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

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

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

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

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Volume 18

ELECTROPHORESIS

A Survey of Techniques and Applications. Part A: Techniques

edited by Z. Deyl
co-editors: F. M. Everaerts, Z. Prusík and P. J. Svendsen

1979 xvi + 392 pages
Price: US $83.00 / Dfl. 170.00
ISBN 0-444-41721-4

This first volume in a two-part set, deals with the principles, theory and instrumentation of modern electromigration techniques. The second part will be concerned with the detailed applications of electromigration methods to all diverse categories of compounds, although to a limited extent some applications are discussed in Part A.

Electromigration methods have been so extensively used in the past for both analytical and preparative separations that several of these methods have become standard procedures. These are discussed in the book together with newer developments in the field. Hints are included to help the reader to overcome the difficulties which often arise fro the lack of available equipment and adequa theoretical background of the individual techniques is also given. A theoretical approach to the deteriorative processes is presented in order to facilitate further development of a particular technique and its application to a special problem.

In each chapter practical realizations of different techniques are discussed an examples are presented to demonstrate th limits of each method. The mathematical an physicochemical background is arranged so as to make it as coherent as possible for both non-professionals, such as post-graduate students, and experts using electromigration techniques.

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.

Volume 17
75 YEARS OF CHROMATOGRAPHY – A HISTORICAL DIALOGUE

edited by L. S. Ettre and A. Zlatkis

1979 xiv + 502 pages
Price: US $54.75 / Dfl. 112.00
ISBN 0-444-41754-0

On the occasion of the 75th anniversary of the invention of chromatography, this book compiles the personal stories of 59 pioneers of the various chromatographic techniques (including five Nobel Prize laureates).

In their contributions to this volume, these pioneers review the events which influenced them to enter the field; explain the background of their inventions; summarize their activities and results during their professional lives; and discuss their interactions with other scientists and other disciplines. The book is completed by a chapter devoted to "Those who are no longer with us".

This book is more than a nostalgic recollection of the past for those who have been in chromatography for some time. It also provides, for the younger generation of chromatographers, a unique record of how present-day knowledge was accumulated as well as an insight into the background of the methods and techniques which they use in their daily work.

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

Volume 16
POROUS SILICA
Its Properties and Use as Support in Column Liquid Chromatography

by K. K. Unger

1979 xii + 336 pages
Price: US $58.50 / Dfl. 120.00
ISBN 0-444-41683-8

Although an enormous amount of literature exists about basic surface chemistry of silica on the one hand, and about its use as packing in high-performance liquid chromatography on the other, no comprehensive and systematic survey has previously been published to cover both subjects.

This book covers both treatments and provides the chromatographer with full information on the properties of silica and its chemically bonded derivatives in context with its chromatographic behaviour. The text is divided into two parts: the first deals with the physical and chemical properties of silica including pore structure, surface chemistry, particle preparation and characterization, while the second surveys the wide-spread application of untreated and chemically modified silica as adsorbent, support and ion exchanger in the four modes of HPLC, i.e. adsorption, partition, ion exchange and size exclusion chromatography. A separate chapter is devoted to packing procedures and performance of silica columns.

CONTENTS: 1. General Chemistry of Silica. 2. Pore Structure of Silica. 3. Surface Chemistry of Porous Silica. 4. Particle Characteristics. 5. Silica Columns – Packing Procedures and Performance Characteristics. 6. Silica and Its Chemically Bonded Derivatives as Adsorbents in Liquid-Solid Chromatography. 7. Silica as a Support in Liquid-Liquid Chromatography. 8. Chemically Modified Silica as Packing in Ion-Exchange Chromatography. 9. Silica as Packing in Size-Exclusion Chromatography. Appendix: Commercially Available Silica Packings. List of Symbols and Abbreviations. Subject Index.

Volume 15
ANTIBIOTICS
Isolation, Separation and Purification

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

1978 x + 772 pages
Price: US $95.00 / Dfl. 195.00
ISBN 0-444-41727-3

This book has been written in response to the great interest currently being shown in modification of some of the older, and many newer

antibiotics to improve upon existing, naturally produced compounds.

Twenty-four eminent scientists in the field of antibiotic isolation have contributed chapters on key chemical families of antibiotics, with emphasis placed on isolation, separation and purification of these substances, many of which can be used as starting materials for further modification. In addition the authors have provided brief summaries of the chemical, physical and biological properties, usage and structural formulae of many of the compounds. Isolation methods include solvent and resin extractions, counter-current distribution, gas-liquid and high-pressure chromatography and electrophoresis. Many of the recently discovered naturally produced antibiotics are also discussed.

CONTENTS: Actinomycins *(A. Mauger and E. Katz)*. Ansamycins *(A. Ganguly)*. Cephalosporin Antibiotics *(R. L. Hamill and L. W. Crandall)*. Coumarin-Glycoside Antibiotics *(J. Berger and A. D. Batcho)*. 2-Deoxystreptamine-Containing Antibiotics *(J. A. Marquez and A. Kershner)*. Griseofulvins *(G. H. Wagman and M. J. Weinstein)*. Lincomycin Related Antibiotics *(T. E. Eble)*. Macrolide Antibiotics *(J. P. Majer)*. Marine-Derived Antibiotics *(L. S. Shield and K. L. Rinehart, Jr.)*. Penicillins and Related Antibiotics *(B. B. Mukherjee and B. K. Lee)*. Peptide Antibiotics *(E. Gross)*. Plant-Derived Antibiotics *(L. A. Mitscher)*. Polyether Antibiotics *(R. L. Hamill and L. W. Crandall)*. Siderochromes *(H. Maehr)*. Streptamine-Containing Antibiotics *(D. Perlman and Y. Ogawa)*. Streptothricins and Related Antibiotics *(A. S. Khokhlov)*. Tetracyclines *(S. Neidleman)*. Subject Index.

Volume 14
RADIOCHROMATOGRAPHY
The Chromatography and Electrophoresis of Radiolabelled Compounds

by T. R. Roberts

1978 x + 174 pages
Price: US $44.00 / Dfl. 90.00
ISBN 0-444-41656-0

The aim of this book is to describe and discuss all of the various radiochromatography and radioelectrophoresis methods in a single volume. For each technique, the historical development is outlined and the relative merits of the radiochemical detection methods currently available are assessed. This is followed by a discussion of the method of choice for any particular application. Each chapter also describes in detail the practical aspects of the various techniques and provides examples of applications taken from the recent literature.

CONTENTS: Introduction. Radioactivity detectors used in chromatography. Radio-paper chromatography. Radio-thin-layer chromatography. Radio-electrophoresis. Radio-column chromatography. Radio-gas-liquid chromatography. Miscellaneous applications related to radiochromatography. Appendix. Subject Index.

Volume 13
INSTRUMENTATION FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

edited by J. F. K. Huber

1978 xii + 204 pages
Price: US $39.00 / Dfl. 80.00
ISBN 0-444-41648-X

A practical guide for all those involved in the application of column liquid chromatography, this book provides a valuable, up-to-date review of the large selection of instrumentation currently available, describing the general design features and the specific technical solutions in the instrumentation for high-performance liquid chromatography. For the purposes of this survey, the chromatographic system has been divided into a number of interacting sub-systems and these are described in detail by experts in the field. Special emphasis is given to discussion of the general principles of design which will remain relevant even if new technical solutions are found in the future.

CONTENTS: The chromatographic apparatus from the viewpoint of system theory *(J. F. K. Huber)*. Pump systems *(M. Martin and G. Guiochon)*. Solvent gradient systems *(M. Martin and G. Guiochon)*. Sample introduction systems *(J. C. Kraak)*. Column design selection *(J. C. Kraak)*. Components and accessories for preparative high-performance liquid chromatography *(A. Wehrli)*. Detectors based on the measurement of optical and electrical properties of the mobile phase *(H. Poppe)*. Electrochemical detectors *(H. Poppe)*. Radiometric detectors *(P. Markl)*. Combination of liquid chromatography and mass spectrometry *(E. Kenndler and E. R. Schmid)*. Specifications of commercial liquid chromatographs *(R. R. Becker)*. Subject index. Manufacturer index.

Volume 12
AFFINITY CHROMATOGRAPHY

by J. Turková

1978 x + 406 pages
Price: US $81.50 / Dfl. 167.00
ISBN 0-444-41605-6

This book reviews the application of affinity chromatography for the isolation of various

biologically active substances. The reviewing table comprises almost 1,400 references and is completed by data on use of solid supports and spacers. Great attention is given to the review of the most commonly used solid supports and to the method of attachment, together with the methods of characterization of both the carriers and the immobilized affinity ligands.

This extensive and up-to-date review is intended mainly for biochemists and biologists. It will be particularly useful to clinicians engaged in human or veterinary medicine, as well as to those dealing with chromatography and industrial chemistry.

CONTENTS: Introduction. The principle, history and use of affinity chromatography. Theory of affinity chromatography. Application of affinity chromatography to the quantitative evaluation of specific complexes. General considerations on affinant-sorbent bonding. Choice of affinity ligands for attachment. Hydrophobic chromatography, covalent affinity chromatography, affinity elution and related methods. Solid matrix supports and the most used methods of binding. Characterization of supports and immobilized affinity ligands. General considerations on sorption, elution and non-specific binding. Examples of the use of affinity chromatography. Immobilized enzymes. Subject Index. List of compounds chromatographed.

Volume 11
LIQUID CHROMATOGRAPHY DETECTORS

by R. P. W. Scott

1977 x + 248 pages
Price: US $41.00 / Dfl. 84.00
ISBN 0-444-41580-7

The rapid development of liquid chromatography over the past decade has been due to the introduction of highly sensitive linear liquid chromatography detectors. This book provides a comprehensive treatment of the function and optimal working conditions of liquid chromatography detectors. It is divided into four parts.
Part 1 includes a detailed discussion of properties of the detecting system that can impair column performance and how these effects can be minimized. In Parts 2 and 3, the various types of detectors that have been developed are described and a detailed treatment given of commercially available detectors. Part 4 discusses the practical operation of liquid chromatography, including methods for quantitative analysis as well as practical hints on detector operation and special detector techniques. The final chapter deals with spectroscopic detectors and provides a detailed description of LC/UV and LC/MS systems.
This work is particularly useful because of the presentation of the necessary detector specifications which enables readers to make a rational comparison of the performance of one detector with that of another.

CONTENTS: Introduction. General characteristics of liquid chromatography detectors. Bulk property detectors. Solute property detectors. The use of detectors in liquid chromatography. Subject Index.

Volume 10
GAS CHROMATOGRAPHY OF POLYMERS

by V. G. Berezkin, V. R. Alishoyev and I. B. Nemirovskaya

1977 xiv + 226 pages
Price: US $50.25 / Dfl. 103.00
ISBN 0-444-41514-9

This book is devoted to the strategy of application of gas chromatography in polymer chemistry and discusses, in detail, the use of gas chromatography in research work and the polymeric compounds industry. It is the second, revised and enlarged edition of the original version published in the USSR in 1972.

Volume 9
HPTLC – HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHY

edited by A. Zlatkis and R. E. Kaiser

1977 240 pages
Price: US $53.75 / Dfl. 110.00
ISBN 0-444-41525-4

HPTLC is the advanced technology of thin-layer chromatography and is defined as the combined action of several variables which include: an optimized coating material with a separation power superior to the best high performance liquid chromatographic separation material; a new method of feeding the mobile phase; a novel procedure for layer conditioning; a considerably improved dosage method and a competent data acquisition and processing system. Thus a complete system and procedure is discussed here. This should be understood as a stepwise improvement of an analytical method,

which has been a powerful tool since the pioneering work of E. Stahl.

CONTENTS: Simplified theory of TLC *(R. E. Kaiser)*. The separation number in linear and circular TLC *(J. Blome)*. Advantages, limits and disadvantages of the ring developing technique *(J. Blome)*. The U-chamber *(R. E. Kaiser)*. Dosage techniques in HPTLC *(R. E. Kaiser)*. High performance thin-layer chromatography: development, data and results *(H. Halpaap and J. Ripphahn)*. Consideration on the reproducibility of TLC separations *(D. Jaenchen)*. Potential and experience in quantitative HPTLC *(U. B. Hezel)*. Application of a new high-performance layer in quantitative TLC *(J. Ripphahn and H. Halpaap)*. Appendix. Index.

Volume 8
CHROMATOGRAPHY OF STEROIDS

by E. Heftmann

1976 xiv + 204 pages
Price: US $44.00 / Dfl. 90.00
ISBN 0-444-41441-X

Although some theory is included, this is mainly a laboratory handbook, arranged according to the steroids analyzed as well as according to the methods used. Currently used techniques are described in detail.

CONTENTS: Introduction. Liquid column chromatography. Paper and thin-layer chromatography. Gas chromatography. Relations between structure and chromatographic mobility. Sterols. Bile acids and alcohols. Estrogens. Androstane derivatives. Pregnane derivatives. Corticosteroids. Miscellaneous steroid hormones. Vitamins D. Molting hormones. Steroid sapogenins and alkaloids. Cardenolides and bufadienolides. List of Abbreviations. References. Subject Index.

Volume 7
CHEMICAL DERIVATIZATION IN LIQUID CHROMATOGRAPHY

by J. F. Lawrence and R. W. Frei

1976 viii + 214 pages
Price: US $44.00 / Dfl. 90.00
ISBN 0-444-41429-0

This book is intended for all investigators concerned with the use of physical separation techniques for solving complex analytical problems. It provides a comprehensive account of modern derivatization in liquid chromatography with special emphasis on the practical aspects.

Volume 6
ISOTACHOPHORESIS
Theory, Instrumentation and Applications

by F. M. Everaerts, J. L. Beckers and Th. P. E. M. Verheggen

1976 xiv + 418 pages
Price: US $78.00 / Dfl. 160.00
ISBN 0-444-41430-4

This book is the only text currently available providing full information on the new separation technique known as Isotachophoresis. The various chapters can be referred to more or less independently by scientists interested in fundamental aspects, by research groups intending to construct an instrument and by workers concerned mainly with the analytical aspects.

Volume 5
INSTRUMENTAL LIQUID CHROMATOGRAPHY
A Practical Manual on High-Performance Liquid Chromatographic Methods

by N. A. Parris

1976 second impression 1979
x + 330 pages
Price: US $48.75 / Dfl. 100.00
ISBN 0-444-41427-4

Available texts on liquid chromatography have tended to emphasize the developments in the theoretical understanding of the technique and methodology or to list numerous applications, complete with experimental details.
This work intends to bridge the gap between these two treatments by providing, with the minimum of theory, a practical guide to the use of the technique for the development of separations. The material is based largely on practical experience and highlights details which may have important operational value for laboratory workers.

CONTENTS: Introduction and historical background. Basic principles and terminology. Chromatographic support and column. Liquid chromatographic instrumentation. Liquid chromatographic detection systems. Nature of the mobile phase. Liquid-solid (adsorption) chromatography. Liquid-liquid (partition) chromato-

graphy. Ion-exchange chromatography. Steric exclusion chromatography. Qualitative analysis. Quantitative analysis. Practical aspects of trace analysis. Practical aspects of preparative liquid chromatography. Published LC applications information. The latest trends and a glimpse into the future. Subject Index.

Volume 4
DETECTORS IN GAS CHROMATOGRAPHY

by J. Ševčík

1976 192 pages
Price: US $34.25 / Dfl. 70.00
ISBN 0-444-99857-8

The first systematic treatment of gas chromatographic techniques, this publication devotes special attention to so-called specific detectors and working conditions which strongly influence results (e.g. gas flow, effect of additives in gases, working temperature, detector form and dimensions).

Volume 3
LIQUID COLUMN CHROMATOGRAPHY
A Survey of Modern Techniques and Applications

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

1975 xxiii + 1176 pages
Price: US $141.50 / Dfl. 290.00
ISBN 0-444-41156-9

The wide selection of applications in various fields of chemistry and biochemistry, written by specialists in the area, makes this volume a necessary reference work for those involved in chromatographic investigations.

Volume 2
EXTRACTION CHROMATOGRAPHY

edited by T. Braun and G. Ghersini

1975 xviii + 566 pages
Price: US $68.25 / Dfl. 140.00
ISBN 0-444-99878-0

This volume is the result of the collective work of many specialists, each responsible for a chapter in which a definite aspect of column extraction chromatography is thoroughly presented and discussed.

Volume 1
CHROMATOGRAPHY OF ANTIBIOTICS

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

1973 x + 238 pages
Price: US $46.25 / Dfl. 95.00
ISBN 0-444-41106-2

This book has been written to aid the identification of very similar compounds by use of specific chromatographic techniques. It contains detailed data on paper and thin-layer chromatography, electrophoresis, counter-current distribution and gas chromatographic systems for over 1,200 antibiotics and their derivatives, and provides information on chromatographic media, solvents, detection methodology and mobility of the antibiotics.

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Proceedings of the 9th International Symposium on Chromatography and Electrophoresis, Riva del Garda, 15-17 May, 1978

edited by A. Frigerio and L. Renoz

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(Biomedical Applications, Vol. 6, No. 2)

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Biomedical Applications

CHROMBIO. 386

SIMPLE METHOD FOR THE DETERMINATION OF CHOLINE AND ACETYLCHOLINE BY PYROLYSIS GAS CHROMATOGRAPHY

YUJI MARUYAMA*, MASARU KUSAKA, JUN MORI, AKIKO HORIKAWA and YOSHIKAZU HASEGAWA

Japan Upjohn Research Laboratories, 168 Oyagi-machi, Takasaki-City, Gunma Prefecture 370 (Japan)

(First received February 13th, 1979; revised manuscript received June 11th, 1979)

SUMMARY

An improved purification procedure is described for the simultaneous assay of endogenous choline and acetylcholine by pyrolysis gas chromatography, particularly for providing a simple and effective method for propionylation of choline in the presence of acetylcholine. The reaction was carried out in acetonitrile solution prepared by dissolving the evaporated residue of the supernatant of brain homogenate. Thus samples for propionylation were prepared without the use of ion-exchange chromatography.

INTRODUCTION

Methods for the simultaneous assay of choline and acetylcholine by gas chromatography (GC) were developed [1—4] after instrumental assay methods for acetylcholine had been established [5—9]. These methods commonly include a demethylation procedure to produce volatile derivatives and acylation of choline to propionylcholine by reaction with propionyl chloride. For simultaneous demethylation of choline and acetylcholine, chemical or pyrolysis methods are generally used for GC and/or gas chromatography—mass spectrometric (GC—MS) analysis. However, chemical demethylation is complex, time-consuming, and requires meticulous anhydrous conditions. Therefore, pyrolysis has been generally applied to the GC separation of non-volatile quaternary compounds. On the other hand, complete esterification of choline with propionyl chloride by allowing the mixture to stand at room temperature for 5—30 min is not always reproducible and successive purification procedures with ion-exchange chromatography are necessary. In this paper, we have

*To whom correspondence should be addressed.

presented an effective method for direct propionylation of the amine in acetonitrile solution and confirmed its reproducibility. Also, samples for propionylation were prepared without the use of ion-exchange chromatography.

MATERIALS AND METHODS

Apparatus and materials

A Model NJE 2601 Metabostat (Shin-Nihon Musen, Japan) was used for microwave irradiation. The output of the device is adjustable from 0 to 5 kW at 2.45 GHz. A stabilized power supply maintains a constant power output from the magnetron 2M12 (New Japan Radio Company) even if input voltages vary by 10% from 200 V a.c. (60 Hz, rms). The magnetron is water-cooled and a thermal switch is used to monitor temperatures. The duration of irradiation can be set from 0.1 to 9.9 sec in 0.1-sec units with high reproducibility. GC analyses were carried out on a Shimazu Model 3BF chromatograph equipped with a hydrogen flame ionization detector, and a Model PYR-MS pyrolyzer developed as a result of cooperative effort between Kotaki-Shoji Co., Tokyo, Japan and the authors' group. The filament consists of 80% platinum and 20% radium, and is coil-shaped to allow the sample to be placed at the point of maximum heating. MS analysis was by a pyrolysis gas chromatograph—chemical-ionization quadrupole mass spectrometer (JEOL-Q1OA PGC/CI/QMS) coupled with a basic JMA-980A computer system and a high-speed graphic output system which includes a KSR-733 silent printer, and multiple ion detection capabilities. Both gas chromatographs were equipped with glass columns, 1.2 m × 3 mm I.D., packed with 5% OV-101, 5% dodecyldimethylenetriamine succinamide (Jenden Phase) on 80—100 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.).

The carrier gas was nitrogen for routine GC analyses and helium for the combined GC—MS studies. The flow-rate for both systems was 25 ml/min. The temperature of the columns was 85°, the injection port was at 130°, and the detector at 200°.

Animals

Sprague—Dawley rats were obtained from Nihon Clea Co. and housed two per cage. The lights were automatically turned on at 8.00 a.m. in 12 h light—dark cycles. The rats were exposed to microwave irradiation at a level of 5 kW for 1.5 sec.

Chemicals

All the common chemicals employed were reagent grade, obtained from either Wako Pure Chemical Industries (Tokyo, Japan) or Sigma (St. Louis, Mo., U.S.A.). Propionyl chloride was obtained from Tokyo Kasei Co. (Tokyo, Japan).

Extraction from tissue

The principle is based on the procedures of Stavinoha et al. [8] and of Maruyama and Hosoya [10, 11] except for the propionylation procedure. An outline of the procedure is given in Fig. 1. Rats were sacrificed by microwave

irradiation and the brains were removed from the skull. Extraction of choline and acetylcholine was carried out with 3 ml of 15% 1 *N* formic acid in acetone, using 100 μl of 0.3 m*M* butyrylcholine as the internal standard, in a cold Polytron homogenizer at 6800 rpm for 20 sec. After standing in ice for 30 min, the homogenates were centrifuged at 16,000 *g* at 0° for 15 min. The supernatant solutions were washed twice with 1 ml of diethyl ether. After discarding the ether the aqueous portion was dried. The residue was dissolved in 100 μl of acetonitrile and 300 μl of propionyl chloride were added. This solution was allowed to stand at 60° for 40 min for complete acylation. The solvent was then evaporated with a nitrogen stream and the residue was dissolved in 200 μl of distilled water. Twenty microlitres of potassium peroxide solution (2 g of KI and 1.8 g I_2 in 10 ml water) were added to the solution and mixed well on the flushing mixer. After centrifugation by Beckman Microfuge for 3 min at 10,000 *g* the supernatants were removed and the precipitates were dissolved in 50 μl of acetonitrile. To remove excess iodide approximately 5 mg of macroporous AGI-X8-Cl^- was added to the solution and the mixture was shaken for 5 sec. For assaying endogenous acetylcholine and choline, 2 μl of the mixture were put on the platinum ribbon of the pyrolyzer and pyrolyzed at 2 A for 7.5 sec.

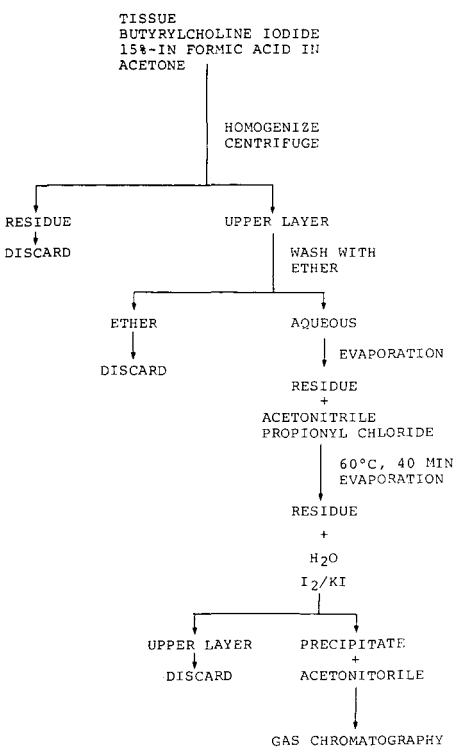

Fig. 1. Extraction procedure for choline and acetylcholine from brain tissue.

RESULTS AND DISCUSSION

Conditions for propionylation of choline

In order to determine the critical conditions for the unsuccessful propionylation of choline, standard samples in each tube were prepared as follows: 300 μl of propionyl chloride were poured into small tubes in which 100 μl of 0.3 m*M* acetylcholine iodide, 100 μl of 0.3 m*M* choline and 100 μl of 0.3 m*M* butyrylcholine iodide, all in acetonitrile, had been placed. The tubes were then allowed to stand at room temperature for 10, 20, 30, 40 and 60 min, respectively, and the percentage yield of the product in each sample was measured by GC after treating the samples with potassium periodide solution to precipitate the quaternary ammonium compounds. The results are shown in Fig. 2. The reaction gradually increased with time and reached a maximum, 92.0%, after 40 min. Next, the samples prepared as above were used to test the effect of temperature on maximum yield as shown in Table I. From this experiment, the reaction was complete after 40 min at 60°. The stability and reproducibility were confirmed by repeating the analysis on samples refrigerated at 4° for two weeks.

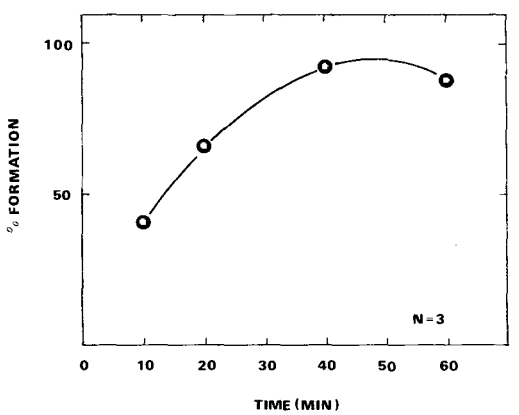

Fig. 2. Effect of reaction time on propionylation at room temperature (23°).

TABLE I

EFFECT OF TEMPERATURE ON PROPIONYLCHOLINE PRODUCTION

Values represent the percentage formation of propionyl ester, mean ± S.E. (n = 3).

Heating time (min)	Temperature (°C)		
	23	40	60
10	35 ± 5.3	45 ± 0.4	76 ± 1.9
40	56 ± 0.6	82 ± 1.1	102 ± 2.2

Sensitivity and standard curves

Using this method, propionyl choline and acetylcholine were separated, and their sharp peaks were observed within 15 min on the gas chromatogram as the pyrolyzed products (Fig. 3). Peaks derived from biological samples were compared with those from the standards by pyrolysis GC—MS analysis [12].

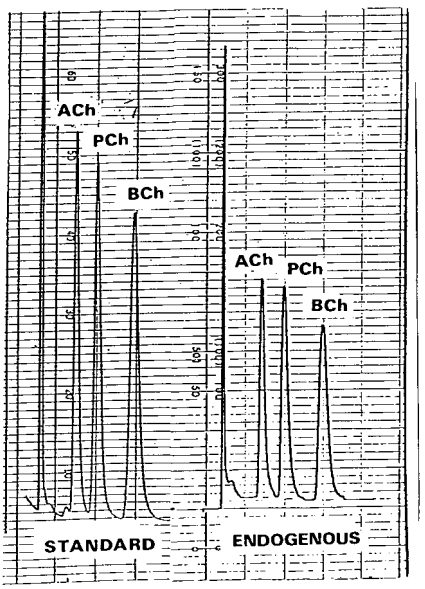

Fig. 3. Typical gas chromatogram of propionylcholine (PCh) and acetylcholine (ACh) as pyrolized esters of dimethylaminoethanol. Peaks of standard compounds of PCh and ACh are seen on the left and those derived from endogenous compounds are shown on the right. Butyryl choline iodide (BCh) is used as internal standard.

The correlation between the ratio and amounts of the compounds was excellent. The measurement for acetylcholine was more sensitive than propionylcholine; however, propionylcholine could be determined down to the 30—240 picomole range.

Recovery from tissue

The efficiency of the assays described was tested by studying the recovery of choline added to brain homogenate. The results of the experiment are shown in Table II. The total recovery for choline represents the 38.0 ± 0.96 nmoles of added standard plus the endogenous amount of the compound extracted from 1 ml of brain homogenate. With the procedure shown in Fig. 1, a normal level of acetylcholine was also assayed without interference. When the added standard was subtracted, endogenous choline was estimated at 8.1 ± 0.98 nmoles/ml of homogenate. The total recovery of added choline by this method was therefore 99.6 ± 4.2%. In order to confirm reproducibility, further experiments assaying the endogenous compounds from rat brain after decapitation or by microwave irradiation were carried out (Table III). Assayed levels after microwave irradiation were 25.5 ± 3.76 for choline and 26.9 ± 1.30 nmoles/g

TABLE II

RECOVERY OF CHOLINE ADDED TO BRAIN HOMOGENATE

Choline and butyrylcholine (30 nmoles) were added to 1 ml of brain homogenate and extracted in the manner shown in Fig. 1. The values (nmoles/ml homogenate) represent the mean ± S.E. from three determinations.

Amines	Normal endogenous	Added	Theoretical total	Assayed total	Recovery (%)
Choline	8.1 ± 0.98	30	38.2 ± 0.96	38.0 ± 0.96	99.6 ± 4.2
Acetylcholine	8.3 ± 0.00	—	—	8.3 ± 0.29	—

TABLE III

ACETYLCHOLINE AND CHOLINE LEVELS (nmoles/g WET WEIGHT) IN RAT WHOLE BRAIN

The values represent the mean ± S.E. of five determinations. Choline as its propionyl ester and acetylcholine were assayed by pyrolysis GC using butyrylcholine as an internal standard.

	Acetylcholine	Choline
Microwave (5 kW for 1.6 sec)	26.9 ± 1.3	25.5 ± 3.8
Decapitation	14.4 ± 1.3	172.3 ± 30.7

tissue for acetylcholine, and those by decapitation were 172.3 ± 30.7 and 14.4 ± 1.29 nmoles/g tissue, respectively. The levels of the amines after microwave irradiation or decapitation were clearly in accord with those reported by Stavinoha [2, 13]; that is, levels of the amines were found in approximately equimolar amounts in rat whole brain, and the variations previously reported [14—16] were not seen.

In summary, we have provided an improved method for the esterification of choline in the presence of acetylcholine and demonstrated the usefulness of pyrolysis gas chromatography for assaying both amines simultaneously in brain tissue.

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Biomedical Applications

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF HYDROCORTISONE AND METHYLPREDNISOLONE AND THEIR HEMISUCCINATE ESTERS IN HUMAN SERUM

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SUMMARY

A high-performance liquid chromatographic method is described for the simultaneous determination of methylprednisolone (MP) and methylprednisolone hemisuccinate (MPHS), or hydrocortisone (HC) and hydrocortisone hemisuccinate (HCHS) in human serum. Reversed-phase liquid chromatography was performed on a microparticulate C_{18} column (Spherisorb, 5 μm) using a mobile phase of 2% glacial acetic acid, 30—35% acetonitrile, 70—65% water with ultraviolet detection (254 nm). The method uses 17α-hydroxyprogesterone as the internal standard for the determination of methylprednisolone and its hemisuccinate ester, or 11-deoxy-17-hydroxycorticosterone as the internal standard for the determination of hydrocortisone and its hemisuccinate ester. The sensitivity is 0.03 μg/ml for HC, 0.07 μg/ml for MP, 0.04 μg/ml for MPHS, and 0.10 μg/ml for HCHS, with a detection limit of 0.02 μg/ml for all four steroids. Calibration curves are linear up to 3 μg/ml for MP or MPHS (as equivalent MP) and up to 4 μg/ml for HC and 7 μg/ml (as equivalent HC) for HCHS. The pooled relative standard deviation for replicate samples for each steroid is <7%. Plasma concentration—time curves are reported for MP and MPHS or HC and HCHS of two human subjects following intramuscular administration of 125 mg of methylprednisolone sodium succinate for injection, U.S.P., or 250 mg of hydrocortisone sodium succinate for injection, U.S.P.

INTRODUCTION

The sodium salts of methylprednisolone hemisuccinate (MPHS) and hydrocortisone hemisuccinate (HCHS) are water-soluble esters of the 21-hydroxycorticosteroids, methylprednisolone (MP) (11β,17α,21-trihydroxy-6α-methyl-1,4-pregnadiene-3,20-dione) and hydrocortisone (HC) (11α,17,21-trihydroxypregn-4-ene-3,20-dione). After parenteral administration of a solution of these esters, they are hydrolyzed to their respective parent corticosteroids. Several methods have been described to determine the parent 21-hydroxycortico-

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steroids, such as the Silber—Porter photometric method [1] or radioimmunoassay [2—4]. However, these methods would require the prior separation of the 21-hydroxycorticosteroid from its hemisuccinate ester and subsequent hydrolysis of the hemiester to its parent steroid. Thin-layer chromatography, which has also been used in the analysis of corticosteroids [5—8], was considered as a possible method for the simultaneous analysis of the hemiester and its parent steroid. However, this method lacked the desired reproducibility.

High-performance liquid chromatography (HPLC) offered the advantage of simultaneous analysis of hemiester and parent steroid [9]. HPLC has been used extensively in the analysis of corticosteroids using either normal phase [10—12] or reversed-phase chromatography [13—15] in biological fluids. This paper reports a method using reversed-phase HPLC with ultraviolet detection for the simultaneous determination of either MP and MPHS, or HC and HCHS, in human serum or plasma.

EXPERIMENTAL

Reagents

Hydroxycortisone hemisuccinate and methylprednisolone hemisuccinate, U.S.P. reference standards, and methylprednisolone and hydrocortisone, N.F. reference standards, and pooled human serum were used to prepare spiked serum samples. 17-α-Hydroxyprogesterone (Upjohn, Kalamazoo, Mich., U.S.A.) and 11-desoxy-17-hydroxycorticosterone (Abbott, North Chicago, Ill., U.S.A.) were used as internal standards. Distilled-in-glass spectroscopic grade ethyl acetate, hexane, methyl alcohol, and acetonitrile (Burdick and Jackson, Muskegon, Mich., U.S.A.) were used without further purification. Glacial acetic acid (Mallinckrodt, St. Louis, Mo., U.S.A.) was used to prepare the 5% acetic acid solution and the mobile phases.

Apparatus

The chromatographic apparatus consisted of a high-pressure liquid chromatograph (Spectra-Physics Chromatronix, Model 3500, Santa Clara, Calif., U.S.A.), equipped with a 100 μl value injection loop, a fixed wavelength detector (Model 8200), and a strip chart recorder (Hewlett-Packard Model 7130A). The column was an ODS reversed stationary phase (Sperhisorb 5 μm ODS, 3 × 250 mm, Spectra-Physics). Peak areas and retention times were determined with an integrator (Autolab Minigrator, Spectra-Physics).

The apparatus used in the extraction procedure was a reciprocal shaker (Eberbach, Ann Arbor, Mich., U.S.A.), a refrigerated centrifuge (Sorvall RC-3, Norwalk, Conn., U.S.A.), and a drying block (Driblock DB-3, Techne, Cambridge, Great Britain).

Mobile phase

The mobile phase was prepared by mixing exact volumes of acetonitrile, filtered distilled water, and glacial acetic acid. The solution was stirred and degassed. For the determination of MP and MPHS, the ratio of acetonitrile—water—glacial acetic acid was 35:65:2. For the determination of HC and HCHS, the ratio of acetonitrile—water—glacial acetic acid was 30:70:2.

Chromatographic operation conditions

The ultraviolet detector was fixed at 254 nm. The sensitivity of the detector was 0.04 a.u.f.s. (absorbance units full scale) for MP and MPHS and 0.08 a.u.f.s. for HC and HCHS. The flow rate was held constant at 1 ml/min. The column pressure was 2000—2500 p.s.i. Chart speed was 0.5 cm/min.

Extraction procedure

One-half milliliter of spiked serum or human sample, 1 ml of 5% acetic acid solution containing 1 μg per ml of internal standard, and 10 ml of hexane were added to a 20 ml screw-cap test tube. The tubes were shaken for 15 min at 100 cycles/min and then centrifuged for 5 min at 1400 *g* at 10°.

The hexane (upper) layer was aspirated off. Six milliliters of ethyl acetate were then added to the remaining aqueous layer. The tubes were shaken at 100 cycles/min for 20 min and centrifuged at 1400·*g* at 10° for 5 min. Five milliliters of the ethyl acetate layer were transferred to a conical tube and evaporated to dryness with filtered air at 40°. The residue was redissolved in 500 μl of mobile phase and then injected on to the column.

Internal standard

17α-Hydroxyprogesterone was used as the internal standard in the procedure for MP and MPHS. 11-Desoxy-17-hydroxycorticosterone was used as the internal standard in the procedure for HC and HCHS. The internal standards were prepared by diluting a methanol solution of each of the above (100 μg/ml) with 5% acetic acid solution to a concentration of 1 μg/ml.

Preparation of spiked serum samples

Methylprednisolone—methylprednisolone hemisuccinate. Methanolic solutions of MP (500 μg/ml) and MPHS (400 μg/ml, equivalent MP) were diluted (8:100) with water and then further diluted with pooled human serum to concentrations from 0.10 to 2.95 μg/ml for MP and 0.08 to 3.20 μg/ml for MPHS. Each spiked sample contained both MP and MPHS. MPHS concentrations are reported as equivalent MP concentrations.

Hydrocortisone—hydrocortisone hemisuccinate. Methanolic solutions of HC (500 μg/ml) and HCHS (800 μg/ml, equivalent HC) were diluted (8:100) with water and then further diluted with pooled human serum to concentrations from 0.08 to 4.05 μg/ml for HC and 0.12 to 6.97 μg/ml for HCHS. Each spiked sample contained both HC and HCHS. HCHS concentrations are reported as equivalent HC concentrations.

RESULTS AND DISCUSSION

In the analysis of MP and MPHS, the parent steroid and its hemiester were resolved from endogenous hydrocortisone, the internal standard and other endogenous components. The retention times were about 3.5, 5, and 7 min for MP, MPHS and internal standard (17α-hydroxyprogesterone). The retention time for HC was about 2.5 min. Chromatograms of extracted serum spiked with MP and MPHS and a blank serum extract containing internal standard only are given in Fig. 1.

In the analysis of HC and HCHS, the parent steroid and its hemiester were resolved from endogenous components and the internal standard (11-desoxy-17-hydroxycorticosterone). The retention times were about 3.5, 5.2, and 6.5 min for HC, HCHS and internal standard. Chromatograms of extracted serum spiked with HC and HCHS and a blank serum extract containing internal standard only are given in Fig. 2. Since all substances identified as true metabolites of hydrocortisone retain the 11β-hydroxyl function, either as such or as a keto group [16], 11-desoxy-17-hydroxycorticosterone should not be a metabolite of hydrocortisone.

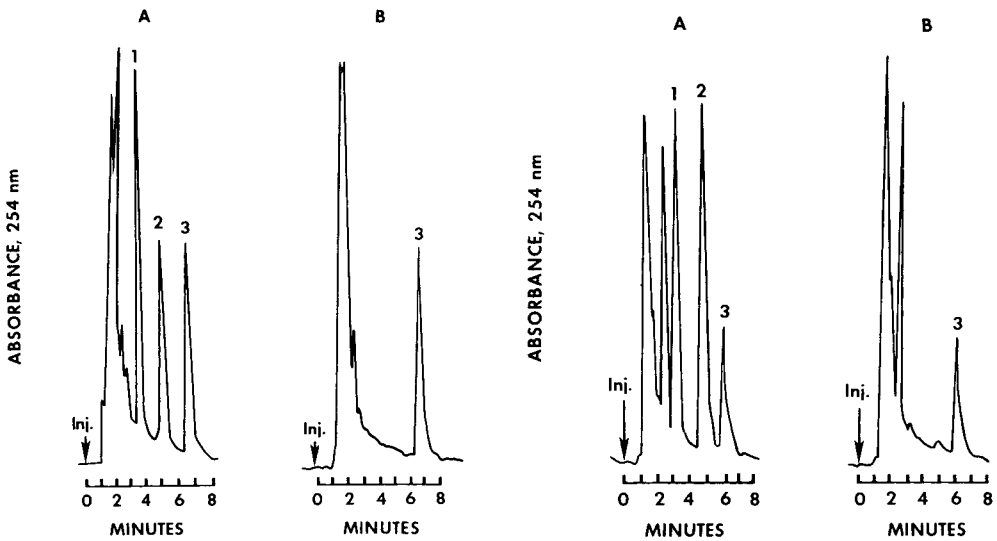

Fig. 1. (A) Chromatogram of extracted human serum spiked with methylprednisolone 2.11 μg/ml (1), methylprednisolone hemisuccinate 1.59 μg/ml (2), and internal standard (3). (B) Extracted "blank" serum containing only internal standard (3); 0.04 a.u.f.s.

Fig. 2. (A) Chromatogram of extracted human serum spiked with hydrocortisone 3.04 μg/ml (1), hydrocortisone hemisuccinate 4.65 μg/ml (2), and internal standard (3). (B) Extracted "blank" serum containing only internal standard (3); 0.08 a.u.f.s.

The peak heights of an extracted serum sample, adjusted for actual volumes, were compared to peak heights of an unextracted aqueous solution of each steroid at known concentrations to determine the percent recovery in the extraction procedure. In the analysis of MP and MPHS, the average percentage recovery was 80% for MP, 94% for MPHS, and 66% for 17α-hydroxyprogesterone. In the analysis of HC and HCHS, the average percentage recovery was 80% for HC, 85% for HCHS and 83% for 11-desoxy-17-hydroxycorticosterone.

The percentage of each steroid partitioning into the hexane layer was also determined. The hexane layer was transferred to a conical test tube, evaporated to dryness, reconstituted with 0.5 ml of mobile phase and injected onto the column. The peak heights were compared to that of an unextracted aqueous solution. In the analysis of MP and MPHS, 16% of the 17α-hydroxy-

progesterone and <1% of MP and MPHS were found in the hexane layer. In the analysis of HC and HCHS, 6% of 11-desoxy-17-hydroxycorticosterone and <1% of HC and HCHS were found in the hexane layer.

Linearity of response and sensitivity

Pooled serum spiked with MP and MPHS from 0.10 to 2.95 μg/ml and 0.8 to 3.20 μg/ml, respectively, were extracted and injected onto the HPLC column. Peak height ratio (peak height of MP or MPHS divided by peak height of internal standard) was used as the response. Least-squares linear regression analysis was used to determine the slope, y-intercept, and correlation coefficient. Using peak height ratio, $Y = 0.007 + 0.752X$ ($r = 0.996$). Using peak area ratio, $Y = 0.02 + 0.592X$ ($r = 0.996$).

Pooled serum spiked with HC and HCHS from 0.08 to 4.05 μg/ml and 0.12 to 6.97 μg/ml, respectively, was extracted and injected onto the HPLC column. Peak heights of HC were corrected for blank serum response representing endogenous HC. Peak heights of HCHS were also corrected for blank serum response representing an endogenous serum component. The corrected peak height ratio was used as the response. Least-squares linear regression analysis was used to determine the slope, y-intercept and correlation coefficient. Using peak height ratio, $Y = 0.004 + 0.831X$ ($r = 0.998$). Using peak area ratio, $Y = 0.04 + 0.587X$ ($r = 0.998$). The concentration of endogenous HC for the pooled serum was calculated to be 0.10 μg/ml.

In the analyses of either MP and MPHS or HC and HCHS, the relationship between serum concentration and peak height ratio was linear up to at least 3 μg/ml of MP and MPHS, 4 μg/ml for HC and 7 μg/ml for HCHS. Peak area ratios were also linear in these ranges. Analysis of 13 standard curves over a period of three weeks indicated that all correlation coefficients of peak height ratio and serum concentration for MP, MPHS, HC and HCHS were 0.99 or greater. The day-to-day coefficient of variation in the slope of the calibration curves was 4.1% for MP, 4.2% for MPHS, 3.2% for HC and 3.5% for HCHS.

The sensitivity of this method was determined to be 0.07 μg/ml for MP, 0.04 μg/ml for MPHS, 0.03 μg/ml for HC and 0.10 μg/ml for HCHS ($p = 0.05$). Sensitivitity is defined as the concentration calculated by linear regression to give a y-response greater than zero 95% of the time. The detection limit was empirically estimated to be 0.02 μg/ml for both steroids and their hemiesters.

Since the serum standards were spiked with both MP and MPHS or HC and HCHS, the effect of the parent steroid response on the hemiester response and the effect of hemiester on the parent steroid response was studied. Serum was spiked with different concentrations of parent steroid only and with hemiester only. These results were compared to standards containing both parent steroid and hemiester. The responses of parent steroid with hemiester were the same as those without hemiester. The responses of hemiester with parent steroid were the same as those without parent steroid. Spiked serum containing only MPHS or HCHS showed no peak at the MP retention time or only endogenous levels of HC.

These results showed that the responses of parent steroid and hemiester were

independent of one another and that MPHS and HCHS were not hydrolyzed to MP and HC during the assay procedure.

Precision and accuracy

The reproducibility of this HPLC method for MP and MPHS and for HC and HCHS was determined. The mean calculated concentration ± S.D. was calculated at each concentration of MP and MPHS as well as HC and HCHS. The results of these calculations are given in Tables I and II. The relative standard deviation was also calculated. Above sensitivity limits, the pooled relative standard deviation is 6.8% for MP, 5.8% for MPHS, 2.9% for HC and 6.1% for HCHS.

The accuracy of this method was determined by spiking serum with MP and

TABLE I

REPRODUCIBILITY IN THE DETERMINATION OF METHYLPREDNISOLONE AND METHYLPREDNISOLONE HEMISUCCINATE IN HUMAN SERUM

Methylprednisolone (μg/ml)		Methylprednisolone hemisuccinate (μg/ml)	
Theoretical	Calculated*	Theoretical	Calculated*
2.95	2.92 ± 0.18**	3.20	3.12 ± 0.07††
2.11	2.15 ± 0.12**	2.23	2.30 ± 0.19**
0.84	0.89 ± 0.04**	1.59	1.58 ± 0.06**
0.42	0.40 ± 0.03**	0.64	0.66 ± 0.05**
0.21	0.20 ± 0.02†	0.32	0.32 ± 0.02†
0.10	0.10 ± 0.03†	0.16	0.15 ± 0.01†
		0.08	0.08 ± 0†

*Mean ± S.D.
**$n = 5$.
†$n = 4$.
††$n = 3$.

TABLE II

REPRODUCIBILITY IN THE DETERMINATION OF HYDROCORTISONE AND HYDROCORTISONE HEMISUCCINATE IN HUMAN SERUM

Hydrocortisone (μg/ml)		Hydrocortisone hemisuccinate (μg/ml)	
Theoretical	Calculated*	Theoretical	Calculated*
4.05	4.02 ± 0.16**	6.97	6.99 ± 0.31**
3.04	3.08 ± 0.16†	4.65	4.66 ± 0.14†
2.43	2.46 ± 0.04†	3.72	3.69 ± 0.06†
1.22	1.22 ± 0.02**	1.86	1.86 ± 0.05**
0.61	0.59 ± 0.01**	0.93	0.91 ± 0.02**
0.30	0.30 ± 0.01**	0.47	0.47 ± 0.01**
0.15	0.16 ± 0.01†	0.24	0.24 ± 0.04†
0.08	0.06 ± 0.02†	0.12	0.12 ± 0.04†

*Mean ± S.D.
**$n = 5$.
†$n = 4$.

MPHS or HC and HCHS at random concentrations within the spiked standard limits. These samples were treated as unknowns and randomly assayed by HPLC. These results are given in Tables III and IV. The average % difference was 3.3% for MP, 4.8% for MPHS, 3.6% for HC and 3.9% for HCHS. It should be noted that spiked plasma samples gave the same response as spiked serum samples.

TABLE III

ACCURACY IN THE DETERMINATION OF METHYLPREDNISOLONE AND METHYLPREDNISOLONE HEMISUCCINATE IN HUMAN SERUM

Methylprednisolone			Methylprednisolone hemisuccinate		
Theoretical concentration (μg/ml)	Calculated concentration (μg/ml)	Difference (%)	Theoretical concentration (μg/ml)	Calculated concentration (μg/ml)	Difference (%)
3.17	3.10	2.2	0.80	0.83	3.8
0.79	0.84	6.3	0.20	0.20	0
0.40	0.42	5.0	0.10	0.11	10
1.06	1.09	2.8	2.41	2.50	3.7
0.53	0.50	5.7	1.20	1.23	2.5
0.13	0.13	0	0.30	0.33	10
3.17	3.24	2.2	2.41	2.57	6.6
1.58	1.55	1.9	1.20	1.18	1.7

TABLE IV

ACCURACY IN THE DETERMINATION OF HYDROCORTISONE AND HYDROCORTISONE HEMISUCCINATE IN HUMAN SERUM

Hydrocortisone			Hydrocortisone hemisuccinate		
Theoretical concentration (μg/ml)	Calculated concentration (μg/ml)	Difference (%)	Theoretical concentration (μg/ml)	Calculated concentration (μg/ml)	Difference (%)
1.00	1.07	7	7.22	7.30	1
0.50	0.55	10	3.61	3.57	1
0.25	0.26	4	1.80	1.71	5
3.0	2.98	1	0.80	0.82	3
0.75	0.74	1	0.20	0.20	0
3.0	3.06	2	4.82	4.70	2
0.75	0.72	4	1.20	1.33	11
1.50	1.50	0	0.40	0.37	8

Human study

Plasma concentration—time curves for MP and MPHS for a single subject following intramuscular administration of 125 mg of Methylprednisolone Sodium Succinate for Injection, U.S.P. (A-methaPred, Abbott, Lot No. 79-822-AR) were determined by the above procedure and are shown in Fig. 3.

Similarly, plasma concentration—time curves for HC and HCHS for a single subject following intramuscular administration of 250 mg of Hydrocortisone Sodium Succinate for Injection, U.S.P. (A-hydroCort, Abbott, Lot No. 79-824-AR) were determined by the above procedure and are shown in Fig. 4.

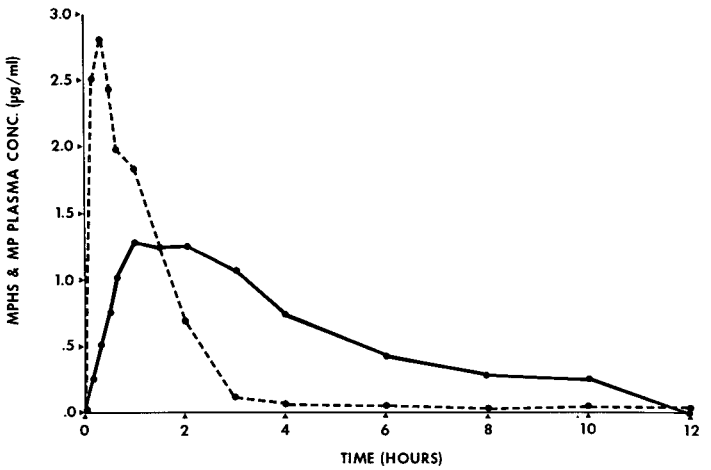

Fig. 3. Plasma concentration—time curve in human for methylprednisolone (——) and methylprednisolone hemisuccinate (- - - -) after a 125 mg intramuscular dose of Methylprednisolone Sodium Succinate for Injection, U.S.P.

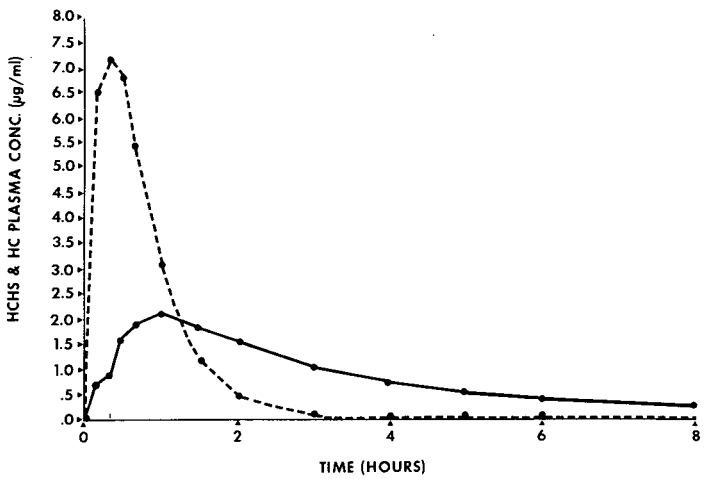

Fig. 4. Plasma concentration—time curve in human for hydrocortisone (——) and hydrocortisone hemissuccinate (- - -) after a 250 mg intramuscular dose of Hydrocortisone Sodium Succinate for Injection, U.S.P.

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CHROMBIO. 378

IDENTIFICATION DE LA 4-O-MÉTHYL DOPAMINE DANS LES TISSUS DE RAT PAR CHROMATOGRAPHIE LIQUIDE EN PHASE INVERSE

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SUMMARY

Identification of 4-O-methyldopamine in rat tissues by reversed-phase liquid chromatography

4-O-Methyldopamine was identified and assayed in tissues from L-dopa treated rats by reversed-phase high-performance liquid chromatography. The initial steps in the separation of catecholamines were performed by alumine, a weak cation-exchange resin, and thin-layer chromatographic techniques.

After L-[^{3}H]dopa administration, the radiochromatogram was superimposed on the fluorochromatogram obtained with authentic marker 4-O-methyldopamine. This metabolite was detected in kidney but not in brain.

The 4-O-methyldopamine:3-O-methyldopamine ratio was 0.032 in kidney. The influence of various treatments on this ratio was investigated. A 160% increase was found after L-dopa administration. This effect was potentiated by nialamide pretreatment (550% increase).

INTRODUCTION

Dans le traitement de la maladie de Parkinson par la L-dopa, il apparait parfois un phénomène de dyskinésie. Dans ce cas, il y a une corrélation entre ce symptôme pathologique et la concentration plasmatique de la O-méthyldopa [1]. D'autre part, l'activité dopaminergique périphérique se reflète au niveau urinaire par la présence de 3-O-méthyldopamine (3 MD) [2]. Cependant, aucune étude sur les métabolites de la L-dopa ou de la dopamine ne signale l'existence dans les tissus de la 4-O-méthyldopamine (4 MD). Théoriquement, cette molécule peut se former dans l'organisme puisque l'on retrouve parmi les produits d'excrétion urinaire un métabolite méthylé en position 4, l'acide isohomovanillique [3]. Dans l'urine des parkinsoniens, le rapport acide isohomovanillique/acide homovanillique est le même que chez les sujets normaux [4, 5]. Toutefois, le traitement par la L-dopa peut modifier ce rapport [6, 7]. Chez le rat, il est plus élevé dans les corps striés que dans l'urine [3].

L'intérêt pour la 4 MD provient du fait que cette substance, injectée à l'animal, possède une action hypokinésique différente de celle de son isomère, la 3 MD [8]. Cette akinésie présente une analogie avec celle observée au cours du syndrome parkinsonien [9].

En ce qui nous concerne, nous avons publié l'action de la S-adénosylméthionine sur le métabolisme de la dopamine et de la L-dopa [10, 11]. Nous avons aussi retrouvé dans l'urine de rat traité par la L-dopa de la 4 MD que nous avons purifiée par chromatographie sur colonne échangeuse d'ions et dosée par fluorimétrie après dérivation [12]. La méthode utilisée était néanmoins difficile à mettre en oeuvre et peu efficace. Une séparation par chromatographie liquide à haute performance (HPLC) des catécholamines formées dans des cultures de tissus de mammifères a été proposée par Stout et al. [13] . Ces auteurs décrivent, entre autres, l'analyse de la MD par chromatographie en phase inverse. Récemment, l'analyse des métabolites de la L-dopa a été réalisée par une méthode impliquant la séparation des catéchols sur une colonne d'alumine puis analyse HPLC [14].

Notre objectif dans le présent travail est de rechercher, "in vivo" chez le rat, la possibilité de formation de la 4 MD. Cette recherche sera faite dans les reins et le cerveau dans une optique physiologique avec surcharge très faible de L-dopa[^{3}H] et, également sous l'angle pharmacologique par traitement de l'animal avec une dose relativement élevée de L-dopa. Afin d'identifier la 4 MD tissulaire, nous utiliserons la méthode HPLC avec appariemment d'ions après séparation des catécholamines par chromatographie échangeuse d'ions et chromatographie couche mince.

MATÉRIEL ET MÉTHODES

Produits chimiques

Ils sont achetés aux sociétés suivantes: L-dopa, sérotonine créatinine sulfate (Fluka, Buchs, Suisse); dopamine-HCl (Serlabo, Paris, France) noradrénaline-HCl, normétanéphrine-HCl, octopamine-HCl, 3-méthoxytyramine-HCl, diméthoxy-3,4-phényléthylamine-HCl, acide dihydroxy-3,4-phénylacétique (Sigma, St. Louis, Mo., É.U.); tyramine-HCl (Prolabo, Paris, France), 4-O-méthyldopamine-HCl; épinine-HCl (Regis, Morton Grove, Ill., É.U.). Tous les produits ont une pureté "pour analyse". L'eau est bidistillée. Le nialamide a été généreusement offert par Pfizer-Clin (Paris, France). La S-adénosylméthionine (SAM) a été préparée au laboratoire selon la technique de Schlenk et al. [15] pour sa synthèse et celle de Haid et Nelboeck-Hochstetter [16] pour sa purification.

Nous avons utilisé l'alumine Woelm N-super I neutre W 200—400 mesh, et l'Amberlite CG 50, activée sous forme H^+ et équilibrée dans un tampon phosphate NaH_2PO_4—K_2HPO_4 0.1 *M* de pH 6.1 (1:1 en volume). La résine Dowex 50 W-X4, 200—400 mesh (Fluka), activée sous forme H^+, est équilibrée dans un tampon phosphate de sodium 0.2 *M* pH 6.5 (1:1 en volume).

La dopa[^{3}H] ou L-3,4-dihydroxyphényl 2,3-[3H] alanine (2.5 Ci/mmole) est fournie par Radiochemical Centre (Amersham, Angleterre). Elle est employée dans les 15 jours après sa réception (conversation á 4° et à l'obscurité). La 3 MD [^{3}H] ou 3-méthoxy-4-hydroxy-β-phényléthylamine 5-[^{3}H] 9.957 Ci/

mmole (NET 321) provient de New England Nuclear (Boston, Mass., É.U.). Le liquide scintillateur pour le comptage est le Picofluor 15 "Packard".

Traitement des animaux

Les expériences sont conduites sur des rats mâles Wistar, souche CF Gif, poids 200 à 250 g. La température de la salle est contrôlée (21° ± 1°); l'alternance jour (7 h—19 h)/nuit (19 h—7 h) est réglée. La nourriture et l'eau sont ad libitum.

Les substances sont administrées par voie intrapéritonéale, en général sous un volume de 1 ml en milieu isotonique (NaCl $9^0/_{00}$). Pour réaliser de fortes concentrations de L-dopa injectable (50—100 mg/kg), celle-ci (200 mg) est solubilisée dans HCl 0.03 *N* (30 ml) par agitation à 50° et à l'obscurité. Après dissolution, le pH est amené à 5.1 par 3 ml de CH_3COONa 1 *M*. Les animaux témoins reçcoivent une quantité équivalente du milieu de dissolution.

Extraits tissulaires

Les reins et le cerveau d'un rat sont prélevés rapidement après décapitation. Toutes les opérations sont réalisées à 4° . Les tissus sont homogénéisés dans 10 ml d'acide perchlorique 0.4 *N*. Les standards internes sont introduits avant cette homogénéisation. Après centrifugation (17,000 *g*, 5 min) le surnageant (fraction I) est conservé à 4°. L'anion perchlorique est alors éliminé par neutralisation à pH 8.3 avec K_2CO_3 6% puis centrifugation (2,000 *g*, 5 min).

Fractionnement sur alumine et amberlite

Le principe de la méthode de fractionnement est celui de Karazawa et al. [17]. On prépare deux colonnes (diamètre 0.6 cm); l'une contient 500 mg d'alumine, la deuxième 0.6 ml de résine amberlite. Elles sont lavées par 20 ml d'eau distillée, puis la colonne d'amberlite est placée au-dessous de la colonne d'alumine. La fraction I précédemment neutralisée à pH 8.3 est alors versée sur la colonne d'alumine. Après écoulement à travers les deux colonnes, on verse 10 ml d'eau. Les colonnes sont séparées, et lavées individuellement par 10 ml d'eau. L'alumine retient les catéchols. Ils sont élués par 3 ml d'HCl 0.2 *N*. La dopamine est ensuite purifiée sur Dowex 50 W X-4 et dosée selon un protocole précédemment décrit [11]. Il est également possible d'analyser cet éluat d'alumine par HPLC selon la technique utilisée ci-après pour la 3 MD et la 4 MD. L'amberlite est traité par 1 ml d'HCl 0.5 *N*; puis une deuxième élution par 2 ml d'HCl 0.5 *N* permet de recueillir la 3 MD et la 4 MD (fraction II).

Concentration

La fraction II est lyophilisée, le résidu repris par 1 ml de méthanol puis 2 ml d'acétate d'éthyle avec agitation à l'aide d'un cyclomixer à température ambiante. Après centrifugation (5,000 *g*, 5 min) le surnageant est débarrassé des sels minéraux. On verse 30 µl d'HCl 0.1 *N* dans la solution méthanol—acétate d'éthyle. L'extrait est évaporé sous pression réduite, à température du laboratoire puis le résidu sec est repris par 200 µl de méthanol—HCl 0.01 *N* (4:1) (fraction III). Cette dernière fraction est suffisamment concentrée et

purifiée pour l'analyse HPLC ou pour l'analyse par chromatographie en couche mince suivie de la HPLC.

Chromatographie liquide à haute performance

La séparation de la 3 MD et de la 4 MD est réalisée sur un appareil Waters Associates Modèle 204 U avec pompe 6000A, injecteur à septum, détecteur UVM 440 avec filtre à 280 nm et enregistreur Omniscribe. La phase stationnaire est une silice greffée μBondapak C_{18} (diamètre 0.39 cm, longueur 30 cm).

La phase mobile est préparée de la manière suivante: l'eau bidistillée et le méthanol contenant chacun $5 \cdot 10^{-3}$ *M* d'acide heptane sulfonique dissout dans l'acide acétique (PIC B 7) sont filtrés sur leurs filtres Millipore respectifs (HAWP 04700 et FHUP 04700). Les deux solutions sont ensuite mélangées afin d'obtenir la proportion méthanol—eau (35:65 en volume). Le pH final est de 3.3. L'élution est de type isocratique avec un débit de 1 ml/min. L'analyse est faite par enregistrement continu d'1 cm/min de la densité optique à 280 nm. La détermination quantitative s'obtient par mesure de la surface des pics par rapport à des standards. Cette analyse est parfois complétée par une détection fluorimétrique et radiochimique. Dans ce cas, à la sortie de l'appareil on collecte des fractions de 0.33 ml (20 sec) qui après addition d'1 ml d'eau sont analysées fluorimétriquement et comptées. Après injection de 5 à 25 μl de la fraction III les pics correspondants à la 3 MD et à la 4 MD sont respectivement appelés fraction $IV_{3\ MD}$ et $IV_{4\ MD}$.

Chromatographie en couche mince puis HPLC

Les plaques de silicagel 60 F 254, sur support plastique 20 × 20 cm (Merck, Darmstadt, R.F.A.) sont développées avec le mélange *n*-butanol—acétate d'éthyle—ammoniac 32% (60:20:20). Après 4 h de développement, les plaques sont séchées et les spots de 3 MD et 4 MD sont visualisés avec la lumière UV à 254 nm (R_F = 0.39 pour 4 MD et 0.53 pour 3 MD). Les zones correspondantes sont découpées et placées dans 2.5 ml d'HCl 0.01 *N*. Après une nuit à 4°, centrifugation et filtration sur millipore AA WP 02500 0.80 μm, on lyophilise. Les résidus (fraction V_A pour la 4 MD et V_B pour la 3 MD) sont repris par 100 μl d'HCl 0.01 *N*. L'analyse par HPLC est faite sur 25 μl de V_A. Pour les mesures de fluorescence et de radioactivité, on collecte des volumes de 0.33 ml. Le pic correspondant à la 4 MD est la fraction $VI_{4\ MD}$.

Il est possible de détecter qualitativement la 3 MD et la 4 MD sur couche mince, par vaporisation d'une solution aqueuse de *p*-nitrobenzène diazonium tétrafluoroborate à 0.1% puis une solution aqueuse de K_2CO_3 10% (sensibilité: 1 nmole pour la 4 MD, coloration violette).

Analyse fluorimétrique

Le spectrofluorimètre est un appareil Farrand MK 1. La relation entre la quantité de produit en fonction de l'intensité de fluorescence est toujours calculée en tenant compte d'un blanc et d'un standard interne. Les diverses fractions sont dosées directement aux longueurs d'ondes: λ_e = 270 nm, λ_f = 315 nm. Cette analyse est non destructive, les fractions sont récupérées pour une purification ultérieure.

Certaines analyses fluorimétriques sont réalisées, après dérivation, selon un dosage spécifique de la 3 MD ou de la 4 MD [12].

Analyse radiochimique

La mesure radiochimique est faite après addition de 10 ml de Picofluor 15 (Packard, Downers Grove, Ill., É.U.) sur un spectromètre à scintillation liquide Packard. L'efficacité du comptage est pour la L-dopa[^{3}H] ou la 3 MD[^{3}H] de 37 à 43% selon les fractions considérées. Les résultats sont corrigés par rapport à un standard interne de L-dopa[^{3}H] pour les fractions I et l'éluat d'alumine, et de 3 MD[^{3}H] pour les autres fractions.

Étude du rendement des standards internes

Les standards internes sont introduits au niveau de l'homogénéisation des tissus. Dans les cas d'échantillon radioactif (L-dopa[^{3}H]) on additonnne aux homogénats 400 nmoles de 4 MD inerte. Lorsque l'étude est réalisée avec injection de L-dopa inerte, on ajoute des surcharges de 4 et 40 nmoles soit de 3 MD, soit de 4 MD à des échantillons provenant de rats témoins.

La comparaison de ces standards internes aux essais correspondants nous permet d'apprécier le rendement de chaque étape de fractionnement. Les analyses sont faites par dosage fluorimétrique ou spectrophotométrique direct par HPLC.

RÉSULTATS

Chromatographie liquide à haute performance

Un chromatogramme caractéristique de plusieurs catécholamines obtenu par HPLC en phase inverse est représenté Fig. 1. Il est à noter la bonne séparation de la 4 MD des autres amines, notamment de la 3 MD, de la tyramine, de la dopamine ou de la normétanéphrine. Afin de ne pas surcharger la figure, nous avons limité le nombre des catécholamines dans le mélange injecté. Les facteurs de capacité obtenus sont donnés dans le Tableau I. Il est évident que les métabolites aminés de la L-dopa sont tous bien séparés de la 4 MD.

Les temps de rétention (t_R) par analyse HPLC ne dépendent pratiquement pas de la surcharge ni de la nature de l'extrait de départ: pour la 4 MD t_R = 10.2 ± 0.2 min et pour la 3 MD t_R = 8.8 ± 0.1 min. Cependant, la résolution entre la 3 MD et la 4 MD est d'autant meilleure que le volume de la fraction III injecté est plus faible.

Sensibilité de la méthode

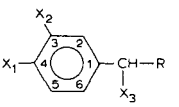
Détection spectrophotométrique. On estime qu'une hauteur de 1 cm, pour la sensibilité maximum de l'appareil, correspond au seuil de détection. En ce cas, l'analyse HPLC étant effectuée sur 1/8ième de la fraction III, la sensibilité de la détection est de 2.9 nmoles de 4 MD ou de 2.2 nmoles de 3 MD dans l'extrait rénal. Pour les blancs (reins de rats contrôles non surchargés en amines), dans la zone de rétention de la 3 MD, la hauteur du pic chromatographique est inférieure au seuil de détection tandis que dans la zone de rétention de la 4 MD l'enregistrement se confond avec la ligne de base.

Détection radiochimique. On estime significative toute radioactivité supérieure à dix fois la racine carrée du mouvement propre. En tenant compte

TABLEAU I

STRUCTURE ET FACTEUR DE CAPACITÉ DES MÉTABOLITES DE LA DOPA

Le facteur de capacité k' est calculé par la relation $k' = (t_R - t_M)/t_M$. t_R = temps de rétention du soluté, t_M = temps de rétention d'un produit non retenu (eau). La formule générale n'est pas applicable à la sérotonine.



Symbole	Nom	X_1	X_2	X_3	R	Facteur de capacité (k')
DOPA	Dihydroxyphénylalanine	OH	OH	H	$CH(NH_2)COOH$	1
DOPAC	Ac dihydroxyphénylacétique	OH	OH	H	$COOH$	1.14
NE	Noradrénaline	OH	OH	OH	CH_2NH_2	1.73
OCT	Octopamine	OH	H	OH	CH_2NH_2	2.05
NM	Normétanéphrine	OH	OCH_3	OH	CH_2NH_2	2.09
DA	Dopamine	OH	OH	H	CH_2NH_2	2.23
EP	Epinine	OH	OH	H	CH_2NHCH_3	2.36
TYR	Tyramine	OH	H	H	CH_2NH_2	2.86
3 MD	3-O-Méthyldopamine	OH	OCH_3	H	CH_2NH_2	3.09
MET	Métanéphrine	OH	OCH_3	OH	CH_2NHCH_3	3.14
5 HT	Sérotonine	—	—	—	—	3.27
4 MD	4-O-Méthyldopamine	OCH_3	OH	H	CH_2NH_2	3.77
DMPEA	Diméthoxy-3,4 phényl-éthylamine	OCH_3	OCH_3	H	CH_2NH_2	5.50

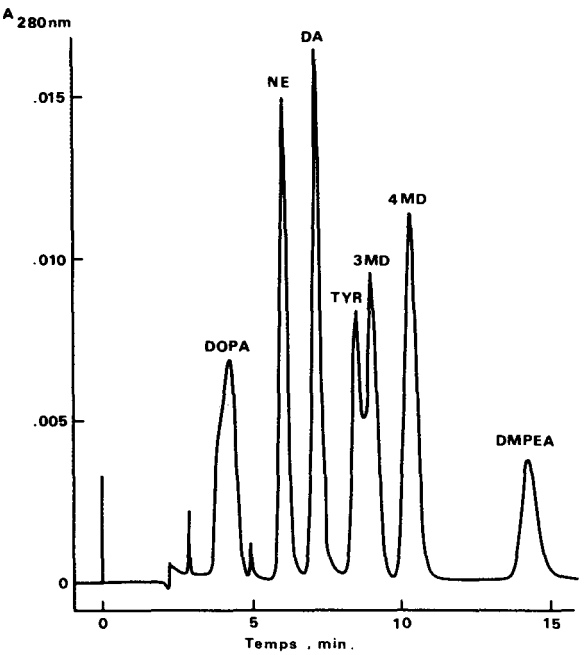


Fig. 1. Représentation d'un chromatogramme HPLC de catécholamines. Colonne: μBondapak C_{18}, 30 × 0.39 cm. Phase mobile: méthanol—eau (35:65 en volume), pic B_7, pH: 3.3, débit: 1 ml/min, pression: 2000 p.s.i. Température ambiante. Échantillon: 25 μl d'un mélange de catécholamines solubilisées dans HCl 0.01 *N*. Les quantités injectées sont les suivantes (en nmoles): dopa: 1.8; NE: 2.11; DA: 2.33; TYR: 2.6; 3 MD: 1.43; 4 MD: 2.14; DMPEA: 1.97. Détection UV à 280 nm; sensibilité DO = 0.02 (pleine échelle).

TABLEAU II

RENDEMENTS (%) DES DIVERSES ÉTAPES DE PURIFICATION DE LA 4 MD

Les rendements en % représentent la moyenne ± SEM de quatre déterminations ($n = 4$), une détermination par rat. Une surcharge de 400 nmoles de 4 MD est ajoutée à chaque homogénat tissulaire. Les déterminations sont fluorimétriques. Signification des fractions: I = homogénat; II = éluat amberlite; III = lyophilisat; $IV_{4\,MD}$ = HPLC (4 MD); V_A = éluat chromatographie sur couche mince (4 MD); $VI_{4\,MD}$ = chromatographie sur couche mince—HPLC (4 MD).

Tissus	Fractions					
	I	II	III → $IV_{4\,MD}$		III → V_A	$VI_{4\,MD}$
Cerveau	100	95 ± 0.9	80.3 ± 1.7	73 ± 2	50.4 ± 2.9	39.8 ± 2.5
Reins	100	87.5 ± 1.8	73.5 ± 3.5	69.5 ± 3.3	49.3 ± 2.0	40.2 ± 2.2

des rendements et de l'efficacité du comptage de la fraction $VI_{4\,MD}$, la radioactivité initiale de la 4 MD[^{3}H] dans la fraction I devra être au minimum de 819 dpm/g pour les reins ou de 935 dpm/g pour le cerveau. Ces valeurs correspondent à des pourcentages minima de 4 MD[^{3}H]:3 MD[^{3}H] détectables de 0.31% dans les reins témoins, 30% dans le cerveau témoin, 0.16% et 0.93% respectivement dans les reins et le cerveau de rats traités au nialamide.

Rendements de la purification de la 4 MD

À titre d'exemple, le Tableau II montre l'évolution du rendement à chaque étape de la purification de la 4 MD. Précisons que l'étape de chromatographie en couche mince a été réalisée avec les 3/4 de la fraction III. Le rendement de la fraction V_A par rapport à III est voisin de 70% . Tous les autres rendements sont supérieurs et se situent entre 80 et 95% . Les différences liées aux tissus, reins ou cerveau, sont négligeables; cependant, le rendement de l'étape I → II est meilleur dans le cas du cerveau. La reproductibilité du rendement (SEM/moyenne) obtenue pour 4 déterminations de 4 MD, respectivement à partir des reins et du cerveau, est de 4.7% et 2.8% avec la fraction IV, et de 5.4% et 6.5% avec la fraction VI.

En ce qui concerne les standards internes (4 ou 40 nmoles de 3 MD ou de 4 MD) introduits au niveau de l'homogénat de reins de rats témoins, les rendements pour la fraction IV sont de 79 ± 2 pour la 3 MD et de 75 ± 2 pour la 4 MD. Ils sont légèrement supérieurs à ceux obtenus pour les échantillons radioactifs étant donné les prélèvements effectuées en vue des mesures, dans ce dernier cas. La détection sur chromatographie sur couche mince avec des surcharges initiales de 4 ou 40 nmoles de 3 ou de 4 MD est à la limite de l'appréciation visuelle. La détermination quantitative n'est plus possible sur les fractions V et VI.

Formation de 4 MD[^{3}H] après administration de L-dopa[^{3}H] (Tableau IV)

Après administration de L-dopa[^{3}H] à des rats, prétraités ou non par un inhibiteur de la monoamine oxydase, la formation possible de 4 MD[^{3}H] et de 3 MD[^{3}H] est étudiée dans les reins et dans le cerveau.

TABLEAU III

RADIOACTIVITÉ SPÉCIFIQUE DES DIVERSES ÉTAPES DE PURIFICATION CONDUISANT À LA 4 MD[^{3}H] À PARTIR DU REIN ET DU CERVEAU DE RAT TRAITÉ PAR LA L-DOPA[^{3}H]

Chaque activité spécifique est la moyenne de deux déterminations (n = 2), une détermination par rat. Une surcharge de 400 nmoles de 4 MD est ajoutée à chaque homogénat tissulaire. Les déterminations sont radiochimiques et fluorimétriques. Signification des fractions: I = homogénat; II = éluat amberlite; III = lyophilisat; $IV_{4\,MD}$ = HPLC (4 MD); V_A = éluat chromatographique sur couche mince (4 MD); $VI_{4\,MD}$ = chromatographie sur couche mince—HPLC (4 MD). Le prétraitement est effectué 5 h avant l'injection de L-dopa[^{3}H]. Les rats sont sacrifiés 30 min après l'injection de L-dopa[^{3}H].

Tissus	Traitement		Activité spécifique (dpm/nmoles de 4 MD)					
	Prétraitement	L-dopa[^{3}H]	I	II	III → $IV_{4\,MD}$	III → V_A	V_A	$VI_{4\,MD}$
Cerveau	NaCl 9‰	1.6 mCi/0.126 mg/kg	1997	13.2	15.3	0	0	0
	Nialamide 100 mg/kg	1.6 mCi/0.126 mg/kg	2520	329	331	4.7	12.2	0
Reins	NaCl 9‰	1.6 mCi/0.126 mg/kg	25,184	1061	1101	35.2	44.7	33.4
	Nialamide 100 mg/kg	1.6 mCi/0.126 mg/kg	22,751	2012	2042	65.1	90.6	62.7

Les mesures de radioactivité et de quantité de 4 MD (standard interne) permettent de déterminer la radioactivité spécifique "As" dans chaque étape du fractionnement (Tableau III). La radioactivité spécifique diminue au cours du fractionnement jusqu'à la fraction $IV_{4\,MD}$. On remarque l'égalité de la radioactivité entre les fractions $IV_{4\,MD}$ et $VI_{4\,MD}$. Cette observation est un argument en faveur de la pureté radiochimique de la 4 MD[^{3}H] dans ces deux fractions. À partir de ces résultats on déduit:

le pourcentage de métabolite 3 MD[^{3}H] + 4 MD[^{3}H] par rapport à la totalité des métabolites tritiés tissulaires R [^{3}H],

le pourcentage de 4 MD[^{3}H] par rapport à la 3 MD[^{3}H].

Ces résultats sont consignés dans le Tableau IV. À titre indicatif figurent également la radioactivité totale R [^{3}H] et celle de la DA[^{3}H] en % de R [^{3}H].

Dans les reins. La formation du dérivé 4-O-méthylé de la dopamine: 4 MD[^{3}H] est identifiée après administration de la L-dopa[^{3}H]. Le rapport 4 MD [^{3}H]:3 MD[^{3}H] est faible 3.2% mais cette valeur est acceptable par rapport au seuil de sensibilité 0.3% . Ce rapport subit une augmentation significative ($p < 0.001$) de 66% lorsque la quantité de L-dopa administrée est 80 fois plus importante. Dans ces conditions l'O-méthylation de la dopamine augmente légèrement tandis que l'accumulation de dopamine dans les reins est très forte (× 12).

L'administration de SAM est sans effet sur la 3 et la 4-O-méthylation de la dopamine dans les reins.

Le nialamide, inhibiteur de la MAO, élève la radioactivité de la 3 MD[^{3}H] (+ 110%). Mais surtout il augmente la 4 MD[^{3}H] de façon parallèle à la 3 MD[^{3}H]. En conséquence le rapport 4 MD[^{3}H]:3 MD[^{3}H] reste sensiblement le même: 3.1% . L'inhibition de la désamination oxydative de la 4 MD[^{3}H] et de la 3 MD[^{3}H] est identique in vivo. Les concentrations de ces amines étant très faibles, l'activité de la monoamine oxydase périphérique est sans effet.

Dans le cerveau. La 4 MD[^{3}H] n'est pas retrouvée dans le cerveau, et cela aussi bien pour les rats témoins ou traités au nialamide. Dans ce dernier cas, nous pouvons affirmer que le rapport 4 MD[^{3}H]:3 MD[^{3}H] est inférieur à 0.9% , pourcentage bien inférieur à celui déterminé dans les reins.

La 3 MD[^{3}H] existe en quantité très faible chez les animaux témoins. Le nialamide modifie beaucoup la concentration de ce métabolite: la radioactivité est multipliée 17 fois.

Mise en évidence de la 4 MD[^{3}H]. L'étude par HPLC de la fraction III ne montre qu'un seul pic de radioactivité dont le volume de rétention correspond à la 3 MD[^{3}H]. Cette analyse démontre l'absence de 3 DA[^{3}H] et la présence exclusive de 3 MD[^{3}H] dans les fractions II et III. Par contre, le pic de 4 MD[^{3}H] est masqué par celui de 3 MD[^{3}H] et par une analyse radiochimique sur des fractions collectées trop importantes. La chromatographie sur couche mince permet d'isoler un spot de 4 MD (fraction V_A). L'analyse par HPLC de cette fraction montre une superposition entre le diagramme de fluorescence (standard interne de 4 MD) et celui de radioactivité 4 MD[^{3}H] en fonction du temps de rétention (Fig. 2): ce type de chromatogramme est un bon argument en faveur de l'identité entre les deux substances. D'autres expériences réalisées avec la L-dopa inerte viendront corroborer ce premier résultat.

TABLEAU IV

DISTRIBUTION DE LA 4 MD[^{3}H], 3 MD[^{3}H], DA[^{3}H] ET DE LA RADIOACTIVITÉ TOTALE DANS LE REIN ET LE CERVEAU DE RAT TRAITÉ PAR LA L-DOPA[^{3}H]

Les abréviations sont les suivantes: R[^{3}H] = radioactivité totale en millier de dpm par g de tissu; DA[^{3}H] = dopamine tritiée analysée après séparation sur Dowex de l'éluat alumine; MD[^{3}H] = 3- et 4-O-méthyldopamine[^{3}H] (fraction II); 3 MD[^{3}H] = 3-O-méthyldopamine[^{3}H]; 4 MD[^{3}H] = 4-O-méthyldopamine[^{3}H] (fraction $VI_{4\ MD}$). Voir le texte pour les détails. Les résultats sont exprimés sous la forme de la moyenne ± SEM de *n* déterminations. *n* correspond au nombre de rats. Le prétraitement est effectué 5 h avant l'administration de L-dopa[^{3}H]. Les rats sont sacrifiés 30 min après l'injection de L-dopa[^{3}H]. Pour le lot L-dopa + S-adénosylméthionine (SAM) ces deux produits sont administrés simultanément. Comparaison des moyennes, pour un même tissu, à celles du lot: L-dopa[^{3}H] 1.6 mCi/0.126 mg/kg.

Tissus	Traitement		*n*	Radioactivité totale R[^{3}H] (dpm 10^{-3} g^{-1})	DA[^{3}H]:R[^{3}H] (%)	MD[^{3}H]:R[^{3}H] (%)	4 MD[^{3}H]:3 MD[^{3}H] (%)
	Prétraitement	L-dopa[^{3}H]					
Cerveau	NaCl 9‰	1.6 mCi/0.126 mg/kg	2	570 ± 42	7.5 ± 0.9	0.7 ± 0.1	0
	Nialamide 100 mg/kg	1.6 mCi/0.126 mg/kg	2	784 ± 125	4.9 ± 0.8	13.0 ± 0.5**	0
Reins	NaCl 9‰	1.6 mCi/0.126 mg/kg	2	6152 ± 203	2.8 ± 0.6	4.2 ± 0.3	3.2 ± 0.1
	Nialamide 100 mg/kg	1.6 mCi/0.126 mg/kg	2	5823 ± 140	4.5 ± 0.3	8.9 ± 0.4*	3.1 ± 0.2
	non	0.8 mCi/10 mg/kg	6	2278 ± 130	33.2 ± 3.1***	5.4 ± 0.3	5.3 ± 0.2***
	non	0.8 mCi/10 mg/kg + SAM 1 mg/kg	6	2337 ± 85	33.7 ± 2.9	5.0 ± 0.3	5.8 ± 0.3

*$p<0.05$.
**$p<0.01$.
***$p<0.001$ (test *t* de signification).

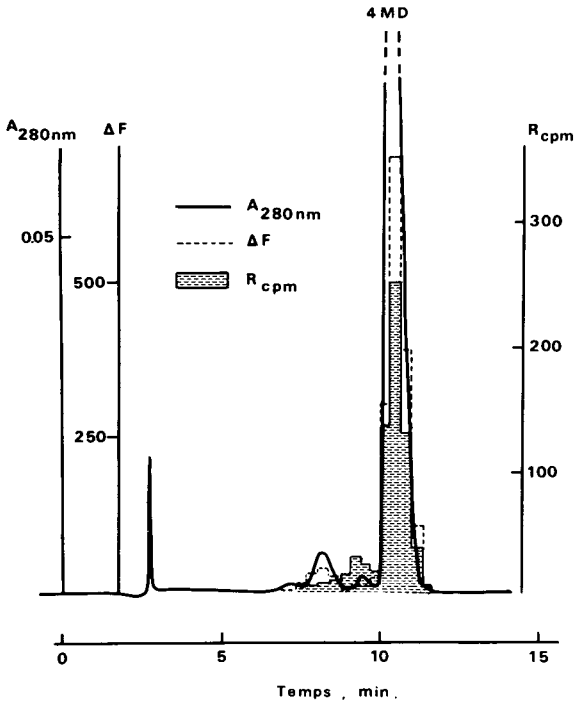


Fig. 2. Chromatogramme HPLC de la fraction $V_{4\,MD}$. Les conditions chromatographiques sont décrites dans la légende de la Fig. 1. Échantillon: 25 μl de la fraction $V_{4\,MD}$ (chromatographie sur couche mince, spot 4 MD). Le volume de cette fraction $V_{4\,MD}$ est de 100 μl. Elle provient de reins de rats traités par la L-dopa[^{3}H] 1.6 mCi/0.126 mg/kg. Le sacrifice des rats est fait 30 min plus tard. 400 nmoles de 4 MD sont ajoutés à l'homogénat de reins. $A_{280\,nm}$: Détection UV à 280 nm; sensibilité DO = 0.1 (pleine échelle). ΔF: Mesure fluorimétrique à λ_e = 270 nm; λ_f = 315 nm des fractions collectées (20 sec). R_{cpm}: Mesure radiochimique des fractions collectées (20 sec).

Influence de la L-dopa et du nialamide sur le taux de 3 MD et de 4 MD dans les reins (Tableau V)

Sans administration de L-dopa, le taux endogène de 3 MD et de 4 MD dans les reins de rats n'est pas mesurable. Le traitement des rats par une dose de L-dopa voisine de celle en usage dans la maladie de Parkinson est donc doublement justifiée: d'abord, en tant que méthode de mise en évidence de cette 4-O-méthylation de la dopamine, et, ensuite comme modèle pharmacologique. Cette deuxième motivation reste valable lorsque les rats sont prétraités par le nialamide. En effet, Birkmayer et al. [18] estiment que la propriété akinésique de la L-dopa est potentialisée par certains inhibiteurs de la monoamine oxydase du type B spécifique de la dopamine.

La preuve de la formation de 4 MD in vivo, dans les reins, après administration de L-dopa, seule ou en présence de nialamide, est apportée dans ce travail pour la première fois. Ainsi, l'analyse HPLC de la fraction III met en évidence 2 pics (Fig. 3). Comparés à des standards, ils présentent toutes les caractéristi-

ques des deux catécholamines O-méthylés recherchées 3 MD et 4 MD:

Temps de rétention

Maximum d'absorption à 280 nm

Maximum de leur spectre de fluorescence directe à λ_e = 270 nm et λ_f = 315 nm

Spectre de fluorescence et maximum à λ_e = 327.5 nm et λ_f = 375 nm après dérivation selon une méthode décrite dans des travaux antérieurs [12]

Le recyclage des fractions composant ces pics pour une nouvelle analyse HPLC permet d'affiner l'identification de ces deux métabolites

De plus, après chromatographie sur couche mince, l'analyse HPLC de la fraction V_A nous permet de retrouver un pic correspondant aux caractéristiques spectrales et de rétention de la 4 MD.

La possibilité d'artefact dans l'identification de la 4 MD a été soigneusement éliminée. Ce métabolite provient bien du traitement à la L-dopa car on ne le retrouve pas chez les témoins. Il ne se forme pas à partir de la 3 MD au cours de la purification comme le démontre un standard interne de 3 MD introduit dans l'homogénat. L'alumine ne le retient pas: ce n'est pas un catéchol. Il est cationique puisqu'il reste fixé sur l'amberlite. Les caractéristiques obtenues

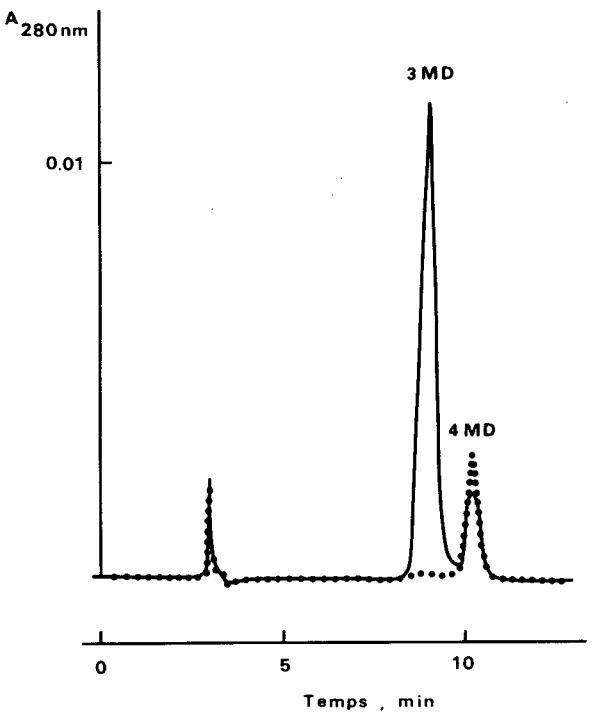


Fig. 3. Chromatogramme HPLC de reins de rats traités par la L-dopa inerte. Les conditions chromatographiques sont décrites dans la légende de la Fig. 1. Sensibilité de la détection UV à 280 nm: 0.02 (pleine échelle). Volume d'échantillon injecté: 5 µl; soit 1/40ième de la fraction III (lyophilisat). —: Reins de rat prétraité au nialamide 100 mg/kg, puis cinq heures après par la L-dopa 100 mg/kg. Les animaux sont sacrifiés 30 min après cette dernière injection. • • • : Reins de rat témoin en présence d'un standard interne de rendement de 40 nmoles de 4 MD au niveau de l'homogénat.

TABLEAU V

EFFET DE L'ADMINISTRATION DE L-DOPA ET DE NIALAMIDE SUR LE TAUX DE DA, 3 MD ET 4 MD DANS LE REIN DE RAT

Les rats sont traités par le nialamide 100 mg/kg intrapéritonéal, 5 h avant l'injection de L-dopa. Le sacrifice est effectué 30 min après administration de L-dopa. Les résultats sont exprimés sous la forme de moyenne ± SEM de quatre déterminations, une détermination par animal. Les dosages sont réalisés par analyse HPLC. Pour 3 MD et 4 MD on utilise l'éluat amberlite lyophilisé (fraction III) et pour DA l'éluat alumine. Comparaison des moyennes avec le lot: L-dopa 100 mg/kg.

Traitement		DA (nmoles/g)	3 MD (nmoles/g)	4 MD (nmoles/g)	4 MD:3 MD (%)
Prétraitement	L-dopa				
non	50 mg/kg	123.4 ± 11.3*	5.0 ± 0.6	N.D.**	N.D.**
non	100 mg/kg	299.4 ± 24.3	6.5 ± 0.4	0.6 ± 0.1	8.3 ± 1.0
Nialamide 100 mg/kg	100 mg/kg	1044 ± 39*	56.9 ± 2.9 *	12.0 ± 1.2*	21.0 ± 1.2*

*$p < 0.001$ (test t de signification).
**N.D. = non détectable.

par chromatographie sur couche mince et par HPLC, de même que les analyses spectrales, l'identifient à la 4 MD.

Dans le Tableau V, l'effet de la L-dopa et du nialamide est étudié sur le taux de 3 MD et de 4 MD dans les reins de rat. L'administration de L-dopa provoque la formation de 3 MD. Le taux de 4 MD dans les reins est très faible, même par traitement avec 100 mg/kg de L-dopa. Dans ce cas, le rapport 4 MD:3 MD est de 8.3% . Lorsque les animaux sont prétraités avec le nialamide, le taux de 3 MD est multiplié par 9. Cette augmentation était moins forte (× 2) par traitement des rats avec de petites quantités de L-dopa[^{3}H] (0.126 mg/kg) (cf. Tableau IV). Mais le phénomène remarquable est la modification du rapport 4 MD:3 MD qui atteint 21% .

DISCUSSION

La séparation de deux phényléthylamines substituées 3 MD et 4 MD par chromatographie liquide en phase inverse, avec une élution par un solvant hydrométhanolique contenant un contre-ion, dépend de la lipophilie du complexe amine—acide heptane sulfonique. Cependant ces deux amines ont un caractère lipophile identique puisque la substitution d'un hydroxyle par un groupement méthoxyle en *méta* ou en *para* ne modifie pas la constante fragmentale hydrophobe [19]. Par contre, la constante σ de Hammett du substituant hydroxylé est plus faible que celle du substituant méthoxylé en position *para*, tandis qu'elle est la même pour ces deux substituants en position *méta* sur le noyau aromatique des phényléthylamines [20]. La constante σ_X de Hammett correspond à la variation de pK provoquée par le remplacement, sur le noyau, d'un H par le substituant X. le pK de la 4 MD sera supérieur à celui de la 3 MD, la dissociation du complexe 4 MD—contre-ion sera plus faible; en conséquence le temps de rétention de la 4 MD est plus important que celui de la 3 MD.

La résolution entre ces deux amines peut être améliorée en réduisant la concentration de méthanol dans la phase mobile (méthanol—eau, 20:80). Toutefois les volumes de rétention augmentent, et la sensibilité de la détection diminue.

Une détection fluorimétrique en continu après dérivation selon la technique précédemment décrite [12], à la sortie de la colonne de μBondapak améliorerait la sensibilité de la détection.

La présence de 4 MD[^{3}H] in vivo dans les reins et son absence dans le cerveau permet de penser à un métabolisme différent dans ces deux organes. Les hypothèses suivantes peuvent être formulées:

(1) L'activité catéchol-O-méthyltransférase n'est pas la même du point de vue de la spécificité de l'O-méthylation en position 3 ou 4 de la dopamine, in situ, dans le cerveau ou dans les reins. Cette différentiation n'est pas surprenante si l'on compare nos résultats avec l'étude de ce type d'activité, réalisée par Bade et al. [21], sur le cerveau, les érythrocytes et le foie de rat. Utilisant comme substrat le dihydroxy-3,4 benzaldéhyde, ces auteurs ont montré que le rapport *méta:para* d'O-méthylation et la constante de Michaelis étaient différentes entre d'une part l'enzyme du cerveau et d'autre part l'enzyme du foie.

(2) Le métabolisme de la 4 MD dans le cerveau ou les reins n'est pas le même. Cette différence ne provient pas de la désamination oxydative puisque le traitement par un inhibiteur de la monoamine oxydase ne provoque pas l'apparition de 4 MD dans le cerveau. Dans cet organe, l'absence de 4 MD pourrait résulter d'une élimination rapide de ce métabolite neurotoxique par une voie différente de celle de la 3 MD.

Le traitement des rats par des quantités importantes de L-dopa, après administration de nialamide, provoque une augmentation considérable de la 4-O-méthylation et du rapport 4 MD sur 3 MD dans les reins. Deux hypothèses peuvent être émises sur cette élévation:

(1) L'inhibition de l'activité monoamine oxydase par le nialamide est plus forte vis-à-vis de la 4 MD que pour la 3 MD. Dans la littérature, des différences d'affinité ont été relevées entre les substrats de la monoamine oxydase: à titre d'exemple, la 4 MD est un meilleur substrat que la diméthoxy-3,4 phényléthylamine pour l'enzyme du cerveau de rat [22]. Dans notre expérimentation, le nialamide agirait plus efficacement sur la désamination de l'isomère 4 et conduirait ainsi à un rapport 4 MD:3 MD plus élevé. Cependant, lorsque la L-dopa est administrée à faible dose, la présence de nialamide n'influe pas sur le rapport 4 MD[^{3}H]:3 MD[^{3}H] (cf. Tableau IV). Ce résultat rend assez fragile cette première hypothèse.

(2) L'inhibiteur de la monoamine oxydase provoque une accumulation de dopamine (× 3.5) dans les reins. La catéchol-O-méthyltransférase, probablement saturée par ce substrat, accélère alors le mécanisme de 4-O-méthylation. Les résultats obtenus par administration de L-dopa[^{3}H] sont en faveur de cette hypothèse.

En définitive, il apparait que la voie de 3-O-méthylation de la dopamine est prépondérante. D'autre part, le rapport 4 MD:3 MD est relativement constant (environ 3.2%) dans des conditions physiologiques. Enfin, par traitement par la L-dopa, ce rapport est modifié au profit de la 4-O-méthylation. Ce phénomène est à rapprocher des travaux de Mathieu et al. [6] qui observent une augmentation du rapport acide isohomovanillique:acide homovanillique dans l'urine de rat. Un hyperfonctionnement de la voie dopaminergique n'est donc pas sans conséquence quant à l'orientation de la méthylation.

La 4 MD ne semble pas provenir de la 4-O-méthyldopa car cette dernière

substance n'est pas détectée dans l'urine humaine ou de rat après traitement par la L-dopa [23]. La dopamine pourrait être son précurseur direct. Quelles que soient les conditions pharmacologiques étudiées, il ne nous a pas été possible de mettre en évidence, la diméthoxy-3,4 phényléthylamine qui serait formée in vitro sous l'influence de la gaïacol-O-méthyltransférase [24].

Le rapport acide isohomovanillique:acide homovanillique trouvé dans les tissus est beaucoup plus élevé que celui de 4 MD:3 MD déterminé dans le présent travail. Ceci suggère l'hypothèse de la formation d'acide isohomovanillique par méthylation en 4 de l'acide dihydroxy-3,4 phénylacétique et non pas à partir de la 4 MD par désamination oxydative. Les travaux de Creveling et al. [25] confortent cette hypothèse: in vitro à pH physiologique, l'O-méthylation *para/méta* de l'acide dihydroxy-3,4 phénylacétique est supérieure à celle de la dopamine.

L'étude de la formation de 4 MD dans le cerveau, après administration de L-dopa inerte, n'a pas été envisagée par suite du manque de sensibilité de la détection spectrophotométrique. Par contre, notre méthodologie devrait permettre d'étudier la formation de 4 MD[^{3}H] dans le cerveau, si toutefois elle existe, en administrant à l'animal de la L-dopa avec une dose traceuse de L-dopa[^{3}H].

Le problème de l'existence de ce métabolite 4-O-méthylé chez l'homme n'est pas encore résolu. Néanmoins, une partie de ce travail pourra être adaptée au dépistage de la 4 MD, en particulier, chez les parkinsoniens traités par la L-dopa.

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RÉSUMÉ

La 4-O-méthyldopamine est identifiée et dosée dans les tissus de rats traités par la L-dopa en utilisant la chromatographie liquide à haute performance en phase inverse. Préalablement, les catécholamines sont séparées sur alumine et résine échangeuse de cation, puis chromatographie sur couche mince.

Après administration de L-dopa[^{3}H], le radiochromatogramme est superposé au chromatogramme obtenu par détection fluorimétrique d'un standard interne de 4-O-méthyldopamine. Ce métabolite est présent dans les reins mais indétectable dans le cerveau.

Le rapport 4-O-méthyldopamine:3-O-méthyldopamine est égal à 0.032 dans les reins. L'effet de différents traitements sur ce rapport est étudié. Une augmentation de 160% est déterminée après administration de L-dopa. Cet effet est potentialisé par un prétraitement au nialamide (550% d'augmentation).

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Biomedical Applications

CHROMBIO. 384

DETERMINATION OF THE NATURALLY OCCURRING MONOACETYL DERIVATIVES OF DI- AND POLYAMINES

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SUMMARY

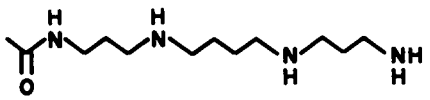
A method is described for the determination of pmol quantities of monoacetylputrescine, N^1-acetylspermidine, N^8-acetylspermidine and related compounds. The method is based on the derivatization of these compounds with 5-dimethylaminonaphthalene-1-sulphonyl-chloride, followed by thin-layer chromatographic separation. Cleanup steps allow the application of the method to urine analyses. From the repeated determination of acetylated polyamines in the urine of healthy individuals it can be concluded that these conjugates are the major excretory form of di- and polyamines.

The cleanup steps used in this procedure and the method described for the stabilization of 5-dimethylaminonaphthalene-1-sulphonyl derivatives on thin-layer plates are advantageous also for the analyses of total polyamines in urine hydrolysates, and in related applications of the dansylation method.

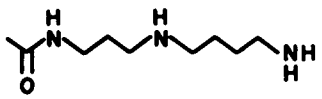
INTRODUCTION

Urinary polyamine analyses have been employed for the early detection of cancer, or for the evaluation of the efficacy of anticancer treatment [1—18]. In all these studies and in related work in which serum [19—21] or cerebrospinal fluid [22—24] polyamines were measured, the samples were hydrolysed with 6 *N* HCl prior to analysis, in order to liberate polyamines from their conjugates. It is known from previous work that acetylputrescine, acetylcadaverine and acetylspermidines are normal urinary constituents [25—31]. Structural formulae and chemical nomenclature of the acetylated polyamines are shown in Fig. 1.

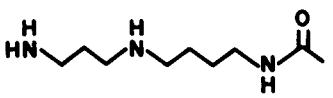
Abdel-Monem and Ohno [30—32] suggested the measurement of these acetylated polyamines as a more specific method than total polyamine determination for the detection of cancer. They were able to show significantly



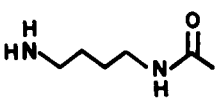
N^1-Monoacetylspermine
(N^1-(3-acetamidopropyl)-N^4-(3-aminopropyl) - 1,4-diaminobutane)



N^1-Monoacetylspermidine
(N^1-(3-acetamidopropyl) - 1,4 - diaminobutane)



N^8-Monoacetylspermidine
(N^1-(4-acetamidobutyl) - 1,3-diaminopropane)



Monoacetylputrescine
(N^1-acetyl - 1,4 - diaminobutane)

Fig. 1. Structural formulae of acetylated di- and polyamines.

increased excretion of N^1-monoacetylspermidine in comparison with N^8-monoacetylspermidine in about half of fifteen cancer patients.

A great variety of methods are presently available for the determination of polyamines [33, 34]. In principle several approaches are feasible for the analysis of both free polyamines and their acetylation products. Ion-exchange column chromatography [25, 26, 35] and coupled gas—liquid chromatography—mass spectrometry [28, 36] can give adequate separations. However, the only method which has been actually used for the quantitative estimation of monoacetylputrescine and the acetylspermidines in urine consists of the following steps [37, 38]: (a) reaction of the urine samples with 5-dimethylaminonaphthalene-1-sulphonyl chloride (Dns-Cl) [39—41]; (b) thin-layer chromatographic (TLC) separation of the Dns derivatives; (c) extraction of the (partially) separated compounds from the thin layer; (d) separation and quantitation of the different compounds by high-performance liquid chromatography, using a silica gel column and a fluorescence flow detector.

In the present paper a method is presented which also relies on the dansylation reaction. Derivatization is followed by TLC and by in situ fluorescence measurement of the separated Dns derivatives. In order to obtain "clean" chromatograms, pre-separation and purification steps have been

worked out. These are applicable to the analysis of free polyamines in hydrolyzed urine samples as well.

MATERIALS AND METHODS

Chemicals

Laboratory chemicals were usually of A-grade. They were purchased from Baker Chemicals (Deventer, The Netherlands) or from Merck (Darmstadt, G.F.R.). Putrescine dihydrochloride, spermidine phosphate ($(C_7H_{19}N_3)_2 \cdot 3H_3PO_4 \cdot 6H_2O$) and spermine phosphate ($C_{10}H_{26}N_4 \cdot 2H_3PO_4 \cdot 6H_2O$) for standardization purposes as well as the corresponding (less pure) hydrochlorides of spermidine and spermine were from Fluka (Buchs, Switzerland).

The acetylated polyamine hydrochlorides and 5-dimethylaminonaphthalene-1-sulphonyl chloride were prepared in our laboratory according to published procedures [39, 42].

Thin-layer chromatography

20 × 20 cm silica gel plates (silica gel 60; Merck,) were used throughout. The plates were developed by ascending chromatography in filter paper lined tanks (Camag, Muttenz, Switzerland).

Quantitative evaluation of the plates

This was achieved by in situ fluorescence scanning, using the TLC scanner of Camag. Fluorescence was activated at 320 nm and total emission was measured, using a 400 nm cut-off filter for the elimination of the UV light. Slit: 6 × 0.8 mm.

Collection of urine samples

Urine samples were collected for 24-h periods from healthy adult drug-free males and females. 50 ml of acetic acid plus 1 g sodium metabisulphite, or 100 ml of 3 *N* HCl were placed in the collection flasks in order to avoid bacterial contamination. Immediately after the collection period, 25-ml aliquots of the urine samples were stored at −20° until they were analyzed.

Pre-separation of urine on Dowex 50W-X8 columns

Purification of the ion-exchange resin (Dowex 50W-X8, 200—400 mesh; Serva, Heidelberg, G.F.R.) was achieved by washings with large volumes of 1 *N* NaOH, water and 6 *N* HCl. It was stored at +3° suspended in 6 *N* HCl. Columns were prepared from 5-ml polypropylene pipettor tips (Gilson, Villier-Le-Bel, Arnouville-Les-Gouesse, France) by adjusting a cotton wool plug into the constricted end of the tip and application of a total of 5 ml of resin, suspended in 6 *N* HCl. The resin columns, usually 12 at a time, were connected by polyethylene tubes with the silicon pumping tubes (1 mm I.D.) of a peristaltic pump (mp 13 or mp 13 GJ-4; Ismatec, Zürich, Switzerland) and washed with distilled water to neutral pH. Aliquots (2 ml) of the urine samples were mixed with 2 ml of ethanol and allowed to stand for 1 h at 3° after which the precipitate was removed by centrifugation. The clear supernatants were applied to the columns by pumping at a rate of about 0.8 ml/min. Subsequently the

columns were washed at the same pumping rate with 25 ml of 2.0 *N* HCl. The polyamine fractions (containing both conjugated and non-conjugated derivatives) were eluted with 25 ml of 6 *N* HCl. They were evaporated to dryness at a pressure of about 15 mm Hg, using a Rotavapor (Büchi, Flawil, Switzerland).

Hydrolysis of urine samples

Mixtures of 5 ml of urine with 5 ml of 12 *N* HCl were sealed in Pyrex glass tubes (15 × 160 mm) and heated for 18 h at 120°. The content of the tubes was evaporated in vacuo, and the residues were dissolved in 5 ml of water. Two millilitres of these solutions were mixed with the same volume of ethanol and subjected to chromatography on Dowex 50 columns, as described for the non-hydrolyzed samples.

Derivatization with 5-dimethylaminonaphthalene-1-sulphonyl chloride (Dns-Cl)

The dried residues of the column eluates were dissolved in 0.4 ml of 0.2 *N* perchloric acid and 0.8 ml of acetone. (This amount of solvent was used to quantitatively transfer the residues of the eluates from a 100-ml round bottom flask into glass tubes (12 × 100 mm).) A solution of 20 mg of Dns-Cl in 0.4 ml of acetone was added; the solutions were saturated with sodium carbonate and reacted overnight at room temperature, as described previously [39–41]. After reaction of the excessive Dns-Cl with proline, the samples were extracted with 4 ml of toluene, and the toluene extracts were evaporated in a stream of air to dryness. Subsequently a solution of 150 µl of 5 *M* KOH in methanol was added, and the samples were heated for 30 min at 50° in a water bath in order to remove easily hydrolyzable Dns derivatives [43]. By addition of 1.5 ml of water and 100 mg of a 1:1 mixture of KH_2PO_4 and Na_2HPO_4, the Dns derivatives were extracted with 4 ml of toluene from the aqueous phase. Usually 20-µl aliquots of these extracts were separated by TLC.

Thin-layer chromatography

Samples were applied manually, using a 20-µl "Pipetman" pipettor (Gilson). The rapid application of 20-µl volumes of the toluene solutions of Dns derivatives does not produce starting zones on silica gel thin layers with a diameter exceeding 2 mm. Only some rapidly migrating side products of the reaction tend to move further, but have no significant influence on the quality of the subsequent chromatographic separations. Normally the spots are applied at a distance of 20 mm from one plate edge and at a distance of 10 mm from each other, i.e. 20 samples can be separated on each plate. Three standards, prepared from authentic, pure compounds and run through the entire procedure, including chromatography on the Dowex columns were normally applied on each plate.

Separation of the acetylated polyamines. The non-polar Dns derivatives, including Dns-ammonia are moved near the solvent front by developing the plates with ethyl acetate (1 run). For the actual separation of the relatively non-polar acetyl derivatives the following solvent was used in a solvent-vapor-saturated tank: chloroform—tetrachloromethane—methanol (70:30:5) (2 runs). Between each run, the plates are dried at room temperature for 5 min. It

should be pointed out that the ethanol usually present in the commercial chloroform has to be removed by distillation.

Free polyamines and total polyamines. The previously described solvents [39—41] for the separation of Dns-polyamine derivatives were used for the separation of both free polyamines and total polyamines, as obtained by hydrolysis of the urine samples with 6 *N* HCl. Chromatography with cyclohexane—ethyl acetate (1:1) (2 runs) followed by cyclohexane—ethyl acetate (3:2) (1 run) was most appropriate in our hands. This solvent separates bis-Dns-cadaverine from bis-Dns-putrescine, however it does not allow one to determine the former compound because it is too close to the large spot of Dns-ammonia. If bis-Dns-cadaverine is to be determined, chloroform—triethylamine (5:1) is preferable. [39—41, 44, 45] In this case, however, the spermine spot is close to side products of the dansylation reaction.

Stabilization of the Dns derivatives

Spraying with a solution of triethanolamine in propanol-2 enhances fluorescence intensity and stabilizes the Dns derivatives on thin layers [39—41]. Recently, spraying with solutions of viscous organic solvents (paraffin oil in cyclohexane or toluene, among others) was suggested for the same purpose [46]. We have tried to avoid this technically difficult process. The following practical solution for this problem was found: the plates are immersed twice for 1 min into a 10% solution of Rhodorsil oil SI 710 (a silicon oil available from Prolabo, Paris, France) in cyclohexane. Between the immersions they are dried for 5 min at room temperature. Fluorescence is stable at least for 1 week if the plates are stored in the darkness. No significant diffusion of the Dns-polyamine spots is observed. One can imagine that paraffin oil similarly applied would be equally useful.

RESULTS

Separation of the monoacetyl polyamines

The solvent mixture consisting of chloroform—tetrachloromethane—methanol (70:30:5) is capable of separating the Dns derivatives of putrescine, cadaverine, spermidine, spermine, monoacetylputrescine, monoacetylcadaverine, N^1- and N^8-monoacetylspermidine and N^1-monoacetylspermine either on the usual 20 × 20 cm silica gel plate or on a 10 × 10 cm high-performance thin-layer plate. This solvent was therefore previously used for the separation of labelled polyamines in metabolic studies, with subsequent preparation of autoradiographs [47]. For the quantitative evaluation of the plates by fluorometry this separation has some disadvantages. (a) Spermine moves close to the solvent front and by-products of the dansylation reaction can interfere with its quantitative evaluation. In this respect the solvent is similar to chloroform–triethylamine (5:1) [39—41] which has been repeatedly used for the analysis of total polyamines in urine [44, 45]. (b) Since the thin-layer scanner has only a limited resolution capacity, one would like to achieve a better separation of the two monoacetylspermidines, and of monoacetylputrescine from monoacetylcadaverine. (c) The two monoacetyl-spermidines move closely behind Dns-ammonia. If the latter is present in large amounts, it interferes with the estimation of the acetylspermidines.

In the case of urine analyses there is normally no restriction in the availability of material. It was decided, therefore, to separate free polyamines (and total polyamines, as obtained by acid hydrolysis of urine) and acetylated polyamines on two plates using two different solvents. Chromatograms showing separations of various sample types in the two solvent systems are shown in Figs. 2 and 3. Typical fluorescence scans of acetylated polyamines and of the amines of a urine hydrolysate are shown in Figs. 4 and 5.

The above-mentioned separation problems with acetylated polyamines were solved by moving all non-polar Dns derivatives, including those of the polyamines and ammonia near the solvent front, by developing the plate first with ethyl acetate. Two successive runs with chloroform—tetrachloromethane—methanol (70:30:5) then adequately separated the acetylated polyamines (Fig. 2). Samples separated by cyclohexane—ethyl acetate (1:1) result in separations shown in Fig. 3. It should be pointed out here that the low background fluorescence achieved in these chromatographic separations is due to the pre-separation of the urine samples on Dowex 50W-X8 columns, and especially due to the exposure of the Dns derivatives to alkaline conditions before separation, as is described in detail in the Methods section.

Urine contains a large number of compounds with primary amino groups. It is therefore difficult to establish the specificity of the method. The criteria for the uniformity of the spots of the Dns derivatives of monoacetylputrescine, and of N^1- and N^8-monoacetylspermidines were: (a) elution of the non-derivatized amines from Dowex 50W-X8 columns with 4 *N* HCl and 6 *N* HCl,

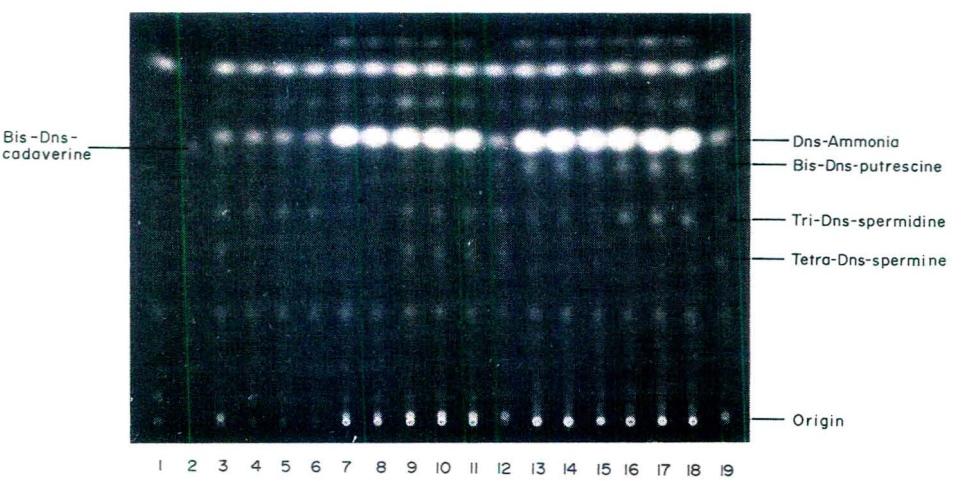


Fig. 2. Thin-layer chromatogram of Dns derivatives, standard samples and dansylated urine fractions. Solvent: 1st run: ethyl acetate; 2nd and 3rd run: chloroform—tetrachloromethane—methanol (70:30:5). 1 = Blank; 2 = tri-Dns-monoacetylspermine; 3—5, 10, 11, 19 = standard mixture containing 50 pmol of mono-Dns-monoacetylputrescine, bis-Dns-N^1-monoacetylspermidine, bis-Dns-N^8-monoacetylspermidine, bis-Dns-putrescine, tri-Dns-spermidine and tetra-Dns-spermine; 6—8 = Dns derivatives of urine samples (equivalent to 10 µl of urine); 12—15 = urine samples corresponding to 6—8, however, with added standard mixture (corresponding to 3—5); 16—18 = urine samples, hydrolyzed prior to derivatization with 6 *N* HCl. The Dns derivatives of the free polyamines move under these chromatographic conditions faster than Dns-ammonia. They cannot be detected on this chromatogram.

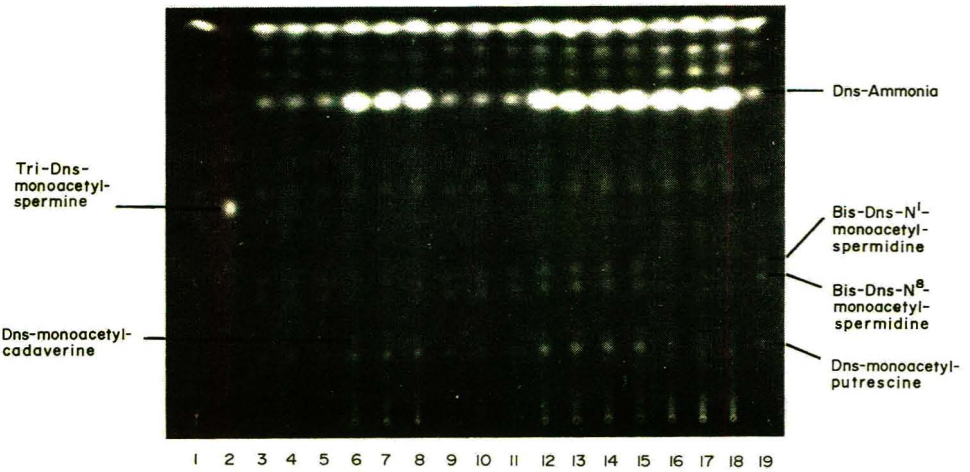


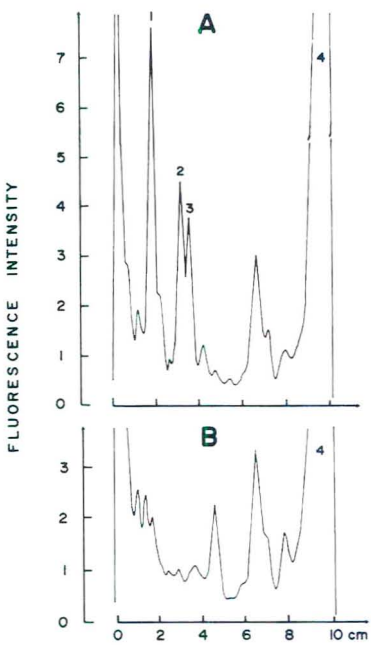
Fig. 3. Thin-layer chromatogram of Dns derivatives, standard samples and dansylated urine fractions. Solvent: cyclohexane—ethyl acetate (1:1) (2 runs) and cyclohexane—ethyl acetate (3:2) (1 run). 1 = Blank; 2 = bis-Dns-cadaverine; 3, 12, 19 = standard mixture (containing 50 pmol of mono-Dns-acetylputrescine, bis-Dns-N^1-acetylspermidine, bis-Dns-N^8-acetyl spermidine, bis-Dns-putrescine, tri-Dns-spermidine and tretra-Dns-spermine); 4—6 = standards with 50 pmol monoacetylputrescine, N^1-monoacetylspermidine and N^8-monoacetyl spermidine, hydrolyzed with 6 *N* HCl prior to derivatization; 7, 8 = non-hydrolyzed urine (corresponding to 10 μl); 9—11 = urine with added standard mixture; 13—15 = urine hydrolyzed with 6 *N* HCl; 16—18 = urine with added monoacetylputrescine, N^1-acetylspermidine and N^8-acetylspermidine, hydrolyzed with 6 *N* HCl prior to derivatization.

respectively; (b) identical mobility of the non-derivatized amines on thin-layer electrophoretograms using pyridine—acetic acid buffer pH 4.8 [47]; (c) identical mobility of the Dns derivatives of these compounds in various solvents, and (d) upon hydrolysis of the urine samples prior to ion-exchange column chromatography with 6 *N* HCl (120°; 12 h) and dansylation, spots did not appear on the chromatograms corresponding with the Dns-acetyl-polyamines (Fig. 2), but rather in zones corresponding to the Dns derivatives of the free polyamines (Fig. 3).

Sensitivity, recovery, reproducibility

As might be expected, the method has the usual sensitivity of procedures using dansylation. With the stabilization of the Dns derivatives by silicon oil, and using the Camag TLC-scanning equipment, it was possible to routinely measure picomole amounts of the various acetylated and free polyamines on normal (20 × 20 cm) thin-layer plates. For the measurement of urine samples the aliquot of the Dns derivatives normally applied on the plates, corresponded to 10 μl of urine and 50 pmol of polyamine.

It is known from previous work [41] that recovery of Dns-polyamines during the derivatization reaction is high. No attempt was made, therefore, to determine the total recovery. However, the recovery of the free and acetylated polyamines from the ion-exchange column chromatographic procedure has been determined. Standard samples, containing 2, 5, 7.5 and 10 nmol



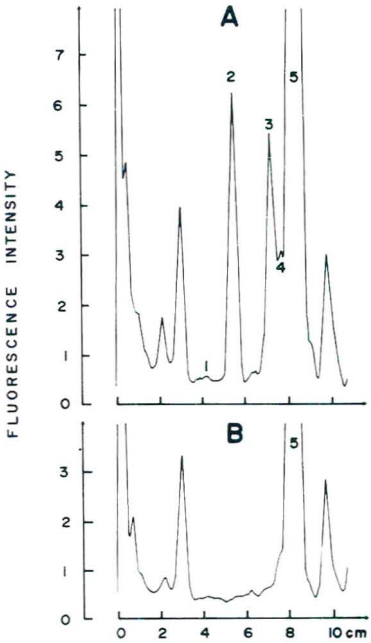


Fig. 4. In situ fluorescence scans of the Dns derivatives of urine constituents. (A) Scan of track 12; (B) scan of track 16 of Fig. 2. 1 = Dns-monoacetylputrescine; 2 = bis-Dns-N^8-acetyl-spermidine; 3 = bis-Dns-N^1-acetylspermidine; 4 = Dns-ammonia. For details see legend to Fig. 2.

Fig. 5. In situ fluorescence scans of the Dns derivatives of urine constituents. (A) Scan of track 13; (B) scan of track 7 of Fig. 3. 1 = Tetra-Dns-spermine; 2= tri-Dns-spermidine; 3 = bis-Dns-putrescine; 4 = bis-Dns-cadaverine; 5 = Dns-ammonia. For details see legend to Fig. 3.

respectively of each amine were applied on the columns; the columns were washed with 25 ml of 2.0 *N* HCl and the amines were then eluted with 25 ml of 6 *N* HCl. After evaporation the samples were dansylated, and similar samples not run through the ion-exchange columns were dansylated in parallel. From the 4 ml of toluene extracts, containing the mixture of Dns-derivatives, 20-μl aliquots were separated by TLC and measured by fluorescence scanning. The standard deviation of the determinations was ± 7% within the range of 10 to 50 pmol. There was no significant difference between the amounts of amines recovered from the ion-exchange columns and those subjected to dansylation directly. This shows that recovery of the acetylated amines from the ion-exchange column was over 90%. The same is true for the free polyamines.

The high recovery of the acetylated polyamines indicates their stability against acid hydrolysis. Indeed, free polyamines were never detected on the chromatograms when the acetylated products were run through the procedure. This can also be seen from Fig. 3 and Fig. 5B which show separations and a scan of a non-hydrolyzed urine sample, respectively. Virtually no free polyamines are detectable in this sample, prior to acidic hydrolysis. In order to

test the capacity of the 5-ml Dowex 50W-X8 columns, increasing volumes of the same urine sample were separated, and polyamines were determined subsequently. Fig. 6 shows the results. It appears from this figure that a linear relationship exists between the volume of the urine sample and the amount of the monoacetylspermidines, at least up to 10 ml of urine. In other words, the 5-ml Dowex 50W-X8 columns quantitatively retain the monoacetylspermidines even of 10 ml of urine. In contrast, monoacetylputrescine is lost from the column if more than 2 ml of urine are applied. This finding is in agreement with the fact that monoacetylputrescine and putrescine are eluted from Dowex 50W-X8 columns with 4 *N* HCl already whereas monoacetylspermidines and the free polyamines (spermidine and spermine) come off the column only with 6 *N* HCl [47].

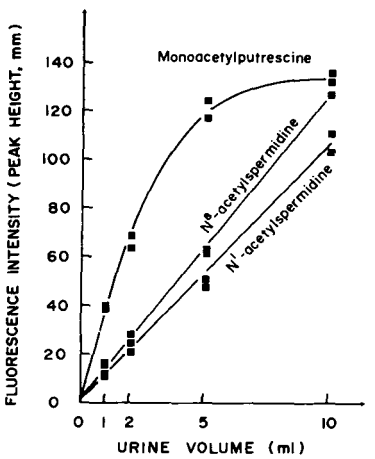


Fig. 6. Relationship between urine sample volume and recovery of monoacetylputrescine, N^1-acetylspermidine and N^8-acetylspermidine, from a 5-ml Dowex 50W-X8 column. In contrast with the acetylspermidines, significant losses of monoacetylputrescine are observed if the sample volume exceeds 2 ml. For details of the washing and elution procedure see Methods section.

In practice the urine sample volume was kept constant at 2 ml. When only the monoacetylspermidines (or non-conjugated spermidine and spermine) are to be analyzed, the column volume can be decreased to 1 ml, with a concomitant decrease of the eluent volumes (10 ml of 2.0 *N* HCl for washing, and 6 ml of 6 *N* HCl for elution).

Recovery of polyamines after hydrolysis with 6 *N* HCl (120°, 18 h) of their acetyl derivatives was estimated by hydrolysis of standard mixtures and by hydrolysis of urine samples to which known amounts of the acetyl derivatives were added. Recoveries in these two types of samples were identical within the precision of the method. The mean recovery for putrescine from acetylputrescine was 94%, for spermidine from N^1- and N^8-monoacetylspermidine, 63%.

Acetylated polyamines in human urine

Five adult healthy males and five adult healthy females participated in this exploratory study. No restrictions were imposed on food or fluid intake. Urine was collected for 24-h periods at two different times, and the urine samples were analyzed as described above.

The results of the acetylpolyamine measurements are shown in Table I. From a further male, 24-h urine was collected on six consecutive days. Again there was no restriction with regard to food or fluid intake. The results of the analyses of this experiment are shown in Table II. It appears from the values of the tables that the daily variation of excretion of acetylated polyamines is of the order of 10—20% of the mean, and that the inter-individual variation is much greater. High excretors may excrete as much as 12—16 μmol/day of the acetylated spermidines, and low excretors only about 50% of this value. In

TABLE I

EXCRETION OF ACETYLATED POLYAMINES IN THE URINE (μmol/24 h) OF MALE AND FEMALE ADULT VOLUNTEERS

Monoacetylputrescine: AcPut; N^1-monoacetylspermidine: N^1-AcSpd; N^8-monoacetylspermidine: N^8-AcSpd. (a) urine collection January 25; (b) urine collection February 13. Mean values of duplicate determinations.

Subject no.		AcPut	N^1-AcSpd	N^8-AcSpd	N^1-AcSpd + N^8-AcSPd	N^1-AcSpd / N^8-AcSPd
Male						
1	a	20.28	6.91	5.75	12.66	1.20
	b	19.05	6.61	5.72	12.33	1.16
2	a	16.70	3.99	3.45	7.34	1.16
	b	13.79	5.50	4.54	10.04	1.21
3	a	27.38	6.35	6.10	12.45	1.04
	b	18.20	7.66	5.22	12.88	1.47
4	a	23.11	10.35	6.30	16.65	1.64
	b	13.23	6.26	6.08	12.34	1.03
5	a	22.65	5.13	4.99	10.12	1.03
	b	16.38	4.57	4.62	9.19	0.99
mean ± S.D.	a	22.0 ± 3.9	6.6 ± 2.4	5.3 ± 1.2	11.8 ± 3.4	1.21 ± 0.25
mean ± S.D.	b	16.1 ± 2.6	6.1 ± 1.2	5.2 ± 0.7	11.4 ± 1.6	1.17 ± 0.19
Female						
6	a	14.49	4.45	2.59	7.04	1.72
	b	12.54	3.34	2.11	5.45	1.58
7	a	22.09	4.52	3.48	8.00	1.30
	b	20.24	3.88	3.27	7.15	1.19
8	a	22.39	3.00	5.93	8.93	0.51
	b	16.45	2.60	4.41	7.01	0.59
9	a	28.97	6.77	4.95	11.72	1.37
	b	18.46	5.31	3.44	8.75	1.54
10	a	17.00	4.71	3.76	8.47	1.25
	b	13.04	4.81	4.23	9.04	1.14
mean ± S.D.	a	21.0 ± 5.6	4.7 ± 1.4	4.1 ± 1.3	8.8 ± 1.8	1.23 ± 0.4
mean ± S.D.	b	16.2 ± 3.4	4.0 ± 1.1	3.5 ± 0.9	7.5 ± 1.5	1.21 ± 0.4
mean of all measurements ± S.D.		18.8 ± 4.6	5.3 ± 1.8	4.4 ± 1.2	9.9 ± 2.8	1.21 ± 0.3

TABLE II

EXCRETION OF ACETYLATED POLYAMINES IN THE URINE (μmol/24 h) OF AN ADULT MALE VOLUNTEER* DURING SIX CONSECUTIVE DAYS

Monoacetylputrescine: AcPut; N^1-monoacetylspermidine: N^1-AcSpd; N^8-monoacetylspermidine: N^8-AcSpd. Mean values of duplicate determinations.

Day	AcPut	N^1-AcSpd	N^8-AcSpd	N^1-AcSpd + N^8-AcSpd	N^1-AcSpd / N^8-AcSpd
1	15.14	3.65	2.84	6.49	1.29
2	15.97	3.96	3.18	7.14	1.25
3	14.39	4.08	3.54	7.62	1.15
4	15.33	4.76	3.82	8.58	1.25
5	15.22	5.70	3.74	9.44	1.52
6	15.48	5.69	3.91	9.60	1.46
Mean ± S.D.	15.26 ± 0.52	4.64 ± 0.89	3.51 ± 0.42	8.15 ± 1.3	1.32 ± 0.14

*Subject No. 11.

repeated urine collections the pattern of excreted polyamine conjugates remaines practically the same, indicating that the method gives consistent results.

Free and total polyamines in human urine

The amounts of non-conjugated putrescine and spermidine were less than 10% of the total amines. In contrast, most of the spermine excreted in human urine seems to be non-conjugated. Total spermine found in the two urine collections from five males was: 2.2 ± 0.9 μmol/24 h and 1.3 ± 0.6 μmol/24 h. The free amine was only 10% less than the total. Interestingly, females excreted significantly less spermine. 0.7 ± 0.4 μmol/24 h were found in the average in the two collections, with only one excretor above 1 μml/24 h.

The values for total spermidine and putrescine (means of five males and five females) after correction for losses during hydrolysis were: putrescine 18.7 ± 6; spermidine 10.2 ± 2. These values are in excellent agreement with the average amounts of acetylputrescine and acetylspermidines, found in the same urine samples (Table I). It can be concluded from this finding that acetylputrescine and the two acetylspermidines are the major excretory forms of putrescine and spermidine, respectively.

DISCUSSION

The method described in this paper is sufficiently rapid and accurate to be routinely applied to the analysis of acetylputrescine, the acetylspermidines and free polyamines in human urine. Monoacetylspermine and monoacetylcadaverine are also sufficiently separated from the other spots to be detected, if present in significant amounts.

The cleanup procedure, using Dowex X8 columns was based on the work of Shimizu et al. [48], who suggested a comparable procedure for the determination of free polyamines from nervous tissue. Another procedure also employing

6 *N* HCl for the elution of free polyamines from Dowex 50W columns has been devised by Fujita et al. [49]. As was shown in the present work, the thin-layer chromatograms of dansylated polyamines can be considerably improved by the cleanup steps. Other methods of polyamine analyses might also profit from this procedure. The impregnation of the plates with a viscous oil by dipping is technically easier than the hitherto used spraying.

The analyses of acetylated polyamines in the urine of only ten adult healthy volunteers (five males and five females) can only be considered as being preliminary. However, these analyses together with the repeated determinations of the 24-h output of a single individual show already that the inter-individual variation is only about ±30% of the mean. They also show that the individual pattern of urinary acetylpolyamines is relatively constant, and not influenced too greatly by food and fluid intake. Abdel-Monem and Ohno [32] reported the following "normal" values for acetylputrescine and the acetylspermidines, based on the analyses of urines from nine individuals: monoacetylputrescine: 11.7 ± 1.5 μmol/24 h; N^1-monoacetylspermidine: 2.9 ± 0.6 μmol/24 h; N^8-monoacetylspermidine: 2.8 ± 0.5 μmol/24 h. These values are at the lower end of the range of reported total urinary excretion of putrescine and spermidine [16]. The values found in this work (Table I) are somewhat higher than these reported values [32], but are in agreement with them, in so far as the ratio of N^1- and N^8-monoacetylspermidine is close to one in both cases.

Hydrolysis of the urine samples showed that total putrescine and spermidine values are close to the value of acetylputrescine and the sum of the acetylspermidines. If more extended experience confirms the impression of the present finding, that virtually all conjugated putrescine and spermidine is represented by the monoacetylderivatives, hydrolysis of the urine samples may become dispensable. This step is one of the major sources for the limited precision of the method. Since detailed information of the polyamine pattern of urine can be obtained by the separation of the dansyl derivatives of non-hydrolyzed samples, if run in two different solvents, our method should be a useful tool in the exploration of the diagnostic value of urinary polyamine analysis in diseased states.

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SIMULTANEOUS GAS CHROMATOGRAPHIC DETERMINATION OF LORCAINIDE HYDROCHLORIDE AND THREE OF ITS PRINCIPAL METABOLITES IN BIOLOGICAL SAMPLES

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SUMMARY

A method is described for the determination of the antiarrhythmic drug lorcainide hydrochloride and its three main metabolites in plasma, urine, faeces and tissues from man and animals. The procedure involves the extraction of the parent drug, its metabolites and the internal standard from the biological materials at different alkaline pH values, back-extraction into sulphuric acid and re-extraction into the organic phase (heptane—isoamyl alcohol). After silylation of the different phenolic and the N-dealkylated metabolites, analyses were carried out by automated gas—liquid chromatography with electron-capture detection. The method has a sensitivity limit of 5 ng for lorcainide, and 10—20 ng for the various metabolites, per millilitre of plasma.

The method was applied to urine, faeces, plasma and tissue samples from man and animals. It was also suitable for automatic sample analysis.

INTRODUCTION

Lorcainide hydrochloride, N-(4-chlorophenyl)-N-[1-(1-methylethyl)-4-piperidinyl] benzeneacetamide monohydrochloride (I; Fig. 1), is a promising new antiarrhythmic agent, effective against ventricular arrhythmias and atrial fibrillation [1—4]. Studies concerned with its metabolic fate have identified the N-dealkylated and three phenolic metabolites in human plasma [5, 6].

This paper describes a sensitive procedure for the simultaneous determination of both lorcainide and the main metabolites in plasma, urine, faeces and tissue samples. The method was used to obtain more detailed information about the pharmacokinetics of the drug in man and animals [7, 8].

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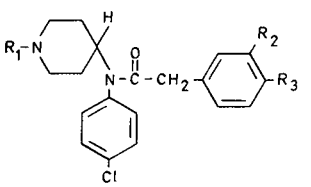
EXPERIMENTAL

Standards and reagents

Lorcainide hydrochloride, nor-lorcainide or N-(4-chlorophenyl)-N-(4-piperidinyl)benzeneacetamide (II), N-(4-chlorophenyl)-4-hydroxy-N-[1-(1-methylethyl)-4-piperidinyl] benzeneacetamide (III), N-(4-chlorophenyl)-4-hydroxy-3-methoxy-N-[1-(1-methylethyl)-4-piperidinyl[benzeneacetamide (IV), and the internal standard, N-(4-chlorophenyl)-N-[1-(3-methylbutyl)-4-piperidinyl] benzeneacetamide hydrochloride (V), were all originally synthesized in our research laboratories and were of analytical grade. Chemical structures are shown in Fig. 1.

Spectrophotometric grade *n*-heptane and acetonitrile were used; methanol and isoamyl alcohol were of analytical grade. The silylating reagent consisted of N,O-bis(trimethylsilyl)acetamide (BSA), to which 1% of trimethylchlorosilane (TMCS) had been added as a catalyst (Aldrich-Europe, Beerse, Belgium).

The inorganic reagents were prepared in double-distilled water. A borate buffer (pH 8.5) was prepared by adding 15.2 ml of 0.1 *M* hydrochloric acid to 50 ml of 0.025 *M* sodium borate decahydrate (borax). A final volume of 100 ml was prepared.



Compound	R_1	R_2	R_3
R 15889 (I)	$CH(CH_3)_2$	H	H
R 15665 (II)	H	H	H
R 39611 (III)	$CH(CH_3)_2$	H	OH
R 41853 (IV)	$CH(CH_3)_2$	OCH_3	OH
R 17251 (V)	$CH_2CH_2CH(CH_3)_2$	H	H

Fig. 1. Chemical structures of lorcainide (R 15889), its principal metabolites (R 15665, R 39611 and R 41853) and the internal standard (R 17251).

Standard solutions

A combined standard solution containing 25 mg of all synthesized drugs (I, II, III and IV) as the free bases was prepared in 25 ml of methanol.

A 10-ml volume of the internal standard solution was also prepared at a

concentration of 1 mg/ml. To spike the samples with the internal standard, an aliquot of this stock solution was further diluted to 2 μg/ml.

All the solutions were stored in a refrigerator at 4° prior to use.

Extraction procedure

Plasma. Plasma samples (1 ml) were pipetted into 15-ml glass centrifuge tubes and spiked with 0.2 μg of the internal standard. The solution was buffered with 2.5 ml of the borate buffer solution (pH 8.5) and 4 ml of heptane—isoamyl alcohol (95:5, v/v) were added. The tubes were carefully rotated for 10 min (35 rpm, Cenco rotary mixer) and then centrifuged (5 min, 1000 *g*). The upper organic layer was transferred to a second centrifuge tube, containing 2 ml of 0.05 *M* sulphuric acid. The organic layer was removed and discarded after shaking and centrifugation. After repetition of this step, the plasma in the first tube was made more alkaline by adding a few drops of 1 *M* sodium hydroxide (pH 11) and extracted twice with 4 ml of the organic solvent mixture. Both upper organic layers were transferred to the second tube and discarded after extraction with the sulphuric acid. The remaining acidic phase was made basic with concentrated ammonia (pH 10) and extracted twice with 2 ml of the heptane—isoamyl alcohol mixture. The combined organic layers were evaporated to dryness under a stream of nitrogen in a water bath at 60°.

Other samples. Human urine and faeces as well as different animal tissues were similarly processed.

Urine samples of 0.1 ml were processed by the present extraction procedure.

Faeces were homogenized (1:5, w/v) in methanol, using an Ultra-turrax TP 18/2 homogenizer. After centrifugation, the precipitates were washed twice with methanol and centrifuged again. The various supernatants of each faeces sample were mixed and the volume was measured. One millilitre of each of these faeces extracts was then submitted to the extraction procedure.

Tissue samples of 2 g were homogenized in 100-ml centrifuge tubes, containing 7.5 ml of distilled water and 2.5 ml of the borate buffer solution. The homogenates were processed as described above.

The extraction procedure is outlined in Fig. 2.

Calibration procedure

Samples of control plasma (1 ml) were spiked with lorcainide and its metabolites at concentrations ranging from 0.01 to 3 μg/ml, and with the internal standard at a fixed concentration of 0.2 μg/ml. The samples were taken through the extraction procedure described previously.

Apparatus

All the analyses were performed on a Varian Model 3700 gas chromatograph equipped with a Varian Model 8000 automatic sample injector and a pulse-modulated constant-current ^{63}Ni electron-capture detector.

The glass column (200 × 0.2 cm) was packed with 3% OV-22 on 80—100 mesh Supelcoport (Supelco, Bellefonte, Pa., U.S.A.). The column temperature was 260° and the injector and detector temperatures were 290° and 320°, respectively. Nitrogen was used as a carrier gas at a flow-rate of 40 ml/min.

Area integrations, calculations and plotting of the chromatograms as well as

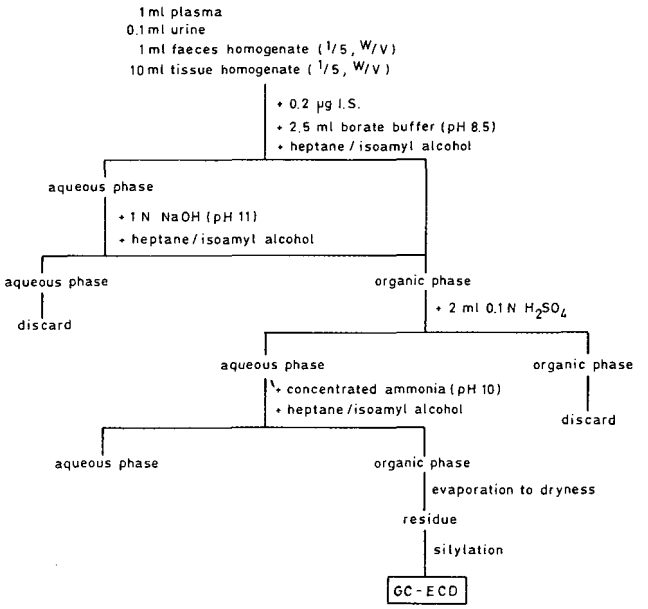


Fig. 2. Extraction scheme for lorcainide and its principal metabolites from plasma, urine, faeces and tissues.

the control of the autosampler functions were carried out by a Spectra-Physics Model 4000 data system.

Derivatization

The extraction residues were dissolved in 10 µl of BSA, containing 1% trimethylchlorosilane, and 100 µl of acetonitrile. The tubes were capped tightly, vortex-mixed, and allowed to stand for 10 min in an oil bath at 90°. After cooling, the samples were transferred to 200-µl microvolume vials and placed in the autosampler. Sample volumes of 1 µl were injected and cleaning of the injection system was achieved by alternating sample vials and vials of pure solvent.

Calculations

Results were calculated by determining the peak area ratios of the different compounds, related to the internal standard and comparing these ratios with the appropriate standard curves.

RESULTS

The recoveries of the extraction procedure, the detection limits and the retention times of lorcainide, its metabolites and the internal standard are summarized in Table I. The plots of the standard curves all passed through zero and were linear over the concentration range 0.01—3.0 µg/ml plasma, having correlation coefficients of 0.999 (Table II).

TABLE I

ANALYTICAL DATA FOR THE REPORTED PROCEDURE

Compound		t_R (min)	RRT**	Percentage recovery*** (mean ± S.D., n = 5)	Detection limit (ng/ml plasma)
R 15665*	(II)	1.88	0.57	85 ± 3	10
Lorcainide	(I)	2.53	0.77	81 ± 4	5
R 17251	(V)	3.28	1.00	88 ± 6	—
R 39611*	(III)	4.28	1.30	78 ± 4	10
R 41853*	(IV)	5.87	1.79	69 ± 8	15

*Analyzed as the trimethylsilyl derivatives.

**RRT = relative retention time of the compounds (relative to the internal standard, R 17251).

***Percentage recovery = recovery of the extraction procedure, obtained after analysis of 1 μg of the appropriate compound added to 1 ml of control plasma.

TABLE II

CORRELATION COEFFICIENTS AND MATHEMATICAL EXPRESSIONS OF THE STANDARD CURVES OF LORCAINIDE AND ITS METABOLITES

R 15889	(I)	$y = 5.097x + 0.039$	$r = 0.9997$
R 15665	(II)	$y = 4.455x - 0.085$	$r = 0.9996$
R 39611	(III)	$y = 4.336x - 0.004$	$r = 0.9998$
R 41853	(IV)	$y = 4.413x - 0.094$	$r = 0.9996$

DISCUSSION

Although the extraction of the unaltered drug and its internal standard was rather simple, an additional extraction step at pH 8.5 was necessary for the optimum recovery of the hydroxylated metabolites III and IV. The formerly used extraction step at pH 11, however, was maintained because it led to a higher recovery for the dealkylated metabolite II. Its recovery was further improved by taking a ratio of 95:5 for the heptane—isoamyl mixture instead of the more commonly used mixture ratio of 98.5:1.5. This improvement was also achieved using hexane—diethyl ether (50:50) and toluene—butanol (90:10) mixtures although both these latter mixtures gave a few more extraneous peaks. No interfering peaks were observed using the heptane—isoamyl alcohol (95:5) mixture.

It is clear that for the determination of lorcainide alone, as, for example, after intravenous administration, the extraction method can be simplified as has been described by Jähnchen et al. [9].

As the metabolites examined are rather polar, they are not very suitable for direct gas–liquid chromatographic analysis and therefore they have to be derivatized to obtain reliable results. Both the N-dealkylated and the hydroxylated metabolites were easily derivatized with dimethylformamide dimethylacetal, but the resulting derivatives still showed some adsorption and yielded larger retention times. Trimethylsilyl (TMS) derivatives were chosen

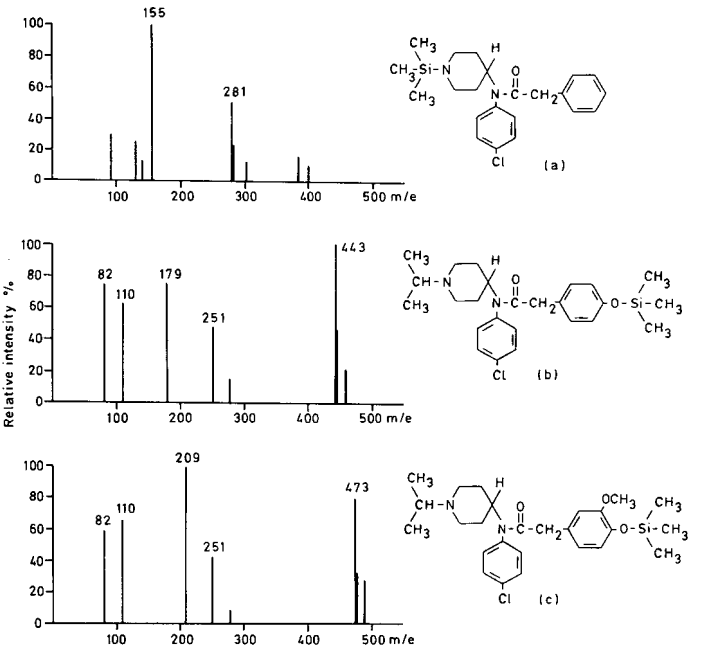


Fig. 3. Mass spectra of the TMS derivatives of compounds II (a), III (b) and IV (c).

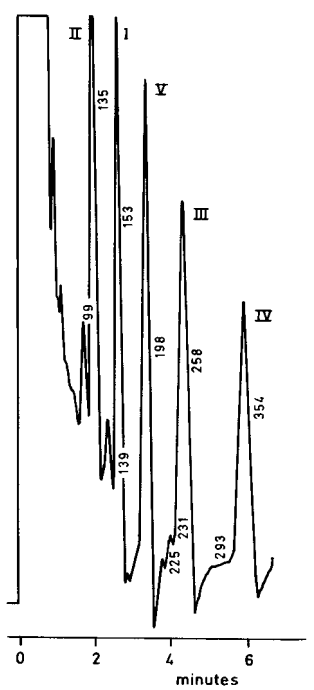


Fig. 4. Chromatogram of a heart (ventriculus cordis) tissue extract from a dog chronically treated with daily oral doses of 10 mg/kg body weight. GLC conditions were as indicated in the text.

because they reacted rapidly, yielding derivatives with much improved column characteristics under the chromatographic conditions described. The O-silylation with BSA occurred instantaneously and quantitatively, the N-silylation yielded at least about 95% after heating the reaction mixture at 90° for 10 min and adding 1% TMCS to the derivatization reagent to catalyze the reaction. Structure identification of the various TMS derivatives was recently confirmed by combined gas chromatography—mass spectrometry and nuclear magnetic resonance spectroscopy [6]. Mass spectra and structures are shown in Fig. 3.

The application of the method to the assay of several hundred specimens of biological origin demonstrated its suitability; other metabolites were easily detected when present and did not interfere with lorcainide or its most important metabolites. The gas chromatographic column has proved to be extremely stable under the conditions used.

APPLICATIONS

The method described has been used successfully for over one year in pharmacokinetic studies on the fate of lorcainide in healthy volunteers and patients as well as in animals [8]. Typical plasma levels from a patient receiving chronic oral administration are presented in Table III. The patient received five consecutive doses of 100 mg at 0, 12, 24, 36 and 48 h and the time course of the plasma levels was then followed up to 32 h after the last dose. After ten days, the same schedule was repeated with doses of 150 mg of lorcainide hydrochloride.

The method has also proved to be valuable in tissue distribution studies of dogs receiving 0.625, 2.5 and 10 mg/kg body weight [7]. A chromatogram of a heart tissue extract, showing all three metabolites, is represented in Fig. 4.

TABLE III

PLASMA LEVELS FOLLOWING CHRONIC ADMINISTRATION OF 100 mg (A) AND 150 mg (B) OF LORCAINIDE HYDROCHLORIDE

Time (h)	Lorcainide hydrochloride (μg/ml plasma)	
	A	B
0	0.214	0.481
2	0.234	0.627
4	0.406	0.873
6	0.285	0.602
8	0.285	0.562
10	0.296	0.437
12	0.215	0.429
24	0.161	0.393
26	0.192	0.421
28	0.171	0.321
30	0.125	0.380
32	0.155	0.294

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DYNAMIC CATION-EXCHANGE SYSTEMS FOR THE SEPARATION OF DRUGS DERIVED FROM BUTYROPHENONE AND DIPHENYLPIPERIDINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND APPLIED IN THE DETERMINATION OF HALOPEMIDE IN PLASMA

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SUMMARY

Dynamic (solvent generated) cation-exchange systems for the separation of drugs and main metabolites derived from butyrophenone and diphenylpiperidine (haloperidol, pimozide, halopemide) were investigated.

The effect of organic modifier, detergent, counter-ion concentration and of the pH on the retention has been determined. The results show that variation of these parameters permits adjustment of the retention of these drugs over a wide range.

The dynamic cation-exchange system developed was applied to the determination of halopemide and its main metabolite in plasma. The precision and detection limit of the method and the extraction efficiency were established. The time course of halopemide and plasma levels of patients chronically receiving halopemide are reported.

INTRODUCTION

The necessity of determining drugs and their metabolites in body fluids has been generally accepted for different reasons (for example, pharmacokinetics, bioavailability, biotransformation studies, patient compliance) [1—3]. Among the variety of analytical methods applied for this purpose, high-performance liquid chromatography (HPLC) has proved to be pre-eminently suited for this problem [4—6]. In most instances reversed-phase systems with alkyl-modified silicas were applied. Recently, however, the so-called solvent generated ion-exchange systems were found to be very suitable for the chromatography

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of basic compounds in particular [7—10]. The selectivity and column performance of these dynamic ion-exchange systems is sometimes significantly better than those obtained with the normal reversed-phase systems [9, 10].

In the present study we report the results of an investigation into the use of solvent generated cation-exchange systems with sodium dodecyl sulfate for the analysis of drugs and their metabolites derived from the butyrophenones and from the diphenylbutylpiperidines, of which haloperidol and pimozide are well known prototypes.

The ability of the solvent generated cation-exchange system developed for the analysis of a new psychotropic drug halopemide [N-{2-[4-(5-chloro-2,3-dihydro-2-oxo-1H-benzimidazol-1-yl)-1-piperidinyl] ethyl} -4-fluorobenzamide] (Fig. 1,I) in plasma of volunteers, and of patients chronically receiving this new drug, is demonstrated.

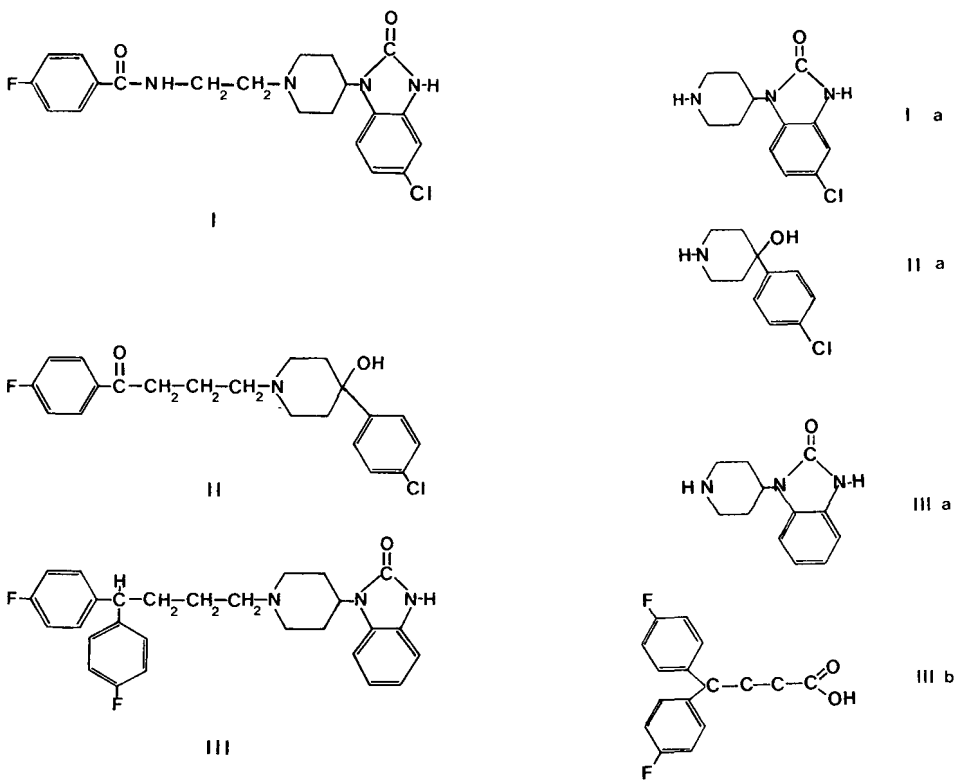

Fig. 1. Structural formulae of the selected compounds. I, Halopemide; Ia, main metabolite of halopemide; II, haloperidol; IIa main metabolite of haloperidol; III, pimozide; IIIa and IIIb, main metabolites of pimozide.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a reciprocating pump (Orlita, Type AE-10-4.4), a Bourdon type manometer, a high-pressure injection valve

(Rheodyne 7105) equipped with a sample loop of 150 μl, an UV spectrophotometer (Perkin Elmer LC-55), a linear potentiometric recorder (Goerz, Servogor RE542) and an electronic integrator (Autolab I). In all of the experiments stainless-steel columns of I.D. 3 mm and length 125 or 150 mm were used. The wavelength of the UV detector was set at 210 nm.

Materials

In all experiments double-distilled water was used. All solvents and chemicals were of analytical grade and used without any further pre-treatment. Sodium dodecyl sulfate (SDS) was obtained from Merck (Darmstadt, G.F.R.). The octyl-modified silica used as column support was RP-8, mean particle size 5 μm (Merck). The drugs and their metabolites were obtained from Janssen Pharmaceutica (Beerse, Belgium). Their structures are given in Fig. 1.

Procedures

Chromatography. The HPLC columns were packed by a pressurized balanced slurry technique [11] (the slurry liquid consisted of a mixture of chloroform and tetrabromoethane of specific gravity of 1.82); they were washed with 100 ml of methanol and then equilibrated with the eluent until constant retention of the compounds under investigation was obtained. Standard solutions of the drug and its metabolites were prepared from stock solutions of the compounds in methanol (5.7 mg/100 ml) and were stored in a refrigerator at 4°.

Sample preparation. Blank pooled plasma and plasma samples containing halopemide were stored at −20°. After thawing, all samples were ultrasonicated for 3 min. To extract halopemide and its metabolite 0.2 ml of 2 *M* NaOH and 10.0 ml of $CHCl_3$ were added to 1 ml of plasma in a glass stoppered centrifuge tube. The tube was shaken for 1 min and centrifuged (5 min at 1730 *g*). The aqueous phase was removed by aspiration and 9 ml of the organic layer were transferred, using a Hamilton syringe (type 1005), to another tube containing 1 ml of 2.1 *M* perchloric acid. The tube was shaken and centrifuged (for 1 min at 1730 *g*). A 500-μl portion of the aqueous phase was transferred to another tube which contained 250 μl of 4 *M* NaOH and 250 μl of this final solution were used to fill the sample loop.

RESULTS AND DISCUSSION

Chromatography

Dynamic (solvent generated) cation-exchange chromatography with sodium dodecyl sulfate (SDS) was chosen as the separation method for the selected drugs and their respective metabolites (Fig. 1). These dynamic cation-exchange systems were found to be very useful for the separation of amino acids [7] and basic substances such as tricyclic antidepressants [9] and catecholamines [10]. Compared to normal reversed-phase systems, dynamic cation-exchange systems exhibit more flexibility for adjusting selectivity and show significantly better column efficiency (for example, symmetrical peak shapes) [9, 10].

In order to find optimal chromatographic conditions for the separation of the selected drugs and possible metabolites, the influence of a number of param-

eters such as the methanol, SDS and counter-ion concentrations and the pH of the mobile phase on the capacity ratio was investigated.

Fig. 2 shows the influence of the methanol content of the mobile phase. The capacity ratio of basic solutes decreases with increasing methanol content, as is commonly found in reversed-phase systems. However, the decrease of the capacity ratio is mainly due to a decrease of the amount of SDS adsorbed at the hydrophobic support (comparable with the ion-exchange capacity) with increasing methanol content [8].

The influence of the SDS content of the mobile phase is shown in Fig. 3. The capacity ratio of the basic compounds increases with increasing SDS content of the mobile phase and levels off at larger amounts because of the shape of the adsorption isotherm of SDS [8]. For compound IIIb only physical adsorption occurs. Dynamic cation-exchange systems behave in a way similar to conventional ion-exchange systems [7, 8, 10]. This means that the capacity ratio can be adjusted in a predictable way by varying the counter-ion concentration (in this study Na^+) as shown in Fig. 4.

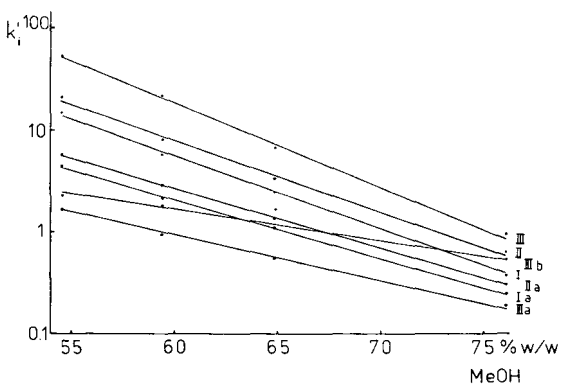

Fig. 2. Influence of the methanol concentration on the capacity ratio (k'_i). Stationary phase: C_8-modified silica. Mobile phase: 0.005 *M* NaH_2PO_4 + 0.1% (w/v) SDS + 0.2 *M* $NaClO_4$ (pH 6.5). Roman numerals as in Fig. 1.

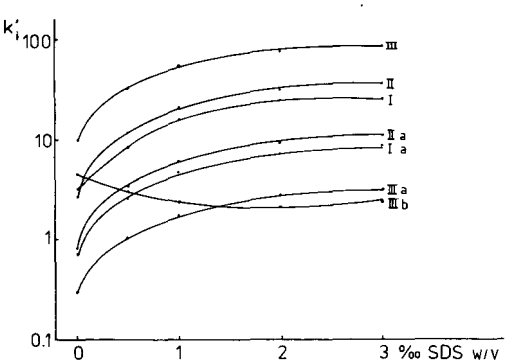

Fig. 3. Influence of the SDS concentration on the capacity ratio. Stationary phase: C_8-modified silica. Mobile phase: 0.005 *M* NaH_2PO_4 + 0.1 *M* $NaClO_4$ + 64.8% (w/w) methanol (pH 6.5). Roman numerals as in Fig. 1.

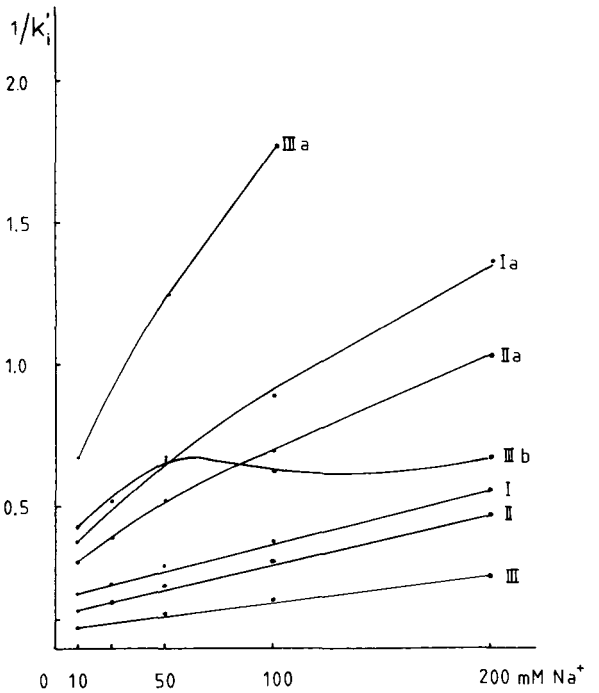

Fig. 4. Influence of the sodium ($NaClO_4$) concentration on the capacity ratio. Stationary phase: C_8-modified silica. Mobile phase: 0.005 *M* NaH_2PO_4 + 0.1% (w/v) SDS + 64.8% (w/w) methanol (pH 6.5). Roman numerals as in Fig. 1.

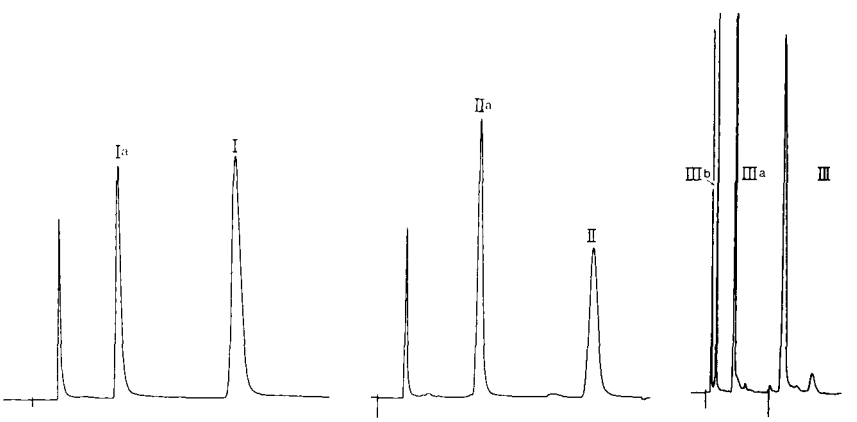

Fig. 5. Separation of test mixtures of selected compounds. Roman numerals are explained in Fig. 1. Stationary phase: C_8-modified silica. Mobile phase: 0.005 *M* NaH_2PO_4 + 0.3% (w/v) SDS + 2 *M* $NaClO_4$ + 59.4% (w/w) methanol (pH 3.00). Flow-rate, 0.60 ml/min.

The influence of the pH of the mobile phase on the capacity ratio was investigated using phosphate-buffered mobile phases of different pH values (range 2.0—6.5). No significant change in capacity ratio could be observed. At pH > 6.5 a significant decrease of the column stability was found. In agreement with previous observations [10] we found a better peak shape and column stability when relatively high sodium perchlorate concentrations were used in the mobile phase.

The results of the influence of methanol, SDS and Na^+ concentration on the capacity ratio as reflected in Figs. 2—4 demonstrate the great flexibility of dynamic cation-exchange systems for the separation of the selected drugs and metabolites. Optimal separation conditions for each of the drugs and its main metabolites can easily be derived from these figures as is demonstrated in Fig. 5.

DETERMINATION OF HALOPEMIDE IN PLASMA

The developed phase system was applied to the analysis of a new psychotropic agent called halopemide, a chemical congener of the neuroleptic benperidol, but with a totally different pharmaceutical and clinical profile [12]. According to the manufacturer this new drug lacks extrapyrimidal side-effects and looks promising as an effective drug for the treatment of psychosis, characterized by autism, emotional withdrawal or apathy.

The metabolism of halopemide has been studied in rats [13]. This study indicates that halopemide is most probably metabolized via an oxidative N-desalkylation into *p*-fluorohippuric acid and compound Ia.

Until now no data about the plasma time course of halopemide in man after a single dose and of plasma levels of patients chronically administered halopemide are known.

The suitability of the dynamic cation-exchange system and of the extraction procedure for the analysis of halopemide in plasma is demonstrated in Fig. 6, which shows chromatograms of extracts of blank and spiked (112 ng halopemide per ml) plasma.

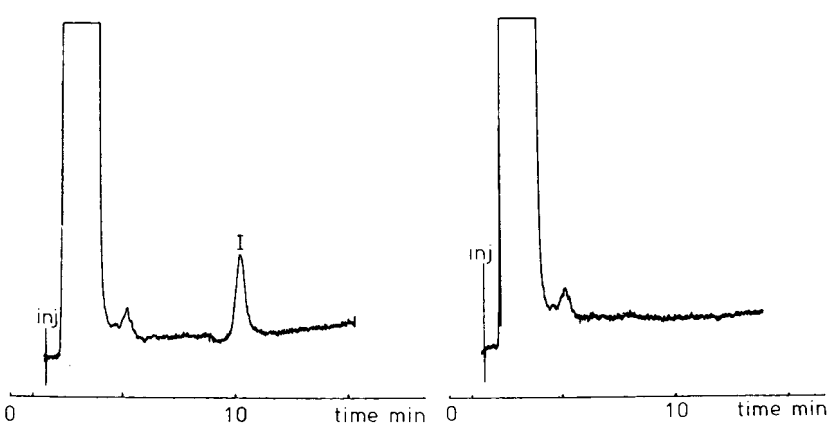

Fig. 6. Chromatogram obtained of an extract of a pooled blank plasma sample and of a plasma sample spiked with 112 ng of halopemide (I). For conditions see Fig. 5.

Quantitative aspects

The precision and linearity of the method were determined by injecting 150 µl of solutions of compounds I and Ia at different concentrations and measuring peak height or peak area. The regression of peak area or peak height vs. injected amount was found to be linear for halopemide up to 540 ng/ml, and up to 400 ng/ml for compound Ia, with a correlation coefficient for both compounds of 0.9988, indicating a high degree of linearity. The precision of the method was estimated from repeated injections (n = 10) of solutions of the compounds at high and low concentration levels. For both compounds the standard deviation, using peak height measurements, was 1.7% when 108 ng were injected and 3.0% with 5.0 ng injected. The peak-to-peak value of the baseline noise was 15×10^{-4} a.u. This led to a calculated limit of detection, for a signal-to-noise ratio of 3, of 1 ng for halopemide and 0.75 ng for compound Ia. This corresponds to a detection limit of 7.4 ng/ml plasma for halopemide and 12.9 ng/ml plasma for compound Ia for a given injection volume of 150 µl.

Recovery of the extraction

The recovery and reproducibility of the extraction were determined by spiking blank plasma samples with different amounts of halopemide and compound Ia and extracting as described under procedures. The recovery of halopemide was 98 ± 3% (n = 4) for halopemide and 43 ± 6% (n = 4) for Ia.

The recovery of compound Ia could be increased to 80% by using a more polar extraction liquid composed of diethyl ether and ethyl acetate (10:2, v/v). However, the greater recovery of compound Ia was accompanied by a much

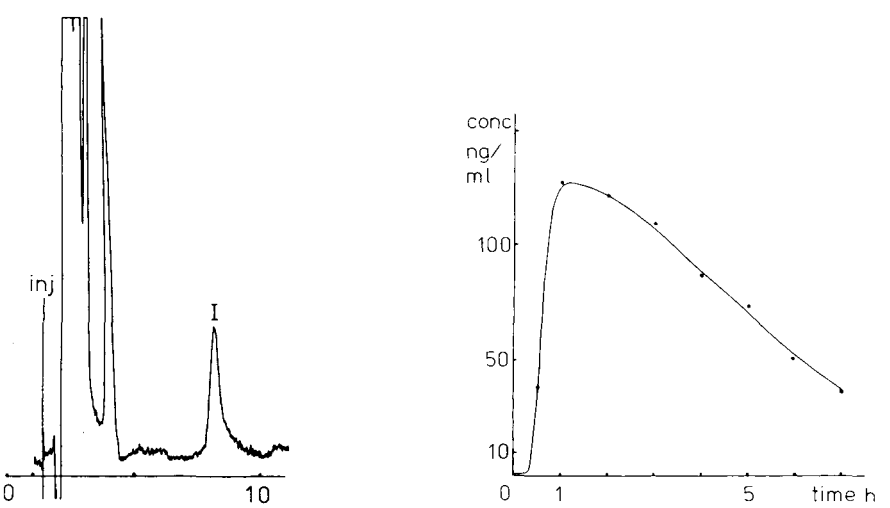

Fig. 7. Chromatogram of an extract of plasma of a patient who chronically received 20 mg of halopemide daily. Comedication was promethazine. For conditions see Fig. 5. Flow-rate 0.48 ml/min.

Fig. 8. Time course of halopemide in plasma of a volunteer after ingestion of a 20-mg dose.

larger background which seriously affects the quantitative determination of both substances. For this reason a milder extraction liquid, despite the low recovery of Ia, was found to be advantageous.

Time course and plasma level of halopemide in volunteers and patients

The developed method for the analysis of halopemide was applied to plasma samples of volunteers and patients. A typical chromatogram of an extract of plasma of a patient is given in Fig. 7. Fig. 8 shows the plasma time course of halopemide in a volunteer after an oral dose of 20 mg. From the data obtained a plasma half-life of 3.5 h could be calculated. The apparent volume of distribution in this case was calculated to be 145 l.

Plasma samples obtained from patients who were chronically treated with halopemide were also determined by HPLC. Promethazine, used as comedication, did not interfere in the analysis. The results of these analyses are given in Table I. In order to determine the main metabolite Ia, a phase system with a 5% lower methanol content was chosen, which guarantees the elution of this compound free from coextracted endogenous plasma constituents.

TABLE I

PLASMA LEVELS DETERMINED BY HPLC OF MALE PATIENTS CHRONICALLY TREATED WITH HALOPEMIDE

Patient No.	Daily dose of halopemide (mg)	Plasma concentration (ng/ml)
1	20	87
2	60	360
3	60	243
4	20	118
5	20	99

In none of the plasma samples of these patients was compound Ia present at levels greater than 12.9 ng/ml (detection limit). This result might indicate a fast elimination rate of this metabolite and/or a slow metabolism of the parent drug.

Analysis of halopemide and this metabolite in urine might give more information about the fate of halopemide in man.

CONCLUSION

Dynamic cation-exchange chromatography was found to be eminently useful for the analysis of the butyrophenon and diphenylalkyl piperidine type psychotropic drugs and their metabolites in plasma.

Plasma levels of halopemide in patients receiving halopemide (20—60 mg daily) chronically, ranged from 87 to 360 ng/ml. Until now no metabolites were found in the plasma at concentrations greater than 12 ng/ml. Future work will therefore be devoted to the analysis of halopemide and its metabolite (Ia) in urine.

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QUANTITATIVE ANALYSIS OF SULPIRIDE IN BODY FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A high-performance liquid chromatographic method for the analysis of sulpiride, N-ethyl-2-(2-methoxy-5-sulphonamido-benzamido-methyl)-pyrrolidine, in body fluids is described. A structurally related compound, N-ethyl-2-(2,4-dimethoxy-benzamido-methyl)-pyrrolidine, was used as internal standard.

A fluorescence detector with excitation maximum at 299 nm and emission maximum at 342 nm was used for the quantitation. The detection limit was about 10 ng/ml in serum and cerebrospinal fluid and about 200 ng/ml in urine. The experimental error was 5—10% in the concentration range 25—100 ng/ml. Some preliminary data from a pharmacokinetic study in healthy volunteers are presented. The half-life for sulpiride in serum was about 8 h. Sulpiride was also measured in cerebrospinal fluid from five drug-treated psychotic patients.

INTRODUCTION

Sulpiride, N-ethyl-2-(2-methoxy-5-sulphonamido-benzamido-methyl)-pyrrolidine (Fig. 1), is a neuroleptic drug with a structure that is different from other antipsychotic agents [1]. Like neuroleptics, phenothiazines and butyrophenones it markedly accelerates dopamine synthesis and metabolism in the brain but it has only a weak effect on dopamine stimulated adenylate cyclase. The frequency of extrapyramidal side effects is reported to be low compared to other neuroleptics [2, 3]. This makes sulpiride a theoretically and clinically interesting drug. The pharmacokinetics of sulpiride in man has not previously been systematically studied.

All the available analytical methods for sulpiride have some disadvantages for pharmacokinetic studies. A very specific mass fragmentographic method has

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been developed [4], but it is not sensitive enough. A spectrophotofluorometric method with high sensitivity has been described [5], but interference from other fluorescent compounds with similar extraction qualities cannot be excluded. A high-performance liquid chromatography (HPLC) separation method has also been developed [6], but this technique has not been used for the quantitative analysis of sulpiride.

To make pharmacokinetic studies of sulpiride possible, we have developed a sensitive and specific analytical method for sulpiride in body fluids using HPLC with fluorescence detection. To compensate for erratical loss of the compound during the extraction procedure an internal standard with a chemical structure and fluorescence qualities very similar to those of sulpiride was used (Fig. 1).

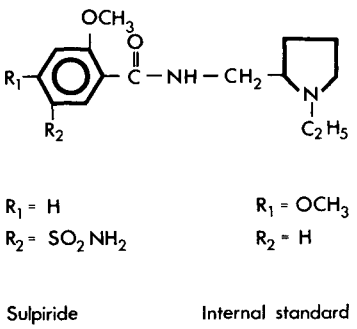

Fig. 1. Chemical structures of sulpiride and the internal standard.

EXPERIMENTAL

Materials

Acetonitrile for liquid chromatography was HPLC grade obtained from Rathburn Chemicals (Walkerburn, Great Britain). Other solvents and chemicals were of p.a. quality. Sulpiride was a gift from Delagrange (Paris, France). The internal standard, N-ethyl-2-(2,4-dimethoxy-benzamido-methyl)-pyrrolidine (Fig. 1), was a gift from Astra (Södertälje, Sweden).

HPLC was performed with a Spectra Physics System 3500B consisting of two solvent delivery pumps, a solvent programmer and a rotary valve injector with a 10-μl loop for syringe injection.

The fluorescence detector was a SFM 22 Model from Kontron (Stockholm, Sweden), equipped with a 10-μl flow-through cell. The recorder was a W+W 600 Tarkan.

A 15 cm × 4 mm reversed-phase column packed with 5 μm C_{18} Nucleosil (Macherey, Nagel & Co., Düren, G.F.R.) was used. The packing of the column was performed principally according to Bristow et al. [7].

Sample preparation

Serum and cerebrospinal fluid samples. The extraction was performed partly according to Kleimola et al. [5]. The internal standard (1 μg in 100 μl methanol) was added to 4.0 ml serum or cerebrospinal fluid (CSF) (or when high

levels were expected 2.0 ml serum) followed by 240 μl or 120 μl 0.5 *N* sodium hydroxide. The sample was mixed and 10.0 ml of chloroform was added. The test-tube was gently turned 10 times manually and centrifuged at 2000 *g* for about 5 min. A 9.0-ml aliquot of the chloroform layer was transferred to another test-tube. The water phase was extracted once more with 5.0 ml chloroform, 4.0 ml of which was combined with the first chloroform phase. If the test-tube with chloroform was contaminated by the water layer, the chloroform was transferred to a clean test-tube. The chloroform was evaporated nearly to dryness in a stream of nitrogen. During the evaporation the test-tube was heated with a hair drier. The residue in the tube was dissolved in about 1 ml methanol and transferred to a small conical tube. The methanol was evaporated to dryness and the residue was dissolved in 50 μl methanol and stored at −20° pending analysis.

Urine samples. To 1.0 ml urine about 1 g sodium chloride was added. The internal standard (5.0 μg in 50 μl methanol) was added and the pH was adjusted to 9.5—10.0 with 0.5 *M* sodium hydroxide (about 100 μl). The sample was extracted twice with 5.0 ml chloroform in the same manner as with the serum. The residue was dissolved in 100 μl methanol.

Preparation of standard curves

Standard curves were prepared by adding internal standard and known amounts of sulpiride in methanol to pool samples of the body fluid to be analysed. Several dilutions of sulpiride were made to keep the volume of methanol added to each sample small, between 25 and 100 μl. The range of the standard concentrations of sulpiride in serum were 10 ng/ml to 2500 ng/ml and for the corresponding urine samples 0.5 μg/ml to 100 μg/ml.

Liquid chromatography

The solvent in pump A consisted of water—acetic acid (99:1) and in pump B of acetonitrile—acetic acid—water (50:1:49). A linear program from 12—60% B was run in 10 min. The flow-rate was adjusted so that the pressure over the column should not exceed 400 p.s.i. (1.2—1.6 ml/min). Ten microliters of the sample were injected and the program was started. When the internal standard had been eluted (10—16 min) the composition of the solvent was changed to 12% B in 1 min. After 5 min equilibration, the next sample could be injected. The detector settings were: excitation at 299 nm and emission at 342 nm.

Sampling of serum

Serum was collected by venipuncture from three healthy male volunteers who had received 100 mg sulpiride by intravenous (i.v.) injection and 4—6 weeks later an oral (p.o.) dose. The compound was given at 9 a.m. with the subjects fasting. The time schedule for sampling is indicated in Fig. 2.

Sampling of cerebrospinal fluid

CSF was collected from psychotic patients by lumbar puncture. Samples were taken before the morning dose after the patients had been treated for 4 weeks with sulpiride, 800 mg/day. All samples were frozen to −20° within an hour.

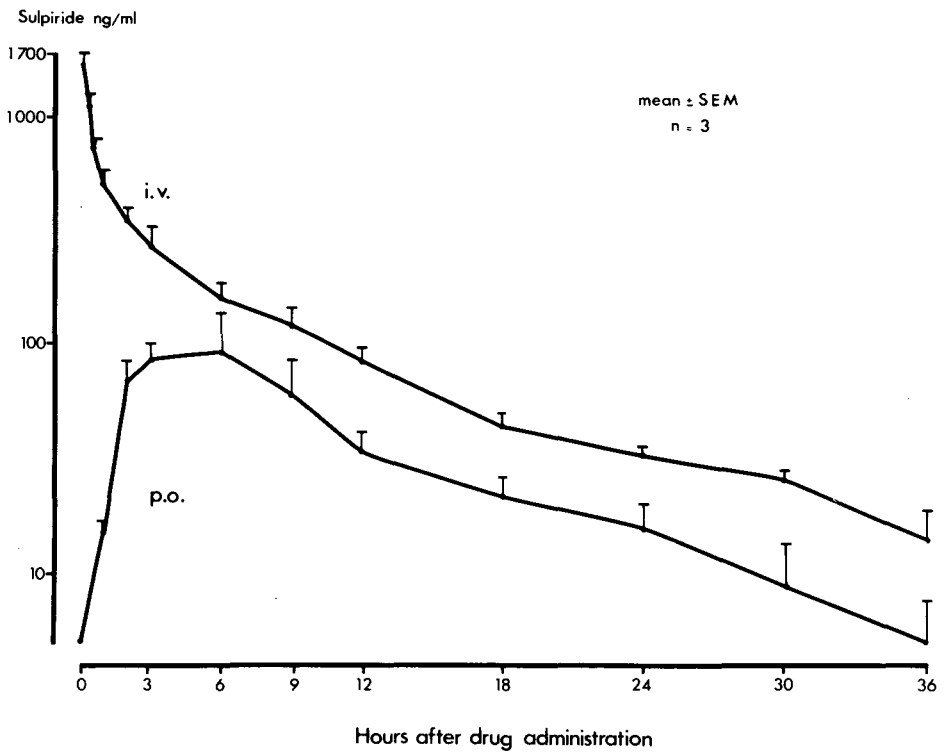

Fig. 2. Drug concentrations in serum from 3 male volunteers after i.v. and p.o. administration of 100 mg of sulpiride.

The experiments were approved by the Ethics Committee of the Karolinska Institute, Stockholm, Sweden.

RESULTS AND DISCUSSION

The recovery of sulpiride added to human serum was almost quantitative (Table I). The chromatographic procedure is very reproducible as the sampling valve delivers exactly 10 μl at each injection and the compound did not seem to be destroyed in the column. These circumstances make it possible to measure sulpiride also without the use of an internal standard.

TABLE I

RECOVERY OF SULPIRIDE AFTER EXTRACTION FROM SERUM

Amount of sulpiride added to 4 ml serum (ng)	Peak height (mm)	Sulpiride* peak height without extraction (mm)	Recovery (%)
200	13	13	100
400	32	31	103
1000	67	79	85
		mean $\overline{X}$	96

*Sulpiride dissolved in methanol to a concentration corresponding to 100% recovery.

The advantage of using an internal standard is that accidental loss of the drug during the extraction and vaporization of the final methanol solutions during storage would then be compensated for. The disadvantage of using the standard is noticeable in the chromatographic part of the analytical procedure. An ideal internal standard should have about the same elution qualities as sulpiride and also have its fluorescence maximum at about the same wavelength. The methoxy analogue used fulfills the fluorescence criterium having an excitation maximum of 293 nm and an emission maximum of 338 nm, values from the uncalibrated Kontron detector (sulpiride: excitation max. 300 nm, emission max. 342 nm). However, it is not as polar as sulpiride, which makes the retention time longer and consequently fewer samples can be run during a day. The experimental error, with and without the standard, was of about the same magnitude (5—10% for 25—100 mg sulpiride added to serum).

The standard curves were linear from the limit of detection (ca. 10 ng/ml) to at least 2 μg/ml (Fig. 3). The correlation coefficients were greater than 0.99. From several experiments we have noticed a tendency to somewhat higher correlation coefficient with the use of the internal standard.

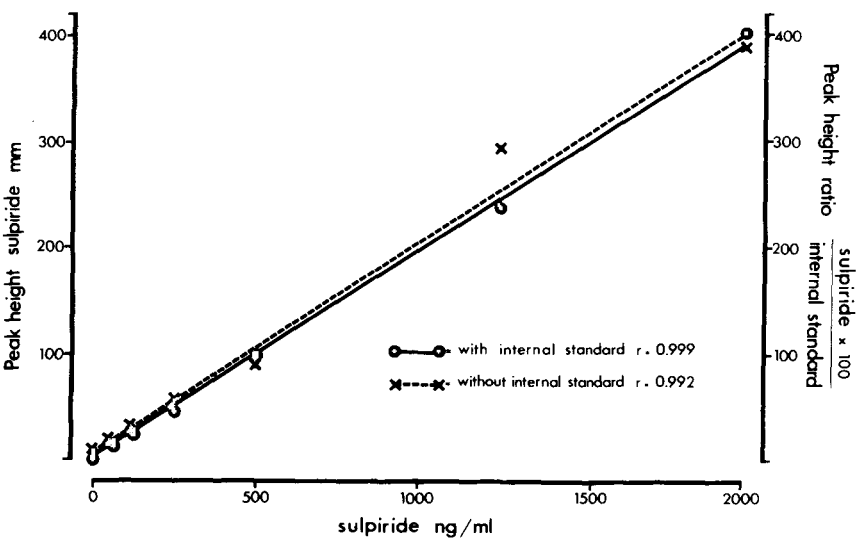

Fig. 3. Standard curves for the analysis of sulpiride in serum with and without the use of internal standard.

Fig. 4 demonstrates a typical chromatogram from the analysis of sulpiride in serum from a subject given 100 mg of sulpiride orally. A small unknown peak is usually seen after the sulpiride peak. In some serum samples there are also peaks just before sulpiride. This is not a problem when analysing samples from sulpiride-treated patients as their serum levels are usually high. However, when the concentration is low, as in the late phase of excretion, after single-dose administration, gradient elution, starting with a low percentage acetonitrile is necessary to get a good separation of the peaks.

The detection limit for sulpiride in CSF was the same as in serum, about 10 ng/ml in a 4-ml sample. In the CSF samples no interfering peaks were observed.

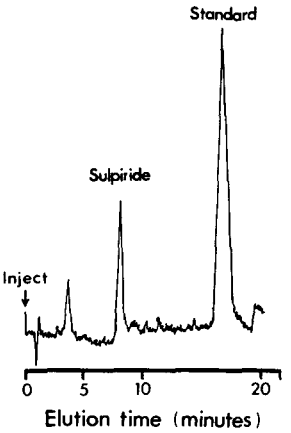

Fig. 4. Chromatogram of a serum sample from a volunteer. The sample was taken 2 h after p.o. administration of sulpiride, 100 mg. The drug concentration in serum was 51 ng/ml.

The chromatograms of urine samples showed several large unknown peaks. The detection limit for sulpiride in urine was about 200 ng/ml due to such interference. It is possible that an improved extraction procedure could lower this limit. However, we have not found it necessary to develop such a procedure as the amounts of sulpiride in urine from drug-treated subjects are very high. The addition of sodium chloride to the urine samples before the extraction is important, as the recovery otherwise could be very variable.

The precision of the method was high as demonstrated from duplicate analyses of 2-ml serum samples from a healthy volunteer after an i.v. injection of 100 mg sulpiride (Table II). The mean percent deviation was 3.3 ± 3.3%.

A pharmacokinetic study of sulpiride in healthy volunteers has recently been initiated. Some preliminary results are shown in Fig. 2. The interindividual variations in serum sulpiride levels were greater after p.o. than after i.v. administration probably due to differences in the absorption and first pass effect. The concentration in serum reached a maximum between 3 to 6 h after

TABLE II

CONCENTRATIONS OF SULPIRIDE IN SERUM FROM A HEALTHY VOLUNTEER AFTER AN I.V. INJECTION OF 100 mg SULPIRIDE

Time from injection		Sulpiride duplicate analysis		Deviation from mean (%)
min	h	ng/ml	ng/ml	
10		2662	2744	1.5
20		1332	1444	4.0
30		1178	966	9.5
	1	713	703	0.7
	2	548	546	0.2
	6	271	294	4.2
	9	159	141	6.0
	12	90	90	0

the intake of tablets. The serum levels found after p.o. administration were in the same range as those found by Kleimola et al. [5]. The half-life of sulpiride in plasma after i.v. administration was about 8 h, which is also in agreement with earlier findings [6].

Drug levels in CSF from a few schizophrenic patients treated with sulpiride have also been analysed (Table III). It is clear that sulpiride passes into the central nervous system and that quite high drug levels are present in the CSF during drug treatment.

The present method will be used in pharmacokinetic and clinical studies on sulpiride in healthy volunteers and schizophrenic patients. The correlation between serum and CSF levels of the drug and the relationships to biochemical and clinical variables will also be investigated.

TABLE III

DRUG LEVELS IN CSF FROM 5 FEMALE SCHIZOPHRENIC PATIENTS TREATED WITH SULPIRIDE (800 mg/DAY) FOR 4 WEEKS

Samples were taken at 8 a.m. before the first daily dose of sulpiride.

Patient No.	Sulpiride in CSF (ng/ml)
1	83
2	88
3	57
4	48
5	50

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DOSAGE DE L'HÉMISUCCINATE DE BENFURODIL DANS LE SANG ET L'URINE PAR CHROMATOGRAPHIE EN PHASE LIQUIDE HAUTE PRESSION

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SUMMARY

High-pressure liquid chromatographic determination of benfurodil hemisuccinate in blood and urine

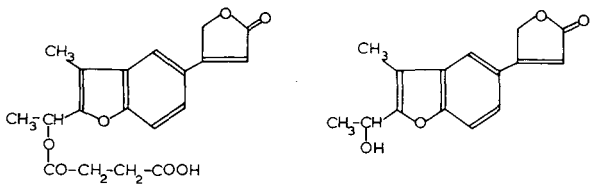
A sensitive and reliable method for quantitative determination of benfurodil hemisuccinate and benfurodil in plasma by high-pressure liquid chromatography on a Zorbax SIL column with a mean particle size of 7 μm and UV detection at 254 nm is described.

Benfurodil hemisuccinate is stable in plasma but not in aqueous solutions. This is explained by its great fixation to plasma proteins which has been shown by equilibrium dialysis.

INTRODUCTION

L'hémisuccinate de benfurodil est un médicament commercialisé en France sous le nom d'Eucilat* comme vasodilatateur périphérique. En l'absence de méthode spécifique de dosage dans les liquides biologiques, aucun contrôle du taux sanguin n'a pu être fait au cours des traitements.

Nous nous proposons de décrire, dans cette note, la technique de dosage de



Hémisuccinate de benfurodil Benfurodil

Fig. 1. Formules chimiques de l'hémisuccinate de benfurodil et du benfurodil.

l'hémisuccinate de benfurodil et du benfurodil (Fig. 1), que nous avons mis au point par chromatographie en phase liquide haute pression.

MATÉRIEL ET MÉTHODES

Appareils

Chromatographe en phase liquide Dupont modèle 841 avec détecteur UV à longueur d'onde fixe (254 nm), équipé d'une colonne de silice Zorbax SIL (Dupont, Wilmington, Del., É.U.) de longueur 25 cm et de diamètre intérieur 4.6 mm. Les microparticules de silice, de forme régulière sont totalement poreuses et de diamètre 6 à 8 μm. Pression 14 MPa (140 bars), vanne d'injection à six voies avec une boucle de 20 μl. Enregistreur Sefram Servotrace (déroulement 5 mm/min). Spectromètre de masse Riber (GCMS R 10-10) équipé d'une sonde d'introduction directe et d'une source à double ionisation (impact électronique et ionisation chimique) couplé à un chromatographe en phase gazeuse (Girdel série 30) équipé d'une colonne de verre SE30 (four 240°).

Dialyseur à l'équilibre Dianorm équipé de cellules de 1 ml et de membranes de cellulose "Spectrapor" (barrière moléculaire 12,000 à 14,000). Vitesse de rotation 16 tours/minute et température de dialyse à 37°.

Réactifs

Phase mobile: hexane, dichlorométhane, chloroforme, alcool isopropylique (Merck, Darmstadt, R.F.A., qualité pour analyses), acide acétique (Prolabo, Paris, France, Normapur) 64, 20, 10, 5, 1. Débit 0.6 ml/min à température ordinaire.

Tampon acétate 0.5 *M* (pH 4.8). Tampon phosphate 0.5 *M* (pH 6.4). Sulfate de sodium. Le N-butyryl *p*-aminophénol à 50 mg/l dans l'acétone est utilisé comme étalon interne.

Technique

Les dosages sont effectués par chromatographie en phase liquide haute pression.

Dosage dans le plasma. On mélange 500 μl de plasma obtenu par centrifugation et 100 μl de solution étalon, puis on ajoute 500 μl de tampon acétate 0.5 *M* (pH 4.8), 10 ml de chloroforme et on agite pendant 1 min. La phase aqueuse est éliminée et la phase organique est séchée sur sulfate de sodium anhydre puis évaporée. Le résidu est repris par 200 μl de mélange d'élution et 20 μl de cette solution sont alors injectés sur la colonne du chromatographe.

Dosage dans l'eau et les urines. Le dosage est effectué selon un protocole très voisin en remplaçant le tampon acétate par un tampon phosphate 0.5 *M* (pH 6.4) afin d'éliminer des pics urinaires interférants avec les produits dosés.

Étude de la stabilité. Au cours des essais préliminaires, nous avons remarqué que l'hémisuccinate de benfurodil s'hydrolyse en benfurodil dans les urines même conservées à 4° alors qu'il est stable dans le plasma. Cette observation nous a conduit à étudier plus en détail la stabilité de ce médicament dans divers milieux (plasma, urines, eaux à différents pH) à 37° et en fonction du temps (0, 30 min, 1 h, 2 h, 4 h).

Étude de la fixation aux protéïnes plasmatiques. Le plasma surchargé en hémisuccinate de benfurodil a été dialysé contre du liquide physiologique, dépourvu de protéïnes. Du liquide physiologique surchargé en hémisuccinate de benfurodil a été dialysé dans les mêmes conditions. Cinq prélèvements ont été effectués aux temps t = 15 min, 30 min, 45 min, 1 h, 1 h 30 min pour la détermination des taux de benfurodil et d'hémisuccinate de benfurodil de part et d'autre de la membrane.

RÉSULTATS

Séparation chromatographique

La chromatgraphie en phase liquide permet, comme le montre la Fig. 2, une

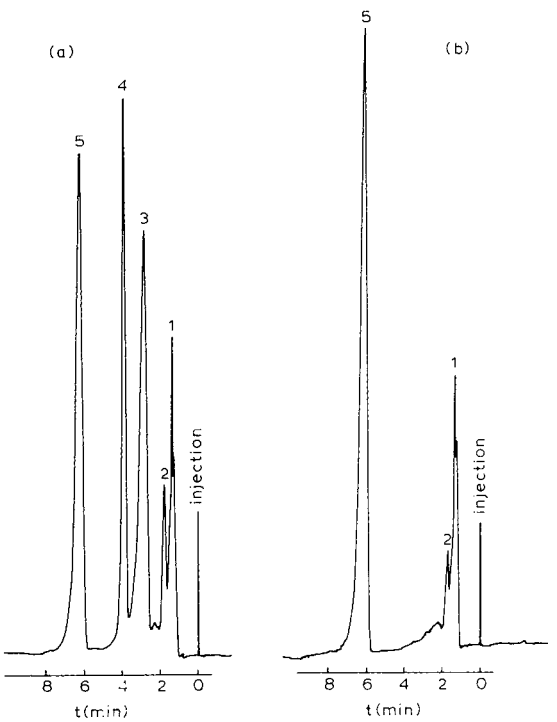


Fig. 2. Chromatogramme de l'hémisuccinate de benfurodil et du benfurodil après extraction plasmatique (a), et d'un blanc plasmatique (b). 1 et 2: pics plasmatiques non identifiés; 3: hémisuccinate de benfurodil 5 mg/l; 4: benfurodil 2 mg/l; 5: N-butyryl *p*-aminophénol, étalon interne. Colonne Zorbax SIL.

bonne séparation de l'hémisuccinate de benfurodil et du benfurodil. La droite d'étalonnage est obtenue, après addition à un plasma témoin de quantités connues d'hémisuccinate de benfurodil (0—20 mg/l) et d'une quantité constante d'étalon interne (N-butyryl *p*-aminophénol), en calculant le rapport des hauteurs de pics respectifs. D'autre part, les chromatogrammes d'extraits plasmatiques ou urinaires de sujets non traités ne présentent pas de pic au niveau de l'étalon, ni au niveau des produits étudiés.

Sensibilité

La sensibilité de la méthode est pour l'hémisuccinate de benfurodil de 100 ng/ml dans le plasma et 300 ng/ml dans les urines et pour le benfurodil de 20 ng/ml dans le plasma et les urines.

Reproductibilité

La reproductibilité a été étudiée en effectuant huit dosages sur deux échantillons de plasma témoin, surchargé l'un par l'hémisuccinate de benfurodil à raison de 10 mg/l, l'autre par le benfurodil à raison de 2.5 mg/l. Nous avons trouvé 9.95 ± 0.32 mg/l pour le premier et 2.5 ± 0.1 mg/l pour le second.

Stabilité

Les résultats, rassemblés dans les Figs. 3 et 4, montrent que l'hémisuccinate de benfurodil est stable dans le plasma, alors qu'il s'hydrolyse en benfurodil dans l'urine et l'eau (environ 60% au bout de 4 h). L'augmentation du taux de benfurodil correspond à la décroissance du taux d'hémisuccinate de benfurodil et la vitesse d'hydrolyse, qui augmente avec l'acidité, devient très importante à

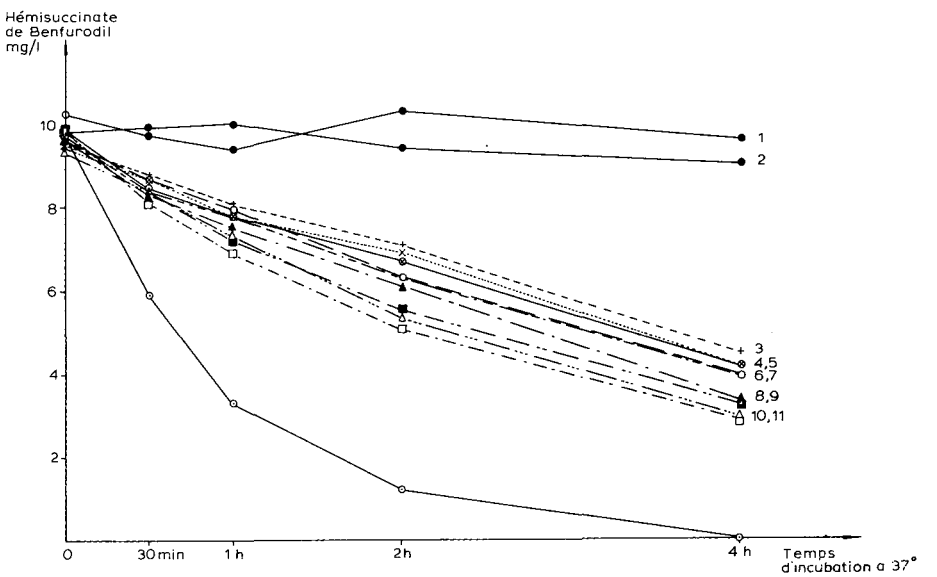


Fig. 3. Étude de la stabilité de l'hémisuccinate de benfurodil dans différents milieux en fonction du temps. 1, 2: plasma; 3: eau (pH 7.0); 4: eau (pH 6.0); 5: urines (pH 7.0); 6: eau (pH 7.4); 7: urines (pH 5.0); 8: eau (pH 5.0); 9: eau + glucose 5%; 10: eau; 11: eau + NaCl 0.9%; 12: eau (pH 1.5).

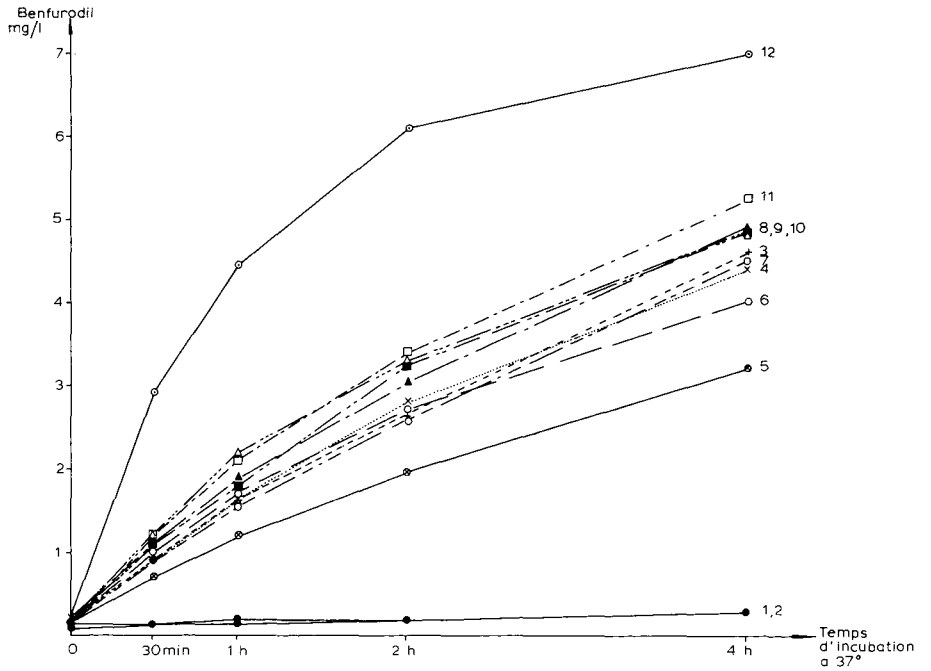


Fig. 4. Taux de benfurodil provenant de l'hydrolyse de l'hémisuccinate de benfurodil dans divers milieux en fonction du temps.

pH 1.5, pH voisin de celui du liquide gastrique.

Dans une expérience similaire, nous avons démontré la stabilité du benfurodil.

Fixation aux protéïnes

Le Tableau I montre que la fraction d'hémisuccinate de benfurodil introduit dans le plasma, qui dialyse en une heure, est voisine de 2% seulement, alors qu'elle atteint près de 50%, c'est-à-dire l'équilibre, après surcharge du liquide physiologique. Ces résultats peuvent s'expliquer par une fixation importante de ce médicament aux protéïnes.

TABLEAU I

ÉTUDE DE LA FIXATION AUX PROTÉÏNES PAR DIALYSE À L'ÉQUILIBRE EN FONCTION DU TEMPS

Temps	Plasma surchargé		Fraction dialysée	
	Hémisuccinate de benfurodil (mg/l)	Benfurodil (mg/l)	Hémisuccinate de benfurodil (mg/l)	Benfurodil (mg/l)
0	10.30	0.03		
15 min	9.70	0.04	0.15	<0.02
30 min	9.95	0.04	0.15	<0.02
45 min	9.75	0.06	0.15	<0.02
1 h	10.15	0.05	0.20	0.02
1 h 30 min	9.25	0.08	0.20	0.02

DISCUSSION

La chromatographie en phase liquide a été préférée à la chromatographie en phase gazeuse car, avec cette technique, les deux produits non dérivés trainent sur la colonne et la sensibilité n'est pas bonne. D'autre part, les essais de

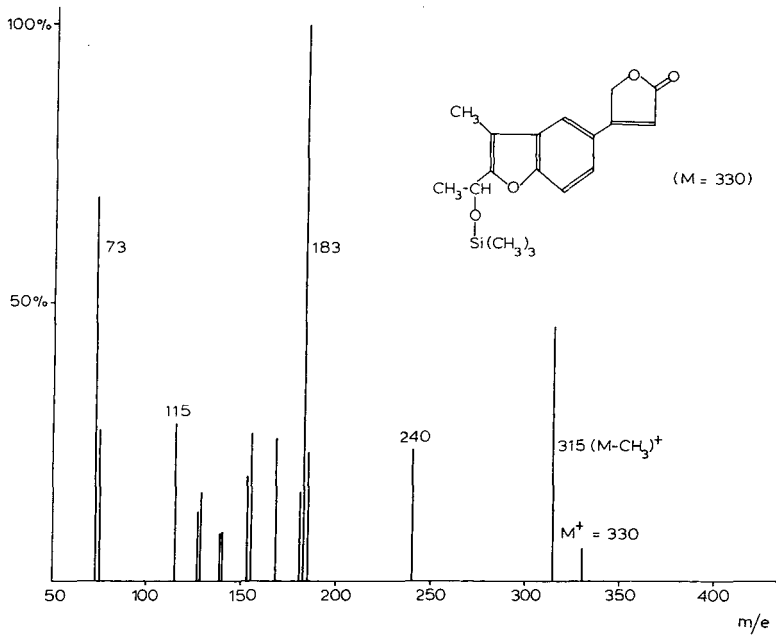


Fig. 5. Spectre de masse du benfurodil silylé obtenu par couplage—impact électronique.

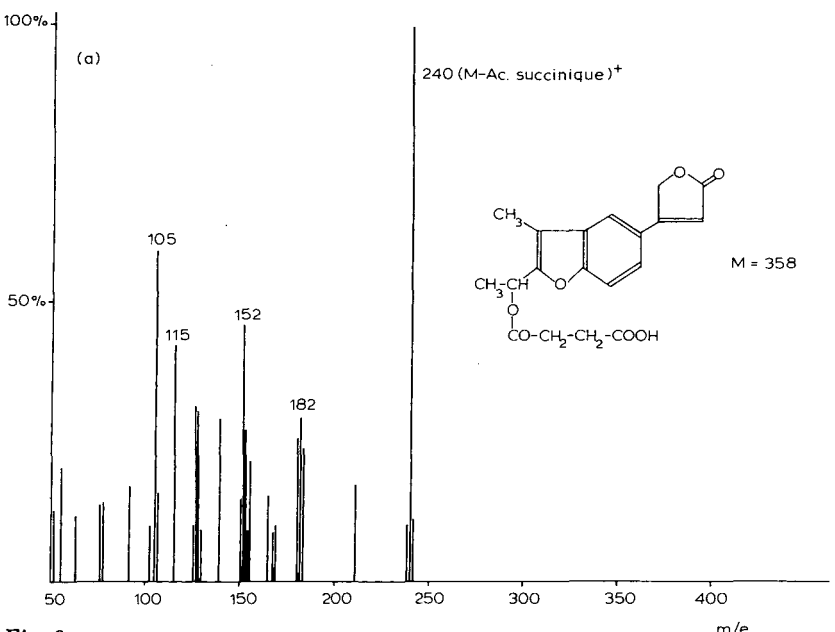


Fig. 6a.

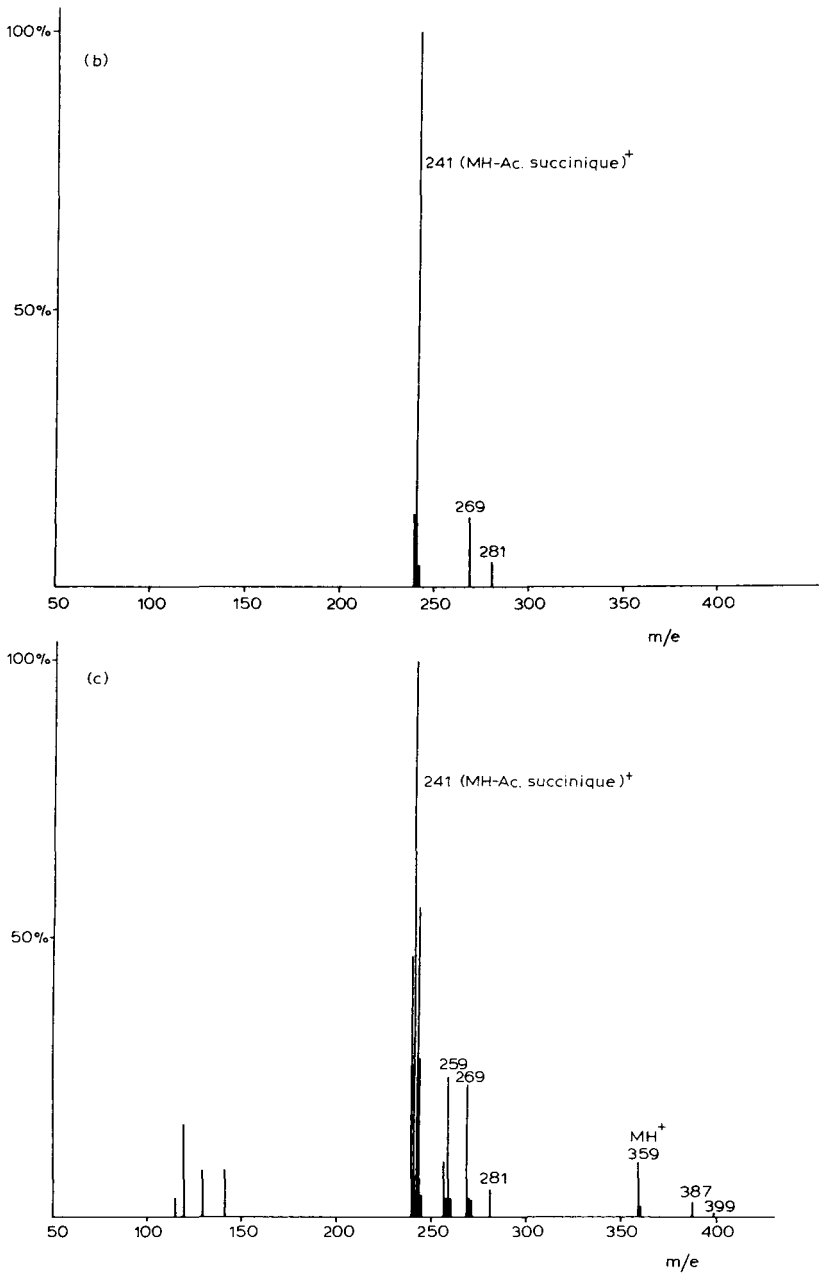


Fig. 6. Spectres de masse de l'hémisuccinate de benfurodil obtenus (a) par couplage—impact électronique; (b) par couplage—ionisation chimique méthane; (c) par introduction directe—ionisation chimique méthane.

dérivation ont montré une décomposition de l'hémisuccinate de benfurodil qui donne plusieurs pics. Toutefois, nous avons pu obtenir les spectres de masse des deux produits sans dérivation pour l'hémisuccinate de benfurodil et après silylation pour le benfurodil.

Le spectre de masse du benfurodil silylé (Fig. 5) présente bien l'ion moléculaire ($M^+ = 330$).

Les spectres de l'hémisuccinate de benfurodil, non dérivé, obtenus par introduction directe et par couplage chromatographie en phase gazeuse—spectrométrie de masse sont indiqués sur la Fig. 6.

En introduction directe, le pic moléculaire de l'hémisuccinate de benfurodil ($M^+ = 358$) n'est pas obtenu en ionisation par impact électronique ni en ionisation chimique par l'ammoniac, mais seulement en ionisation chimique par le méthane.

En couplage chromatographie—spectrométrie de masse, on n'obtient pas le pic moléculaire ($M^+ = 358$) ni en ionisation par impact électronique, ni en ionisation chimique, mais le spectre du produit extrait du plasma de deux sujets traités est absolument identique à celui de l'hémisuccinate de benfurodil pur, préparé dans les mêmes conditions.

L'hémisuccinate de benfurodil apparaît donc comme un produit instable qui s'hydrolyse en milieu aqueux et se décompose au cours des essais de séparation par chromatographie en phase gazeuse. La chromatographie en phase liquide se révèle donc la méthode de choix pour sa séparation et son dosage. Sa stabilité dans le plasma par rapport à l'eau s'explique très vraisemblablement par sa grande fixation aux protéïnes plasmatiques que nous avons pu mettre en évidence.

La Fig. 7 montre l'évolution des concentrations sanguines d'hémisuccinate de benfurodil après administration orale de 150 mg de ce produit chez un individu normal, le benfurodil n'apparaissant qu'à l'état de traces.

Les résultats de l'étude pharmacocinétique de l'hémisuccinate de benfurodil sont détaillés dans une autre publication [1].

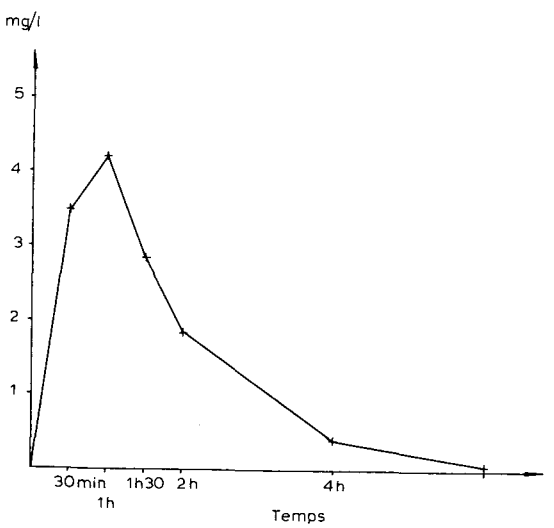


Fig. 7. Concentrations sanguines de l'hémisuccinate de benfurodil après administration orale de 150 mg.

REMERCIEMENTS

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RÉSUMÉ

Nous proposons une méthode simple et reproductible de dosage de l'hémisuccinate de benfurodil et du benfurodil par chromatographie en phase liquide haute pression sur colonne Zorbax SIL et détection UV à 254 nm.

L'hémisuccinate de benfurodil est stable dans le plasma alors qu'il s'hydrolyse en benfurodil en milieu aqueux. Cette stabilité peut s'expliquer par la fixation importante de ce médicament aux protéïnes plasmatiques.

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DETECTION OF ENDOGENOUS SALSOLINOL IN NEONATAL RAT TISSUE BY A RADIOENZYMATIC—THIN-LAYER CHROMATOGRAPHIC ASSAY

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SUMMARY

A sensitive radioenzymatic—thin-layer chromatographic assay for the quantitative analysis of the tetrahydroisoquinoline alkaloid, salsolinol, in plasma and neonatal rat tissue is described. The assay involves the enzymatic O-methylation of salsolinol by catechol-O-methyltransferase in presence of [^{3}H]S-adenosylmethionine, and subsequent separation by thin-layer chromatography of the resultant [^{3}H]O-methyl-salsolinol from the O-methylated derivatives of dopamine, epinephrine and norepinephrine. The method allows the detection of as little as 100 pg salsolinol per g tissue, and the accurate quantitation of as little as 100 pg/ml plasma and 500 pg/g tissue. This assay permitted the detection of trace amounts of endogenous salsolinol in neonatal rat tissue (< 500 pg/g tissue).

INTRODUCTION

Recent evidence in both laboratory animals and in humans suggests that tetrahydroisoquinoline (TIQ) and tetrahydro-β-carboline (TBC) alkaloidal metabolites, formed endogenously from interactions between acetaldehyde and catecholamines or serotonin during alcohol consumption, may play a role in the development of physical dependence to ethanol (for reviews see refs. 1 and 2). Since a number of TIQs and TBCs have psychotomimetic activity, and the TIQ alkaloid, tetrahydropapaveroline, is a precursor of morphine in plants, it has further been speculated that physical dependence to ethanol or opiates may share a common biochemical basis.

In in vivo studies involving acute or chronic administration of ethanol [2],

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formation of TIQs in the brain could only be demonstrated under pharmacological conditions which enhanced the biosynthesis of TIQs or inhibited their metabolic degradation. In order to explore the possible formation of the TIQ, salsolinol (the condensation product of endogenous dopamine with alcohol-derived acetaldehyde), in brain tissue under conditions of physical dependence to ethanol without intervening pharmacological manipulations, we developed a gas chromatographic—electron capture (GC—EC) assay for salsolinol capable of detecting as little as 8 ng of the alkaloid per g of brain tissue [3]. Using this assay, we were unable to detect the formation of brain salsolinol in alcoholic mice [4], when conversion of 1% or more of endogenous dopamine to salsolinol could not have escaped detection. Our negative findings were subsequently confirmed by other investigators [5, 6] in mice and rats. Nevertheless, it has recently been demonstrated that intracerebral administration of TIQs or TBCs in minute amounts to rats mimics ethanol dependence and increases free-choice ethanol consumption over water [7—11]. It has thus become evident that the question of in vivo formation of brain TIQs in alcoholic animals (not subjected to pharmacological manipulations which enhance the biosynthesis of TIQs or which delay their degradation) needs re-examination with more sensitive analytical methods for detecting minute amounts of TIQs.

Another contemporary aspect of ethanol toxicity concerns its teratogenic action, which has been established in laboratory animals and in humans (for review see ref. 12). Although no mechanism has been proposed to explain the teratogenicity of ethanol, evidence has been presented in one human study indicative of interference with cellular migration in the brain during embryogenesis [13]. We have speculated that the teratogenic anomalies produced by ethanol could be in part the result of interference with embryonal catecholaminergic or serotoninergic mechanisms as a consequence of aberrant biosynthesis of TIQ or TBC alkaloids. Teratogenicity of opiates and psychoactive drugs has already been reported in the literature [14, 15]. A study to validate this hypothesis is in progress in our laboratory, and mandated the development of a sensitive analytical assay for the detection and quantitation of the TIQ, salsolinol.

The present report outlines the development of a sensitive radioenzymatic assay for salsolinol in plasma and neonatal rat tissue. A future communication will deal with the detection and quantitation of brain salsolinol. Since it has been reported [16] that salsolinol is a substrate for mammalian catechol-O-methyltransferase (COMT) as well as a competitive inhibitor of the O-methylation of catecholamines, the possibility of development of a sensitive thin-layer chromatographic—radioenzymatic (TLC—RE) assay for detection, separation and quantitation of trace amounts of salsolinol and catecholamines became apparent. This endeavor was greatly facilitated by the recent commercial availability of a radioenzymatic assay kit (CAT-A-KIT, Upjohn Diagnostics, Kalamazoo, Mich., U.S.A.) for catecholamines, which is based on the method of Passon and Peuler [17]. The method described below is a modification of the commercial kit, adapted for the incorporation of salsolinol into the assay. Salsolinol (SAL), dopamine (DA), epinephrine (EPI), and norepinephrine (NE) are simultaneously converted to their corresponding *meta* ^{3}H-methoxy

derivatives ([^{3}H]salsoline, [^{3}H]3-methoxytyramine, [^{3}H]metanephrine, and [^{3}H]normetanephrine, respectively) by the catalytic action of COMT in the presence of [^{3}H]S-adenosylmethionine (^{3}H-SAM). The ^{3}H-O-methylated derivatives are extracted and separated by TLC. [^{3}H]Normetanephrine and [^{3}H]metanephrine (but not [^{3}H]salsoline or [^{3}H]3-methoxytyramine) are susceptible to periodate oxidation which converts the former two derivatives into [^{3}H]vanillin; this additional chemical characteristic serves to differentiate between the ^{3}H-O-methylated derivatives of EPI and NE on the one hand and those of DA and SAL on the other.

MATERIALS AND METHODS

Chemicals and equipment

The following principal chemicals were used: CAT-A-KIT (catecholamines radioenzymatic assay kit [^{3}H], Upjohn Diagnostics), salsolinol HBr (Aldrich, Milwaukee, Wisc., U.S.A.), salsoline HCl (ICN Pharmaceuticals, Plainview, N.Y., U.S.A.), and Liquifluor (New England Nuclear, Boston, Mass., U.S.A.).

Vacutainer tubes, containing 100 μl of a solution (pH 6—7) composed of 90 mg EGTA per ml and 60 mg reduced glutathione per ml, were purchased from Upjohn Diagnostics. Glassware was siliconized by immersion for 1 min in a 1% solution of Prosil (VWR Scientific, Columbus, Ohio, U.S.A.), and then rinsed in distilled water and dried at 150°.

Prescored silica gel GF TLC plates, 20 × 20 cm, 250 μm thick (Analabs, North Haven, Conn., U.S.A.) were used in conjunction with a 16-channel TLC Multispotter (Analytical Instrumentation Specialties, Libertyville, Ill., U.S.A.).

Reagents

Reagents 1—9 are CAT-A-KIT reagents [18].

Reagent 1 (catecholamines standard solution): Each ml contains 100 μg each of *l*-NE, *l*-EPI, and DA in acid glutathione solution. Diluted 1:10,000 with demineralized double-distilled water before use.

Reagent 2 (stabilizing solution): Acidic glutathione solution, diluted 1:10,000 with demineralized double-distilled water before use.

Reagent 3 (buffer solution): For buffering the enzyme reaction in the assay. Contains tromethamine, EGTA, and magnesium chloride.

Reagent 4 (^{3}H-SAM, methyl donor): S-Adenosyl-L-methionine (^{3}H-methyl), 5 μCi ^{3}H/10 μl in dilute sulfuric acid—ethanol (acetaldehyde-free).

Reagent 5 (COMT enzyme preparation): Rat liver COMT (in excess of assay needs), tromethamine, glutathione, benzylhydroxyamine HCl, and dithiothreitol.

Reagent 6 (stopping/carrier solution): Contains 4 m*M* each of normetanephrine, metanephrine, and methoxytyramine, in pH 11 borate buffer containing EDTA.

Reagent 7 (oxidizing reagent): Sodium metaperiodate 4% w/v solution.

Reagent 8: Glycerol 10% v/v solution.

Reagent 9 (control human plasma): Contains assayed levels of catecholamines (DA, EPI, and NE), EGTA and glutathione.

Separation of O-methylated derivatives of SAL, DA, EPI and NE by TLC

In order to determine whether the O-methylated derivative of salsolinol (salsoline) was separable by TLC from the O-methylated derivatives of EPI, NE, and DA, 5 ml of Reagent 6 were spiked with 4.6 mg of salsoline HBr to provide a 4 m*M* concentration of salsoline. The resulting mixture of salsoline, methoxytyramine, metanephrine and normetanephrine was extracted [19] into 2 ml of toluene—isoamyl alcohol (3:2), and the aqueous and organic phases separated. The O-methylated compounds were extracted from the organic phase into 0.1 ml of 0.1 *N* acetic acid. The acid layer was separated and washed with 1 ml of toluene—isoamyl alcohol (3:2), and the organic wash discarded. Absolute ethanol (0.1—0.15 ml) was added to the acid extract to clear the solution. The ethanolic—acid extract was spotted on the silica gel TLC plates using the TLC Multispotter set at low speed and 60°. The distance between the solvent front and points of application was set at 16 cm. After allowing to cool, the plates were developed in 44 ml of *tert.*-amyl alcohol—toluene—40% methylamine (6:2:3) for approximately 2.5 h in developing tanks lined with Whatman No. 1 filter paper and pre-equilibrated for 10 min with the developing solvent. The plates were allowed to dry, and the four zones were visualized under UV light (254 nm).

Radioenzymatic reaction

The following radioenzymatic assay is a modification of the method of Peuler and Johnson [19]. Aliquots (50 μl) of each sample (e.g. plasma, tissue) to be analyzed for SAL, DA, EPI and NE, were mixed with 10 μl of diluted acidic glutathione stabilizing solution (Reagent 2) and 40 μl of a reagent mixture composed of equal parts (10 μl each) of distilled water, buffer solution (Reagent 3), ^{3}H-SAM (Reagent 4), and COMT (Reagent 5). The mixtures were incubated at 37° for 60 min in a metabolic shaker. To each mixture was then added 50 μl of the buffered carrier solution (Reagent 6) and 5 μl of a salsoline carrier solution (freshly prepared by dissolving 46 mg salsoline HCl in 5 ml of pH 11 borate buffer containing 27.8 mg EDTA per ml). The ^{3}H-O-methylated derivatives and their non-radioactive carriers were then extracted and separated by TLC as described above. The radioactive zones on the TLC plates were scraped into individual scintillation vials.

[^{3}H]Metanephrine and [^{3}H]normetanephrine were eluted from the silica in their respective scintillation vials by vigorous mixing with 1 ml of 0.05 *M* ammonium hydroxide. [^{3}H]Metanephrine and [^{3}H]normetanephrine were then each converted to [^{3}H]vanillin by periodate oxidation as described by Peuler and Johnson [19] and in the CAT-A-KIT Procedures Manual [18]. Following the oxidation, 1 ml of 0.1 *M* acetic acid was added to each vial with vigorous mixing. To each vial were then added 10 ml of toluene—Liquifluor (1000:50, v/v) scintillation cocktail with vigorous mixing, and the radioactivity counted in a Beckman LS-35 liquid scintillation counter with an efficiency for ^{3}H of 58%.

[^{3}H]Salsoline and [^{3}H]methoxytyramine were eluted from the silica in their respective scintillation vials by vigorous mixing with 1 ml of 0.05 *M* ammonium hydroxide. Ten ml of toluene—isoamyl alcohol—Liquifluor (700:300:50, v/v/v) scintillation cocktail were added to each vial with vigorous shaking, and the

radioactivity counted in a Beckman LS-35 liquid scintillation counter with an efficiency for ^3H of 39% (38.7—40%).

Aqueous standard curve

A salsolinol HBr stock solution was prepared (10 μg SAL per 10 ml distilled water, with 60 mg reduced glutathione per ml), and various dilutions of this stock solution were made from which 50-μl aliquots were assayed by the radioenzymatic procedure described above. The concentrations analyzed were 0, 50, 500, 1000 and 1500 pg salsolinol per 50-μl sample. Each sample was analyzed in triplicate.

Plasma standard curve

A salsolinol HBr stock solution was prepared (2.5 mg SAL per 10 ml distilled water, with 60 mg reduced glutathione per ml). Various dilutions of this stock solution were made from which 2-μl aliquots were used to spike 50-μl samples of human plasma (Reagent 9) which were subsequently assayed by the radioenzymatic procedure described above. The amounts of salsolinol added to the 50-μl plasma samples were 0, 5, 30, 50, 230, and 500 pg. Each sample was analyzed in duplicate to quadruplicate. An aliquot of an appropriate blank, which consisted of the contents of the vacutainer tubes (see Chemicals and equipment) used for plasma collection diluted with distilled water, was also assayed without salsolinol spiking. In all plasma samples, the ^3H-O-methylated derivatives of endogenous DA, EPI and NE were also assayed and compared to the standard values provided with the CAT-A-KIT. Adult rat plasma was analyzed similarly.

Tissue standard curve

Sprague-Dawley neonatal rats were used to prepare the tissue standard curve. The pups were immersed in liquid nitrogen, and stored at −20° until used. At the time of assay, each frozen pup was weighed, minced, and homogenized in 8 ml of 0.1 *N* $HClO_4$ (containing 5 m*M* glutathione) by use of a Polytron tissue shearer. The tissue homogenates were spiked with various concentrations of salsolinol (with a volume not exceeding 150 μl). The concentrations of salsolinol ranged from 100 pg to 48 ng per g tissue. The Polytron was rinsed with an additional 3 ml of 0.1 *N* $HClO_4$ which was then added to the spiked homogenate. Each homogenate was centrifuged at 100,000 *g* at 4° for 1 h. The supernatant was decanted and retained. The pellet was rehomogenized in 6 ml of 0.1 *N* $HClO_4$, recentrifuged at 100,000 *g* at 4° for 1 h, and the supernatant decanted. The two supernatants were combined and stored at −20° until assayed. Upon thawing, the supernatant was subjected to a final centrifugation at 39,000 *g* for 1 h at 4° and any residue discarded. Duplicate aliquots of the supernatants were assayed according to the radioenzymatic procedure described above. Perchloric acid blanks (containing 5 m*M* reduced glutathione) without salsolinol spiking nor tissue were also assayed.

Verification of the identity of trace amounts of tissue salsolinol

Three sets of experiments were designed to verify the identity of [^3H]-

salsoline, particularly at the lower limits of sensitivity of the radioenzymatic assay for tissue salsolinol:

(a) The first set of experiments was designed to ascertain that the radioactivity detected on the TLC plate zone corresponding to the R_F of salsoline, at the lower limits of sensitivity of the tissue standard curve, was contributed exclusively by a COMT-dependent end-product (presumably [^{3}H]salsoline). Neonatal rat homogenates were prepared as described above and spiked with salsolinol to give a concentration range of 100—2000 pg salsolinol per g tissue. The samples were subjected to the radioenzymatic assay described above in presence of ^{3}H-SAM but with the omission of COMT. After extraction and development on TLC plates as described above, the zones on the TLC plates corresponding to the R_F of salsoline were scraped, eluted, and counted, in order to determine if a significant amount of radioactivity (above background) could be detected as compared to similar samples which had been O-methylated in presence of COMT.

(b) The second set of experiments was designed to further ensure that the tissue-extracted radioactivity on the TLC plate at the R_F corresponding to salsoline was contributed by a single product ([^{3}H]salsoline). Two-dimensional TLC in two additional solvent systems was performed on two salsolinol-spiked tissue samples (1000 pg salsolinol per g tissue) which were taken through the entire radioenzymatic assay procedure (in presence of ^{3}H-SAM and COMT) as described above. The TLC plates were then developed first in *tert.*-amyl alcohol—toluene—40% methylamine (6:2:3) solution as previously described, and the zones corresponding to the tritiated O-methylated derivatives of SAL, DA, EPI and NE visualized under UV light. The plates were allowed to dry and then turned 90° and re-developed in one of the following solvent systems: isopropanol—*n*-butanol—water—formic acid (60:20:19:1) or 1-butanol - methanol—1 *N* formic acid (60:20:20), and the solvent front allowed to travel a distance of 16 cm. The plates were allowed to dry and again visualized under UV light. The fluorescent spot in the salsoline lane was scraped from each plate, as were additional 1-cm increments of silica below and above the fluorescent spot, from the origin to the solvent front. Elution and counting of radioactivity were performed as described above.

(c) Finally, a third set of experiments was designed to determine if any endogenous salsolinol was present in the neonatal rat, which may contribute to the [^{3}H]salsoline counts resulting from exogenously added salsolinol in the assay. Nine tissue homogenates, with no exogenously added salsolinol, were taken through the entire radioenzymatic procedure (with ^{3}H-SAM and COMT). Duplicate 50-μl aliquots of each tissue homogenate were subjected to the methylation, extraction, and TLC separation procedures as described above.

Presentation of data

All data are presented as gross disintegrations per minute (DPM), as well as gross counts per minute (CPM) ± S.E.M. — the latter to facilitate comparison with published results for catecholamines [17—19].

RESULTS AND DISCUSSION

Separation of O-methylated derivatives of SAL, DA, EPI and NE by TLC

Fig. 1 illustrates the separation of the O-methyl derivatives of NE, EPI, DA and SAL in four samples, by the TLC method described under Materials and methods. The four distinct spots observed under UV light represent normetanephrine, metanephrine, 3-methoxytyramine, and 7-O-methylsalsolinol (salsoline) with average R_F values of 0.22, 0.35, 0.49, and 0.54, respectively.

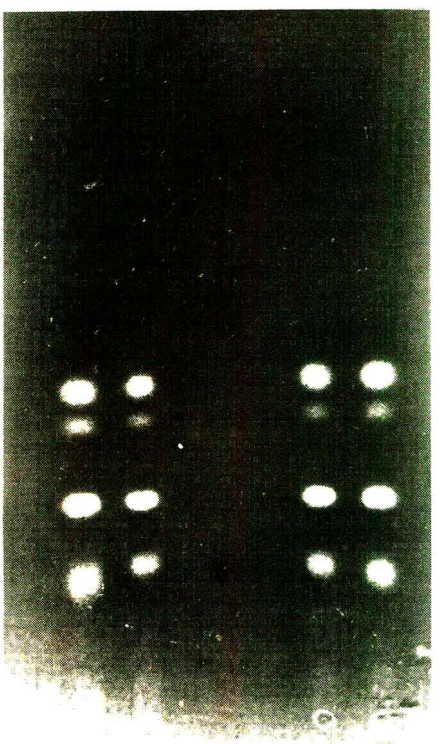

Fig. 1. TLC separation of normetanephrine (R_F = 0.22), metanephrine (R_F = 0.35), 3-methoxytyramine (R_F = 0.49), and salsoline (R_F = 0.54). A total of 20 experiments were performed, including the four shown in the figure. (Reverse negative taken under UV light; the points of application are at the bottom of the photograph.)

Aqueous standard curve for salsolinol

Fig. 2 represents the standard curve for varying concentrations of aqueous salsolinol solutions expressed in terms of DPM and CPM of [^{3}H]salsoline formed. The data demonstrate linearity of the assay from the highest tested concentration of 1500 pg salsolinol per 50-μl sample (30 ng/ml) to the lower limit of sensitivity of less than 50 pg salsolinol per 50 μl (< 1 ng/ml). The aqueous blank (with no salsolinol) averaged 198 ± 4 CPM per 50 μl (508 ± 9 DPM per 50 μl) for the area on the TLC plate corresponding to the R_F of

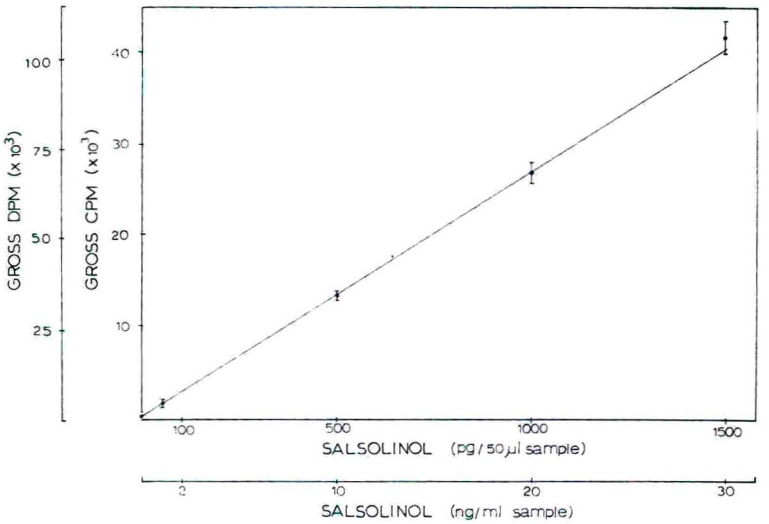

Fig. 2. Aqueous standard curve for salsolinol, expressed in terms of DPM or CPM of [^{3}H]-salsoline formed. Each point represents the mean of three observations. Standard errors are given only for CPMs (those for the DPMs being smaller on the DPM scale).

salsoline. As reported by Upjohn [18] and as observed with our use of the CAT-A-KIT, it is common to obtain a blank value of as much as 185 CPM per 50-μl sample for the area on the TLC-plate corresponding to the R_F of the O-methylated derivative of DA, as compared to a blank of 30—40 CPM per 50-μl sample for the areas on the TLC plates corresponding to the O-methylated derivatives of EPI and NE. This is likely due to the fact that [^{3}H]-methoxytyramine is extracted from a basic solution whereas [^{3}H]metanephrine and [^{3}H]normetanephrine are extracted from acidic solution. Since salsoline is a dopamine derivative, and since [^{3}H]salsoline and [^{3}H]methoxytyramine are extracted by the same procedure and counted in the same cocktail, it was not surprising that the blank for [^{3}H]salsoline resembled that of [^{3}H]methoxytyramine.

Plasma standard curve

Fig. 3 demonstrates the linearity of the human plasma standard curve for salsolinol (measured in terms of [^{3}H]salsoline formed) over the tested range of 5 pg per 50 μl (100 pg/ml) to 500 pg per 50 μl (10 ng/ml). Similar results were obtained with adult rat plasma. The plasma blank (with no salsolinol) averaged 219 ± 13 CPM per 50-μl sample (561 ± 32 DPM per 50 μl) (n = 4) for the area on the TLC plate corresponding to the R_F of salsoline — a value which is not significantly greater than that observed for the aqueous blank (198 ± 4 CPM per 50 μl).

For technical control purposes, the NE, EPI and DA contents of the plasma samples were also determined following the conversion of these catecholamines to their ^{3}H-O-methylated derivatives, and the values obtained were well within the ranges reported by Upjohn [18].

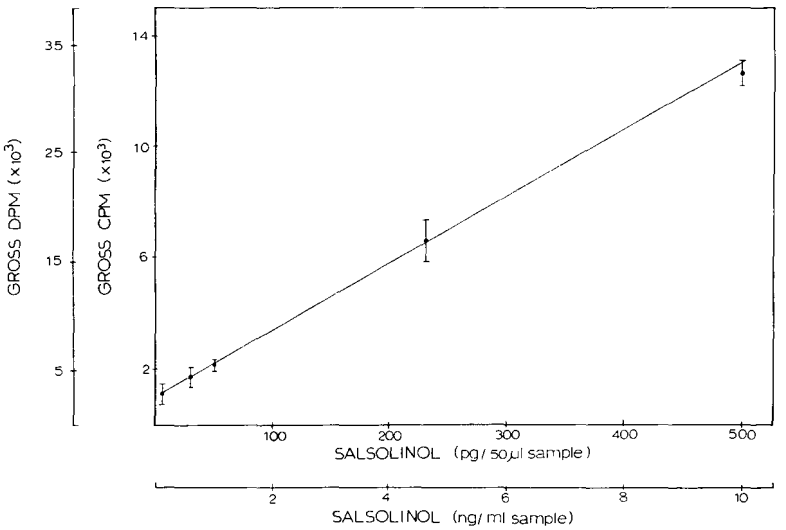

Fig. 3. Plasma standard curve for salsolinol, expressed in terms of DPM or CPM of [^{3}H]-salsoline formed. Each point represents the mean of two to four observations. Standard errors are given only for CPMs (those for the DPMs being smaller on the DPM scale).

Tissue standard curve

Fig. 4 demonstrates the linearity of the tissue standard curve for salsolinol (measured in terms of [^{3}H]salsoline formed) over a range of 500 pg salsolinol per g tissue to 48 ng salsolinol per g tissue. At the lower portion of the curve (< 500 pg salsolinol per g tissue) salsolinol can still be detected but its accurate quantification is no longer evident. The perchloric acid blank (with neither

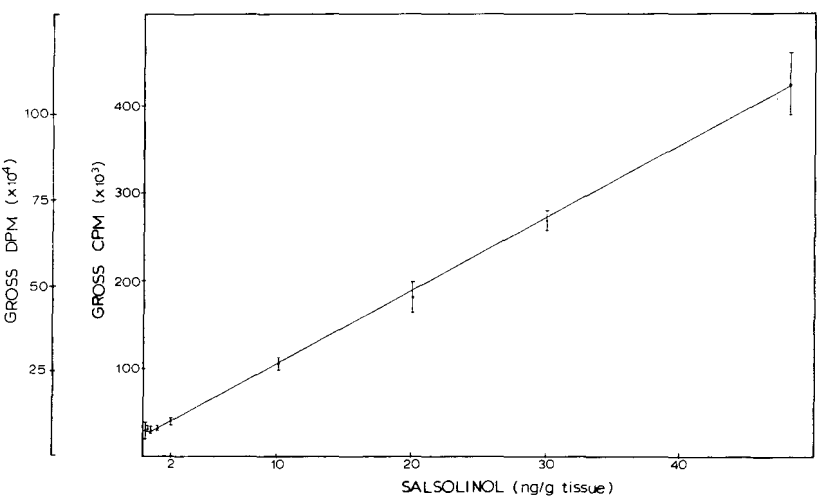

Fig. 4. Tissue standard curve for salsolinol, expressed in terms of DPM or CPM of [^{3}H]-salsoline formed. Each point represents the mean of six observations. Standard errors are given only for CPMs (those for the DPMs being smaller on the DPM scale).

tissue nor salsolinol) averaged 140 ± 7 CPM per 50-μl sample (358 ± 18 DPM per 50 μl) for the area on the TLC plate corresponding to the R_F of salsoline (n = 5).

Verification of the identity of trace amounts of tissue salsolinol

In the Materials and methods section, three sets of experiments were described aimed at verifying the identity of [^{3}H]salsoline, particularly at the lower limits of sensitivity, and at detecting endogenous tissue salsolinol.

(a) Table I shows the results of the first set of experiments, designed to verify the COMT-dependency of the radioactivity detected on the TLC plate zone corresponding to the R_F of salsoline at the lower level of sensitivity of the tissue standard curve (Fig. 4). The results shown in Table I demonstrate that, as compared to tissue samples processed in the same manner in the presence of COMT (Fig. 4), the exclusion of COMT resulted in insignificant radioactivity in the TLC zone corresponding to the R_F for salsoline. It is thus evident that the radioactivity detected at the lower limits of sensitivity in the tissue

TABLE I

RADIOENZYMATIC ASSAY IN THE ABSENCE OF COMT

Concentration of salsolinol added to neonatal tissue (pg per g tissue)	Gross CPM per g tissue*	Gross DPM per g tissue
100	5916 ± 233	15,169 ± 597
500	6069 ± 35	15,561 ± 89
1000	4383 ± 175	11,238 ± 448
2000	5992 ± 264	15,364 ± 677

*Mean value (± S.E.M.) of two observations. These mean values represent 84—120 CPM per 50-μl sample, and correspond to the value of the perchloric acid blank (with neither tissue nor salsolinol).

standard curve (Fig. 4) is only contributed by a tritiated O-methylated product (presumably [^{3}H]salsoline) and is not due to the formation of any COMT-independent reaction product which may have utilized ^{3}H-SAM as a ^{3}H-methyl donor. It should also be mentioned that the TLC zones corresponding to the R_F values of methoxytyramine, metanephrine, and normetanephrine were likewise lacking in any significant radioactivity above background values.

(b) The second set of experiments, using two-dimensional TLC in two additional solvent systems, was designed to further ensure that the tissue-extracted radioactivity on the TLC plate at the R_F corresponding to salsoline was contributed by a single product ([^{3}H]salsoline). The results of these two-dimensional chromatographic studies demonstrated that radioactivity on the TLC plate in the zone corresponding to the R_F of salsoline was contributed by a single product ([^{3}H]salsoline), since the two-dimensional development in the two additional solvent systems did not resolve the [^{3}H]salsoline spot into any additional components. It should be pointed out that if any [^{3}H]isosalsoline (6-O-methylsalsolinol) was formed along with the [^{3}H]salsoline (7-O-methyl-

salsolinol), then the former must have an identical mobility as the latter in all three solvent systems used in these experiments. It has been reported previously [5, 16] that isosalsoline and salsoline exhibit identical retention times in gas chromatographic procedures.

(c) Finally, in the third set of experiments, which was designed to determine if any endogenous salsolinol was present in the neonatal rat, an average of 19,944 ± 677 CPM/g tissue (51,138 ± 1735 DPM) was found in the zones on the TLC plates corresponding to the R_F of salsoline. Since this value falls on the non-linear lower portion of the tissue standard curve (Fig. 4), the amount of endogenous salsolinol present in neonatal rats cannot be accurately quantitated, but it is significantly less than 500 pg/g tissue ($P < 0.05$ by Student t test) and significantly greater than the values reported in Table I ($P < 0.001$ by Student t test). These findings are indicative of the presence of traces of endogenous salsolinol in neonatal rats who themselves and their parents had never been exposed to exogenous ethanol. An explanation for this finding may reside in the observation that acetaldehyde is endogenously formed in the rat gut [20], and it is possible that, following absorption, this endogenous acetaldehyde may condense with dopamine to form traces of salsolinol. Such reactions could conceivably occur in the fetus or be passively acquired through placental transfer of either acetaldehyde or salsolinol.

CONCLUSIONS

The radioenzymatic assay for salsolinol described in this report enables detection of trace amounts of salsolinol in tissue (0.1 ng/g), although accurate quantitation of this alkaloid can only be achieved at concentrations of 0.5 ng/g tissue or above. Accurate quantitation of plasma salsolinol can be achieved at concentrations of the alkaloid as low as 0.1 ng/ml. This radioenzymatic method is, therefore, more sensitive than the previous GC—EC assay for salsolinol developed in our laboratory [3], which had a lower sensitivity limit of 8 ng/g tissue. The radioenzymatic method is also more sensitive than the GC—EC procedure of Hamilton et al. [5] which is similar in sensitivity to our previous GC—EC method [3], and is likewise more sensitive than the high-performance liquid chromatographic assay described by Riggin and Kissinger [6] which has a sensitivity of 2 ng salsolinol per g tissue. With the use of the radioenzymatic procedure described in this manuscript, we were able to demonstrate the presence of endogenous salsolinol, in concentrations of < 0.5 ng/g tissue, in neonatal rat tissue in the absence of prior exposure to ethanol.

At the time of submission of this manuscript, Dean et al. [21] reported an apparently similar radioenzymatic assay for salsolinol as the one reported here.

ACKNOWLEDGEMENTS

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Biomedical Applications

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Note

Gas chromatography of urinary N-phenylacetylglutamine

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N-Phenylacetylglutamine (PAG) is a normal constituent of human urine [1]. In untreated phenylketonuria (PKU) its excretion is greatly increased, reflecting secondary endogenous metabolism of phenylalanine [2, 3]. Patients with malabsorptive disease may also excrete large amounts of N-phenylacetylglutamine as a result of excessive bacterial metabolism of unabsorbed phenylalanine in the intestinal lumen [4].

When screening for heritable organic acidurias in our laboratory, the acidified urine samples are extracted with ethyl acetate and the organic acids so obtained are analysed as the corresponding trimethylsilyl (Me_3Si) derivatives by gas chromatography (GC) [5]. For PKU patients and patients with malabsorptive disease, a peak with a relatively high retention time is observed in the chromatograms, which turned out to be a non-trimethylsilylated thermal degradation product of PAG.

EXPERIMENTAL

Synthetic N-phenylacetylglutamine was a gift of Dr. R.J. Kleipool (CIVO-TNO, Zeist, The Netherlands). N-Acetylglutamine was obtained from Serva (Heidelberg, G.F.R.).

For the extraction of organic acids, 5 ml of urine were mixed with 5 ml of a saturated NaCl solution and 0.5 mg of 2-phenylbutyric acid (internal standard). The solution was acidified to pH 1—2 with concentrated HCl and, after the addition of 10 mg of vitamin C (antioxidant), extracted twice with 20 ml of ethyl acetate. The combined ethyl acetate phase was dried over anhydrous Na_2SO_4 and then evaporated to dryness under reduced pressure at 40°.

Trimethylsilylation of compounds was performed with 0.5 ml of N,O-bis-(trimethylsilyl)acetamide (BSA) in chloroform (1:3, v/v) at 37° for 30 min.

For the preparation of methyl esters of synthetic N-acyl amino acids, the substances were dissolved in 1 ml of absolute methanol and esterified with diazomethane in ether for 5 min at room temperature.

Gas chromatography (GC) was carried out on a Varian Aerograph 3700 instrument equipped with a dual flame ionization detector and glass columns (8 ft. × 1/8 in.) packed with 5% GESE-52 on Chromosorb W AW DMCS, 100—120 mesh (HP). The column oven temperature was held at 67° for 10 min followed by an increase of 2°/min up to 220° and finally 15 min at 220°. The gas flow-rate for nitrogen was 30 ml/min, the injection port temperature 210° and the detector temperature 310°.

Mass spectra at 70 eV were recorded on a Jeol JGC-20 KP/JMS-D100/W-JMA combination at an ion-source temperature of 150°, an accelerating voltage of 3 kV and an ionizing current of 300 μA. Both the gas chromatograph and direct inlet were used. For routine analysis of Me_3Si derivatives, the GC conditions were the same as described above.

RESULTS AND DISCUSSION

In Fig. 1 a gas chromatogram of the urinary organic acids (Me_3Si derivatives) of a patient with phenylketonuria, having an unknown peak with t_R = 83.72 min, is presented. The product responsible for this peak could be removed from the ethyl acetate extract by anion-exchange chromatography on 100—200 mesh Dowex 2-X8 (OH^-). When the urine was acidified to pH⩾4, the compound could not be extracted. These observations suggested the presence of a carboxyl function in the original substance. Alkaline hydrolysis of the urine (after removal of phenylpyruvic acid with dinitrophenylhydrazine), followed by acidification and subsequent ethyl acetate extraction showed the accumulation of phenylacetic acid, whereas the unknown was no longer detected.

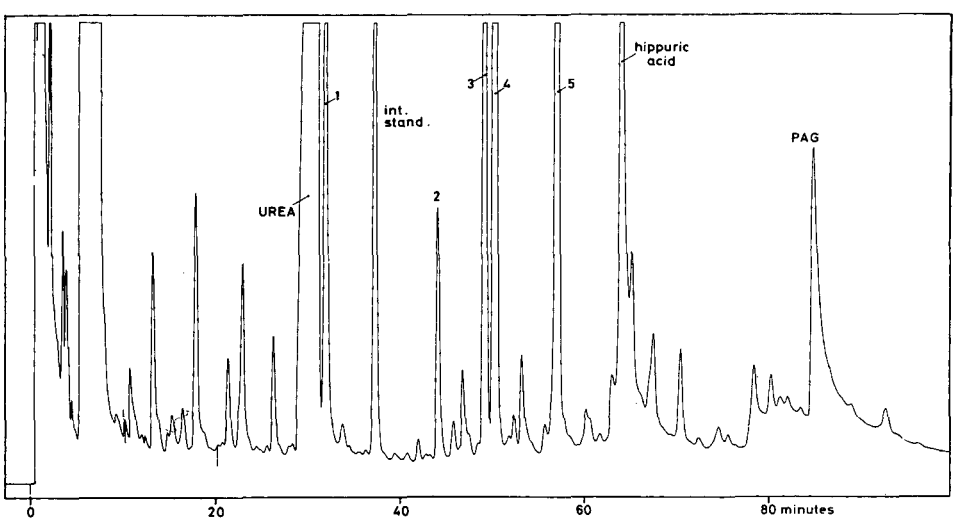

Fig. 1. Gas chromatogram of urinary organic acids (Me_3Si derivatives) of a patient with PKU. 1 = Phenylacetic acid; 2 = mandelic acid; 3 = *o*-hydroxyphenylacetic acid; 4 = phenyllactic acid; 5 = phenylpyruvic acid; internal standard = 2-phenylbutyric acid. The thermal degradation product of PAG is indicated as PAG.

The mass spectrum of the unknown, obtained by combined gas chromatography—mass spectrometry (GC—MS) of the trimethylsilylated urinary extract is given in Fig. 2. The very low abundance of the ions *m/e* 73 and *m/e* 147 indicated that the compound did not contain Me_3Si groups. The abundant ions at *m/e* 91, 92 and 118 were in accordance with the occurrence of a phenylacetyl group. These peaks shifted to higher masses when patients with PKU were loaded with partially deuterated phenylalanine (a mixture of 50% nondeuterated, 40% monodeuterated and 10% dideuterated L-phenylalanine). The various fragment ions present in the mass spectrum suggested the structure of PAG Me_3Si ester, of which Me_3SiOH has been eliminated (M = 246). The mass spectrum was identical to that of synthetic PAG treated with BSA. Because of the silylation procedure used, the formation of a Me_3Si ester has to be expected (see also below for N-acetylglutamine).

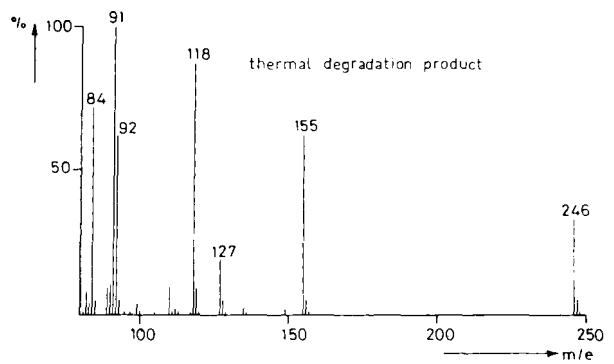

Fig. 2. Mass spectrum at 70 eV of the thermal degradation product of N-phenylacetylglutamine.

To investigate the problem of Me_3SiOH elimination, synthetic PAG was studied in more detail. Using the direct inlet of the mass spectrometer, PAG and its methyl ester showed at low probe temperatures (ca. 80°) the expected mass spectra (acid, Fig. 3; methyl ester, Fig. 4). At higher probe temperatures

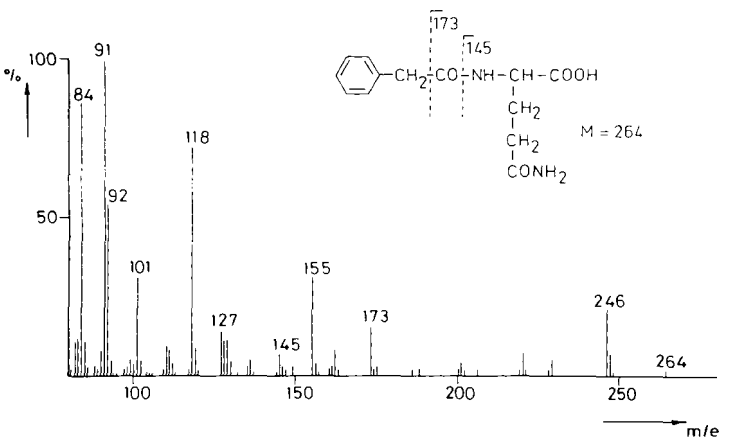

Fig. 3. Mass spectrum at 70 eV of N-phenylacetylglutamine.

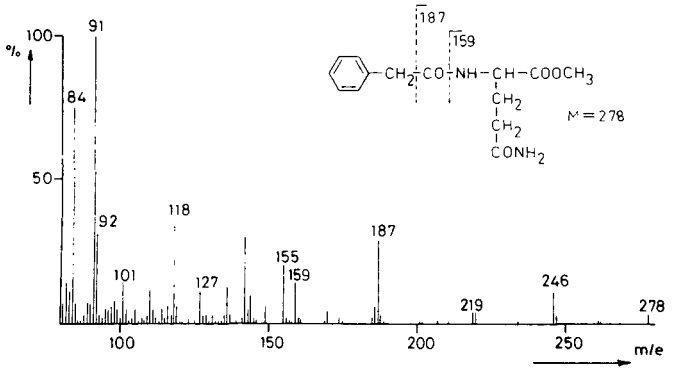

Fig. 4. Mass spectrum at 70 eV of N-phenylacetylglutamine methyl ester.

both mass spectra became identical to that shown in Fig. 2. For practical reasons the Me_3Si ester was not studied using the direct introduction technique. When analysed via the GC inlet system, PAG as well as the corresponding Me_3Si and methyl ester gave rise to the same mass spectrum (Fig. 2). Thermal degradation of organic compounds is known to occur in the ion source of the mass spectrometer. However, from the GC (—MS) data it can be concluded that in the latter cases thermal degradation of PAG (loss of H_2O), its Me_3Si ester (loss of Me_3SiOH) and its methyl ester (loss of CH_3OH) has to take place in the injection port of the gas chromatograph. Especially, PAG itself is not volatile enough to be analysed by GC (m.p. 105°—107°).

In addition, also the behaviour of N-acetylglutamine was studied. Analysis of N-acetylglutamine (M = 188) and its methyl ester (M = 202), using the direct introduction method, showed the expected corresponding mass spectra. The occurrence of a thermal degradation product could not be demonstrated clearly. N-Acetylglutamine, analysed via the GC inlet gave rise to a mass spectrum corresponding to the same type of degradation product (M = 170) as observed for PAG. For N-acetylglutamine methyl ester a broad and a sharp peak were seen on the gas chromatogram. The broad peak with lower retention time led to the mass spectrum of the substance M = 170, whereas the sharp peak gave the expected mass spectrum of the methyl ester (M = 202). As was evident from its mass spectrum obtained via the GC inlet, N-acetylglutamine treated with BSA gave the bis-Me_3Si derivative (M = 332); also the free amide function bears a Me_3Si group. The degradation product could not be detected. In this context it is unknown why for PAG a bis-Me_3Si derivative could not be detected.

In conclusion, GC analysis of the studied glutamine derivatives not protected at the amide function gives rise to the observed degradation; a free amide function seems to be essential. Two possibilities of elimination can be considered, namely that leading to a ketene structure or to an imide structure, as given in Fig. 5 for the degradation product of PAG (M = 246). To discriminate between these structures further investigations are necessary, as are preparative gas chromatography followed by more sophisticated mass spectrometry and nuclear magnetic resonance spectroscopy.

Finally, it has to be noted that our screening method [5] is not

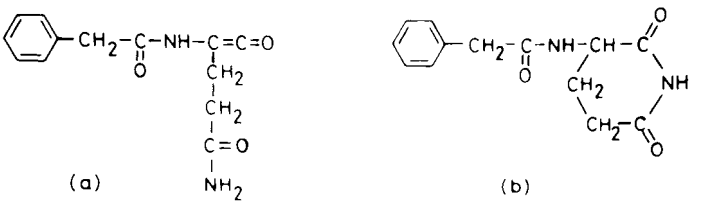

Fig. 5. Ketene (a) and imide (b) structures deduced from PAG.

recommended for the quantitative determination of urinary PAG. Although the extraction yield is 70%, the response with the flame ionization detector is very low. Only a significant asymmetric peak is obtained when the excretion is greatly increased: normal excretion levels are below the level of detection. Therefore, the determination of PAG can be carried out in a more reliable way, using alkaline hydrolysis [3, 4] followed by determination of liberated phenylacetic acid. Publication of our observations were thought to be useful for those who deal with screening for organic acidurias, as elevated PAG concentrations are encountered rather frequently.

ACKNOWLEDGEMENT

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Note

Use of 1-octadecanol as an internal standard for plasma lipid quantitation on chromarods

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The potential use of flame ionisation detection (FID) for the quantitation of lipids, separated by thin-layer chromatography (TLC) on silica gel coated rods, represents an important advance in analytical methodology [1, 2]. This technique combined with the chemical analysis of either individual lipids or of total lipids, can be applied to the determination of the absolute lipid concentrations [2]. The drawbacks of this method are the limitations of the chemical analysis in terms of sensitivity and sample amount. These limitations can partially be avoided by the use of a suitable internal standard. The aim of this report is to introduce 1-octadecanol as an internal standard for the direct quantitation of the major lipid classes and to compare the sensitivity and reproducibility of this technique to that of conventional chemical analysis.

MATERIALS AND METHODS

Lipid standards

Cholesterol was purchased from UCB (Brussels, Belgium), cholesterol oleate from Schuchard (Munich, G.F.R.) and triolein from Sigma (St. Louis, Mo., U.S.A.). Egg lecithin was purified by column chromatography [3]. The purity of the lipids was checked by thin-layer chromatography on silica gel plates. 1-Octadecanol from Serva (Heidelberg, G.F.R.) was used as internal standard.

Plasma samples

To compare the data obtained from chemical and chromatographic analysis, clinical chemistry control sera (lot No. NO4, Hyland Co.) as well as fresh

EDTA-treated plasma samples from normal fasting individuals and from type II hyperlipidemic patients were used.

Lipid analysis by TLC—FID

Internal standard. For the purpose of standardization, a number of compounds structurally related to the lipids were tested. These include oleic acid, tocopherol, octadecanol, undecanoic acid and pregnanediol.

Extraction procedure. For the extraction of the plasma samples a modification of the method previously described was introduced [2]. Plasma (50 μl) was extracted with 1.2 ml of chloroform—methanol (2:1, v/v) containing 50 μg of octadecanol corresponding to a final concentration of 1 mg/ml plasma. The mixture was washed with 0.5 ml of 0.2% $CaCl_2 \cdot 2\ H_2O$ [2] and centrifuged for 10 min at 1000 *g*. The organic phase was dried under nitrogen at 40° and the lipids redissolved in 10 μl of chloroform.

Thin-layer chromatography and FID. Aliquots (1—2 μl) of the chloroform extract were applied to the silica gel coated glass rods (chromarods). TLC of neutral lipids was carried out in petroleum ether (b.p. 60—80°)—diethyl ether—formic acid (85:15:0.1) on the Iatroscan TH-10 (Iatron Lab.) [2]. The peak areas were quantitated by triangulation.

RESULTS

Internal standard

Among the compounds tested, 1-octadecanol appeared to be the most suitable internal standard for lipid analysis by TLC—FID. Its selection was based on its stability and solubility in both the extraction mixture and the chromatographic solvent, and on its R_F value. 1-Octadecanol can be stored at 4° for at least two weeks in the extraction mixture at a concentration of about 1 mg/ml. The separation of the various lipid fractions in the presence of octadecanol is depicted in Fig. 1. The migration of the internal standard in a peak well separated from cholesterol and free fatty acids avoids any contamination of the lipid peaks.

Lipid calibration curves

In order to draw the calibration curves, various amounts of the lipid stock solutions (0.5 mg/ml in chloroform) were added to 1.2 ml of chloroform—methanol (2:1, v/v) containing 50 μg of 1-octadecanol. This mixture was washed with 0.5 ml of 0.2% $CaCl_2 \cdot 2\ H_2O$ and subsequently treated as described above.

The calibration curves span a concentration range of 0.1—2 mg/ml for free cholesterol, 0.15—4 mg/ml for triglycerides and 0.5—5 mg/ml for cholesterol oleate (expressed as mg esterified cholesterol) and for lecithin. The standard curves for free cholesterol, triglycerides and phospholipids are linear in these concentration ranges listed above.

For cholesterol esters the standard curve diverges from linearity at concentrations below 1.5 mg/ml. It could be fitted to a linear equation for concentrations corresponding to normal and pathological plasma samples (1.5—5 mg/ml). These calibration curves were fitted to a linear model by least-squares analysis.

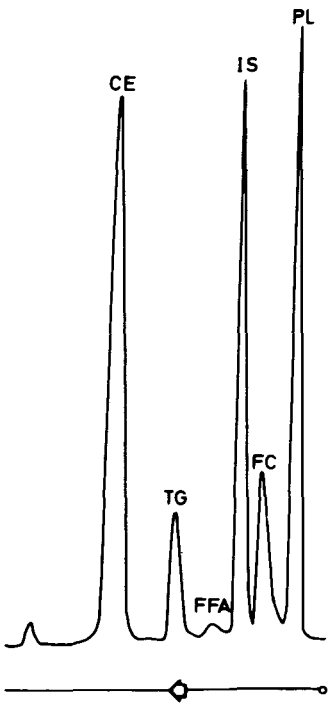

Fig. 1. Separation of plasma lipids and 1-octadecanol on chromarods, in petroleum ether—diethyl ether—formic acid (85:15:0.1). PL, phospholipids; FC, free cholesterol; IS, 1-octadecanol, internal standard; FFA, free fatty acids; TG, triglycerides; CE, cholesterol esters.

The equation parameters together with the correlation coefficients are summarized in Table I.

These data show that the correlation coefficients are higher than 0.98 for the four calibration curves, which validates the use of this particular internal standard. The lower correlation coefficient, for the phospholipids, was due to a loss of accuracy in the triangulation of the sharp phospholipid peak. The use of an integrator would improve the phospholipid quantitation.

Under the experimental conditions described above the detectable amount of each lipid is about 0.1 μg in a working range between 1 and 25 μg of lipid.

TABLE I

CHARACTERIZATION OF THE LIPID CALIBRATION CURVES

The curves were characterized according to the equation: area ratio (lipid/IS) = slope × concentration ratio (lipid/IS) + intercept.

Lipid*	Slope	Intercept	Correlation coefficient
CE	1.62	0.98	0.991
FC	1.43	0.12	0.996
TG	0.86	0.13	0.991
PL	0.83	0.11	0.984

*CE, cholesterol esters (expressed as cholesterol); FC, free cholesterol; TG, triglycerides; PL, phospholipids; IS, internal standard (1-octadecanol).

Validity of the internal standardization procedure

The extent of lipid recovery as a function of the extraction time was checked on plasma samples extracted on a Vibromix for 15, 30, 60 and 180 sec. The concentrations measured were independent of the length of the extraction and the samples were routinely extracted for 30 sec. The absolute concentration values provided with the control serum are strongly dependent on the chemical methods applied to the quantitation of total cholesterol and triglycerides. Table II summarizes the values for total, free and esterified cholesterol, phospholipids and triglycerides, obtained from a control serum, a pooled plasma sample and from two hyperlipidemic type IIA and IIB samples. The results obtained by TLC—FID are compared to those of conventional chemical analysis and to the values provided by Hyland (Costa Mesa, Calif., U.S.A.), together with the control serum. Standard deviation for the TLC—FID was estimated from ten successive analyses performed on the control serum and the pooled sample.

Compared to the values obtained by the chemical methods routinely used in our laboratory [4—9] the TLC—FID values are higher for total cholesterol and lower for phospholipids and triglycerides. Comparison of the two techniques remains valid up to cholesterol values of 4.5 mg/mg and triglyceride values of 3.4 mg/ml. The precision given by the standard deviation (estimated from ten successive analyses) is comparable to that of the normal samples, as are the differences between chemical and chromatographic values, indicating that linearity of the standard curves is acceptable in this concentration range.

The precision of the TLC—FID technique is comparable to that of the chemical analysis and amounts to about 10% for each lipid. The accuracy could be improved by use of an electronic integrator.

The method proposed is therefore comparable to conventional chemical analysis as regards the absolute lipid concentration values, the precision and the detection limits.

CONCLUSION

The introduction of an internal standard for the quantitation of plasma lipids by TLC with FID enables a one-step quantitation of cholesterol, cholesterol esters, triglycerides and phospholipids in normal and hyperlipidemic samples. In comparison with the technique previously proposed [2] it represents a major improvement by avoiding any chemical analysis step, being based on an extraction and a chromatographic separation only. This means a considerable time saving together with avoiding the risk of any limitations or contamination by lack of specificity of either colorimetric or enzymatic procedures. This procedure is applicable to microquantities of either plasma or lipoprotein fractions, can be carried out in the presence of high salt, buffers, etc., without any previous dialysis step, and could be valuable for clinical and research purposes. It could also be applied to the characterization of lipid mixtures from cell extracts or tissues such as fish lipids [10] or sterol mixtures as 1-octadecanol migrates close to free cholesterol. This particular standard compound can also be used with other non-polar solvents such as benzene—chloroform (80:20) that have been proposed for lipid separations.

TABLE II

COMPARISON OF THE TLC—FID PROCEDURE WITH CHEMICAL ANALYSIS

Lipid*	Sample**	Chemical analysis***	TLC—FID
TC	A	115 ± 12[a]	153 ± 15
		117 ± 11[b]	
		153 ± 15[c]	
	B	142 ± 14[a]	178 ± 18
	C	447 ± 50[a]	455 ± 43
	D	371 ± 28[a]	350 ± 37
CE	A	83 ± 10	112 ± 10
	B	95 ± 12	131 ± 13
	C	312 ± 39	305 ± 32
	D	260 ± 16	243 ± 25
FC	A	32 ± 5[d]	41 ± 4
	B	47 ± 6[d]	46 ± 5
	C	135 ± 11[d]	140 ± 10
	D	111 ± 12[d]	107 ± 9
TG	A	74 ± 12[a]	39 ± 3
		45 ± 10[f]	
		91 ± 15[g]	
	B	98 ± 18[e]	78 ± 8
	C	261 ± 32[e]	230 ± 21
	D	373 ± 48[e]	340 ± 37
PL	A	154 ± 15[h]	111 ± 12
		143 ± 28[i]	
	B	197 ± 20[h]	174 ± 18
	C	514 ± 48[h]	460 ± 50
	D	402 ± 43[h]	385 ± 32

*TC, total cholesterol; CE, cholesterol esters; FC, free cholesterol; TG, triglycerides; PL, phospholipids; expressed in mg/dl ± 2 S.D.

**A, Clinical Chemistry Control Serum (CCS); B, plasma pool of normal fasting patients; C, type IIA hyperlipidemic patient; D, type IIB hyperlipidemic patient.

***a, Cose et al. [4]; b, enzymatic method (Q-Pak I, Hyland); c, Pearson et al. [5] (Q-Pak I, Hyland); d, Roeschlau et al. [6]; e, Giegel et al. [7]; f, Kessler and Lederer [8] (Q-Pak I, Hyland); g, enzymatic method (Q-Pak, Hyland); h, Zilversmit and Davis [9]; i, Molybdate, vanadate—phosphate complex (Q-Pak, Hyland).

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Note

Rapid determination of diazoxide in plasma and urine of man by means of high-performance liquid chromatography

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The non-diuretic benzothiazine, diazoxide, is used as an antihypertensive drug, especially for the intravenous treatment of hypertensive crises. It is recommended to administer the drug as a bolus injection, 300 mg within 10—30 sec [1], but recently it was shown that also an infusion during 20—30 min was effective [2] and could avoid hypotension and consequent myocardial ischaemia. Orally administered diazoxide is seldom used for chronic treatment of severe hypertension.

No clear relationship has been established between plasma concentration, half-life ($T_{1/2}$) of elimination and vascular activity [1]. In hypertensive patients, the $T_{1/2}$ of elimination varies markedly between 21 and 50 h [1, 3,4]. The large

variation in pharmacokinetics observed may be as much related to analytical problems as to inherent variations in the patients. Currently analysis is carried out by a simple UV method [1, 3, 5], which is of low specificity since possible metabolites may have the same absorption characteristics. Sadee et al. [6] developed an excellent gas chromatographic—mass spectrometric (GC—MS) method for measuring diazoxide plasma concentrations but access to such equipment and the availability of the deuterated internal standard makes the assay difficult for routine applications. Grevink and Fleuren [7] developed a gas—liquid chromatographic method, which required derivatization of diazoxide as the methyl derivative. Both methods are quite laborious and the large series of samples that are required in kinetic studies or with therapy compliance control pose considerable methodological problems.

High-performance liquid chromatography (HPLC) combines simple UV detection with chromatographic specificity. The method is somewhat less specific than GC—MS but its simplicity, speed and the possibility of analysing large series of blood samples are advantages. An HPLC method for the analysis of diazoxide was developed for the purpose of studying pharmacokinetics of diazoxide in healthy human volunteers, hypertensive patients and control of therapy compliance, and some results of the pharmacokinetics of diazoxide in healthy volunteers are reported.

MATERIALS AND METHODS

Apparatus

A Spectra Physics 3500B high-performance liquid chromatograph was used, equipped with a spectrophotometric detector (Model 770). The detector was connected to a 1-mV recorder (BD 7, Kipp en Zonen, Emmen, The Netherlands). A stainless-steel column (10 cm × 4.6 mm I.D.) was packed with LiChrosorb RP-8, particle size 5 μm, obtained from Chrompack (Middelburg, The Netherlands). An injection loop of 100 μl was used. Detection of diazoxide was effected at 270 nm. The detection limit of diazoxide is 0.1 μg/ml.

Solvents

For diazoxide as a simple drug the solvent was a degassed mixture of 300 ml water and 200 ml methanol. The solvent flow-rate was 1 ml/min, at a pressure of 125 atm. (k' diazoxide = 4.4.)

For diazoxide with comedication of chlorthalidon, the solvent was a degassed mixture of 325 ml of 0.005 *M* sodium acetate and 175 ml of methanol. (k' diazoxide = 5.75; k' chlorthalidon = 3.25.)

Sample preparation

Plasma. Plasma (100 μl) is mixed with 400 μl of perchloric acid (0.33 *N*) on a Vortex mixer. Deproteinization is completed after standing for 5 min. The mixture is centrifuged for 5 min at 4000 rpm (2600 *g*) in a Heraus Christ centrifuge; 100 μl of the supernatant are injected onto the column.

Urine. Urine (10 μl) is mixed with 500 μl of perchloric acid (0.33 *N*) on a Vortex mixer; 100 μl of the mixture are injected onto the column.

Drugs

Diazoxide was obtained from Schering (Essex Nederland B.V., Amstelveen, The Netherlands).

Subjects and patients

Two healthy Caucasian subjects, both employees of the Department of Nephrology, volunteered for this study. Blood samples for routine control or pharmacokinetics studies in hypertensive patients were obtained from patients in the Department of Nephrology. Doses of 25, 100, 100 and 200 mg diazoxide were administered intravenously (i.v.) to the volunteers. Blood samples of 1 ml were taken at regular time intervals by venipuncture.

Spontaneously voided urine was collected for 60 h. One volunteer excreted the drug under acidic urinary conditions achieved by the daily intake of 8 g of ammonium chloride. The other volunteer excreted diazoxide under alkaline urinary conditions maintained by the intake of 10 g of sodium bicarbonate per day.

Recovery

The recovery of diazoxide from plasma was 83.7 ± 1.2% (S.D.) at a concentration of 10 μg/ml and 83.1 ± 2.8% (S.D.) at 5 μg/ml; from urine it was 98.1 ± 1.2% (S.D.).

The calibration curves were obtained by adding known amounts of diazoxide to human plasma and urine samples. They were linear for the concentration range 0.10–50 μg ($r = 0.998$). The sensitivity limit was 0.1 μg/ml.

RESULTS

Fig. 1 shows a chromatogram of two different human plasma samples containing diazoxide. The second peak (×) is an endogenous compound as it appeared in the plasma of both volunteers and patients, and is sometimes present in markedly varying concentrations in plasma of patients and volunteers who did not receive diazoxide.

Fig. 2 shows the plasma concentration, salivary concentration and renal excretion rate—time profiles in a volunteer after an intravenous dose of 100 mg of drug under alkaline urinary conditions. The half-life of elimination in the volunteers varies from 15 to 20 h. The ratio between the concentration of diazoxide in plasma and saliva is found to be constant in each volunteer, but varies between 1.5 and 4.8.

The two human volunteers excreted about 20% of the drug unchanged after all doses (Table I). The relationship of renal excretion rate (μg/min) to the plasma concentration (μg/ml) appeared to be linear, showing an average renal clearance constant of 5.36 ml/min, as shown in Fig. 3. The renal clearance of diazoxide was calculated from each urine sample and corresponding plasma sample and plotted against urine flow or urinary pH.

When the urinary pH is not modified by the intake of ammonium chloride or sodium bicarbonate the renal clearance—urinary pH relationship shows a relatively high correlation coefficient (Table I). In both cases the renal clearance is also dependent on the urine flow (Fig. 4). The average renal

clearance over the whole time course of the elimination process of diazoxide is low (2—6 ml/min). The calculated pharmacokinetic parameters of diazoxide are summarized in Table I.

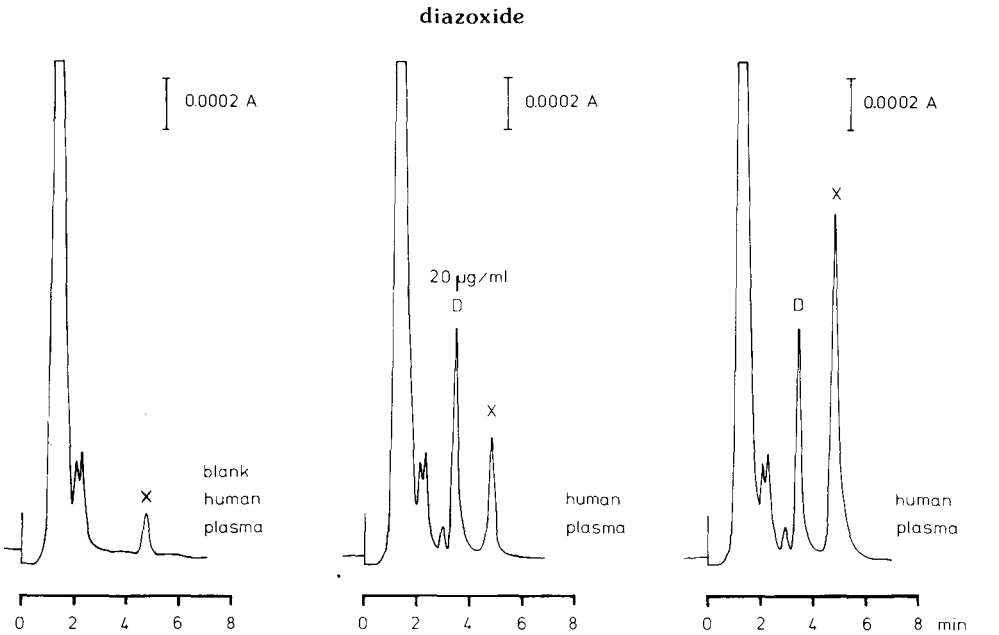


Fig. 1. Chromatogram of diazoxide (D) as single medication in a human plasma sample. Compound X is an endogenous compound present in markedly varying concentrations. Solvent: 300 ml water—200 ml methanol. Solvent flow-rate: 1 ml/min. Column: LiChrosorb RP-8, 5 µm particle size.

DISCUSSION

The HPLC method is found to be reliable and extremely simple and it exhibits a good sensitivity limit of 0.1 µg/ml. This value is between the sensitivity limit of the UV method of Symchowicz et al. [4] and that of the GC—MS method of Sadee et al. [6] (10 ng/ml). Even with a subclinical dose of 25 mg i.v., the plasma concentration elimination curve is followed for 60 h, which is long enough for estimation of $T_{1/2}$ values.

The low renal clearance of the unchanged drug implies high protein binding, strong tubular reabsorption and therefore dependency on urinary pH and urine flow. Because of the low renal clearance, the main route of elimination therefore must be oxidation to a carboxylic acid, followed by glucuronidation and renal excretion.

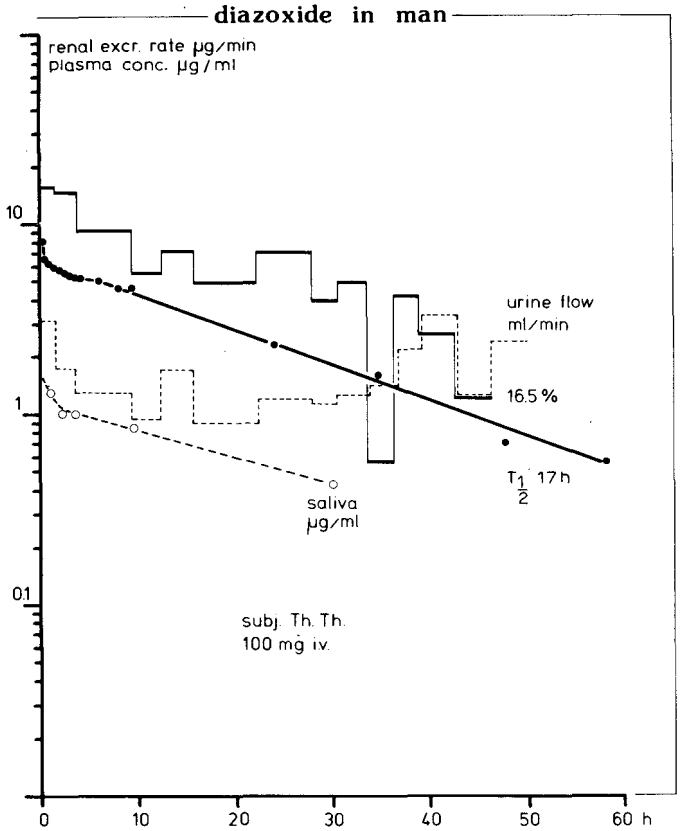


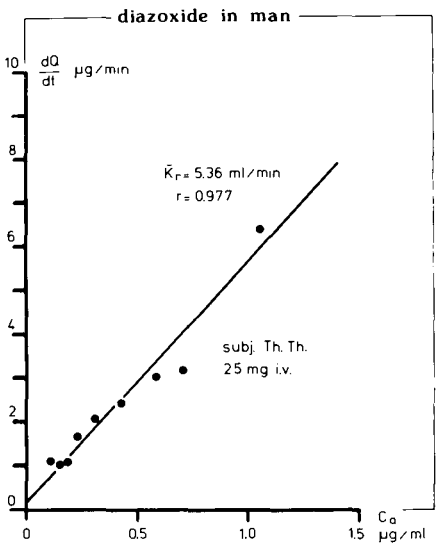
Fig. 2. Plasma and saliva concentrations and renal excretion rate—time profiles of diazoxide after an i.v. dose of 100 mg in a volunteer. There is a constant ratio (4.8) between the plasma and saliva concentration. The urinary pH in this experiment has been kept alkaline [pH 7.70 ± 0.49 (S.D.)].

TABLE I

SOME PHARMACOKINETIC PARAMETERS OF DIAZOXIDE IN MAN

Subject	Dose (mg, i.v.)	Urine flow (ml/min ± S.D.)	Urine pH (± S.D.)	$T_{½}$ (h)	Percentage excreted*	Renal clearance (ml/min ± S.D.)
T.Th.	25	0.99 ± 0.30	6.19 ± 0.28	17	27.8	6.70 ± 1.79
T.Th.	100	1.69 ± 0.76	7.70 ± 0.49	15	16.5	2.05 ± 0.79
F.H.	200	1.45 ± 0.89	6.42 ± 0.59	20	20.4	3.50 ± 1.76
F.H.	100	2.61 ± 1.81	5.34 ± 0.17	16	14.7	3.25 ± 1.96

*Percentage of the dose excreted in the urine unchanged.
**Correlation coefficient relationship between urinary pH and renal clearance.
***Correlation coefficient relationship between urine flow and renal clearance.



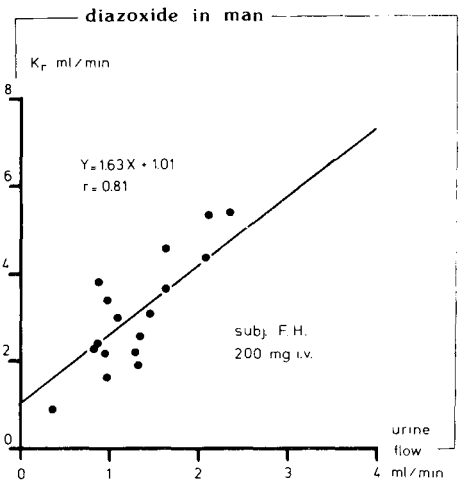


Fig. 3. Linear relationship between the renal excretion rate dQ/dt (µg/min) and plasma concentration C_a (µg/ml) of diazoxide in man with uncontrolled urinary pH. The average renal clearance (K_r) over the whole excretion period is 5.36 ml/min.

Fig. 4. Relationship between the renal clearance of diazoxide (K_r) and the urine flow in a volunteer receiving 200 mg iv. The urinary pH is 6.42 ± 0.59 (S.D.).

The half-life of elimination in the two healthy volunteers did not vary much. The $T_{1/2}$ may be dependent on the renal function, since with impaired kidney function the $T_{1/2}$ is prolonged to 40—50 h [8], but it also depends on the degree of hypertension [6].

The availability of a simple, reliable and fast analytical method for the measurement of diazoxide in human plasma samples may reveal the relationships between dose, speed of injection, plasma concentration and blood pressure lowering effects.

Ratio of plasma/saliva concentration	r	
	pH—K_r**	flow—K_r***
—	0.46	0.72
4.8	0.22	0.38
1.5	0.62	0.81
3.5	0.020	0.22

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Note

Simultaneous, rapid high-performance liquid chromatographic microanalysis of plasma carbamazepine and its 10,11-epoxide metabolite

Applications to pharmacokinetic studies in humans

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Carbamazepine is an anticonvulsant used in the treatment of grand mal, psychomotor epilepsy and also in trigeminal neuralgia. Side-effects are fairly common and therapeutic drug monitoring should be carried out during treatment.

Plasma concentrations of carbamazepine may be closely related to its therapeutic as well as toxic effects [1]. Carbamazepine exerts a useful therapeutic effect at plasma concentrations of 2—5 μg/ml [2, 3]. Plasma levels higher than 12 μg/ml are often associated with adverse effects. Carbamazepine 10,11-epoxide, the major metabolite in plasma [4], is as potent as the parent compound in preventing electroshock-induced seizures in the rat [5]. It is necessary, therefore, to measure the parent drug and its active metabolite, as both may contribute to the therapeutic effect of the drug [6].

Gas—liquid chromatographic (GLC) [7, 8], thin-layer chromatographic [9], spectrophotometric [10], fluorescence [11], and high-performance liquid chromatographic (HPLC) [12, 13] methods have been used to determine carbamazepine alone or in combination with other agents.

All proposed methods suffer from one more of the following requirements: an excessive amount of manipulation time; extraction (either single or multiple steps) to clean the sample; sample dilution; a fairly large volume of plasma; chemical derivatization (in GLC). Furthermore, the poor stability of carbamazepine and its 10,11-epoxide at high temperatures (GLC) may cause errors in the determination of true plasma levels.

The method developed has none of these drawbacks. Quantitation can be achieved within 15–20 min after obtaining the plasma sample. No derivatives are made, and no extraction used. This method is ideally suited for the simultaneous determination of the two compounds in plasma using as little as 50 μl of plasma, and was used to investigate the kinetics of carbamazepine and its metabolite in humans.

EXPERIMENTAL

Reagents

Acetonitrile (HPLC grade) was supplied by Merck (Darmstadt, G.F.R.). Carbamazepine and its 10,11-epoxide were obtained from Ciba-Geigy (Basel, Switzerland) by generous gift. Nitrazepam was supplied by Roche (Basel, Switzerland).

Apparatus and chromatographic conditions

An LC3 liquid chromatograph (Pye Unicam, Cambridge, Great Britain) equipped with a variable-wavelength detector (Pye Unicam) was used in a reversed-phase system with Partisil ODS-2 as the stationary phase (250 mm × 4.6 mm I.D.; particle size 10 μm; Whatman), and acetonitrile–water (50:50, v/v) as the mobile phase. Assays were performed using column thermoregulation (25°). The flow-rate of the mobile phase was maintained at 0.9 ml/min (±0.02). The effluent stream was monitored at 288 nm. The volume of sample injected was 20 μl (Rheodyne injector).

Standard curve and sample preparation

A standard curve was prepared for carbamazepine and its 10,11-epoxide in the following manner. Aliquots of 200 μl of pooled human plasma were spiked with various quantities of methanolic stock solution (1 g/l) of each compound. The stock solutions were kept refrigerated and sealed until use (stable for 2 months at +4°). A plasma that was known to be free of the compounds to be analysed and five plasma samples, with concentrations ranging from 1 to 20 μg/ml of carbamazepine and its epoxide, were Vortex mixed for about 20 sec. These samples were deproteinized by the addition of 200 μl of a freshly prepared (daily) acetonitrile solution of internal standard, nitrazepam, at 4 μg/ml (methanolic stock solution of 1 g/l, stored at +4° in the dark up to one month), Vortex mixed 10 sec and centrifuged for 5 min at 650 *g*. Aliquots of 20 μl of the clear supernatant were chromatographed. Peak heights were used for quantitation.

Drug recovery study

Five samples each of 200 μl of water and pooled human plasma were spiked

with a stock solution of carbamazepine, its 10,11-epoxide and nitrazepam to give a final concentration of 5 μg/ml and analysed as described above.

Drug interference study

Many compounds were tested for possible interference. A reference toxicological plasma was used (Hyland, Costa Mesa, Calif., U.S.A.).

In vivo study

Six in-patients participated voluntarily in the study. Carbamazepine, 3 mg/kg or 6 mg/kg of body weight in capsule form, was administered as a single oral dose at 8.00 a.m. No food was allowed for 12 h before dosing. Venous blood samples were collected in heparinized vials at various times and were centrifuged within 10 min at 650 *g* (+4°) to obtain plasma fractions (stored at −20° until analysis).

RESULTS AND DISCUSSION

Method

The standard curves of carbamazepine and its 10,11-epoxide were linear in the concentration ranges studied. For example, the carbamazepine plot linear regression line was $y = 0.322x - 0.037$ ($r = 0.9999$), and that for the epoxide $y = 0.179x - 0.019$ ($r = 0.9977$), where y is the ratio of the peak height (in centimeters) of carbamazepine or the epoxide to that of nitrazepam, and x is the concentration in μg/ml.

The peak height measurements were found to be superior to peak area estimation. For example, the reproducibility, estimated by the ratio (S.D.) (peak of carbamazepine)/(mean)(peak height or area), was 0.016 for peak height and 0.0304 for peak area measurements.

Reproducibility was calculated by 30 replicate analyses for each concentration on the standard curve (Table I) and was satisfactory.

Recovery was essentially complete: 99.8% ± 0.2 (mean ± S.D., $n = 30$). Under the conditions of this analysis, the minimum concentration that can be

TABLE I

REPRODUCIBILITY OF CARBAMAZEPINE (I) AND ITS 10,11-EPOXIDE METABOLITE (II) FROM PLASMA

Each value represents the mean of 30 experiments (± S.D.).

	Amount added to plasma (μg/ml)	(peak height I or II)/(peak height IS) ± S.D.	Amount found (μg/ml) ± S.D.
I	2.0	0.474 ± 0.009	1.90 ± 0.40
	5.0	1.446 ± 0.027	5.15 ± 0.12
	10.0	2.644 ± 0.019	9.76 ± 0.08
	20.0	6.043 ± 0.067	20.06 ± 0.74
II	2.0	0.350 ± 0.008	1.98 ± 0.30
	5.0	0.915 ± 0.020	5.05 ± 0.15
	10.0	2.066 ± 0.024	9.80 ± 0.10
	20.0	4.087 ± 0.059	20.30 ± 0.62

measured accurately is about 200 ng/ml of carbamazepine and of the epoxide. This value for carbamazepine is well below the therapeutic level.

Lower concentrations give poor accuracy because of error in peak height measurement. Carbamazepine exhibits maximal absorption at around 285 nm and its epoxide at 288 nm. Detection at 288 nm was adequately sensitive and endogenous and exogenous interferences were minimized.

Column regulation permits no variation in retention time and accurate reproducibility of the peak height ratio of drug to internal standard (IS). Retention times for carbamazepine and the 10,11-epoxide were 7.4 min and 8.4 min, respectively (IS = 6.6 min). The flow-rate of 0.9 ml/min was optimal. The efficiencies (plates per metre) were determined using various flow-rates. At 0.9 ml/min, the number of plates per metre was 4772 for carbamazepine and 5088 for the 10,11-epoxide with resolution factor $R = 1.04$. A typical chromatogram is shown in Fig. 1.

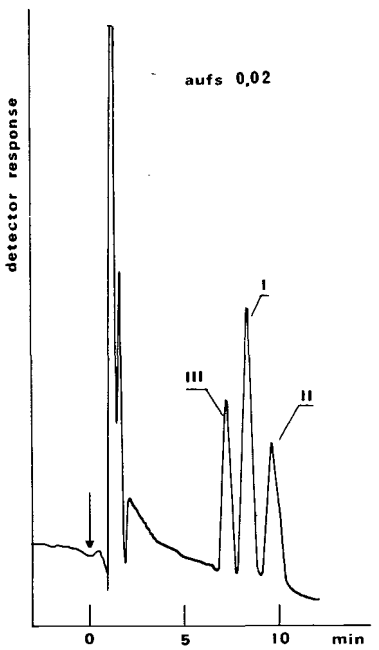

Fig. 1. Chromatogram (HPLC) of carbamazepine (I) and its 10,11-epoxide metabolite (II) (5 μg/ml of each), and nitrazepam (III), (internal standard, 4 μg/ml), using pooled, spiked human plasma.

The drug interference study showed that only phenytoin interfered with carbamazepine but at a concentration much higher than would usually be encountered clinically. Pooled human plasma spiked with 5 μg/ml of carbamazepine and 30 μg/ml of phenytoin gives 5.27 μg/ml for carbamazepine (recovery 105.2%, error 5.2%).

After centrifugation the clear supernatant may be stored in a sealed glass culture tube for 2—4 days without significant change.

In vivo study

The data for humans are graphically presented in Fig. 2.

The various pharmacokinetic parameters (and their mean values) calculated are set out in Table II.

The mean values of K and $T_{1/2}$ obtained in the present study are reasonably similar to these reported by others [7, 13—15]. Dose dependence of K_{el} and V_d was studied in our laboratory and the results [16] suggest that the elimination rate constant tends to increase with increasing drug dose.

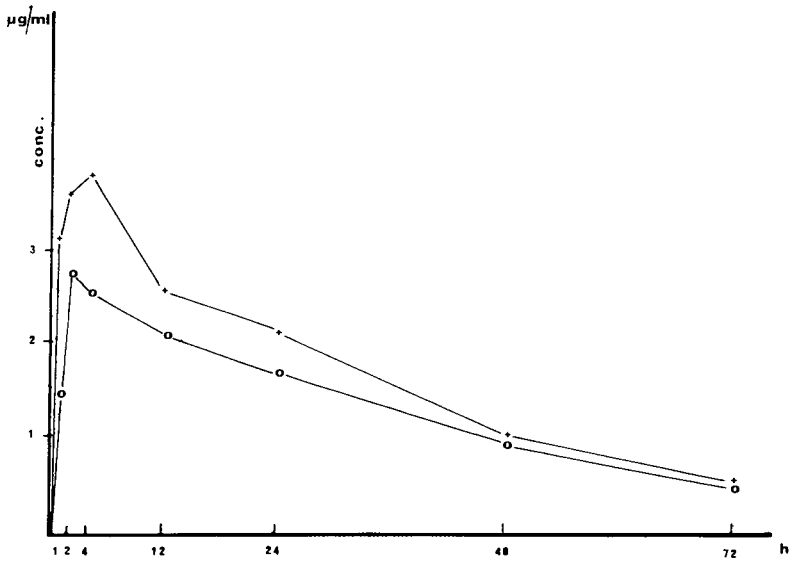

Fig. 2. Plasma levels of carbamazepine after a single oral dose (○, 3 mg/kg; +, 6 mg/kg) of carbamazepine to human volunteers.

TABLE II

PHARMACOKINETICS PARAMETERS* AND THEIR VALUES FROM SIX VOLUNTEERS

Dose	Patient	$T_{1/2}$ (h^{-1})	K_{el} (h^{-1})	AUC_0^∞ (mg l^{-1} h)	V_d (l kg^{-1})
3 mg/kg	Del.	50.4	0.0134	273	0.78
	Ric.	35.0	0.0198	190	0.85
	Bon.	30.5	0.0227	130	0.99
Mean		38.6	0.0186	197	0.87
S.D.		± 10.4	± 0.0047	± 71	± 0.11
6 mg/kg	Lab.	25.9	0.0267	151	1.46
	Zel.	43.3	0.0160	225	1.65
	Mar.	22.9	0.0302	98	1.10
Mean		30.7	0.0243	158	1.40
S.D.		± 11.0	± 0.0074	± 63	± 0.28

*$T_{1/2}$, elimination half-life; K_{el}, first-order elimination rate constant; AUC_0^∞, area under the plasma concentration curves; V_d, apparent volume of distribution.

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Note

Direct thin-layer densitometric determination of pharmacological concentrations of furosemide in plasma and urine

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Furosemide is one of the most commonly used diuretics. Therapeutic doses vary enormously, from 20 mg in elderly patients suffering from congestive heart failure to one or more grams in for instance uraemic patients and some patients with the nephrotic syndrome.

The clinical effect is easily registered by the diuretic response and since furosemide is widely non-toxic, monitoring of the plasma concentrations is not used in daily clinical treatment. However, measurements of furosemide concentrations in plasma and urine are necessary in studies of the renal effect in different pathophysiological states.

Several methods are available for estimation of furosemide in biological fluids. Both the original colorimetric [1] and the fluorometric methods [2—4] have the disadvantage that they are non-specific and estimate both furosemide and its pharmacologic inactive metabolite 4-chloro-5-sulfamyolanthranilic acid (CSA). The gas chromatographic method has a high specificity [5] but is very time-consuming and has a very low analytical capacity. A specific direct thin-layer fluorometric method with a very high sensitivity has recently been described but only for plasma measurements [6]. The method described needs 1000 μl of plasma for duplicate measurements.

During the last few years we have used a direct thin-layer densitometric assay based on a colour reaction with Ehrlich's reagent for estimation of furosemide in both plasma and urine. It seemed preferable to use the same method for both plasma and urine in order to minimize errors, because a direct comparison

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of the concentrations in plasma and urine is required for determination of the renal clearance of the drug. Since we have been interested in studying renal furosemide clearance in anaemic uremic patients, it was further desirable to reduce the amount of plasma needed.

MATERIALS AND METHODS

Apparatus

A Zeiss spectralphotometer with thin-layer chromatographic TLC-scanning equipment KM 3 (Carl Zeiss, Oberkochen, G.F.R.) linked to a Servogor Sb RE 646 recorder (Goerz Electro, Vienna, Austria) was used. The apparatus was equipped with two photomultipliers for the simultaneous measurement of remission and transmission.

Chemicals

All reagents were of guaranteed reagent grade and were used without further purification. Furosemide and CSA were obtained by courtesy of Hoechst Pharmaceuticals (Frankfurt/M, G.F.R.).

The composition of Ehrlich's reagent: 1 g of *p*-dimethylaminobenzaldehyde dissolved in 50 ml of hydrochloric acid (25%) and 50 ml of ethanol (96%).

TLC plates

Pre-coated silica gel 60 glass plates without fluorescent indicator, 20 × 10 cm, with a layer thickness of 0.25 mm (Merck, Darmstadt, G.F.R.) were used. Before chromatography the plates were washed with acetone for 15 min and dried for 15 min at 80°.

Standard solutions

Furosemide (100 mg) was dissolved in 100 ml of ethanol (99%). This solution was diluted with distilled water. Standards with known amounts of furosemide were prepared from furosemide-free pooled plasma and urine spiked with 20 μl solution per ml plasma or urine. Standard solutions were prepared once a month.

Extraction

Samples were stored deep-frozen until analysis. A 250-μl aliquot of plasma acidified with 50 μl of hydrochloric acid (3 *M*) or 150 μl of urine acidified with 75 μl of phosphate buffer (0.2 *M*, pH 2.0) was extracted with 1.5 ml of chloroform for 5 min using a mechanical shaker. The two phases were separated by centrifugation for 5 min at 3000 *g*. 1.2 μl of the organic phase was transferred to a conical glass tube and evaporated to dryness under a stream of nitrogen at 40°. Residuals were redissolved in 100 μl of methanol and the evaporation to dryness was repeated. The residue was dissolved in 10 or 20 μl of ethylene chloride—methanol (3:1) and 5 μl of this extracted material was spotted under nitrogen onto the TLC plate.

Thin-layer chromatography

TLC was conducted with the exclusion of light in a tank lined with Whatman

No. 2 chromatography paper. The solvent was chloroform—methanol—glacial acetic acid (89:6:2.5). In this solvent furosemide has a R_F value of 0.25—0.29, CSA of 0.11.

Seventeen samples were spotted onto each plate. Ten to seven of the unknown urine or plasma samples were applied onto one plate together with the appropriate reference spots from standard solutions.

Staining

After chromatography the spots of furosemide were coloured in situ by the following procedure: The TLC plates were dipped in Ehrlich's reagent—ethanol (99%)—hydrochloric acid (25%) (20 ml:40 ml:40 ml) for 7 sec and excess of reagent was removed by pressing the wet layer onto filter paper No 617. The TLC plates were then immediately placed in an oven at 50° for 10 min. To ensure uniform heating of the TLC plate it was placed on a 3 mm thick copper plate. After heat treatment the TLC plates were left at room temperature for 15 min before scanning, to ensure full development of the spots, which then remain stable for at least one week.

Measurement and quantitation

The monochromator was set at 480 nm and the remission/transmission ratio used was 100:25. The scanning speed was 120 mm/min. Measurements on the plate can be taken either in the direction of the solvent flow or perpendicular to the solvent flow across the furosemide spots. The latter procedure is much less time-consuming and gives the same results as the former.

The amount of furosemide was calculated by comparison of the peak areas (integrator counts) for samples and standards. The standard curve was fitted with a polynomium of the form $y = ax^b$ where y is the peak area and x is the concentration. The correlation coefficients obtained were 0.99 or better.

RESULTS AND DISCUSSION

Plasma and urine from subjects who had not ingested furosemide showed a variable but always negligible peak in the TLC scan (Fig. 1). As shown in Fig. 2 furosemide is separated from the metabolite CSA. After a single administration of furosemide CSA could not be detected in either plasma or urine. The TLC scan from an uraemic patient given 1000 mg furosemide orally, showed no interfering substances and no CSA in the chromatogram (Fig. 3).

The lower limit for reliable quantification of furosemide in plasma and in urine was 0.1 μg/ml. Reproducibility studies were performed on plasma and urine samples spiked with furosemide and the results are outlined in Table I. The areas of the standards differed from day to day and to calculate the reproducibility the different areas were calculated in per cent of the area of 8 μg/ml (plasma) or 10 μg/ml (urine) of the furosemide standard. The coefficient of variation of day-to-day estimations decreased with increasing furosemide concentrations (Table II). The recovery of furosemide in plasma was 97.0% (S.D. ± 3.4) and in urine 103.7% (S.D. ± 4.5) and for both plasma and urine independent of the furosemide concentration within the range studied (plasma 0.3—7.0 μg/ml, urine 0.3—9.0 μg/ml).

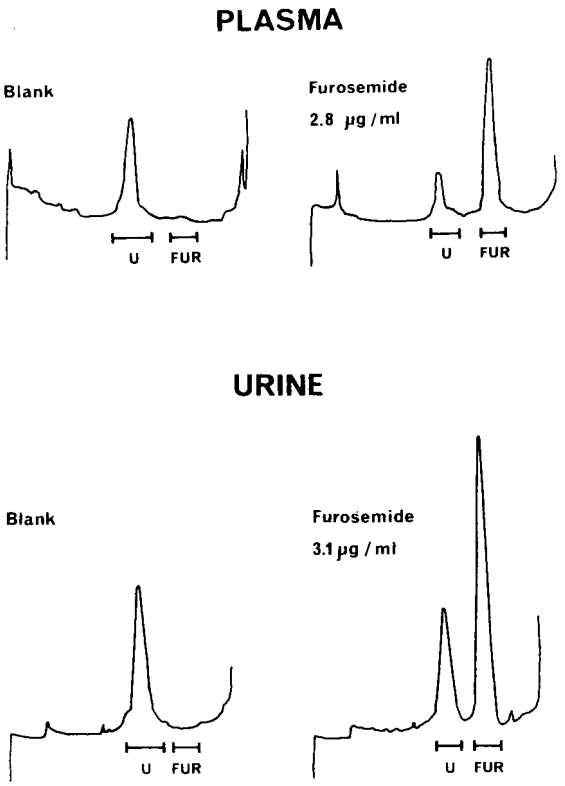

Fig. 1. TLC scans of plasma and urine from patients before and during intraveneous infusion of furosemide. FUR = furosemide, U = unknown substance.

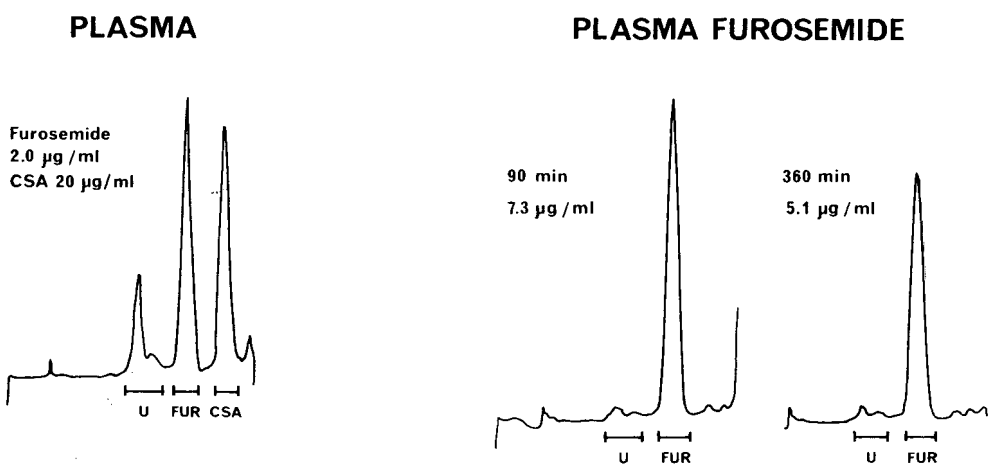

Fig. 2. TLC scan of plasma spiked with furosemide (FUR) and CSA. U = unknown substance.

Fig. 3. TLC scans of plasma from a uraemic patient (creatinine clearance 0.1 ml/sec) 90 and 360 min after oral administration of 1000 mg furosemide (FUR). U = unknown substance.

TABLE I

REPRODUCIBILITY OF THE ANALYSIS

Sample	Amount of furosemide added (μg/ml)	Area $\bar{x}$*	S.D.	S.E.M.
Plasma	0	1.53	0.39	0.11
	0.1	3.32	0.44	0.13
	0.25	5.85	0.49	0.14
	0.5	9.49	0.82	0.24
	1.0	17.70	0.69	0.20
	2.0	32.01	1.56	0.41
	4.0	57.71	2.39	0.69
Urine	0	3.05	0.30	0.09
	0.1	3.89	0.28	0.09
	0.25	5.05	0.25	0.08
	0.5	6.39	0.19	0.06
	1.0	9.65	0.45	0.14
	4.0	27.95	1.21	0.38
	10.0	10.07	2.68	0.85

*n = 12 for plasma; n = 9 for urine.

TABLE II

DAY TO DAY VARIATIONS AT DIFFERENT FUROSEMIDE CONCENTRATIONS

Single determinations.

Sample	Concentration of furosemide (μg/ml)	Coefficient of variation (%)	n
Plasma	0.38	9.1	10
	0.97	6.8	12
	7.20	4.0	10
Urine	0.19	9.0	8
	1.87	6.2	10
	9.26	3.6	7

So far no interfering compounds have been found in samples from patients undergoing multi-drug therapy.

CONCLUSION

A convenient and adequately sensitive direct thin-layer densitometric method has been developed for measurements of furosemide in both plasma and urine. The method is specific which is a necessary requirement for clinical pharmacological studies of furosemide.

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Note

Fluorimetric determination of oxprenolol in plasma by direct evaluation of thin-layer chromatograms

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Oxprenolol, 2-hydroxy-3-(*o*-allyloxyphenoxy-)propylisopropylamine, a beta-adrenergic blocking agent, is used frequently in the treatment of angina pectoris and arterial hypertension. Methods for the determination of oxprenolol by gas—liquid chromatography was described by Nambara et al. [1], and by Jack and Riess [2], the latter method being modified by Degen and Riess [3]. Oxprenolol is determined as its N,O-bis(trifluoro)acetyl derivative. All these methods need several extraction steps to obtain extracts free from interfering substances. They are therefore relatively time-consuming. The limit of detection is 10 ng/ml if 2 ml of plasma are extracted [2, 3].

Fluorimetric methods exist for several other beta-blocking drugs such as atenolol [4, 5], bufuralol [6], propranolol [7—11] and sotalol [12]. The spontaneous fluorescence of these drugs is measured either in the cuvette without further purification or following chromatographic separation on thin-layer plates or by high-performance liquid chromatography. Separation from interfering substances extracted from plasma by thin-layer chromatography (TLC) usually avoids a re-extraction step. Therefore, measurement of spontaneous fluorescence of drugs following TLC separation is a specific and less time-consuming technique.

Oxprenolol does not show intensive fluorescence on TLC plates either in the visible region or in the ultraviolet. Therefore, derivatisation of oxprenolol to a fluorescing compound is necessary. 1-Ethoxy-4-(dichloro-*s*-triazinyl)naphthalene (EDTN) was selected as derivatising agent. Chayen et al. [13] have described an assay of corticosteroids, and Stopher [14] reported the estimation of tolamolol using EDTN as the fluorescence reagent.

EXPERIMENTAL

Instrument

A chromatogram spectrophotometer KM 3 from Carl Zeiss with a Perkin-Elmer recorder Model 56 was used.

Standard and reagents

Oxprenolol hydrochloride and metoprolol tartrate (internal standard) were obtained from Ciba-Geigy (Basle, Switzerland). EDTN was supplied by BDH (Poole, Great Britain). Solvents (analytical grade) and TLC plates (silica gel 60 without fluorescence indicator) were obtained from Merck (Darmstadt, G.F.R.).

Method

Extraction. To 1 ml of plasma 10 μl of a methanolic solution containing 100 ng of metoprolol tartrate as internal standard are added; then 0.5 ml of 1 *N* NaOH, 250 mg NaCl and 5 ml of dichloromethane—diethyl ether (1:4) are added. After shaking for 15 min in a horizontal position the tubes are centrifuged briefly to separate the layers. The organic phase is transferred to another tube and evaporated to dryness at 50° under a gentle stream of nitrogen.

Derivatisation and chromatography. A 40 μl volume of a solution of 1 mg of EDTN in 5 ml of ethyl acetate is added to the solid residue. The stoppered tube is heated to 50° for 15 min. After cooling to room temperature the reaction mixture is taken up with a 100 μl Hamilton syringe. The whole solution is applied with a Linomat III (Camag, Switzerland) onto a TLC plate taking care to keep the spot size less than 4 mm in diameter. Together with a series of plasma samples with unknown amounts of oxprenolol, three standards are spotted per plate. The standards are plasma samples spiked with definite identical amounts of oxprenolol hydrochloride. These standards are carried through analysis together with the other samples.

The chromatograms are developed in an unlined glass tank containing the solvent system chloroform—ethyl acetate (95:5). The solvent (freshly prepared each day) is allowed to ascend about 15 cm above the origin. The R_F values for the derivatives of oxprenolol and the internal standard are 0.62 and 0.41, respectively.

After drying in the air the plate is dipped into a solution of 2% paraffin in cyclohexane. The fluorescence is measured after another drying period of at least 15 min.

Measurement and evaluation. The measurements are performed using the monochromator sample mode of the scanner. Fluorescence of the spots due to the derivatives of oxprenolol and metoprolol is measured with the 365 nm line of a medium pressure lamp St 41 and a 436 monochromatic filter. The slit is 0.5 × 6 mm. The plates are scanned at 100 mm/min.

The peak height ratios oxprenolol/metoprolol are calculated. The amount of oxprenolol in the plasma samples is calculated from the peak height ratios of the standards (mean of three determinations).

Reaction conditions

Oxprenolol hydrochloride and metoprolol tartrate (1 μg of each) were derivatised with EDTN solutions containing 1, 4 and 20 mg of EDTN per 10 ml of ethyl acetate. Three series of samples with 0, 100, 500 and 1000 ng of oxprenolol hydrochloride and 300 ng of metoprolol tartrate (both dissolved in methanol) were reacted with 40 μl of EDTN solution (2 mg per 10 ml) at 35, 40, 50 and 70° for 15 min. Another series of samples with 1 μg of both oxprenolol hydrochloride and metoprolol tartrate was heated to 50° for 10, 15, 30 and 60 min. All further analysis steps were carried out as described above.

Standard curves

Standard curve A. To samples containing 0, 1, 3, 5, 7, 10, 30, 70, 100, 300, 700 and 1000 ng of oxprenolol hydrochloride, 100 ng of metoprolol tartrate were added. After the addition of 40 μl of EDTN solution the samples were derivatised, chromatographed and measured as described above.

Standard Curve B. Blank plasma was spiked with 0, 5, 10, 30, 70, 100, 300, 700 and 1000 ng of oxprenolol hydrochloride; 100 ng of metoprolol tartrate were added as internal standard to each sample. The plasma samples were analysed as described above.

Recovery from plasma

Blank plasma was spiked with 500 ng of oxprenolol hydrochloride per ml. Extraction and concentration was carried out as described above; 1000 ng metoprolol tartrate were added to the solid residue. Standards were prepared by adding 1000 ng of metoprolol tartrate to 500 ng of oxprenolol hydrochloride. The further steps of analysis were performed as described above. Recovery of oxprenolol was calculated by comparing the peak he ght ratios of the plasma samples to the peak height ratios of the pure standards (mean of four determinations).

Reproducibility studies

Reproducibility studies were performed at three concentrations by performing five replicate analyses of blank plasma which had been spiked such that the concentrations of oxprenolol hydrochloride were 30 ng/ml in the first series and 100 and 300 ng/ml in series 2 and 3. To each sample were added 100 ng of metoprolol tartrate as internal standard. Standards were prepared by spiking three blank plasma samples with 50 ng (series 1) or 200 ng (series 2 and 3) of oxprenolol hydrochloride.

Drug interference studies

A number of other drugs were tested for potential interference in the assay. Aliquots of stock solutions of the compounds were heated with EDTN, dissolved in ethyl acetate and chromatographed.

RESULTS

The reaction of oxprenolol, base or salt, with EDTN leads to a single blue

fluorescent product. The same reaction occurs with metoprolol. The fluorescence spectrum of the derivative of oxprenolol on a thin-layer plate is shown in Fig. 1.

The reaction of oxprenolol and metoprolol was carried out with varying amounts of EDTN, between 10 and 60 min and 35 to 70°. In all samples the same relative peak size was obtained, which suggests that the reaction remained constant within this range of conditions. The TLC separation of the EDTN derivatives of oxprenolol and the internal standard, extracted from spiked plasma samples, is demonstrated in Fig. 2. The concentrations are 0 and 30 ng of oxprenolol hydrochloride and 50 ng of metoprolol tartrate. No interference from normal plasma constituents was observed. The minimum detectable amount of oxprenolol is 5 ng.

The linearity of standard curves of pure substances as well as of oxprenolol from spiked plasma samples is excellent ($r>0.9992$ in any experiment) from 10 to 1000 ng oxprenolol (based on the hydrochloride salt). Both curves pass through the origin. It is therefore sufficient to determine only one point on the calibration curve (mean value of three determinations) and to connect this point to zero. The content of unknown samples may be determined by calculation or graphically.

As no radioactive oxprenolol was available, recovery of oxprenolol from plasma could not be determined directly. Our recovery experiments were based upon the assumption that oxprenolol and oxprenolol hydrochloride are derivatised to the same extent with EDTN. Under these conditions recovery from plasma was calculated to be 81.8%. Reproducibility studies were performed at three concentrations in the therapeutic range. The standard deviation was independent of the oxprenolol concentration in the plasma sample. It was calculated to be 4.1%.

A number of other basic drugs were tested for potential interference in the

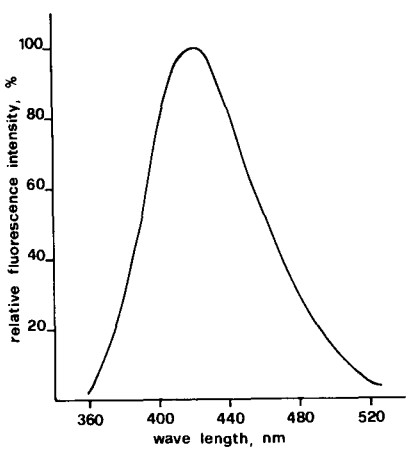

Fig. 1. Emission spectrum of oxprenolol derivatised with EDTN, chromatographed on silica gel 60 plates with the solvent system chloroform—ethyl acetate (95:5). After drying the plate was dipped into a solution of 2% paraffin in cyclohexane. Excitation was with the 313 nm line of a medium-pressure lamp.

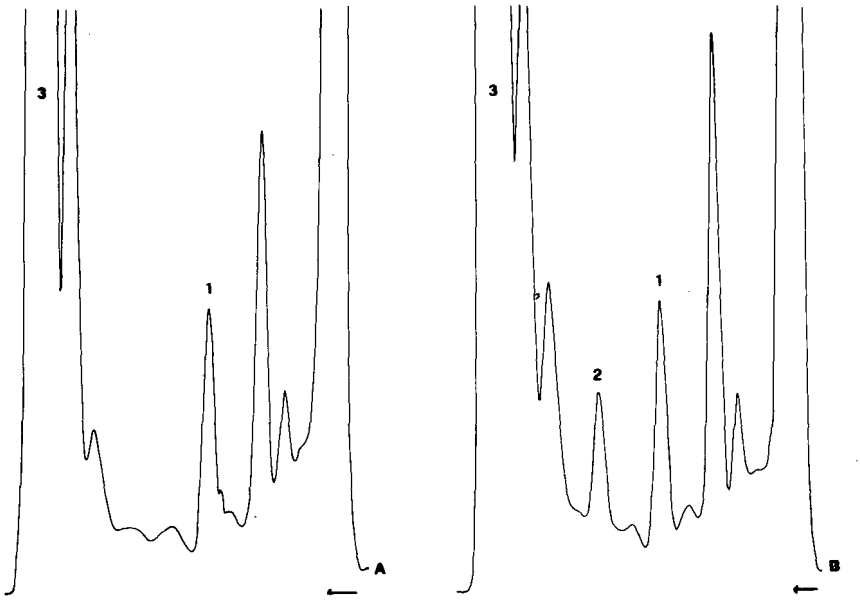

Fig. 2. Scans of chromatograms of 1 ml of blank plasma (A) and 1 ml of blank plasma spiked with 30 ng of oxprenolol hydrochloride (B). Both samples were spiked with 50 ng of metoprolol tartrate. The plasma samples were analysed as described in the text. 1, Derivative of metoprolol; 2, derivative of oxprenolol; 3, excessive EDTN.

assay but none of the compounds investigated in this experiment (the cardioactive agents atenolol, propranolol, digitoxin and chinidin) were found to interfere with the analysis of oxprenolol. Other compounds that were also shown not to impair the usefulness of the assay were guanethidine, hydralazin, triamteren and diazepam.

DISCUSSION

The specificity and sensitivity of the described method is satisfactory for the measurement of blood levels during chronic therapy and for pharmacokinetic measurements where extremely low plasma levels may occur. Its advantage over the gas chromatographic methods described previously is the linearity over a wide range, which includes the therapeutic range of plasma levels. Therefore, it is not necessary to produce a complete calibration graph, but it is sufficient to determine just one point on the curve. The method requires also only 1 ml of plasma for each determination.

Another advantage is that this method is less time-consuming than the gas chromatographic ones, because purification of the plasma extract by re-extraction is avoided. The coefficient of variation of this method is higher than that of the gas chromatographic method described by Degen and Riess [3], 4.1% instead of 3.4%, but the precision is sufficient for the determination of the drug in biological material.

Using EDTN as fluorescence reagent overcomes the disadvantages of using

dimethylaminonaphthalene sulphonyl (Dns) chloride; namely, the requirement of another extraction step after the reaction, the long reaction time (more than 12 h) and the instability of the Dns-amide. Fluorescence of the EDTN derivatives is stable for several days on the thin-layer plate after fixation of the fluorescence by dipping into a paraffin solution. The limit of detection of oxprenolol (5 ng) is slightly higher than the limit of detection Stopher [14] reported for tolamolol (2 ng). Extraction of oxprenolol with dichloromethane—diethyl ether leads to extracts free of interfering substances. Another clean-up by dissolving the dry residue in methanol and shaking with heptane, as described by Stopher, is not necessary in the oxprenolol assay.

In the same way, determination of metoprolol in plasma is possible using oxprenolol as internal standard.

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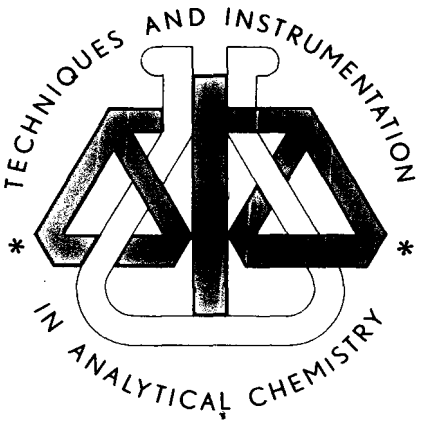

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