. Box 330, 1000 AH Amsterdam, The Netherlands.

Advertisements. Advertisement rates are available from the publisher on request. The Editors of the journal accept no responsibility for the contents of the advertisements.