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BIOMEDICAL APPLICATIONS

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Journal of Chromatography	130 131	132/1 132/2 132/3	133/1 133/2	134/1 134/2	135/1 135/2	136/1 136/2 136/3	137/1 137/2	138/1 138/2	139/1 139/2	140/1 140/2 140/3	142 144/1	144/2 144/3
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Chromatographic Reviews				141/1				141/2				141/3

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.

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(Biomedical Applications, Vol. 1, No. 6)

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Isotachophoresis

Theory, Instrumentation and Applications

by F.M. EVERAERTS, J.L. BECKERS *and* TH. P.E.M. VERHEGGEN.

JOURNAL OF CHROMATOGRAPHY LIBRARY, Vol. 6

1976 xiv + 418 pages
US $61.50/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, which competes with liquid and gas chromatography. All kinds of ionic materials can be separated and several classes of components can be analysed in quick succession as a proper rinsing of the equipment is all that is needed between separations. The various chapters of the book can be referred to more or less independently by scientists interested in fundamental aspects, by researchers intending to construct an instrument and by workers mainly concerned with analytical aspects.

CONTENTS: 1. Historical review. THEORY: 2. Principles of electrophoretic techniques. 3. Concept of mobility. 4. Mathematical model for isotachophoresis. 5. Choice of electrolyte systems. INSTRUMENTATION: 6. Detection systems. 7. Instrumentation. APPLICATIONS: 8. Introduction. 9. Practical aspects. 10. Quantitative aspects. 11. Separation of cationic species in aqueous solutions. 12. Separation of anionic species in aqueous solutions. 13. Amino acids, peptides and proteins. 14. Separation of nucleotides in aqueous systems. 15. Enzymatic reactions. 16. Separations in non-aqueous systems. 17. Counter flow of electrolyte. APPENDICES: A. Simplified model of moving-boundary electrophoresis for the measurement of effective mobilities. B. Diameter of the narrow-bore tube, applied for separation. C. Literature. Symbols and abbreviations. Subject index.

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1937 E

HPTLC
High Performance Thin-Layer Chromatography

edited by **A. ZLATKIS,** University of Houston, Houston, Texas, and **R.E. KAISER,** Institute of Chromatography, Bad Dürkheim

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HPTLC is the advanced technology of TLC 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. The potential and scope of this new technique is discussed and specific examples of biological samples are given. Speed, precision, quantitation, sensitivity and automation are described in detail.
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Biomedical Applications

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A NON-ENZYMIC PROCEDURE FOR THE QUANTITATIVE ANALYSIS OF (3-METHOXY-4-SULPHOXYPHENYL)ETHYLENE GLYCOL (MHPG SULPHATE) IN HUMAN URINE USING STABLE ISOTOPE DILUTION AND GAS CHROMATOGRAPHY—MASS SPECTROMETRY

STEPHEN MURRAY, THOMAS A. BAILLIE and DONALD S. DAVIES

Department of Clinical Pharmacology, Royal Postgraduate Medical School, Ducane Road, London (Great Britain)

(Received January 17th, 1977)

SUMMARY

A method is described for the quantitative analysis of (3-methoxy-4-sulphoxyphenyl)-ethylene glycol (MHPG sulphate) in human urine, based on selected ion monitoring gas chromatography—mass spectrometry and using a specifically deuterium-labelled analogue of MHPG sulphate as internal standard. The procedure involves extraction of the urine sample on Amberlite XAD-2, followed by isolation of MHPG sulphate by column chromatography on Sephadex LH-20. Cleavage of the sulphate conjugate and formation of the MHPG tris(trifluoroacetate) derivative are carried out in a one-step reaction, without recourse to enzymic hydrolysis.

INTRODUCTION

Studies on the metabolic fate of noradrenaline in man have shown that (3-methoxy-4-hydroxyphenyl)ethylene glycol (MHPG) is a major metabolite [1,2] and that this compound is excreted in urine mainly as its sulphate and glucuronide conjugates [1,3]. Whereas MHPG appears to be conjugated mainly with glucuronic acid in peripheral tissues, it has been suggested that sulpho-conjugation of this compound takes place predominantly in the brain [4]. If this is the case, measurement of urinary MHPG sulphate should provide a convenient, non-invasive technique for the assessment of central noradrenergic function in man [4].

A number of methods have been reported for the analysis of free and conjugated MHPG in urine, based on spectrophotometry [5—8], gas—liquid chromatography [4, 9—15] and, more recently, on gas chromatography—mass spectrometry (GC—MS) [16—19]. Of these techniques, the latter combines highest specificity and sensitivity and permits the use of stable isotope-labelled analo-

gues of the compounds of interest as internal standards [20].

MHPG sulphate may be hydrolysed by the action of acid [16], but since free MHPG is itself unstable under these conditions [5, 7, 9], methods for the measurement of the sulphate conjugate rely on the "selective" action of sulphatase enzymes; unconjugated MHPG in urine is determined before and after hydrolysis and the levels of MHPG sulphate are obtained by difference. Although enzymic hydrolysis is a powerful technique for the qualitative study of conjugated metabolites, the use of enzymes for quantitative determinations on a routine basis suffers from a number of disadvantages. First, most sulphatase preparations contain low, and often variable, amounts of β-glucuronidase and, if steps are not taken to selectively inhibit β-glucuronidase activity [21], erroneously high values for MHPG sulphate will be obtained. This problem may, however, be overcome if MHPG sulphate is separated from the corresponding glucuronide prior to enzymic hydrolysis. Chromatographic systems based on thin-layer [22], paper [3] and ion-exchange chromatography [22] have been described for the separation of MHPG and its conjugates, but these have not been adopted for routine use.

A second disadvantage associated with the use of enzymic hydrolysis is that hydrolytic activity may be diminished by the presence in urine samples of endogenous inhibitors of enzyme action [3] or by the use of a large excess of the enzyme in the incubation [4]. Problems of this nature can be overcome satisfactorily only through the use of an appropriate, sulpho-conjugated internal standard. Finally, certain enzyme preparations have been shown to contain traces of MHPG itself and of other interfering substances [23], thus necessitating the use of a "blank" incubation with each batch.

In view of the above disadvantages, we have investigated alternative approaches to the analysis of MHPG sulphate in human urine. In the present communication, we report the development of an improved stable isotope dilution assay for urinary MHPG sulphate, of which a specifically deuterium-labelled analogue has been synthesized for use as internal standard. Following extraction of MHPG and its conjugates from urine, MHPG sulphate is isolated by liquid—gel chromatography and is converted to a derivative suitable for GC—MS analysis by a novel procedure which does not entail hydrolysis by an aryl sulphatase.

EXPERIMENTAL

General

Amberlite XAD-2 (BDH, Poole, Great Britain) was washed exhaustively with acid, alkali, water and solvents [24]. The resin was stored in water and was used as described by Bradlow [25]. Sephadex LH-20 (Pharmacia, Uppsala, Sweden) was washed in turn with 5% acetic acid in ethanol, water, ethanol, chloroform and finally methanol. Trifluoroacetic anhydride was purchased from Sigma (London, Great Britain), as were aryl sulphatase (Type H-1, containing 18,600 units/g sulphatase and approx. 300,000 Fishman units/g β-glucuronidase) and β-glucuronidase (Type II, approx. 40,000 units/g). All solvents employed in the analytical procedure were of analytical grade and were redistilled before use. Ethyl acetate and dimethylformamide were dried over calcium hydride and redistilled.

Acetovanillone, benzyl chloride and [O-^{2}H]ethanol were purchased from Koch-Light Labs. (Colnbrook, Great Britain). Sodium borodeuteride and sodium deutero-oxide in deuterium oxide (40%, w/w) were obtained from Merck Sharp and Dohme (Montreal, Canada) and dicyclohexylcarbodiimide from BDH. A sample of the potassium salt of MHPG sulphate was kindly donated by Hoffmann-La Roche (Basle, Switzerland) while further quantities were purchased from Fluka (Buchs, Switzerland). The piperazine salt of unconjugated MHPG was obtained from Sigma. Thin-layer chromatography (TLC) was carried out using glass plates (5 × 20 cm), precoated with 0.25-mm layers of silica gel G_{F254} (Merck, Darmstadt, G.F.R.).

Infrared (IR) spectrometry. IR spectrometry was carried out on Nujol mulls using a Perkin-Elmer 157G instrument.

Nuclear magnetic resonance (NMR) spectrometry. NMR was performed at 60 MHz on a Hitachi Perkin-Elmer R-24 instrument. Spectra were obtained on solutions in deuterochloroform, except where otherwise stated.

Gas chromatography—mass spectrometry. A Finnigan Model 3200 instrument was used, equipped with a 5 ft. × 2 mm I.D. glass column packed with 3% OV-17 on Gas-Chrom Q (100—120 mesh), and operated at 115° with helium (30 ml/min) as carrier gas. Under these conditions, the retention time of the MHPG tris(trifluoroacetate)(tris-TFA) derivative was approximately 5 min. The mass spectrometer was operated in the electron impact mode with an electron energy of 25 eV, emission current of 400 μA and continuous dynode electron multiplier voltage of 1.3 kV. The pre-amp range was 10^{-7} A · V^{-1} for recording of reference spectra and 10^{-8} A · V^{-1} for analysis of urinary extracts. Data acquisition and reduction was performed by a Finnigan Model 6000 interactive data system, using revision H software.

Synthesis

3-Methoxy-4-benzyloxy-ω-bromoacetophenone (III). This compound was prepared from acetovanillone (I; 10.0 g, 60.2 mmole) by the method of Hegedüs [26]. The crude product was recrystallised from ethanol to give III as fine crystals (yield = 8.0 g, 40%). TLC (chloroform): single spot, R_F = 0.57. MS: *m/e* 334 (M^+), 255, 241, 214 and 91 (base peak). IR: 3065 (w), 1760 (s), 1580 (s), 1510 (s), 1260 (s) and 1155 cm^{-1} (s). NMR: 3.93 (3H, s), 4.36 (2H, s), 5.23 (2H, s), 7.15 (3H, m) and 7.36δ (5H, broad s).

1-(3-Methoxy-4-benzyloxyphenyl) [1,2,2-$^2H_3$] ethylene glycol (V). To a solution of the bromide III (7.5 g, 22.4 mmole) in [O-^{2}H]ethanol (100 g) was added anhydrous potassium acetate (4.39 g) and the mixture was heated under reflux for 19 h. The reaction product was allowed to cool to room temperature, when sodium borodeuteride (1 g, 23.8 mmole) was added in portions with stirring. After 3 h at room temperature, the mixture was treated with a solution of sodium deutero-oxide in deuterium oxide (40% w/w; 5 ml), followed by a further 10 ml of deuterium oxide and allowed to equilibrate for 1 h. The reaction mixture was then poured into water (200 ml) and extracted into diethyl ether (three 150-ml portions). The combined ether extracts were washed with water (two 200-ml portions), dried (sodium sulphate), filtered and evaporated under reduced pressure to give V as an oil. Crystallisation from benzene—

hexane afforded 4.94 g of product, containing benzene of crystallisation [26]. TLC (ethyl acetate): single spot, R_F = 0.29. MS: *m/e* 277 (M^+), 259, 244 and 91 (base peak). NMR: 2.8 (2H, very broad s, —OH), 3.97 (3H, s), 5.27 (2H, s), 7.0—7.8δ (14H, m, includes one molecule of benzene of crystallisation).

1-(3-Methoxy-4-benzyloxyphenyl) [1,2,2-$^2H_3$] ethylene glycol diacetate (VI). A portion of the labelled glycol V (150 mg) was acetylated in acetic anhydride—pyridine (4:1, v/v; 2.5 ml) for 16 h at room temperature. The mixture was then poured into ice-water (20 ml) and the product was extracted with diethyl ether (twice 15 ml). The combined ether extracts were washed with 1 *N* hydrochloric acid (twice 10 ml), saturated sodium hydrogen carbonate solution (20 ml), dried (sodium sulphate) and filtered. Evaporation of the solvent gave VI as a pale yellow oil (yield: 170 mg). TLC (chloroform): single spot with R_F = 0.48. MS: *m/e* 361 (M^+), 300, 258, 211, 209, 159, 157, and 91 (base peak). NMR: 2.03 (3H, s), 2.08 (3H, s), 3.87 (3H, s), 5.12 (2H, s), 6.82 (3H, broad s) and 7.33δ (5H, broad s).

1-(3-Methoxy-4-hydroxyphenyl) [1,2,2-$^2H_3$] ethylene glycol diacetate (VII). The diacetate VI (170 mg, 0.471 mmole) was dissolved in methanol (30 ml) and 5% palladium on charcoal (100 mg) was added. This mixture was hydrogenated for 2.5 h in a Parr shaker, using a hydrogen pressure of 30 p.s.i. The resulting mixture was filtered and evaporated under reduced pressure to yield VII as a colourless oil. TLC (chloroform): single spot, R_F = 0.30. MS: *m/e* 271 (M^+), 210, 196, 168, 154, 94 and 43 (base peak). NMR: 1.99 (3H, s), 2.03 (3H, s), 3.87 (3H, s), 5.55 (1H, very broad s, —OH) and 6.80δ (3H, s).

1-(3-Methoxy-4-sulphoxyphenyl) [1,2,2-$^2H_3$] ethylene glycol potassium salt (IX). The product from the above reaction was dissolved in anhydrous dimethylformamide (5 ml), dicyclohexylcarbodiimide (700 mg) was added and the resulting solution was cooled to 0°. To this solution was added 1.5 ml of a mixture of concentrated sulphuric acid (0.45 ml) and anhydrous dimethylformamide (7.7 ml), which had previously been cooled to 0°. The reaction was allowed to proceed for 2 h at 0° and was quenched by the addition of 75% aqueous ethanol (20 ml). The pH of the mixture was adjusted to 7.5 by the addition of a few drops of 2 *N* potassium hydroxide solution. The mixture was centrifuged and the supernatant was removed. The residue was extracted with 75% aqueous ethanol (thrice 20 ml), and the combined supernatant and extracts were taken to dryness in vacuo.

The residue was redissolved in 5 ml chloroform—methanol (1:1, v/v, 0.01 *M* with respect to potassium chloride) and applied to a column of Sephadex LH-20 (20 g), packed in and eluted with the same solvent system. The effluent between 210 and 270 ml was collected and evaporated to dryness to give VIII as a white solid (103 mg), which was hydrolysed for 3 h at 80° in 1 *M* aqueous potassium hydroxide (3 ml). The resulting solution was neutralized by the careful addition of hydrochloric acid and was lyophilized to yield 306 mg of solid material, of which approximately 10% by weight corresponded to the labelled sulphate conjugate IX. NMR (^2H_2O): 3.90 (3H, s) and 7.20δ (3H, m).

Treatment of a portion of the above product with trifluoroacetic anhydride—ethyl acetate (1:1, v/v) for 90 min at room temperature and subsequent GC—MS analysis showed the formation of a single derivative whose

mass spectrum was almost identical to that previously published for the tris-TFA derivative of [$^2H_3$]MHPG [18]. Deuterium content of this derivative, as measured from the molecular ion cluster, was found to be as follows (atoms % excess): 0.03% $^2H_0$, 0.37% $^2H_1$, 10.60% $^2H_2$ and 88.99% $^2H_3$.

The deuterated sulphate IX was stored at −20° and stock solutions in distilled water (concentration approx. 250 μg/ml) were prepared as required. To aliquots of these solutions was added a fixed amount of unlabelled MHPG sulphate to serve as internal standard and the mixtures were derivatised with trifluoroacetic anhydride in ethyl acetate. The resulting MHPG tris-TFA derivatives were analysed by selected ion monitoring GC—MS, when the ratios of peak heights in the recordings for *m/e* 358 and 360 were used to calculate the concentration of [$^2H_3$]-MHPG sulphate in the original stock solutions.

Urine samples

Twenty-four-hour urine samples were collected from healthy laboratory personnel who were not receiving any medication, and were stored at −20° until analysed.

Analytical procedures

Urinary MHPG sulphate. To an aliquot (5 ml) of a 24-h urine collection is added [$^2H_3$]MHPG sulphate (approx. 25 μg) in distilled water (100 μl). The sample is mixed thoroughly on a Vortex mixer and is passed through a column (10 × 2 cm) of Amberlite XAD-2. The column is washed in turn with water (10 ml) and hexane (10 ml) and the washings are discarded. MHPG sulphate is then eluted with methanol (30 ml) and the solvent is evaporated under reduced pressure. The residue is taken up in 2 ml chloroform—methanol (1:1, v/v, 0.01 *M* with respect to sodium chloride) and is applied to a column (28 × 1 cm) of Sephadex LH-20 (5 g), prepared in and eluted with the same solvent system. The fraction corresponding to 70—100 ml of effluent is taken to dryness under reduced pressure and is transferred with methanol (two 0.5-ml portions) to a screw-capped Reactivial®. The solvent is evaporated under a stream of nitrogen and the residue is further dried for 2 h in a vacuum desiccator in the presence of phosphorous pentoxide.

Anhydrous ethyl acetate (250 μl) and trifluoroacetic anhydride (350 μl) are added to the sample and the vial is securely capped and shaken for 2 h at room temperature. Residual sodium chloride from the LH-20 chromatography solvent system is removed by brief centrifugation and the clear supernatant is transferred to a second Reactivial and evaporated to dryness under a stream of dry nitrogen. The residue is taken up in anhydrous ethyl acetate—trifluoroacetic anhydride (95:5, v/v, 20 μl) and aliquots (2 μl) are injected into the GC—MS instrument, which is focused to monitor the ions at *m/e* 358 and 360.

The concentration of endogenous MHPG sulphate in the urine sample is determined by reference to a standard curve of peak height ratio (*m/e* 358: 360) versus quantity of unlabelled MHPG sulphate. Standard curves, which are prepared with each batch of samples, are linear over the range of MHPG sulphate concentrations encountered in normal subjects and are constructed from known mixtures of the reference (unlabelled) and deuterated conjugate.

Free and "total" MHPG in urine. Measurement of free and "total" urinary MHPG is also carried out by GC—MS using a modification [27] of the procedure described by Bertilsson [28] for cerebrospinal fluid samples. [$^2H_2$]MHPG (prepared by reduction of vanillylmandelic acid with lithium aluminium deuteride) serves as the internal standard in both assays. The volume of urine required for estimation of free MHPG is 200 μl, while that for "total" MHPG is 10 μl. Cleavage of MHPG conjugates is carried out using Sigma Type H-1 enzyme, a preparation containing both aryl sulphatase and β-glucuronidase activity.

RESULTS AND DISCUSSION

Synthesis of [$^2H_3$]MHPG sulphate (Fig. 1)

Preparation of the internal standard, [$^2H_3$]MHPG sulphate, was carried out by modification of a published procedure for the synthesis of the corresponding unlabelled conjugate [26]. Acetovanillone (I) was converted to the bromo derivative III, which was then heated under reflux with potassium acetate in monodeuteroethanol. This treatment gave the acetoxy ketone IV with simultaneous incorporation of two atoms of deuterium in the side-chain. Reduction of IV in situ with sodium borodeuteride, followed by alkaline hydrolysis, afforded the trideuterated glycol derivative V. Acetylation of V and subsequent removal of the benzyl protecting group yielded [$^2H_3$]MHPG diacetate (VII), which was converted to the corresponding sulphate conjugate VIII as described by Mumma [29]. This compound was purified as its potassium salt by chromatography on Sephadex LH-20 [30] and finally hydrolysed under alkaline conditions to give [$^2H_3$]MHPG sulphate (IX).

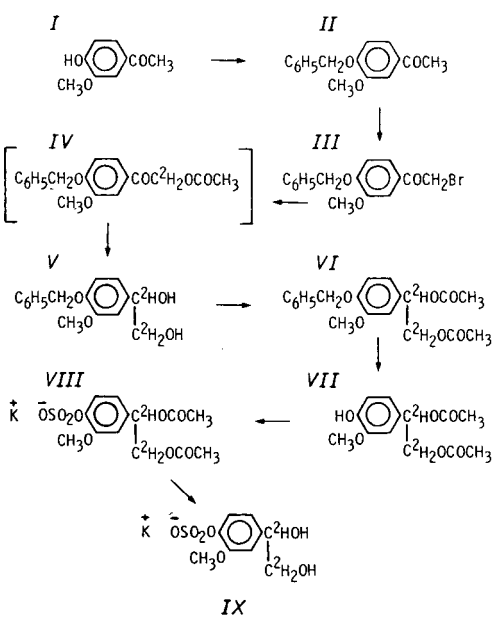

Fig. 1. Synthesis of [$^2H_3$]MHPG sulphate.

MS analysis of compounds V and IX (the latter following trifluoroacetylation) indicated that the deuterium atoms had been completely retained during the latter stages of the synthesis.

Analysis of urinary MHPG sulphate

In the first stage of the assay procedure, a known amount of the labelled internal standard, [$^2H_3$]MHPG sulphate, is added to a 5 ml aliquot of a 24-h urine collection. Free MHPG and its conjugates are then extracted from urine by chromatography on a small column of Amberlite XAD-2. MHPG sulphate is only partially retained on the resin, and, although overall recoveries from the column are poor (yield approximately 30%), this has not proved to be a serious drawback in the analysis.

MHPG sulphate is next separated from its free and glucuronide-conjugated forms by liquid—gel partition chromatography on a column of Sephadex LH-20. The use of this lipophilic gel with methanol—chloroform (1:1, v/v) as the mobile phase has been widely employed for the isolation of steroid sulphates from lipid extracts of biological material [30]. In this system, the presence or absence of a sulphate group determines chromatographic mobility and enables group separations to be carried out, based on conjugate class. Calibration of the LH-20 column with a sample of authentic MHPG sulphate indicated that this conjugate is strongly retained on the gel and is eluted slightly later than the majority of steroid monosulphates, which confirms the relative insensitivity of the LH-20 system towards structural modifications in the lipophilic region of the molecule. In an experiment designed to establish the chromatographic behaviour of MHPG glucuronide on LH-20, an XAD-2 extract of human urine was applied to a column containing 5 g of the gel and the column was eluted with methanol—chloroform (1:1, v/v, 0.01 *M* with respect to sodium chloride). Fractions (10 ml) of the effluent were collected and each fraction was split into three equal parts and taken to dryness. One aliquot was derivatized directly with trifluoroacetic anhydride, while the other two aliquots were incubated with β-glucuronidase and arylsulphatase, respectively, prior to derivatization. The amount of MHPG tris-TFA formed in each case was determined by GC—MS and the results are summarized in Fig. 2. This shows that MHPG glucuronide has an elution volume intermediate between those of free MHPG and its sulphate conjugate and that the LH-20 column fraction (between 70 and 100 ml of effluent) collected in the assay of MHPG sulphate will not contain any of the glucuronide.

In the final stage of the analytical procedure, MHPG sulphate is converted to a derivative suitable for GC—MS analysis by reaction at room temperature with a mixture of trifluoroacetic anhydride and ethyl acetate. This mild treatment has been found to result in quantitative conversion of MHPG sulphate to the MHPG tris-TFA derivative in a single step. A similar type of reaction was observed when isoetharine sulphate [31] and isomeric dopamine sulphates [32] were treated with trimethylsilylating reagents, and when a number of steroid sulphates were reacted with heptafluorobutyric anhydride in benzene [33]; in each case, the sulphate ester is displaced and a derivative of the resulting alco-

TABLE I

VALUES FOR THE EXCRETION OF FREE MHPG, MHPG SULPHATE AND TOTAL MHPG (FREE + CONJUGATED) IN 24-H URINE COLLECTIONS FROM HEALTHY VOLUNTEERS

Subjects	Age (years)	mg MHPG/24 h			mg MHPG/g creatinine		
		Free	Sulphate*	Total*	Free	Sulphate*	Total*
Males							
ARB	28	0.10	1.57	3.11	0.06	0.89	1.76
TB	28	0.04	0.55	1.54	0.03	0.43	1.20
CD	31	0.06	1.05	2.51	0.04	0.74	1.77
HJ	29	0.07	0.89	1.82	0.04	0.50	1.03
SJ	23	0.09	1.19	2.35	0.05	0.68	1.34
SM	28	0.14	1.55	4.21	0.07	0.81	2.20
JR	32	0.09	1.35	2.83	0.04	0.66	1.38
Females							
CH	29	0.11	1.12	2.42	0.09	0.94	2.03
BN	23	0.05	0.55	1.25	0.05	0.52	1.18
PT	33	0.06	0.65	2.25	0.05	0.49	1.71
Mean		0.08	1.05	2.43	0.05	0.67	1.56
± S.D.		± 0.03	± 0.38	± 0.84	± 0.02	± 0.18	± 0.39

* Values expressed in terms of free MHPG present in conjugated form.

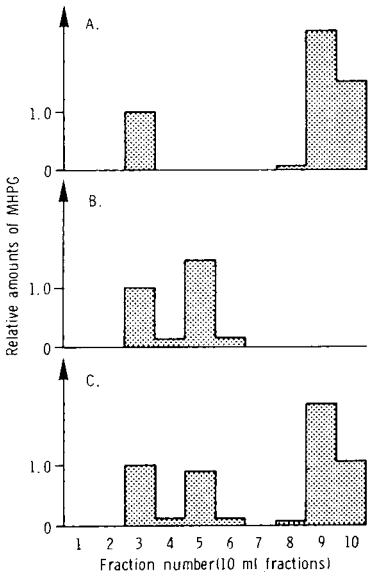

Fig. 2. Chromatographic separation of free MHPG (fr. 3), MHPG glucuronide (fr. 5) and MHPG sulphate (fr. 9—10) on Sephadex LH-20 (mobile phase: methanol—chloroform, 1:1, v/v; 0.01 *M* in NaCl) from an extract of urine. The amount of MHPG in each fraction was determined by GC—MS, (A) following direct derivatization with trifluoroacetic anhydride—ethyl acetate, (B) after incubation with β-glucuronidase, extraction into ethyl acetate and derivatization and (C) after incubation with aryl sulphatase (containing β-glucuronidase activity), extraction into ethyl acetate and derivatization.

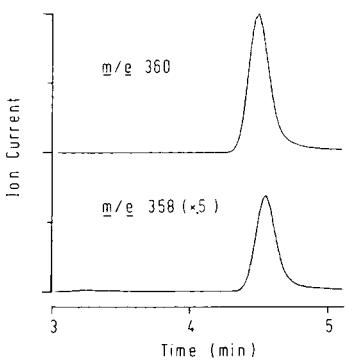

Fig. 3. Selected ion recordings obtained from a urinary extract of MHPG sulphate; *m/e* 358, MHPG tris-TFA derivative; *m/e* 360, internal standard. The concentration of MHPG sulphate in this sample was 0.48 μg (as free MHPG)/ml urine.

hol is formed. Although we have not investigated the general applicability of this reaction, it would appear to provide an attractive alternative to the use of sulphatases, particularly in cases where the conditions employed for acid hydrolysis result in destruction of the liberated alcohol. As is evident from Fig. 2, the glucuronide conjugate of MHPG is stable towards trifluoroacetic anhydride and does not give rise to MHPG tris-TFA under the derivatization conditions employed.

Quantification of endogenous MHPG sulphate is based on ratios of peak heights in the selected ion records for *m/e* 358 (unlabelled MHPG derivative) and *m/e* 360 (internal standard) (Fig. 3). These fragments correspond to loss from the molecular ions of the elements of trifluoroacetic acid and monodeutero-trifluoroacetic acid, respectively [28] Monitoring of the molecular ions, at *m/e* 472 and 475, may also be used for quantitative measurements, although monitoring of these masses gives a lower response per unit mass of sample injected.

In a study of the precision of the analytical procedure, the concentration of MHPG sulphate was determined in five 5-ml aliquots of a 24-h urine collection, when each sample was analysed twice by GC—MS. The mean value obtained was 0.70 μg/ml, with a standard deviation of ±2%.

Levels of MHPG sulphate in 24-h urine collections from ten healthy volunteers are given in Table I. For comparison, corresponding values for free and "total" MHPG, the latter obtained using a conventional enzymic hydrolysis, are also shown. Mean figures for the excretion of MHPG sulphate and of free and "total" MHPG in these subjects were found to fall within the range reported by other authors [3, 4, 16], although it should be noted that literature values for the excretion of these compounds vary widely.

Application of the present method to studies of the effect of antihypertensive drugs on the turnover of noradrenaline in the central and peripheral nervous system is currently in progress.

ACKNOWLEDGEMENTS

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Biomedical Applications

CHROMBIO. 065

FRACTIONATION AND CHARACTERIZATION OF ACIDIC OLIGOSACCHARIDES AND GLYCOPEPTIDES FROM NORMAL AND PATHOLOGICAL URINES

GÉRARD STRECKER*

Laboratoire de Chimie Biologique de l'Université des Sciences et Techniques de Lille I and Laboratoire Associé au C.N.R.S. No. 217, B.P. No. 36, 59650 — Villeneuve d'Ascq (France)

and

ANICK LEMAIRE-POITAU

Institut de Recherches sur le Cancer de Lille (Institut Jules Driessens) and Unité 124 de l'I.N.S.E.R.M., B.P. No. 3567, Lille Cédex (France)

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SUMMARY

A general procedure is described for the isolation of urinary acidic oligosaccharides and glycopeptides resulting from catabolism of glycoproteins. This procedure has been applied to normal urine and to urine from patients with diseases of the metabolism, including mucolipidosis and fucosidosis.

INTRODUCTION

In previous papers [1, 2], we described a procedure for the fractionation of urinary fucose-containing oligosaccharides, using charcoal—Celite chromatography. This procedure was also applied to the isolation of oligosaccharides accumulated in the urine of patients with Sandhoff disease [3] and mannosidosis [4]. We later modified the experimental conditions in order to study glycopeptides and acidic oligosaccharides resulting from the catabolism of glycoconjugates. We applied this procedure to cases of fucosidosis, mucolipidosis II (I-cell disease) and two new types of mucolipidosis recently described by Mande and Durand (unpublished results).

*To whom correspondence should be addressed.

EXPERIMENTAL

Chromatographic and electrophoretic analysis

Descending paper chromatography was carried out on Whatman No. 3 paper (46 × 56 cm), using the solvent systems S1, 1-butanol—acetic acid—water (4:1:5); and S2, ethyl acetate—pyridine—acetic acid—water (5:5:1:3). Paper electrophoresis was conducted on Whatman No. 3 paper using a pyridine—water (15:1935) buffer adjusted to pH 5.4 with acetic acid, in an electric field of 10 V/cm and with a separation time of 16 h. Sugars were stained with aniline oxalate reagent (aniline—ethanol—2.5% oxalic acid aqueous solution, 2:100:150, v/v/v) and glycopeptides and amino acids with 1% ninhydrin solution in acetone.

Monosaccharide and amino acid analysis

Monosaccharides obtained after acid hydrolysis (4 *N* CF_3COOH; 100°; 4 h) were identified by paper chromatography in solvent S2. Molar ratios of monosaccharides were determined by combining colorimetric methods [5] and the gas—liquid chromatography [6] of methylglycosides obtained by methanolysis of oligosaccharides and glycopeptides. Molar ratios of hexosamines and amino acids were determined using a Beckman Multichrom Autoanalyser after acid hydrolysis (4 *N* HCl for 4—24 h at 100°).

Fractionation of acidic oligosaccharides from normal urine

Urine was collected from a 30-year-old man (blood group A Lewis b). Bacterial growth was prevented by the addition of sodium azide (1 part per 10,000). Demineralization was performed batchwise by successive treatments with a cation exchanger (Dowex 50-X8, H^+; 25—50 mesh) and an anion exchanger (Dowex 1-X8, $HCOO^-$; 25—50 mesh) (approximately 300 g of resin per litre of urine). After filtration, the effluents corresponding to 20 l of urine were pooled and concentrated to 2 l under vacuum at 35°. This solution was submitted to adsorption chromatography using a 50 × 50 cm column of charcoal—Celite prepared according to Whistler and Durso [7]. Trace amounts of hydrochloric acid, used for deactivation of charcoal, were eliminated before packing the column by ten successive washings with water (under vacuum) and dryings at 40°. Monosaccharides were eluted from the column with 6 l of water and oligosaccharides and glycopeptides were desorbed with 4 l of ethanolic solutions, the concentration of which varied discontinuously from 3.5 to 50% (ethanol—water) (see Fig. 2). Each eluted fraction was dried under vacuum, the residues were dissolved in 50—100 ml of water, and the resulting solutions were chromatographed on two parallel columns of ion exchanger (20 × 2 cm Dowex 50-X2, H^+, 200—400 mesh; 20 × 2 cm Dowex 1-X2, CH_3COO^-, 200—400 mesh). After washing with 300 ml of water, the columns were separated and the anionic exchanger was eluted with a discontinuous gradient of pyridine acetate (pH 5.4) varying from 1 to 500 m*M* (see Fig. 4). One litre of each solution was directly collected and concentrated to 1 or 2 ml. Acidic oligosaccharides of these different fractions were submitted to preparative chromatography

on Whatman No. 3 paper using the solvents described above. After localization of oligosaccharides by staining lateral bands, the products were eluted with water and the solutions were lyophilized.

Fractionation of acidic oligosaccharides and glycopeptides from pathological urines

With pathological urines, which generally contain large amounts of sugars, a simplified procedure, outlined in Fig. 1, was used.

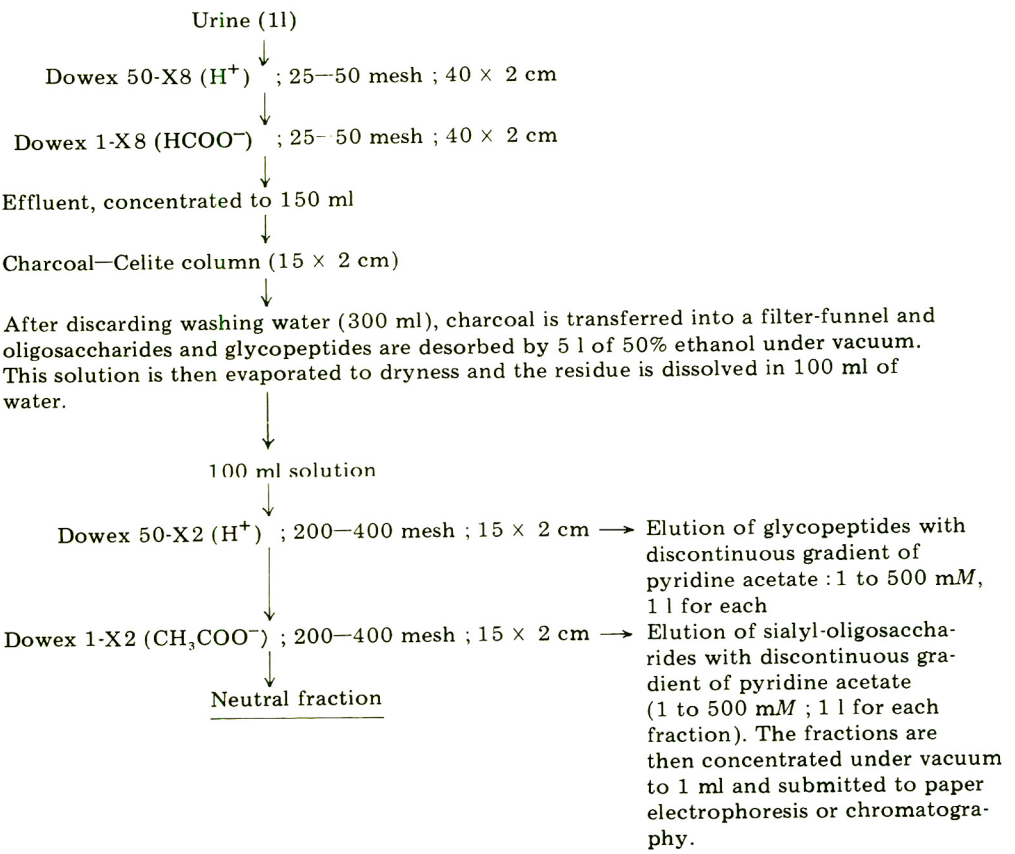

Fig. 1. Scheme for the fractionation of urinary sialyl-oligosaccharides and glycopeptides.

RESULTS

Fractionation of acidic oligosaccharides from normal urine.

Fig. 2 reveals the complex composition of urinary oligosaccharides. Only ethanolic fractions eluted after a 7.5% concentration contain acidic oligosaccharides, which were characterized by paper electrophoresis in pyridine acetate buffer (pH 5.4). The fractionation of these oligosaccharides on an anion ex-

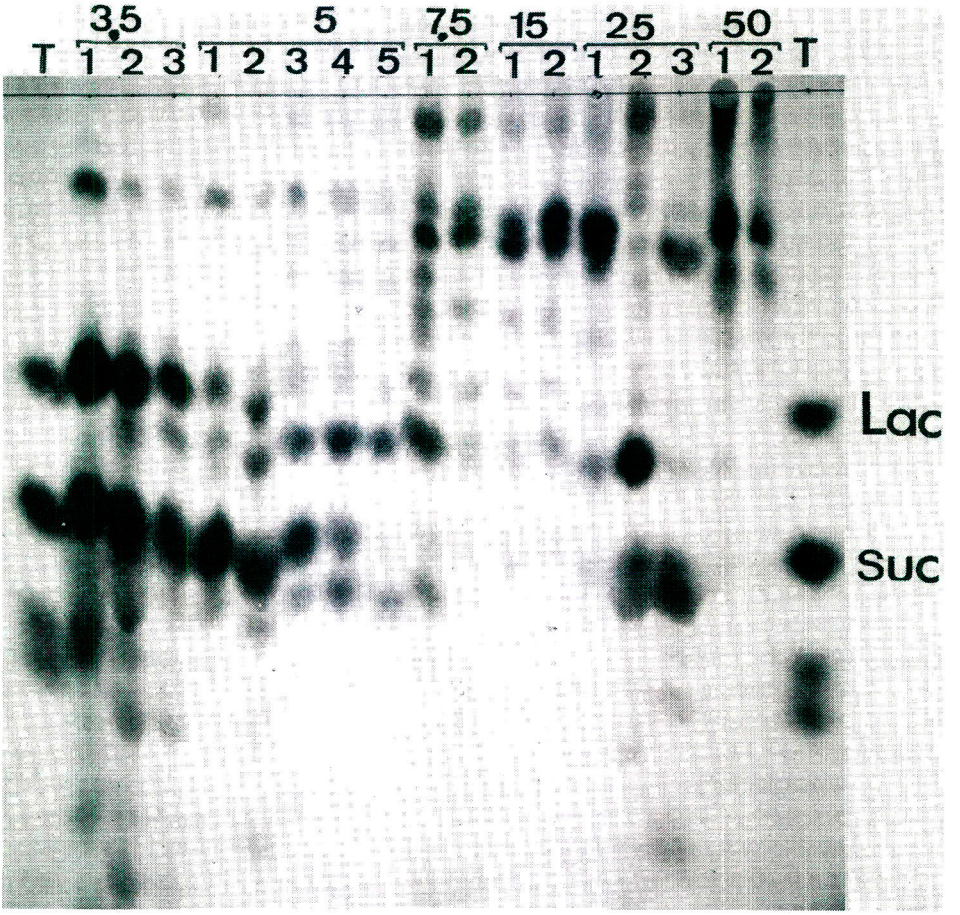

Fig. 2. Normal urine: paper chromatography of oligosaccharides desorbed from a charcoal—Celite column with discontinuous gradient of ethanol (3.5—50%). Solvent S1, development for 5 days. Standard(T): lactose (Lac) and sucrose (Suc).

changer leads to the isolation of a large number of components, as is shown in Figs. 3 and 4. The volume of each sample submitted to paper chromatography varies from 0.5 to 2 ml and corresponds to a 10,000 to 40,000-fold concentrated urine. Finally, preparative paper chromatography using solvent S2 for 2—20 days furnished 45 components with satisfactory purity, as can be seen in Figs. 5 and 6. These different fractionation steps are described in Figs. 2—6.

Oligosaccharides 1—16 were isolated from 7.5, 15 and 25% ethanol fractions. Compounds 17—45 were obtained from the 50% ethanol fraction which was submitted, in a second step, to anion-exchanger separation using the following pyridine acetate concentrations: compounds 17 and 18, 2 m*M*; compounds 19—26, 5 m*M*; compounds 27—31, 10 m*M*; compounds 32—36, 20 m*M*; compounds 37—44, 50 m*M*; and compound 45, 100 m*M*.

Oligosaccharides 2—16 are di- and trisaccharides, as shown in Table I and

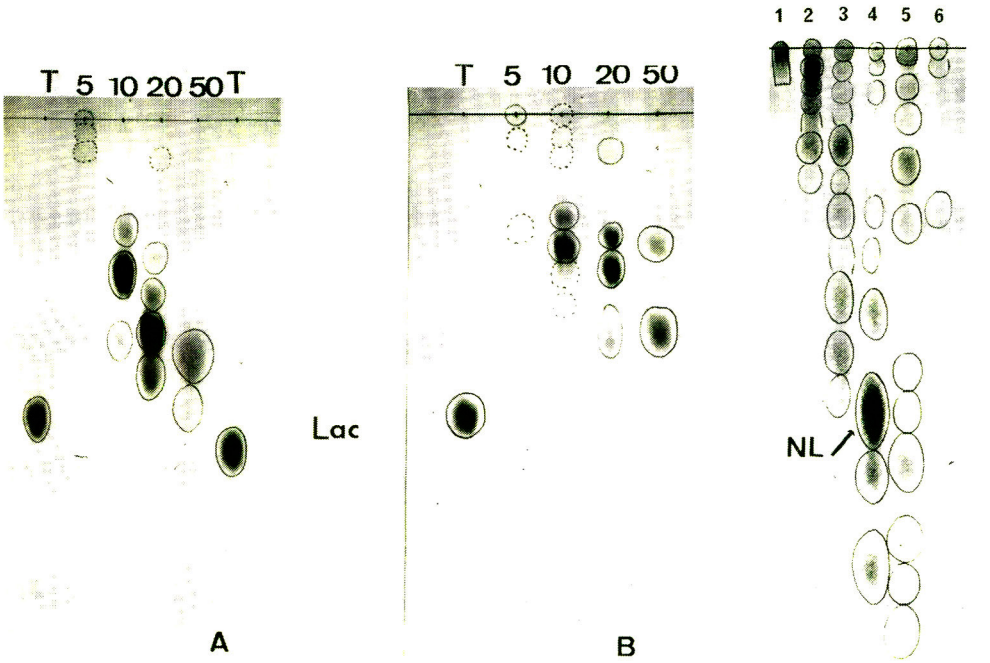

Fig. 3. Normal urine: paper chromatography of acidic oligosaccharides eluted from charcoal with 7.5% ethanol (A) and 15% ethanol (B) and fractionated on an anion exchanger. Pyridine acetate concentrations: 5, 10, 20 and 50 m*M*. Solvent S2, development for 20 h. Standard (T): lactose (Lac).

Fig. 4. Normal urine: paper chromatography of acidic oligosaccharides eluted from charcoal with 50% ethanol and fractionated on an anion exchanger. Pyridine acetate concentrations: 1, 2 m*M*; 2, 5 m*M*; 3, 10 m*M*; 4, 20 m*M*; 5, 50 m*M*; 6, 100 m*M*. Solvent S2, development for 5 days. Samples were concentrated 20,000-fold compared with the volume of treated urine. NL = 3′-neuraminyllactose.

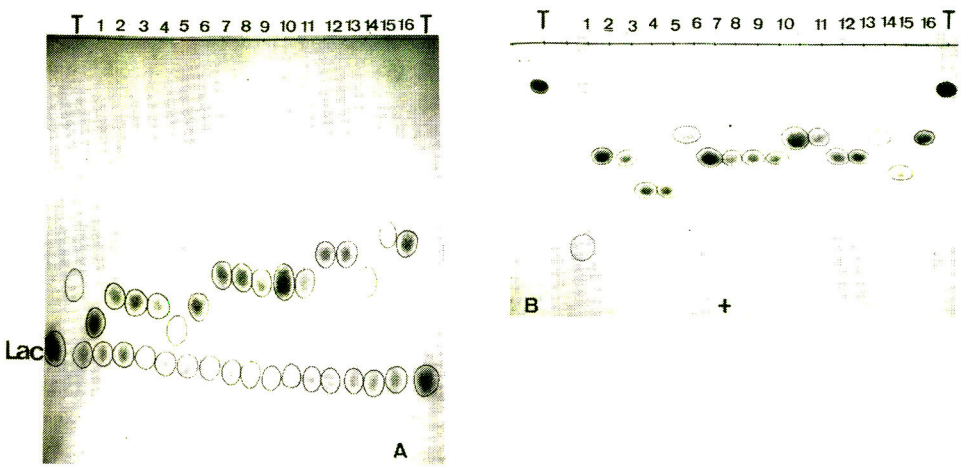

Fig. 5. Normal urine: paper chromatography (A) and electrophoresis (B) of oligosaccharides 1—16 isolated from 20 l of urine. A, Solvent S1, development for 20 h; B, buffer of pH 5.4. Standard(T) = lactose (Lac).

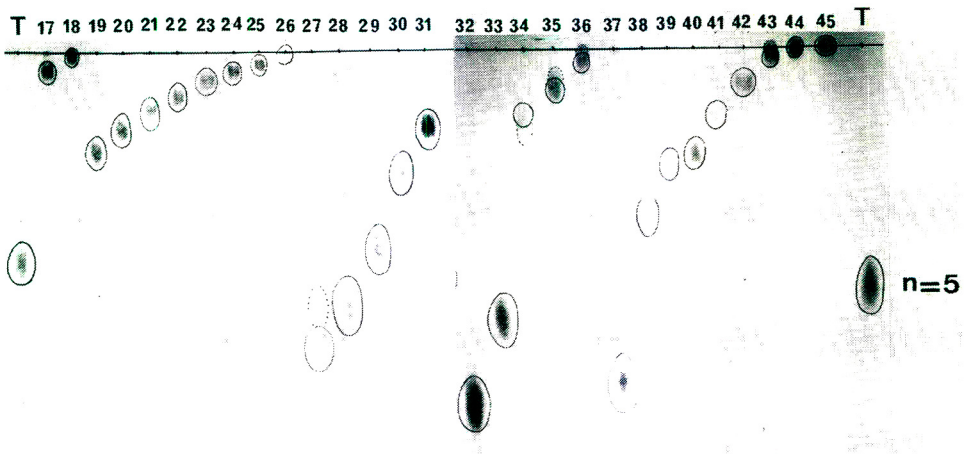

Fig. 6. Normal urine: paper chromatography of oligosaccharides 17—45 isolated from 20 l of urine. Solvent S2, development for 6 days. Lateral standard (T) is a pentasaccharide.

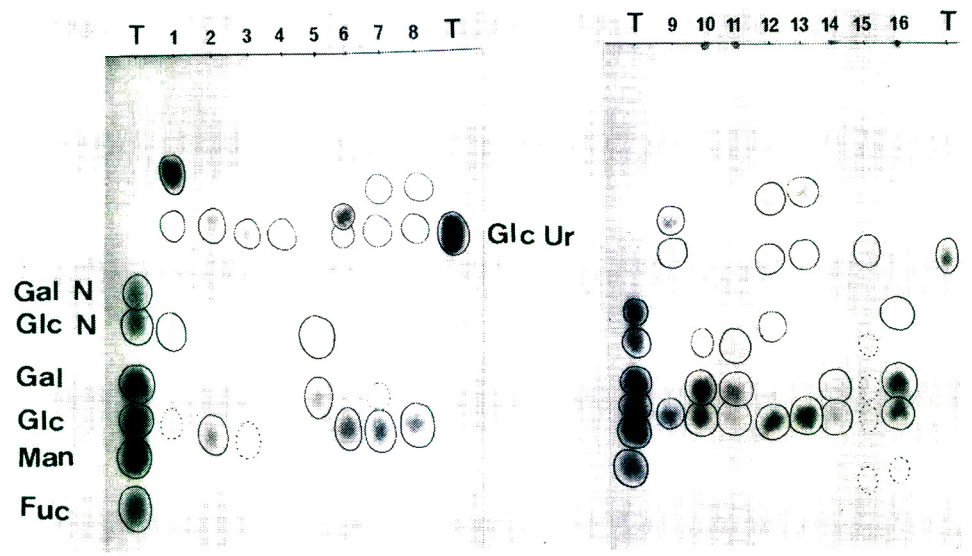

Fig. 7. Normal urine: paper chromatography in solvent S2 of trifluoroacetic acid hydrolysates of oligosaccharides 1—16. Standard (T): galactosamine (GalN); glucosamine (GlcN); galactose (Gal); glucose (Glc); mannose (Man); fucose (Fuc); glucuronic acid (GlcUr).

Fig. 7. Only compounds 1, 2, 6, 7, 8, 9 and 13 were obtained absolutely pure. Their structures are now under investigation.

Compounds 17—45 can be divided into three groups (Fig. 8 and Table I):

(i) neuramic acid and glucose-containing oligosaccharides (27—33), the sugar composition of which is identical with that of glycolipids; (ii) neuramic acid and mannose-containing oligosaccharides (17—26, 34—36 and 43—45), which are related to the glycoprotein catabolism and all of which possess an N-acetyl-

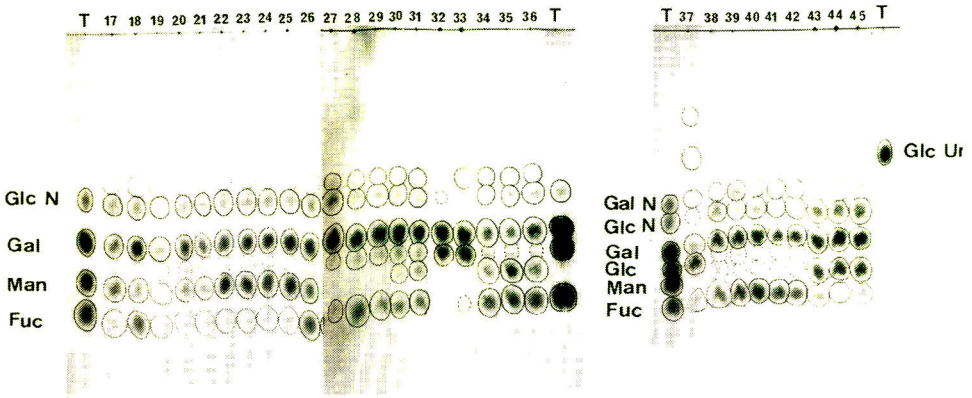

Fig. 8. Normal urine: paper chromatography of trifluoroacetic acid hydrolysates of oligosaccharides 17—45. Solvent S2. Standards: see Fig. 7.

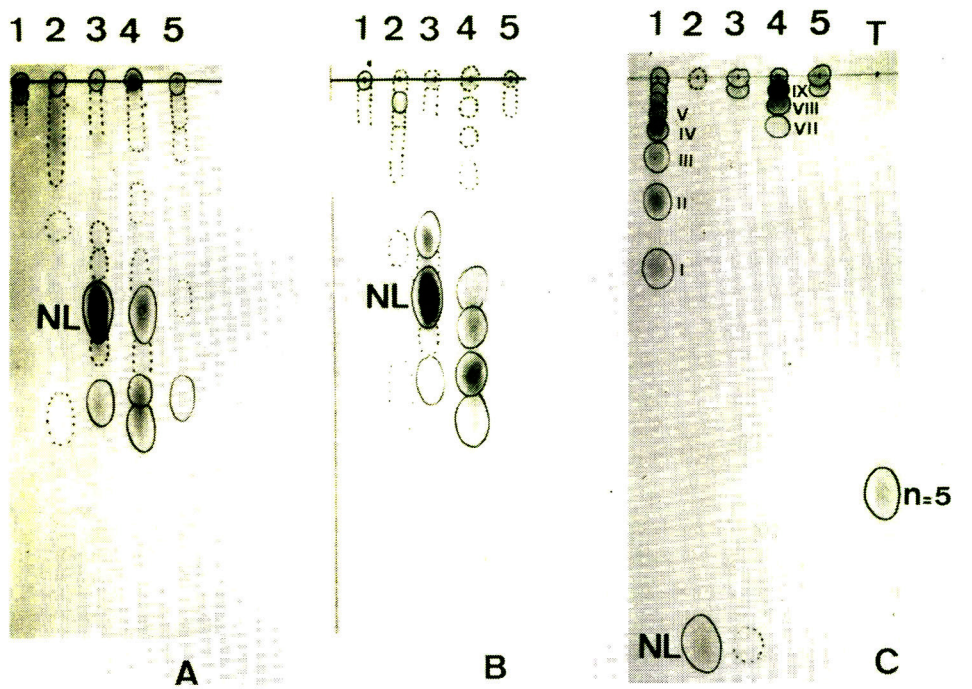

Fig. 9. Urine of mucolipidosis II: paper chromatography of sialyl-oligosaccharides eluted from an anion exchanger with a discontinuous gradient of pyridine acetate: 1, 5 m*M*; 2, 10 m*M*; 3, 20 m*M*; 4, 50 m*M*; 5, 100 m*M*. A and B, normal urines; C, mucolipidosis II. Solvent S2, development for 48 h (A and B) and 4 days (C). Samples of normal urine were concentrated 10-fold compared with samples of mucolipidosis II. NL = 3-neuraminyllactose.

glucosamine residue at the reducing end; and (iii) neuramic acid-free glycopeptides (38—42). These last compounds are characterized by the absence of mannose and by the presence of N-acetylgalactosamine, N-acetylglucosamine and galactose. Hence they are products of the catabolism of "mucine-like" constituents.

TABLE I

COMPOSITION OF ACIDIC OLIGOSACCHARIDES AND GLYCOPEPTIDES ISOLATED FROM NORMAL URINE

Gal = galactose; Glc = glucose; Man = mannose; Fuc = fucose; GalNAc = N-acetylgalactosamine; GlcNAc = N-acetylglucosamine; NANA = N-acetylneuraminic acid; GlcUr = glucoronic acid.

Compound	Amount isolated from 20 l of urine (mg)	Molar ratio											Sugar in reducing position
		Gal	Glc	Man	Fuc	GalNAc	GlcNAc	NANA	GlcUr	Asp	Ser	Thr	Gly	
1	35						0.91		1					GlcNAc
2	10		1.12						1					Glc
3	34								+					
4	26								+					
5	57	1					1.05	0.90						GlcNAc
6	53		1.16						1					Glc
7	20		1.05						1					Glc
8	9		1.15						1					Glc
9	10		1.18						1					Glc
10	205	1	0.85				0.20	0.90						Glc + GlcNAc
11	9	1	0.70				0.35	0.95						Glc + GlcNAc
12	9		1.02				0.2		1					Glc + GlcNAc
13	5		1.05						1					Glc
14	4.2	1	1.12					0.85						Glc
15	15								+					
16	41	1	1.06			0.2		0.90						Glc
17	3	1.5		2.85	0.80	0.1	4.08	1						GlcNAc
18	9.5	7.14		2.96	5.60	2.2	8.5	1						GlcNAc
19	2	2	0.4	0.94	0.60		2.1	1						GlcNAc
20	3.5	1.5	0.3	1.12	0.3		1.85	1						GlcNAc
21	2.1	1.80	0.2	1.75	0.09		2.10	1						GlcNAc
22	4.3	1.60	0.2	2.2	0.3		2.50	1						GlcNAc
23	5.2	1.95	0.3	2.85	0.4		3.01	1						GlcNAc
24	3.5	2.1		2.85	0.60		3.25	1						GlcNAc
25	5	2.70		3.30	0.85		3.85	1						GlcNAc

26	5.6	2		1.46	2.2	1	2.4	0.1	0.3	0.5	0.3	0.1	ND
27	3												
28	4	1	0.08		0.1	0.1	0.1	1					GlcNAc
29	4	1.25	0.45		0.25	0.1	1.05	1					GlcNAc
30	4.5	1.70	0.5	0.3	0.4	0.6	0.9	1					ND
31	4.6	2.50	0.95	1	0.85	0.8	1.30	1					GlcNAc
32	60	1	0.9				0.06	1					Glc
33	6.5	1.1	0.90		0.05	0.15		1					Glc
34	3.4	1		0.8	1.1	0.6	1.70		1				
35	2	1.12		2.1	1.9	0.6	1.90		1				
36	6.2	1.9		1	1.9	0.80	2.10		1				
37	1.5												
38	3.5	1	0.1	0.1	1	0.6	0.7		0.7	0.7	0.1		
39	2	1	0.12	0.1	1.50	0.90	0.60		0.65	0.6	0.5	0.4	
40	4	1.1	0.09	0.09	2	0.4	1.05		0.85	0.42	0.7	0.2	
41	2.4	1	0.1	0.1	2	0.8	0.9		1.05	0.9	0.3	0.1	
42	4	2	0.15	0.10	2	1.08	1.63		1.11	0.8	0.7	0.4	
43	5.5	3.15		3.15	1.25	0.3	5.1	2					GlcNAc
44	13	2.90		2.91	0.95	0.2	4.90	2					GlcNAc
45	12	3		2.90	0.90	0.09	5.05	1.85					GlcNAc

Pathological urines

The application of the procedure outlined in Fig. 1 led us to characterize sialyl-oligosaccharides or glycopeptides accumulated in the urine of patients with inborn metabolic diseases.

(i) Mucolipidosis II, or "I-cell disease" [8]. We studied the urine of seven patients with I-cell disease and, in all instances, we found an important excretion of sialyl-oligosaccharides (Figs. 9 and 10 and Table II), the level of which was 30—100-fold the normal values. The structures of nine of these compounds were determined [9, 10] and are shown in Fig. 11.

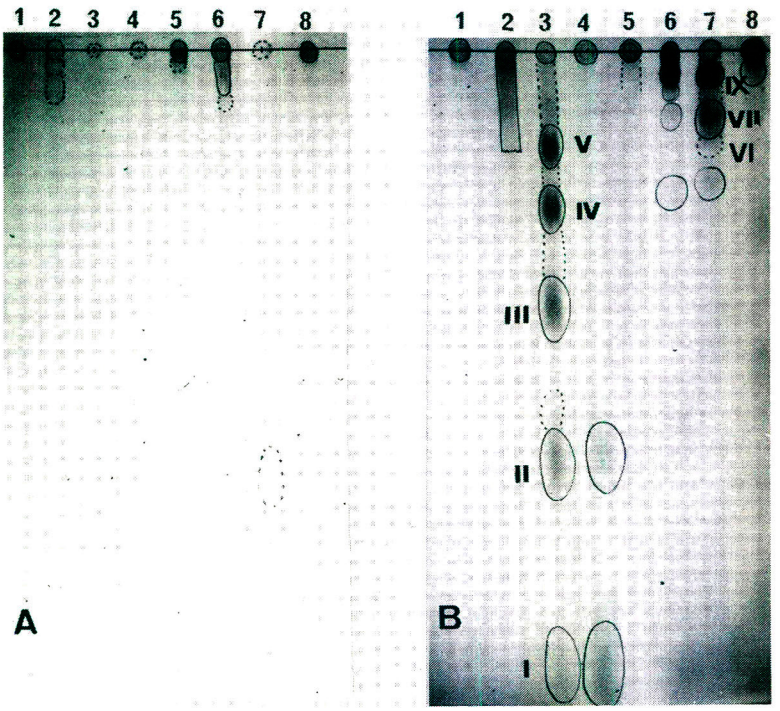

Fig. 10. Urine of mucolipidosis II: paper chromatography of acidic oligosaccharides in a second case of mucolipidosis II. Solvent S2, development for 14 days. A, normal urine; B, mucolipidosis II. Pyridine acetate concentrations: 1, 1 m*M*; 2, 2 m*M*; 3, 5 m*M*; 4, 10 m*M*; 5, 20 m*M*; 6, 50 m*M*; 7, 100 m*M*; 8, 200 m*M*.

(ii) New types of mucolipidosis. Mucolipidosis W. (Mande, unpublished observation) was observed in two children (Laura and Pierre Alexandre W.) and is very similar to I-cell disease, except the fact that the activities of many hydrolases increase in the cells but are normal in the serum. Neuraminidase activity in leucocytes was 2—4% of the normal level.

Mucolipidosis De P. (Durand, unpublished observation) is characterized only by a red spot in the bottom of the eyes and by a dyschromatopsy. The patients (22 and 9 years' old) do not exhibit other significant clinical symptoms or mental retardation. Neuraminidase activities in leucocytes were 18 and 22% of the normal levels in these patients.

TABLE II

SUGAR COMPOSITION OF OLIGOSACCHARIDES ISOLATED FROM URINE OF A PATIENT WITH MUCOLIPIDOSIS II

Abbreviations as in Table I.

Oligosaccharides*	Amount (mg)**	Molar ratio***				Monosaccharide in reducing position
		Gal	Man	GlcNAc	NANA	
I	8	0.91	2	2.07	1.05	GlcNAc
II	11	1.04	2	1.78	1.02	GlcNAc
III	14	0.95	3	2.28	0.95	GlcNAc
IV	15	1.69	3	2.90	0.95	GlcNAc
V	12	1.89	3	3.09	0.96	GlcNAc
VI	5	1.72	2	2.95	1.96	GlcNAc
VII	7	1.75	3	2.92	1.89	GlcNAc
VIII	16	1.91	3	2.89	1.91	GlcNAc
IX	23	2.12	3	3.08	1.96	GlcNAc

* See Fig. 10.
** Milligrams of oligosaccharide isolated per litre of urine by paper chromatography. The amount of isolated product is approximately 60% of urinary material. The level of these oligosaccharides in normal urine is less than 0.5 mg/l.
*** On the basis of 2 or 3 mannose residues.

These two new types of mucolipidosis are characterized by an important excretion of sialyl-oligosaccharides, 300- to 500-fold the normal value (Fig. 12 and Table III), the structures of which are identical with those of I-cell disease (Fig. 11).

(iii) Fucosidosis [11]. With fucosidosis, accumulated material was found mainly in "glycopeptidic fractions" eluted from the cation exchanger and in the "neutral fraction". Electrophoresis of "glycopeptidic fractions" showed abnormal constituents eluted by 1.2 and 5 m*M* pyridine acetate (Fig. 13). Paper chromatography of these fractions furnished five glycopeptides in a pure state (Fig. 14 and Table IV). The neutral fraction (Fig. 15) contained a glycopeptide (GP-6), which remained at the starting point and was purified by paper chromatography in 40 days. The structures of these components have been previously described [12] and are shown in Fig. 16.

DISCUSSION

The fractionation procedures described allow us to characterize, in urine, carbohydrate compounds resulting from the catabolism of glycoconjugates. In normal urine, the level of each component (less than 0.2 mg/l) was too low for structural studies to be undertaken.

These procedures were applied in studies of mucolipidosis and fucosidosis and can be used as a method of diagnosis. Mucolipidosis II, W. and De P. are characterized by a partial or total lack of neuraminidase activity [9, 13] and

I α-NANA-(2 → 3)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 2)-α-Man-(1 → 3)-β-Man-(1 → 4)-GlcNAc

II α-NANA-(2 → 6)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 2)-α-Man-(1 → 3)-β-Man-(1 → 4)-GlcNAc

III α-NANA-(2 → 6)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 2)-α-Man-(1 → 3)
β-Man-(1 → 4)-GlcNAc
α-Man-(1 → 6)

IV α-NANA-(2 → 3)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 2)-α-Man-(1 → 3)
β-Man-(1 → 4)-GlcNAc
β-Gal-(1 → 4)-β-GlcNAc-(1 → 2)-α-Man-(1 → 6)

V α-NANA-(2 → 6)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 2)-α-Man-(1 → 3)
β-Man-(1 → 4)-GlcNAc
β-Gal-(1 → 4)-β-GlcNAc-(1 → 2)-α-Man-(1 → 6)

VI α-NANA-(2 → 6)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 2)
α-Man-(1 → 3)-β-Man-(1 → 4)-GlcNAc
α-NANA-(2 → 3)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 4)

VII α-NANA-(2 → 3)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 2)-α-Man-(1 → 3)
β-Man-(1 → 4)-GlcNAc
α-NANA-(2 → 3)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 2)-α-Man-(1 → 6)

VIII α-NANA-(2 → 6)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 2)-α-Man-(1 → 3)
β-Man-(1 → 4)-GlcNAc
α-NANA-(2 → 3)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 2)-α-Man-(1 → 6)

IX α-NANA-(2 → 6)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 2)-α-Man-(1 → 3)
β-Man-(1 → 4)-GlcNAc
α-NANA-(2 → 6)-β-Gal-(2 → 4)-β-GlcNAc-(1 → 2)-α-Man-(1 → 6)

Fig. 11. Structure of the nine major oligosaccharides isolated from the urine of mucolipidosis II, W and De P. [9, 10].

by a correlative accumulation of sialyl-oligosaccharides in urine. The structures of nine of them have been elucidated, but more than 20 different components remain to be identified. If we assume that these oligosaccharides originate from all of the glycoproteins of the organism, we should be able to predict all of the possible structures of glycans.

It is also interesting to note that all of these oligosaccharides possess an N-acetylglucosamine residue in the reducing position. This result is in good agreement with the hypothesis of the existence of a β-endo-N-acetylglucosaminidase which is able to split glycans even if they are sialylated [14, 15], as with glycans of the "oligomannosidic type" [14, 16]. This enzyme remains to be characterized among mammalians.

TABLE III

SUGAR COMPOSITION OF OLIGOSACCHARIDES ISOLATED FROM URINE OF A PATIENT WITH A NEW TYPE OF MUCOLIPIDOSIS (LAURA W.)

Abbreviations as in Table I.

Oligosaccharides*	Amount* (mg/l)	Molar ratio			
		Gal	Man	GlcNAc	NANA
I	28	1.02	2	2.12	0.94
II	125	1.01	2	1.89	0.96
III	15	1.06	3	1.87	1.11
IV	5	2.24	3	3.20	0.96
V	10	2.20	3	3.00	1.10
VI	15	1.72	2	2.95	2.18
VII	24	1.74	3	2.76	1.95
VIII	48	1.72	3	2.84	2.20
IX	160	2.12	3	2.99	2.08
X	17	2.84	3	3.66	2.66
XI	8	3.20	3	3.75	2.90

* See Fig. 12.

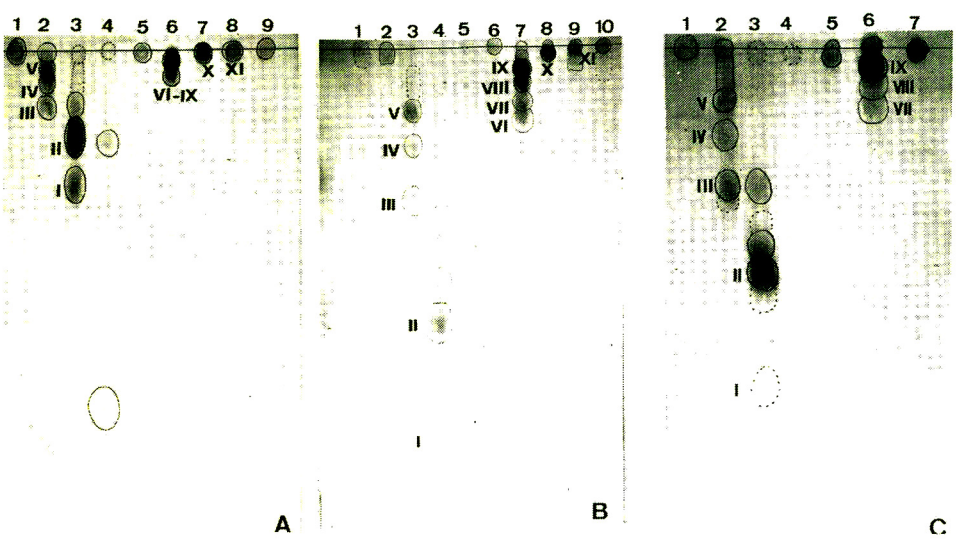

Fig. 12. Paper chromatography of acidic oligosaccharides characterized in urine of patients with two new types of mucolipidosis. A and B, mucolipidosis W (Laura and Pierre Alexandre W.); C, mucolipidosis De P. Solvent S2, development for 6 days (A) or 14 days (B and C). Samples were concentrated 300-fold compared with the initial volume of urine. Pyridine acetate concentrations, see Fig. 10.

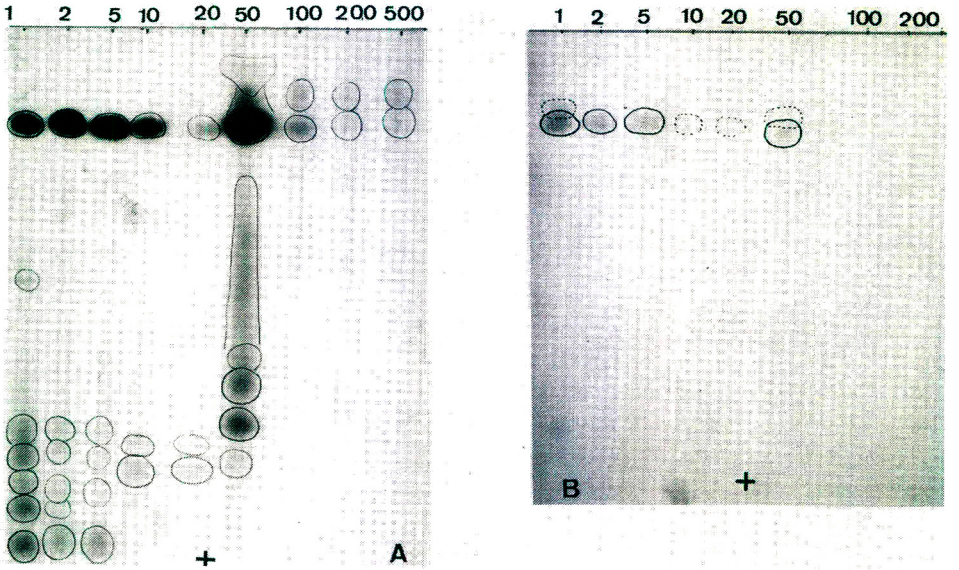

Fig. 13. Urine of fucosidosis: paper electrophoresis of "glycopeptidic fractions" eluted from a cation exchanger with a discontinuous gradient of pyridine acetate (1—500 m*M*). A, Stained with ninhydrin; B, stained with aniline oxalate.

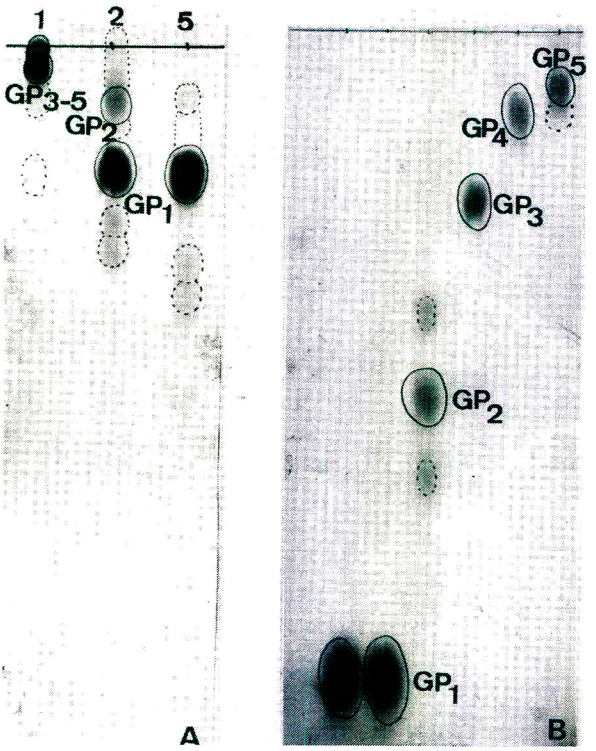

Fig. 14. Urine of fucosidosis: paper chromatography of glycopeptides. A, Fractions eluted from a cation exchanger with 1, 2 and 5 m*M* pyridine acetate; B, glycopeptides isolated by paper chromatography. Solvent S2, development for 20 h (A) or 4 days (B).

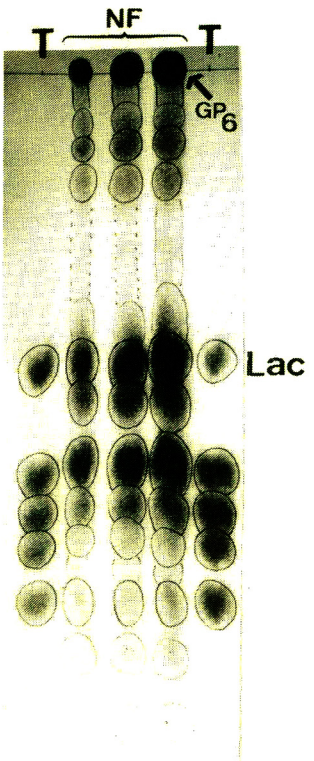

Fig. 15. Urine of fucosidosis: paper chromatography of "neutral fraction" (NF). Solvent S2, development for 18 h. The arrow indicates glycopeptide GP-6. T = standard. Lac = lactose.

TABLE IV

SUGAR COMPOSITION OF GLYCOPEPTIDES ISOLATED FROM URINE OF A PATIENT WITH FUCOSIDOSIS

Abbreviations as in Table I.

Glycopeptide	Amount* (mg/l)	Molar ratio**				
		Gal	Glc	Man	Fuc	GlcNAc
GP-1	96	—	—	—	1.05	0.96
GP-2	7	0.88	0.07	—	0.96	0.90
GP-3	22	—	—	1.88	0.99	1.82
GP-4	10	0.95	—	1.94	1.86	2.84
GP-5	7	1.14	—	1.92	1.96	2.90
GP-6	98	3.90	—	3.05	5.02	6.05

* See Table II.
** On the basis of 1 aspartic acid residue.

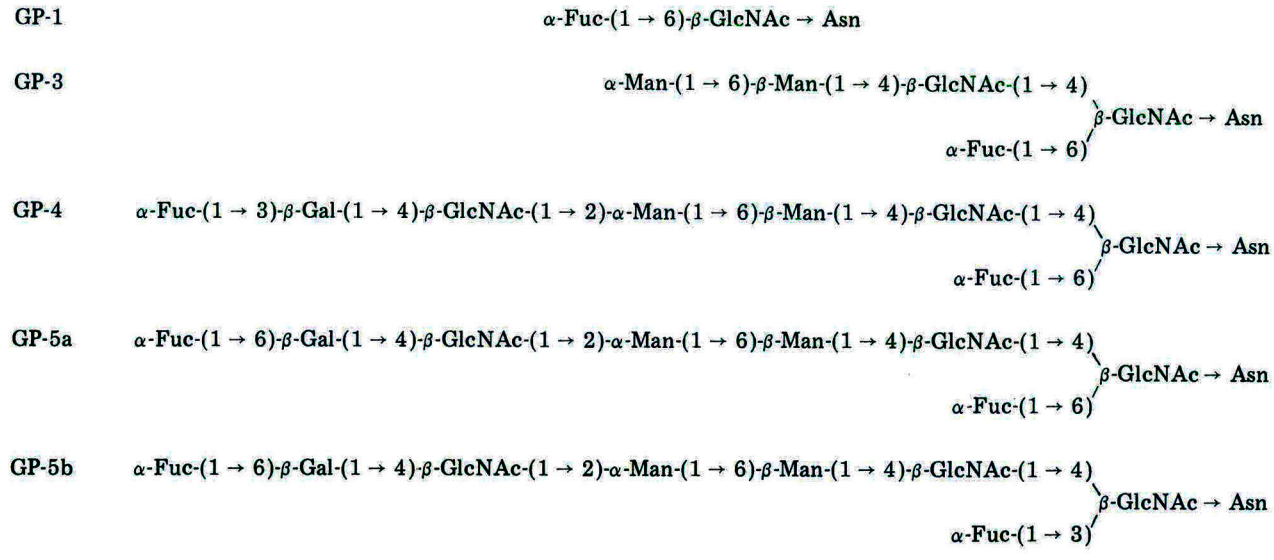

Fig. 16. Structure of glycopeptides isolated from the urine of fucosidosis [12].

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MASS FRAGMENTOGRAPHIC DETERMINATION OF LOFEPRAMINE AND ITS METABOLITES IN HUMAN PLASMA AND URINE USING DEUTERATED INTERNAL STANDARDS

KYUICHI MATSUBAYASHI, HIDEO HAKUSUI and MITSUJI SANO

Research Institute of Daiichi Seiyaku Co., Ltd. 2810 Minamifunabori-cho, Edogawa-ku, Tokyo (Japan)

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SUMMARY

A sensitive and specific method for the determination of lofepramine and its metabolites, desipramine and 2-hydroxydesipramine, in human plasma and urine is described. Lofepramine, desipramine and 2-hydroxydesipramine were derivatized to ethyl *p*-chlorobenzoate, the bis(heptafluorobutyryl) derivative and the N,O-bis(trifluoroacetyl) derivative, respectively, and then analysed by gas chromatography—mass fragmentography. Corresponding deuterated compounds were used as internal standards. Determination was possible at levels as low as 2 ng/ml for lofepramine and desipramine and 20 ng/ml for 2-hydroxydesipramine.

INTRODUCTION

Lofepramine [N-methyl-N-(4′-chlorophenacyl)-3-(10,11-dihydro-5H-dibenz-[*b*, *f*]azepin-5-yl)propylamine hydrochloride] is a tricyclic antidepressant drug, an imipramine analogue in which the amino group of the side-chain is substituted by a *p*-chlorophenacyl group [1]. This compound is apparently more lipophilic than imipramine and is expected to be absorbed and distributed faster into the body [2, 3]. Clinical reports have shown that lofepramine has similar antidepressive activities to imipramine, but a lower toxicity [4]. These properties indicate that lofepramine would be a more desirable antidepressant drug. In recent years, the determination of plasma levels of the drug in man after a single oral administration or during chronic treatment has become more important, because it affords valuable information concerning the bioavailability of the drug and its therapeutic and toxic thresholds. The method for the determination of lofepramine and its metabolite, desipramine, in plasma by gas chromatography has been already reported [5]. However, the method requires skilful technique to control the conditions of the procedure. In the work described here, a mass fragmentographic method with deuterated internal standards was devised for the sensitive and specific determination of lofe-

pramine, desipramine and 2-hydroxydesipramine in plasma and urine. 2-Hydroxydesipramine was shown, in a preliminary metabolic experiment, to be the main unconjugated metabolite of lofepramine in human urine.

EXPERIMENTAL

Reference drugs

The hydrochlorides of lofepramine, desipramine and 2-hydroxydesipramine were gifts from AB Leo (Hersinborg, Sweden). *p*-Chlorobenzoic acid was purchased from Tokyo Kasei (Tokyo, Japan).

Chemicals

Pentadeuteriochlorobenzene and deuterium oxide were purchased from Merck Sharp & Dohme (Quebec, Canada); 10, 11-dihydro-5H-dibenz[*b*,*f*]-azepine from Aldrich (Milwaukee, Wisc., U.S.A.); heptafluorobutyric anhydride and trifluoroacetylimidazole from Tokyo Kasei; and deuterium gas from Showa Denko (Tokyo, Japan).

Deuterated internal standards

4-Chloro-2,3,5,6-tetradeuteriobenzoic acid (p-chlorobenzoic acid-d_4). This compound was prepared according to the method of Gross et al. [6]. Pentadeuteriochlorobenzene (isotopic purity: >99 atom-% ^{2}H) was allowed to react with dichloromethylenedioxybenzene in methylene chloride in the presence of anhydrous aluminium trichloride. The reaction product was hydrolysed with 25% potassium hydroxide solution to give *p*-chlorobenzoic acid-d_4. 2-Chloro-3,4,5,6-tetradeuteriobenzoic acid appeared not to be formed as a by-product. The product was characterized by thin-layer chromatography (TLC) and, after derivatization to its methyl ester, by gas chromatography—mass spectrometry (GC—MS). The isotopic distribution was as follows: d_4, 72.2%; d_3, 23.1%; d_2, 4.3%; d_1, 0.4%; d_0, 0.03%.

10,11-Dideuteriodesipramine (DMI-d_2) hydrochloride. 10,11-Dihydro-5H-dibenz[*b*,*f*]azepine was converted into 5H-dibenz[*b*,*f*]azepine by the method of Schindler and Blattner [7]. 5-(N-Methylaminopropyl)-5H-dibenz[*b*,*f*]azepine (dehydro-DMI) hydrochloride was synthesized from 5H-dibenz[*b*,*f*]azepine as described by Geigy [8]. DMI-d_2 hydrochloride was obtained as follows. Dehydro-DMI hydrochloride in deuterium oxide was stirred under deuterium gas (isotopic purity: >99.5%) in the presence of 5% palladium on carbon. The reaction mixture was frequently monitored by TLC during hydrogenation. After 6 h, the reaction was stopped, the catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was recrystallized from ethanol—light petroleum (b.p. 30—70°). The product was characterized as DMI-d_2 hydrochloride by TLC and GC—MS. The isotopic distribution was as follows: d_2, 81.6%; d_1, 9.0%; d_0, 0.6%.

2-Hydroxy-10,11-dideuteriodesipramine (2-OH-DMI-d_2). This compound was obtained as a metabolite of 10,11-dideuteriolofepramine from rat urine as follows. A suspension of 10,11-dideuteriolofepramine hydrochloride in 0.5% CMC(sodium carboxy methyl cellulose) solution was administered orally to male Wistar rats. The urine was collected during 24 h after administration and

extracted with chloroform under alkaline medium. From the extract, 2-OH-DMI-d_2 was separated and purified by preparative TLC. The product was characterized by TLC and, after preparation of its N,O-bis(trifluoroacetyl) derivative, by GC—MS. The isotopic distribution was as follows: d_2, 77.2%; d_1, 13.0%; d_0, 2.5%. 10, 11-Dideuteriolofepramine hydrochloride was prepared from DMI-d_2 by the method of Ericksoo and Rothe [1].

Gas chromatography—mass fragmentography

A Hitachi Model RMU-6MG mass spectrometer equipped with a gas chromatograph was used. The GC conditions for ethyl *p*-chlorobenzoate (derived from lofepramine) were as follows: glass column (2 m × 3 mm I.D.) containing 2% PEG-20M on Gas-Chrom Q (80—100 mesh); temperatures of the oven, injection port and separator, 110°, 210° and 250°, respectively. The GC conditions for the derivatives of DMI and 2-OH-DMI were as follows: glass column (1 m × 3 mm I.D.) containing 1% OV-101 on Gas-Chrom Q (80—100 mesh); temperatures of the oven, injection port and separator, 220°, 260° and 265°, respectively. The carrier gas (helium) flow-rate was 30 ml/min in all instances. The MS conditions in all instances were as follows: ionization voltage, 30 eV; target current, 80 μA; ion source temperature, 230°; multiplier potential, 1.5—1.7 kV. For mass fragmentography, multiple ion detection was employed. The following ion *m/e* focusing was used: *m/e* 139 for ethyl *p*-chlorobenzoate; *m/e* 143 for ethyl *p*-chlorobenzoate-d_4; *m/e* 459 for the bis(heptafluorobutyryl) derivative of DMI; *m/e* 461 for the bis(heptafluorobutyryl) derivative of DMI-d_2; *m/e* 320 for the N,O-bis(trifluoroacetyl) derivative of 2-OH-DMI; and *m/e* 322 for the N,O-bis(trifluoroacetyl) derivative of 2-OH-DMI-d_2 (see Figs. 1—3).

Analytical procedure

Lofepramine. To 1 ml of heparin-treated plasma, which had previously been acidified with 0.06 ml of 2 *M* hydrochloric acid and stored at −20°, 0.2 ml of 0.5 *M* sodium hydroxide solution and 1 ml of 0.2 *M* sodium hydrogen carbonate—sodium carbonate buffer (4:6) pH 10, were added. The solution was extracted with 5 ml of *n*-hexane by shaking for 10 min on an automatic shaker. After centrifugation, 4 ml of the organic phase were transferred into a glass tube and the solvent was evaporated under reduced pressure. To the residue, 1 ml of ethanol containing 0.1 ml of 30% hydrogen peroxide solution was added, and the mixture was allowed to stand for 1 h at room temperature. To the reaction mixture, 0.5 ml of distilled water and 50 μl of the internal standard solution (4 μg/ml of *p*-chlorobenzoic acid-d_4 in ethanol) were added. The mixture was concentrated under reduced pressure to a volume of about 0.5 ml, then 2 ml of 1 *M* hydrochloric acid and 5 ml of benzene were added. The tube was shaken mechanically for 10 min. After centrifugation, the organic phase was transferred into a glass-stoppered tube and the solvent was evaporated. The residue was dissolved in 0.2 ml of ethanol and 1 ml of ethanol saturated with hydrogen chloride gas. The mixed solution was heated at 60° for 1 h, then the reaction mixture was cooled in ice-cold water and 0.2 ml of isoamyl alcohol was added. The mixture was concentrated under reduced pressure to a volume of about 0.3 ml at room temperature. To the concentrate

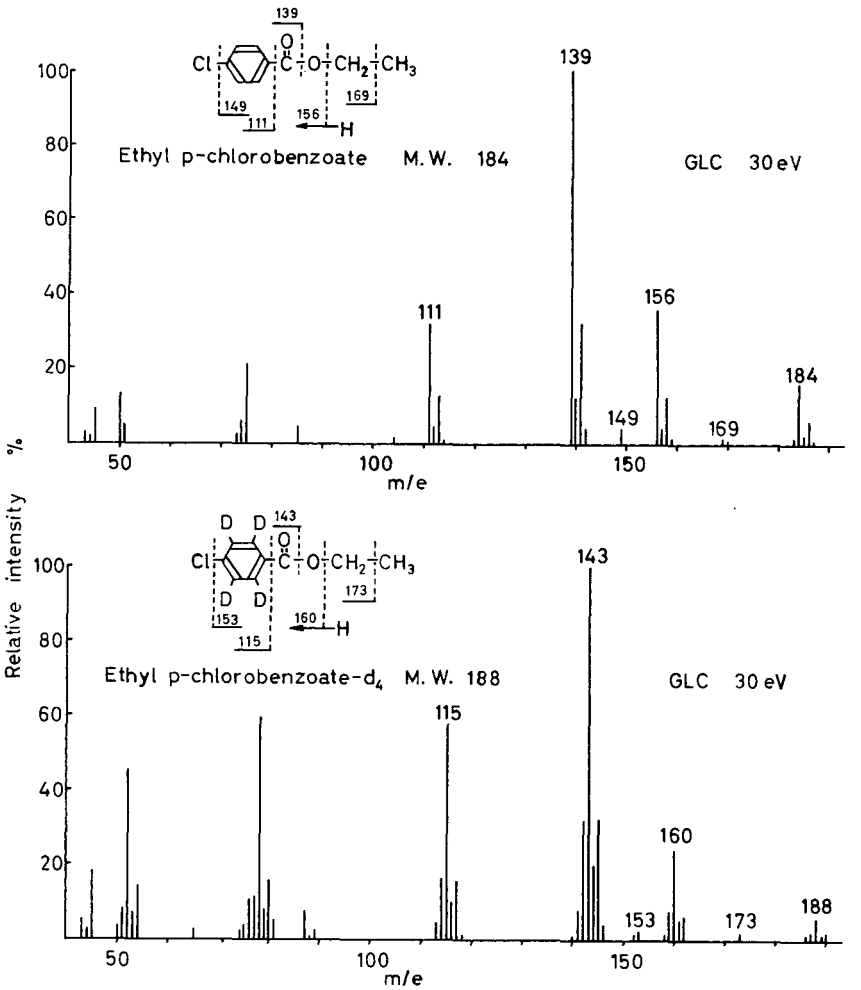

Fig. 1. Mass spectra of ethyl *p*-chlorobenzoate (derived from lofepramine) (above) and ethyl *p*-chlorobenzoate-d_4 (below).

5 ml of *n*-hexane and 2 ml of 5% sodium hydrogen carbonate solution were added. The tube was carefully shaken for 2 min and the organic phase was transferred into a glass tube and concentrated to a volume of about 50 µl under reduced pressure. A volume of 3 or 4 µl of this solution was injected into the GC column.

To 1 ml of urine sample, stored at −20°, 1 ml of the carbonate buffer was added, the mixture was extracted with 5 ml of *n*-hexane, and then processed as described for the plasma sample.

Desipramine. To 1 ml of plasma sample, 20 µl of the internal standard solution (10 µg/ml of DMI-d_2 hydrochloride in 0.1 *M* hydrochloric acid) and 1 ml of 0.2 *M* hydrochloric acid were added. The solution was shaken with 5 ml of dichloroethane for 10 min and, after centrifugation, the organic phase

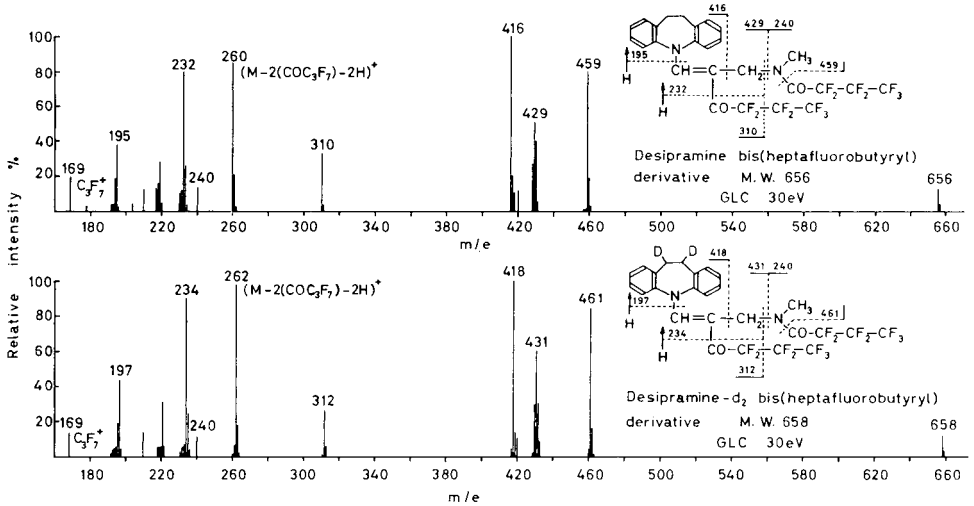

Fig. 2. Mass spectra of desipramine bis(heptafluorobutyryl) derivative (above) and desipramine-d_2 bis(heptafluorobutyryl) derivative (below).

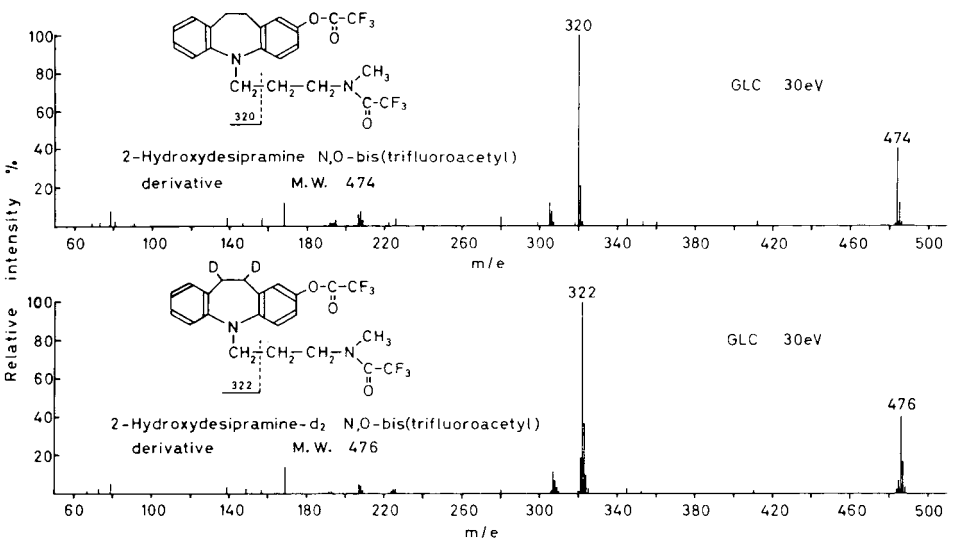

Fig. 3. Mass spectra of 2-hydroxydesipramine N,O-bis(trifluoroacetyl) derivative (above) and 2-hydroxydesipramine-d_2 N,O-bis(trifluoroacetyl) derivative (below).

was removed and discarded. The aqueous phase was made alkaline by adding 0.5 ml of 5 *M* sodium hydroxide solution and extracted with 5 ml of *n*-hexane, and then the mixture was centrifuged. All glassware in the following procedure was rinsed with isoamyl alcohol prior to use. The organic phase was dried over 1 g of anhydrous sodium sulphate and then evaporated to dryness under reduced pressure. To the residue, 200 μl of ethyl acetate and 40 μl of heptafluorobutyric anhydride were added and the reaction mixture was allowed to stand overnight in a tube with a stopper at room temperature. The mixture was evaporated to dryness under reduced pressure. The residue was re-dissolved

in 50 μl of ethyl acetate and an aliquot of 2—4 μl was injected into the GC column.

To a 3-ml urine sample, 50 μl of the internal standard solution as mentioned above were added. The solution was made alkaline by addition of 0.5 ml of 5 *M* sodium hydroxide solution and extracted twice with 3-ml portions of *n*-hexane. After centrifugation, the extracts were combined and then processed as described for the plasma sample.

2-Hydroxydesipramine. To 1 ml of urine sample, 10 μl of the internal standard solution (15 μg/ml of 2-OH-DMI-d_2 in methanol) were added and the pH was adjusted to 10 with 1 ml of 0.2 *M* carbonate buffer. The solution was shaken with 5 ml of dichloroethane for 10 min. After centrifugation, the organic phase was transferred into a glass tube and evaporated to dryness under reduced pressure. The residue was dissolved in 200 μl of acetonitrile, mixed with 20 μl of N-trifluoroacetylimidazole and stood at 80° for 3 h. An aliquot of 1—4 μl of the reaction mixture was used for GC.

Synthesis and spectral data of authentic 10,11-dihydro-5-[3-(N-heptafluorobutyryl-N-methylamino)-2-heptafluorobutyryl-1-propenyl]-5H-dibenz[b,f]azepine

To a suspension of 100 mg of desipramine hydrochloride in 2 ml of ethyl acetate, 1 ml of heptafluorobutyric anhydride was added. The reaction mixture was allowed to stand for 15 h at room temperature, and then evaporated to dryness under reduced pressure. The residue was chromatographed on 4 g of silica gel and eluted with benzene. From the main fraction, 65 mg of the bis-(heptafluorobutyryl) derivative were obtained as pale yellow crystals, m.p. 68—70°; infrared (film), 1680, 1640 (C=O), 1560 cm^{-1} (C=C and C=O); nuclear magnetic resonance ($CDCl_3$), 8.13 (s. 1H, vinylic), 3.85 (s. 2H, $-CH_2-N$), 2.7—3.8 (m. 4H, $-CH_2-CH_2-$), 3.0 (broad s. 3H, $-N-CH_3$), 7.28 ppm (s. 8H, aromatic); ultraviolet λ_{max}. (ethanol) 319 nm ($\epsilon = 4.7 \cdot 10^4$). The mass spectrum is shown in Fig. 2. The elemental composition was confirmed by high-resolution MS.

RESULTS AND DISCUSSION

The GC analysis of lofepramine itself has not been successful because it is decomposed at the high temperatures usually employed. Lundgren et al. [5] reported a method for the determination of lofepramine, in which *p*-chlorobenzaldehyde derived from lofepramine was detected by GC with an electron-capture detector. In this method, the steam-distillation procedure for cleaning up samples had to be operated with much caution because of the high volatility of the aldehyde.

In our study, it was found that lofepramine was very unstable under oxidation conditions and readily formed *p*-chlorobenzoic acid in hydrogen peroxide solution in 80% yield (standard deviation 5%). By determining the *p*-chlorobenzoic acid thus generated, the amount of lofepramine could be calculated. The oxidation reaction was completed in 1 h. The resulting *p*-chlorobenzoic acid was converted into its ethyl ester and determined by mass fragmentography, monitoring the *p*-chlorobenzoyl ion, *m/e* 139 (the base peak ion).

The mass spectrum of the methyl ester gave an analogous pattern to that of the ethyl ester but, as long as *m/e* 139 ion was monitored, the methyl ester was found to be an unsuitable derivative for this analysis because methyl *p*-chlorobenzoate-d_4, used as an internal standard, also gave an ion at *m/e* 139 due to the fragment $[M—Cl]^+$. As shown in Fig. 1, such a disturbance was not encountered with the ethyl ester (Fig. 4A).

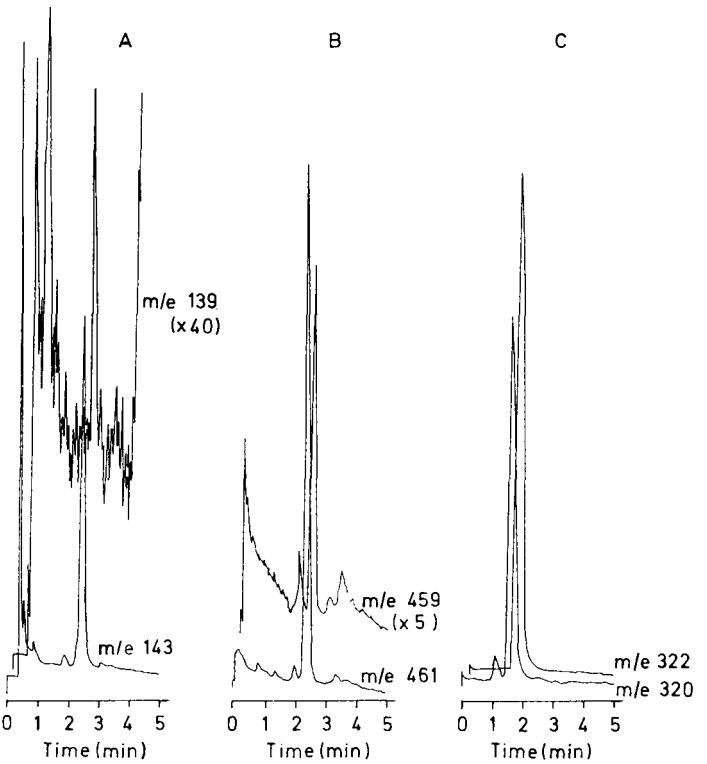

Fig. 4. Mass fragmentograms obtained from plasma or urine of a healthy volunteer dosed with lofepramine. (A) Lofepramine in plasma; (B) desipramine in plasma; (C) 2-hydroxy-desipramine in urine. Samples were processed as described under *Analytical procedure*.

The kinetics of the esterification reaction in ethanol saturated with hydrogen chloride are shown in Fig. 5. The peak-height ratio was linear over the range 2—80 ng/ml in plasma or urine (Fig. 7A). A typical mass fragmentogram obtained from human plasma containing lofepramine is shown in Fig. 4A. The overall recovery of lofepramine in this procedure was about 60%.

Although the internal standard was added after oxidation, the determination of lofepramine was performed accurately because the recovery of lofepramine during the extraction procedure was shown to be more than 95% in preliminary experiments in which known amounts of lofepramine hydrochloride were added in 0.1 *M* borate buffer (pH 10) and extracted with *n*-hexane, the recovery being determined by ultraviolet spectrophotometry [9]. The concomitant metabolite, *p*-chlorobenzoic acid, in biological material remained in the aqueous phase under these extraction conditions.

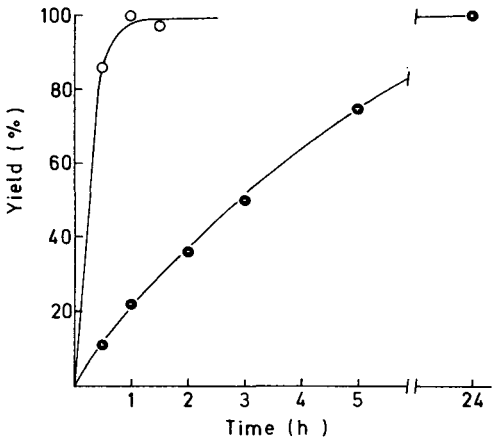

Fig. 5. Yields from the esterification reaction of *p*-chlorobenzoic acid at (•) 25° and (○) 60°.

Desipramine is the active metabolite of lofepramine that retains antidepressant activity. Therefore, the determination of plasma levels of desipramine would also afford valuable biological information. Several methods for the GC determination of desipramine in biological fluids have been described [10—15]. In those methods, desipramine was converted into its acyl derivative in order to avoid adsorption on the stationary phase. It has been shown that the N-heptafluorobutyryl derivative (monoacyl derivative) of DMI is more effective for avoiding adsorption than the acetyl or the trifluoroacetyl derivative. However, under the usual reaction conditions for heptafluorobutyrylation, the bis(heptafluorobutyryl) derivative of desipramine was also formed in considerable amounts and it was difficult to control the reaction to form the N-heptafluorobutyryl derivative selectively. By using ethyl acetate as the reaction solvent, the bis(heptafluorobutyryl) derivative could be obtained more easily and quantitatively. This bis(heptafluorobutyryl) derivative was found to be far less adsorbed than the N-heptafluorobutyryl derivative on the GC column and gave a symmetrical peak in the gas chromatogram. It was also stable to moisture.

These properties of the bis(heptafluorobutyryl) derivative suggested that it was the most suitable derivative for the GC analysis of desipramine. The infrared, nuclear magnetic resonance and ultraviolet spectra of this compound were similar to those of the bis(trifluoroacetyl)derivative of desipramine reported by Walle et al. [16]. The mass spectrum is shown in Fig. 2. These spectral data suggest that the chemical structure of the bis(heptafluorobutyryl) derivative is 10,11-dihydro-5-[3-(N-heptafluorobutyryl-N-methylamino)-2-heptafluorobutyryl-1-propenyl]-5H-dibenz[*b*,*f*]azepine, the compound in which the trifluoroacetyl groups in Walle et al. 's compound are replaced with heptafluorobutyryl groups. The kinetics of the derivatization reaction are shown in Fig. 6. The reaction was completed in 6 h. The ion corresponding to $[M—C_3F_7CO]^+$, *m/e* 459 for the sample and *m/e* 461 for the internal standard, was monitored (Fig. 4B). The ratio peak height of DMI to that of DMI-d_2 was linear over the range 2—60 ng/ml in plasma or urine (Fig. 7B).

For the selective detection of desipramine in biological fluids, it was nec-

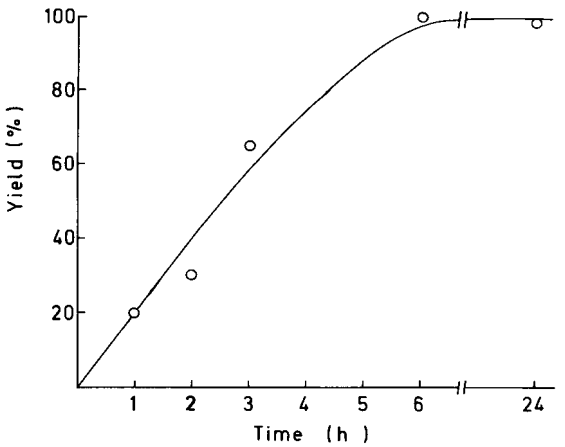

Fig. 6. Yields from the bis(heptafluorobutyryl) derivative formation reaction of desipramine.

essary to remove lofepramine, which readily liberated desipramine during the cleaning up process. Lofepramine was removed by extracting the sample with dichloroethane just after DMI-d_2 was added as an internal standard. Addition of the deuterio-labeled internal standard at the beginning of the procedure contributed to a more precise and accurate determination of plasma or urine levels of DMI.

When 2-OH-DMI was subjected to reaction with N-trifluoroacetylimidazole at 80° for 3 h, the N,O-bis(trifluoroacetyl) derivative was formed quantitatively. When examined by GC, the product gave a single symmetrical peak. With 2-OH-DMI, the tris(heptafluorobutyryl) derivative corresponding to the bis(heptafluorobutyryl) derivative of DMI was found to be unstable under the GC conditions. The mass spectra of the N,O-bis(trifluoroacetyl) derivatives of 2-OH-DMI and of 2-OH-DMI-d_2 are shown in Fig. 3. In the mass fragmentographic determination, the ions monitored were *m/e* 320 for the sample and *m/e* 322 for the internal standard (Fig. 4C). The standard graph was linear over the range 20 ng/ml to 1 μg/ml in urine (Fig. 7C).

The utility of these methods was demonstrated by applying them to clinical

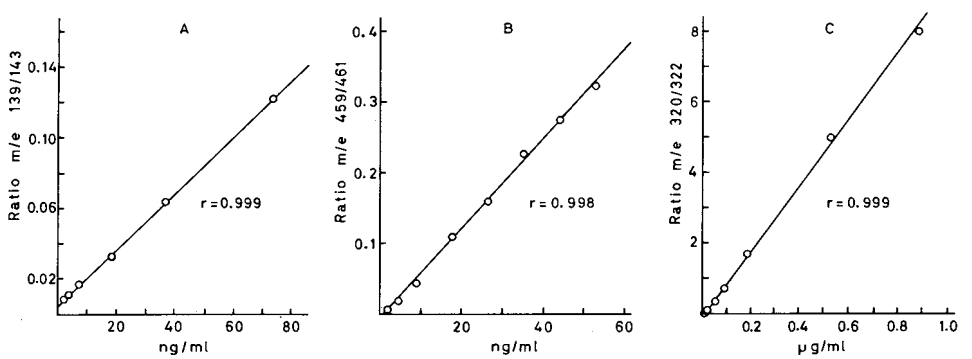

Fig. 7. Standard graphs: (A) lofepramine in human plasma; (B) desipramine in human plasma; (C) 2-hydroxydesipramine in human urine. The standard graphs were obtained by adding known amounts of lofepramine, desipramine or 2-hydroxydesipramine to 1 ml of human plasma or urine and processing them as described under *Analytical procedure*.

experiments with human volunteers receiving single or multiple doses of lofepramine. The clinical significance of these results has been discussed previously [17]. Representative results are shown in Fig. 8.

The results demonstrate that the technique described here seems to be suitable for determining the plasma levels and urinary excretion of lofepramine in depressed patients undergoing chronic treatment.

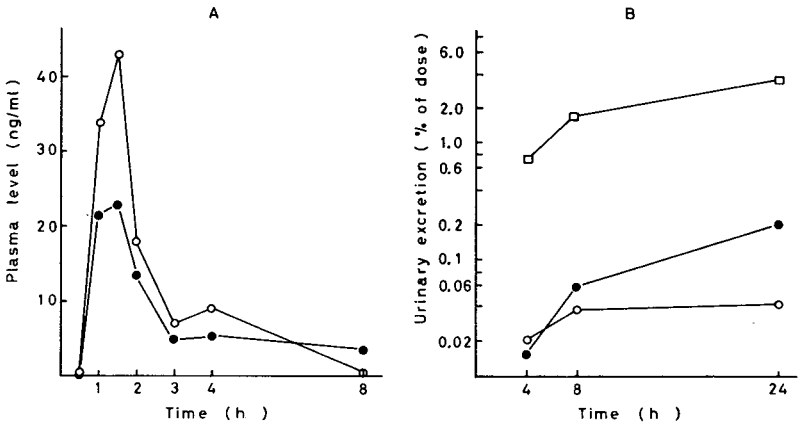

Fig. 8. (A) Plasma levels of lofepramine and desipramine in a healthy volunteer (subject K.O., male, age 27, weight 55 kg) after a single oral dose of 50 mg of lofepramine. (B) Urinary excretion of lofepramine and its metabolites in the same experiment. ○, Lofepramine ●, desipramine; □, 2-hydroxydesipramine.

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Biomedical Applications

CHROMBIO. 070

RAPID DETERMINATION OF DIAZEPAM AND NORDIAZEPAM IN PLASMA BY ELECTRON CAPTURE GAS—LIQUID CHROMATOGRAPHY

APPLICATION IN CLINICAL PHARMACOKINETIC STUDIES

ROBERT E. WEINFELD, HOWARD N. POSMANTER, KO-CHIN KHOO and CARL V. PUGLISI

Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche Inc., Nutley, N.J. 07110 (U.S.A.)

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SUMMARY

A rapid method was developed for the determination of diazepam and nordiazepam (N-desmethyldiazepam) in human plasma using electron capture gas—liquid chromatography (GLC—ECD). The concentration of diazepam and nordiazepam is determined using 0.5 ml of plasma extracted with 1.0 ml of benzene containing 25 ng/ml of methylnitrazepam as the internal standard. The benzene extract is removed and an aliquot is subjected to automated GLC—ECD analysis. The method has a sensitivity limit of 5 ng diazepam and 10 ng nordiazepam per milliliter of plasma. The method was used to determine the plasma levels in man following the first 5-mg diazepam dose, as well as during chronic oral administration of 5 mg diazepam three times daily and 15 mg diazepam once a day.

INTRODUCTION

The determination of drug concentration in the numerous plasma samples generated in bioequivalency and clinical pharmacokinetic studies in man, has created a need for simple, rapid and sensitive assays. Diazepam (the active drug substance in Valium, marketed by Hoffmann-La Roche, Nutley, N.J., U.S.A.), a member of the 1,4-benzodiazepine class of compounds (Fig. 1), is used in the relief of tension, anxiety and skeletal muscle spasms [1—8]. In clinical practice, single oral doses of diazepam range typically from 2 to 10 mg and are administered 2—4 times daily. In previous studies in man, peak concentrations of 221—400 ng/ml of plasma [9] and 137—189 ng/ml of whole blood [10] were

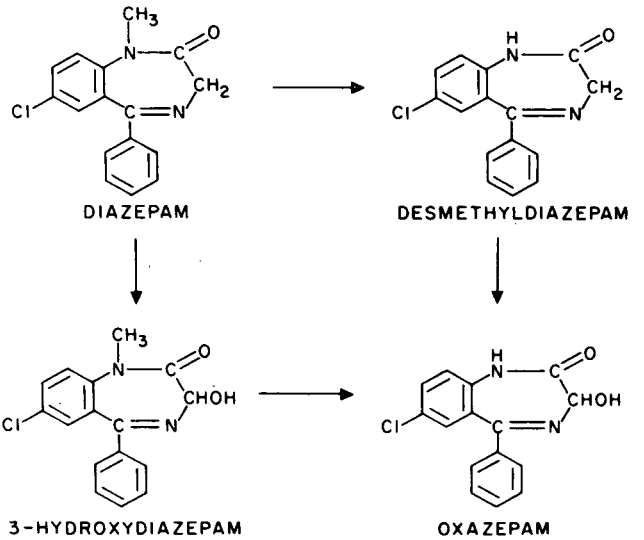

Fig. 1. Major pathways of diazepam metabolism in humans.

measured after single oral administration of 10 mg of diazepam. Chronic oral administration of 5 mg of diazepam three times daily resulted in steady state plasma levels of 230—440 ng/ml [11], while chronic oral administration of 10 mg of diazepam once daily resulted in blood levels of about 200 ng/ml [10]. Concentrations of the major metabolite of diazepam, nordiazepam (N-desmethyldiazepam) (Fig. 1), can range from 10 to 20 ng/ml of blood at the time of peak diazepam levels, following single oral administration of 10 mg of diazepam and reach steady-state concentrations of 100—150 ng/ml of blood during chronic administration of 10 mg of diazepam daily [10].

Assays for the determination of diazepam and nordiazepam in blood and plasma following therapeutic doses of the drug have required the sensitivity and specificity of electron capture gas—liquid chromatography (GLC—ECD).

Numerous GLC—ECD procedures have been developed to measure diazepam and nordiazepam [11—18]. The earlier assays required the conversion of diazepam and nordiazepam to their corresponding *o*-aminobenzophenone derivatives by acid hydrolysis prior to GLC—ECD analysis [12]. The more recent procedures were able to quantitate diazepam and its metabolites by GLC—ECD without derivatization employing the liquid phase OV-17 and a ^{63}Ni electron capture detector (ECD) [11, 13—18].

The previously published procedures are useful in the measurement of diazepam and nordiazepam in studies involving a limited number of specimens for analysis. They are, however, time consuming and impractical when large scale human studies are required which involve the analysis of hundreds of specimens on a routine basis in order to demonstrate bioequivalency of formulations or in clinical pharmacokinetic studies. Moreover, most of the previously reported assays involve many tedious steps including multiple extractions and transfers, clean-up steps, solvent evaporation, and manual injection into the gas chromatograph.

The procedure described herein was developed in response to the need for a rapid, sensitive, specific and reproducible assay for diazepam and nordiazepam in plasma, permitting the preparation of large numbers of extracts in a single day for GLC analysis, and the subsequent automation of sample injection, peak area integration and computation of results.

The concentration of diazepam and nordiazepam in plasma following single and during chronic oral administration of diazepam have been determined successfully by this rapid assay.

EXPERIMENTAL

Parameters for GLC analysis

Column. A U-shaped borosilicate glass column, 1.2 m × 4 mm. I.D., containing a pretested preparation of 3% OV-17 on 60—80 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.) was used. The column was conditioned as previously described[13].

Instrumental parameters. A Tracor Model 222 gas chromatograph (Tracor, Austin, Texas, U.S.A.) equipped with a 15-mCi ^{63}Ni ECD, and an automatic sampler (Model 7671A, Hewlett-Packard, Avondale, Pa., U.S.A.) was used. Argon—methane (90:10) gas mixture (Matheson Gas Products, East Rutherford, N.J., U.S.A.), oil-pumped and dry, at 40 p.s.i.g. and a flow-rate of 70 ml/min through the column, was used as carrier gas. The purge gas flow-rate was 20 ml/min through the detector. The temperature settings were: column, 235°; injection port, 275°, detector, 350°. The flow-rate and oven temperature were adjusted so as to obtain retention times (t_R values) of approximately 3.9, 5.5, 8.6 and 13.0 min for diazepam, nordiazepam and the internal standards, methylnitrazepam and griseofulvin, respectively. An electron capture linearizer (Tracor, Model No. 114460-001) operated the detector in the "constant current pulsed mode". The linearizer parameters were set as follows: standing current, 0.3×10^{-9} A; relative pulse width, 0.18 (0.75 μ sec actual pulse width); attenuator, 8. A 1.0-mV recorder (Model 7127A, Hewlett-Packard) was operated at a chart speed of 0.25 in/min. Under these conditions 1.0 ng of diazepam and 2.0 ng of nordiazepam injected gave nearly full-scale responses, and 0.25 ng methylnitrazepam and 1.0 ng griseofulvin injected gave about 50% f.s.d. The minimum detectable amounts of diazepam and nordiazepam were 5 ng and 10 ng/ml of plasma, respectively.

A mini-computer based data system (Hewlett-Packard, 3352B Laboratory Data System) was interfaced with the electron capture linearizer and automatic sampler.

Reagents

The following reagents were used: Saturated potassium chloride (analytical-reagent grade) (approx. 4.8 *M*), prepared in distilled, deionized water; benzene (Nanograde, Mallinckrodt, St. Louis, Mo., U.S.A.); and absolute ethanol (Publicker, Linfield, Pa., U.S.A.)

Glassware

Special extraction tubes, 125 mm × 13 mm O.D., round-bottom, 9-ml capacity with a 10/18 ℧ joint, were constructed (Fig. 2)*. Tubes and stoppers were washed with a non-corrosive surfactant cleaning solution (Micro, International Products Corp., Trenton, N.J., U.S.A.) in a 50° ultrasonic bath, subsequently rinsed with distilled, deionized water and dried in an oven. Sample vials (Hewlett-Packard) were rinsed with distilled, deionized water and dried in an oven prior to use.

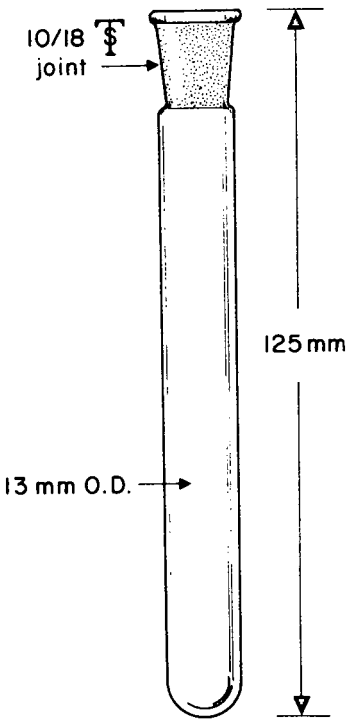

Fig. 2. Special extraction tube.

Standard solutions

Stock solutions of diazepam and nordiazepam were prepared as follows: 10.00 mg of each compound was weighed out in separate 10-ml volumetric flasks, dissolved in 1.0 ml of absolute ethanol and made up to volume with benzene, to yield 1 mg/ml solutions. These stock solutions were diluted 1:100 with benzene to yield 10 μg/ml solutions of diazepam and nordiazepam. A series of mixtures of diazepam and nordiazepam containing 0.05, 0.1, 0.3, 0.5, 1.0, 2.0, 3.0, 4.0, 4.5 and 5.0 μg/ml benzene of each compound was prepared by suitable dilutions of the 10 μg/ml standard solutions for use as calibration standards by addition to plasma.

* A similar tube, Corning No. 9810, is commercially available, and would be a suitable substitute.

Methylnitrazepam, 7-nitro-5-phenyl-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one, was used as the internal standard. Methylnitrazepam (10 mg) was weighed out and placed in a 100-ml volumetric flask, dissolved in 10 ml of absolute ethanol and made up to volume with benzene to yield a 100 μg/ml stock solution. A 10-μg/ml solution was prepared by diluting a 1.0-ml aliquot of the stock solution to 10 ml in a volumetric flask. This solution was used to prepare a 25-ng/ml methylnitrazepam solution in benzene used for sample extraction, by diluting a 250-μl aliquot to 100 ml in a volumetric flask. The diluted solution was stored in an amber 100-ml conical vessel with a 24/40 ℧ ground glass joint. A 1.0-ml dispenser device with a glass joint (Cat. No. 3001-G, Repipet, Lab Industries, Berkeley, Calif., U.S.A.) was used to dispense the extraction solvent.

Griseofulvin (Calbiochem, Los Angeles, Calif., U.S.A.) was used as alternative internal standard. A stock solution was prepared as for methylnitrazepam. The working dilution was prepared by transferring a 200-μl aliquot of the stock solution to a 100-ml volumetric flask and making up to volume with benzene to yield a 200-ng/ml solution for extraction.

Procedure

Preparation of samples and calibration standards. Into the special extraction tube (Fig. 2), 0.5 ml of plasma and 0.5 ml of saturated potassium chloride solution were added and mixed, followed by the addition of 1.0 ml of benzene containing 25 ng of methylnitrazepam/ml. The tube was sealed tightly with a PTFE stopper (Chemplast, Wayne, N.J., U.S.A.) by twisting into the glass joint of the tube. The samples were placed in a suitably sized test tube support and clamped in a mechanical rotator (Model RD-250, Kraft Apparatus, Mineola, N.Y., U.S.A.), and rotated for 10 min at 30 rpm. The samples were centrifuged at 10° for 15 min at 1000 *g* in a refrigerated centrifuge (Model PR-J with a No. 253 rotor and No. 381 sample cups; Damon/IEC Division, Needham, Mass., U.S.A.) and approximately 0.6—0.75 ml of the organic phase was transferred into a 2-ml glass sample vial using a disposable 9-in. Pasteur capillary pipet. The vial was capped with a Teflon-faced rubber septum aluminum seal (Hewlett-Packard) using a hand-operated crimper (Wheaton Scientific, Millville, N.J., U.S.A.). The prepared samples were then ready for automatic injection into the gas chromatograph. Extracts should be chromatographed the same day they were prepared to avoid the possibility of sample degradation.

Along with unknown samples, five plasma calibration standards containing 50 ng of diazepam and 50 ng of nordiazepam were prepared by evaporating 100 μl of the 0.5-μg/ml standard mixture in benzene to dryness under a nitrogen stream (in a special extraction tube) and adding 0.5 ml of control human plasma. These calibration standards were then processed along with the unknowns.

Calibration of the data system. Using the GLC conditions initially set at the parameters outlined (see *Parameters for GLC analysis*), 10 μl of one of the above prepared calibration standard extracts containing 50 ng of diazepam and 50 ng of nordiazepam added to 0.5 ml control human plasma was injected. A typical chromatogram is shown in Fig. 3. This sample served to establish the re-

tention times of the 3 known peaks (diazepam, nordiazepam and methylnitrazepam) and identify any chromatographic problems (e.g. poor peak shape, poor response, abnormal sample impurities, interfering peaks or other disturbances) that could invalidate the results.

The software of the computer-based data system was used to prepare a method defining the parameters illustrated in Fig. 4.

A second extraced plasma calibration standard was chromatographed, and the peak area data were used to establish the response factors for diazepam and nordiazepam required for the internal standard method of calculation of the unknowns, with the results reported in ng/ml of plasma (shown in Fig. 4). The report format for such a calibration standard is shown in Fig. 5.

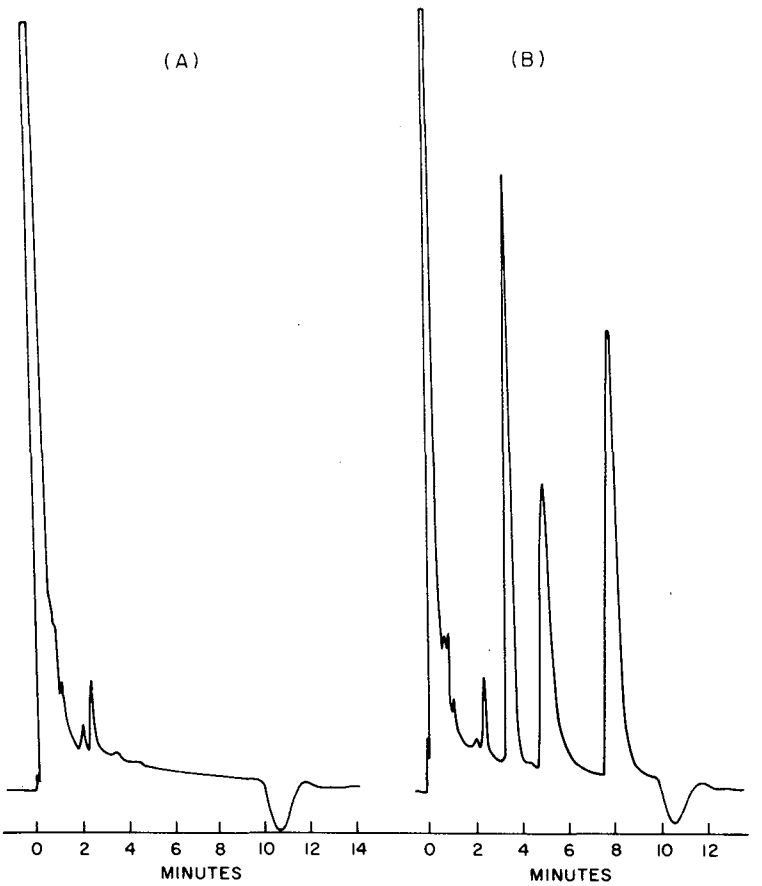

Fig. 3. Chromatograms of the GLC—ECD analysis of plasma benzene extracts. (A) Human control plasma; (B) human control plasma containing 50 ng diazepam (t_R = 3.9 min), 50 ng nordiazepam (t_R = 5.5 min) and 25 ng methylnitrazepam (internal standard, t_R = 8.6 min) per 0.5 ml of plasma. Injection volume: 10 μl of benzene from each extract. Attenuation: × 8.

```
1. CHAN,PROC,RPRT,RDVC
 10, ISTD, ME, T3

2. SAMP,UNTS,TITLE
PLASMA  , NG/ML , PLASMA DIAZEP-NORDIAZEP

3. #PKS,RTM,PRG
 15,  16.00, NO,

4. MIN AR,MV/M,DLY,DVT,DIL-FTR%
 100,   .100, 3.00, 0.00, 100.00

5. REF-RTW,%RTW,ID-LVL,RF-UNK
 .50, 10,    100, 1.000

6.# KWN PKS
  3

  #     TIME  AMT          FACTOR        NAME
  1     3.90  5.0000E  1   =3.1617E  0   DIAZEPAM
  2     5.49  5.0000E  1   =3.1232E  0   NORDIAZEP
  3     8.64  2.5000E  1   =1.0000E  0   &METHYLNIT

7. # EVENTS
  0
```

Fig. 4. The format of a typical computer-based data system method showing the parameters used in the analysis of diazepam and nordiazepam in plasma. The parameters defined and illustrated above included: method of calculation (PROC), format of report (RPRT), reporting device (RDVC), type of sample (SAMP), concentration units to be used for reporting results (UNTS), length of chromatographic run time (minutes) prior to reporting of data (RTM), minimum area (μV-sec) which must be exceeded before a peak will be reported (MIN AR), slope sensitivity (mV/min) threshold for the detection of peaks (MV/M), integration delay (minutes) which should expire after sample injection before integration should begin (DLY), parameters relating to identification of peaks by comparison with retention times of known peaks (REF-RTW), identification level for peaks having an area greater than this level to be considered for identification as a known peak (ID-LVL), retention times, amounts, response factors and names of known peaks (#KWN PKS).

The remaining plasma calibration standards were interspersed among the unknown samples in the automatic sampler tray and served to monitor the GLC—ECD system for accuracy, precision and changes in response during the unattended run.

Procedure for processing samples using the automatic liquid sampler (ALS). The ALS sequence was prepared using the computer-based software of the data system. The sequence contained the information defining instructions for processing the sample vials, and included the parameters illustrated in Fig. 6. In this manner, up to 36 sample vials could be processed unattended per run and the results were reported via teleprinter (Hewlett-Packard Model HP 2752A) for each vial processed. The series of sample vials prepared were then placed in the sampler tray in the assigned position defined in the ALS sequence, including three benzene wash vials and the system was then placed in the automated mode by user command. The system continued to operate unattended by automatic sample injection in the sequence specified and reported the results in ng of diazepam and nordiazepam per ml of plasma in each vial. The teletype report format for an unknown sample is illustrated in Fig. 5.

```
REPORT NO.    606                              PLASMA DIAZEP-NORDIAZEP

CHAN# 10        METHOD: DIAZEP        SAMPLE: RC100          BOTTLE  1

ISTD =  25.000   NG/ML

  RT          AREA       NG/ML       NAME

  3.90        11329 BB    99.779    DIAZEPAM
  5.49        12052 BB   100.491    NORDIAZEP
  8.64        16411 BB               &METHYLNIT

TOTAL AREA =          39792



REPORT NO.    622                              PLASMA DIAZEP-NORDIAZEP

CHAN# 10        METHOD: DIAZEP        SAMPLE: A3242          BOTTLE 17

ISTD =  25.000   NG/ML

  RT          AREA       NG/ML       NAME

  3.88        17269 BB   155.101    DIAZEPAM
  5.63        14469 BB   123.032    NORDIAZEP
  8.58        16092 BB               &METHYLNIT

TOTAL AREA =          47830
```

Fig. 5. The typical format of the data system report is illustrated for a diazepam and nordiazepam calibration standard (100 ng/ml of plasma) prepared as described in the text and injected by the automatic sampler (Report No. 606, upper) and a typical report for an unknown plasma extract is shown in Report No. 622 (lower).

RESULTS AND DISCUSSION

Numerous sensitive and specific GLC—ECD assays have been developed for the determination of diazepam and its metabolites in human plasma and other biological fluids [11, 13—18]. These assays are based on the intrinsic electron-capturing properties of diazepam and its metabolites, which permit detection of picogram quantities under the proper gas chromatographic (GC) conditions. The assay described herein also uses GLC—ECD and has a sensitivity limit comparable to that of previously reported procedures, but has significantly reduced the biological sample preparation time by modification of the extraction step and elimination of solvent evaporation steps. The procedure is essentially a one-tube, one-step extraction, requiring only one transfer into a sample vial for injection into the gas chromatograph. As an illustration of the ease of performing this assay, one person can prepare 36 samples for chromatography in 4 h. The only limitation on the number of samples prepared for GLC is the automatic sampler capacity (i.e., 36 vials). Following sample preparation the remaining analytical procedures, including sample injection, integration of peak areas, and data reduction reporting nanograms diazepam and nordiazepam per milliliter of plasma have been automated.

Several significant instrumental and electronic developments have recently become available that made this method practicable. ALS devices for GC have

```
*LI,ALS,9

1. 1ST BTL,#BTLS,RCAL,INJ/RC
     1,   33,    1,   37

2. WSHS,PMPS,STOP,#INJ
     1,    6,    3,    1

3. CTM,WBTL,ISO
    18.0, YES, YES

4. METHD
   DIAZEP

5. NAMES,WGHTS
BTL   1: RC100    ,  25.000
BTL   2: CONT PRE,  25.000
BTL   3: A3158    ,  25.000
BTL   4: A3165    ,  25.000
BTL   5: A3172    ,  25.000
BTL   6: A3179    ,  25.000
BTL   7: A3186    ,  25.000
BTL   8: A3193    ,  25.000
BTL   9: A3200    ,  25.000
BTL  10: A3207    ,  25.000
BTL  11: A3214    ,  25.000
BTL  12: A3221    ,  25.000
BTL  13: IS100    ,  25.000
BTL  14: CONT PL  ,  25.000
BTL  15: A3228    ,  25.000
BTL  16: A3235    ,  25.000
BTL  17: A3242    ,  25.000
BTL  18: A3256    ,  25.000
BTL  19: A3263    ,  25.000
BTL  20: A3270    ,  25.000
BTL  21: A3277    ,  25.000
BTL  22: A3284    ,  25.000
BTL  23: A3291    ,  25.000
BTL  24: A3298    ,  25.000
BTL  25: A3305    ,  25.000
BTL  26: IS100    ,  25.000
BTL  27: A3312    ,  25.000
BTL  28: A3319    ,  25.000
BTL  29: A3326    ,  25.000
BTL  30: A3253    ,  25.000
BTL  31: A3260    ,  25.000
BTL  32: IS100    ,  25.000
BTL  33: CONT PL  ,  25.000
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Fig. 6. The format of a typical computer-based data system automatic liquid sampler sequence is illustrated listing the parameters used in the analysis of diazepam and nordiazepam. The parameters defined are: first vial in the sequence (1 ST BTL), total number of vials in the sequence (#BTLS), location of the calibration standard vial used for automatic recalibration of response factors (RCAL), number of wash cycles before each sample injection (WSHS), number of pump cycles before each sample injection (PMPS), injection volume (STOP), number of injections to be performed on each vial (#INJ), total cycle time (minutes) between sample injections.

been available for a number of years [19] and are generally reliable, reproducible and capable of sampling from microliter capacity vials. However, without development of a reliable electron capture linearizer [20, 21] automated GLC—ECD would be limited to accurate quantitation of only a narrow range of concentrations of injected samples due to the non-linear response characteristics of ECDs at high concentrations injected [22]. The electron capture linearizer has extended reliable quantitation of electron capturing compounds, including diazepam and nordiazepam, over a range of at least 10^4 in sample concentration injected and eliminates the need for sample dilution and re-injection, which would otherwise defeat the purpose of automatic injection systems. By interfacing the ^{63}Ni ECD, electron capture linearizer and ALS with a mini-computer based automatic data acquisition and analysis system, the means of fully automating the chromatographic and computation processes were accomplished. The method described was developed for the analysis of diazepam and nordiazepam levels following single or during chronic oral administration of therapeutic doses of diazepam for the purpose of demonstrating bioequivalency of formulations and defining pharmacokinetic profiles. The method was designed to specifically quantitate diazepam and nordiazepam in plasma. However, 3-hydroxydiazepam and oxazepam (Fig. 1), if present, are chromatographically resolved from diazepam and nordiazepam, having t_R values of 2.8 and 8.7 min, respectively, and can also be quantitated by this method.

In the development of a rapid assay procedure that required minimal sample handling prior to GLC analysis using an automatic liquid sampler, it was necessary to establish certain guidelines at the outset. A great deal of work has been established by other workers regarding simplified extraction techniques [11, 16, 18, 20, 23, 24], thus, one objective of this work was to further simplify the assay by extraction with a minimal volume of solvent and to eliminate the need to evaporate the solvent prior to GC analysis. Benzene was ideally suited as the extraction solvent since pesticide-grade solvent gave an extremely low ECD response when injected on to the OV-17 phase and more importantly extracted very few impurities and no interfering substances from plasma as shown in Fig. 3. Although a benzene extract of plasma had a yellowish coloration, a 10-μl aliquot injected into the gas chromatograph gave rise to very little ECD responsive material (Fig. 3). Equally important was the fact that diazepam and nordiazepam, although they are relatively polar basic compounds, partition into benzene from plasma to an extent exceeding 90% at physiological pH [11, 16—18, 25]. This property of diazepam and nordiazepam was used to great advantage in the development of a rapid procedure for determining the compounds in plasma by GLC—ECD.

In order to process all samples using a fixed volume of solvent, it was necessary to establish the linearity and recovery of the extraction of diazepam and nordiazepam from plasma into 1.0 ml of benzene under the conditions described. The recoveries were determined from a comparison of the peak areas of ten concentrations (in duplicate) of the standards (5—500 ng) added to plasma prior to extraction with the areas of the same ten concentrations of standards added to control plasma extract residue after extraction. This technique was used to overcome the pronounced tailing and loss of response of in-

jected pure nordiazepam standards in benzene under the GC conditions described. The occurrence of such peak tailing with polar compounds is common in spite of the use of highly inert, silane-treated column supports to minimize such effects. By use of this procedure, "apparent" recoveries of over 100% of added compound from plasma extracts was eliminated. This phenomenon was presumably related to the deactivation of adsorption sites on the column support [26], similar to the phenomenon reported using lecithin, a synthetic phospholipid, as a priming agent in the GLC—ECD analysis of steroids [27]. The average recoveries for diazepam and nordiazepam are summarized in Table I and are shown to be 98.2 ± 3.3% and 94.8 ± 1.5%, respectively. The extraction was found to be linear over this concentration range as shown in Fig. 7. The precision of the extraction procedure was determined based on the analysis of six separate samples of control plasma containing 100 ng diazepam and 100 ng nordiazepam per milliliter and found to be 0.98 ng/ml for diazepam and 2.07 ng/ml for nordiazepam.

Concentrations exceeding 800 ng/ml plasma are very seldom encountered in controlled clinical studies using normal subjects, even during chronic oral administration of up to 15 mg diazepam daily [11]. The steady state plasma levels of patients during chronic oral administration of 30 mg of diazepam daily, were reported to be between 1.0 and 2.0 μg/ml [28]. In such cases, an aliquot of 100 μl (or less) of plasma can be taken for assay, or the volume of benzene used to extract the unknown sample can be increased to 2.0 ml or greater. Whole blood and serum were also tried by the method described and found to work equally well. Slight differences in the chromatograms of control blood compared with plasma were seen, but there were no interferences in the region of diazepam and nordiazepam. In addition, a volume of plasma or blood of up to 1.0 ml can be used in this procedure with no modification. However, plasma is the preferred biological specimen since plasma concentrations of diazepam are nearly twice the corresponding blood concentrations (the blood/plasma concentration ratio is reported to be 0.58). [9].

TABLE I

RECOVERY OF DIAZEPAM AND NORDIAZEPAM FROM PLASMA

Amount of diazepam and nordiazepam added (ng/0.5 ml plasma)	Recovery (%)	
	Diazepam	Nordiazepam
5.0	92.6	N.D.*
10.0	92.7	97.8
30.0	97.5	94.3
50.0	99.5	96.0
100.0	101.2	94.8
200.0	100.8	93.8
300.0	99.9	93.8
400.0	101.6	96.1
450.0	96.6	92.9
500.0	99.6	93.9
Mean ± S.D.	98.2 ± 3.3	94.8 ± 1.5

*N.D. = Non-detectable.

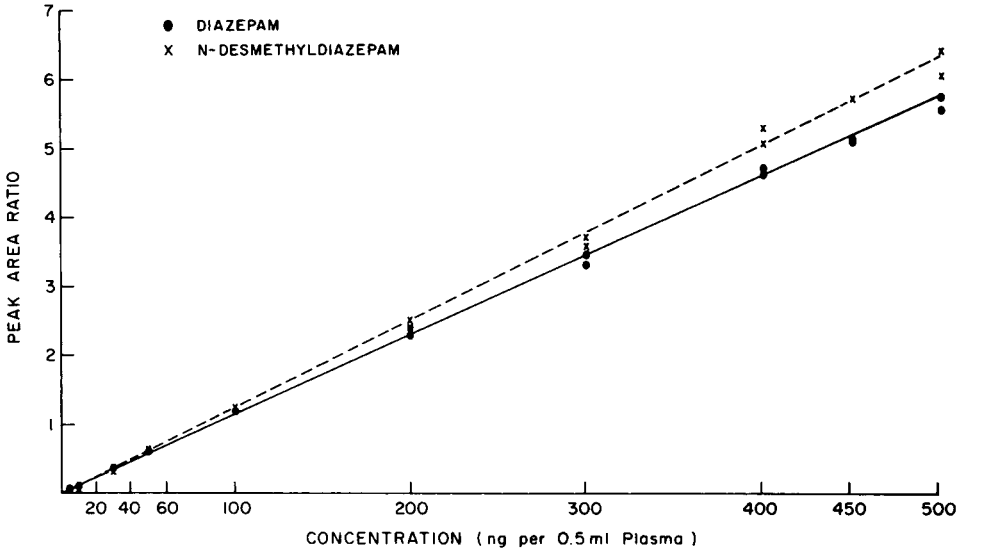

Fig. 7. Calibration curves for the GLC—ECD analysis of diazepam and nordiazepam (N-desmethyldiazepam) added per 0.5 ml of plasma and analyzed by the rapid method described.

A radioimmunoassay (RIA) was reported for diazepam in blood and plasma [29]. The advantages cited for the RIA technique was speed of analysis, ability to perform a large number of determinations in a single day by one person and sensitivity of the assay, requiring as little as 50 μl of plasma. The disadvantage of the RIA procedure was that it required two separate antibodies in order to determine both diazepam and nordiazepam in plasma. The GLC—ECD procedure described here has all of these advantages and measures both compounds simultaneously.

The extraction tubes used were designed (Fig. 2) to allow for efficient mixing of the plasma sample and organic solvent, and avoidance of emulsion formation. The tube is made so that 1.0 ml of benzene occupies approximately 1.5 cm in height, thus allowing for easy transfer of the benzene extract (following centrifugation) uncontaminated by interface material or aqueous phase.

During the development of this method, several problems became apparent. The glass vials used in the automatic injection system are capped with a Teflon-faced rubber septum aluminum seal, which is penetrated by the syringe needle for injection into the gas chromatograph. The vial can be used for only one injection. Repeated injections from the same vial showed a contaminant with a retention time almost identical to diazepam. The source of this contaminant was traced to the septa of the vials, which are susceptible to chemical attack by benzene once penetration of the Teflon liner has occurred. The identity of this interfering substance was not investigated, but several instances of similar interferences have been reported and traced to various plasticizers present in rubber products [30—32]. To avoid erroneous results due to an increased integrated area for diazepam caused by the contaminant, each sample vial is injected only once. It is extremely important to avoid contamination of the benzene solvent and extracts by contact with materials such as rubber and plastics. Only Teflon

and glass should come in contact with the benzene. Another septum material (black septum, Hewlett-Packard) was evaluated and found to be unsuitable due to interfering peaks in the area of 5.5—6.5 min.

Specificity of the assay

Diazepam is known to undergo the metabolic pathways shown in Fig. 1. The parent compound and nordiazepam are the major measurable plasma components following single and chronic oral administration of diazepam in man [10, 11, 14, 25, 33, 34]. However, the presence of the two hydroxylated metabolites (Fig. 1), 3-hydroxydiazepam and oxazepam (3-hydroxynordiazepam) in plasma after administration of diazepam has been reported [14, 34]. The reported plasma concentrations of 3-hydroxydiazepam and oxazepam were as high as 180 ng/ml and 290 ng/ml, respectively, during chronic administration of 20 mg diazepam three times daily for 10 weeks [14]. To determine if the presence of these hydroxylated metabolites caused any interference in the analysis of diazepam and nordiazepam, known amounts of authentic standards of these two compounds were added to control human plasma and assayed by the method described, with the exception of the internal standard. Both hydroxylated metabolites are chromatographically resolved from diazepam and nordiazepam, having t_R values of 2.8 min (oxazepam) and 8.7 min (3-hydroxydiazepam). The recovery of the two hydroxylated metabolites from plasma was determined to be approximately 100% (3-hydroxydiazepam) and 50% (oxazepam). The sensitivity limits would be only 50—100 ng of each compound per milliliter of plasma under conditions described.

The presence of 3-hydroxydiazepam (t_R 8.7 min) in an extract would interfere with the internal standard used in this method. However, in the routine analysis of two thousand plasma samples collected following single and during chronic oral administration of diazepam in normal, healthy male subjects, no evidence of either hydroxylated metabolite was detected. The use of griseofulvin as the internal standard (t_R 13.0 min), is suggested to avoid possible interference from 3-hydroxydiazepam.

Application of the method to biological specimens

The plasma levels of diazepam and nordiazepam were determined in six healthy male subjects in a study comparing the steady state pharmacokinetic profiles of diazepam and nordiazepam when the total daily dose of diazepam was divided into three oral administrations (5 mg three times daily at 7 a.m., 12 noon and 5 p.m.) for a period of 14 days followed by 15 mg single oral daily dosing for a period of 10 days [35]. During the three times daily dosing period, plasma levels were measured following all three doses on day 1 and following the first and third doses during the remainder of that dosing regimen. During the once a day dosing treatment, plasma levels were measured following each administration. The diazepam and nordiazepam plasma concentration-time curves for one subject are shown in Fig. 8.

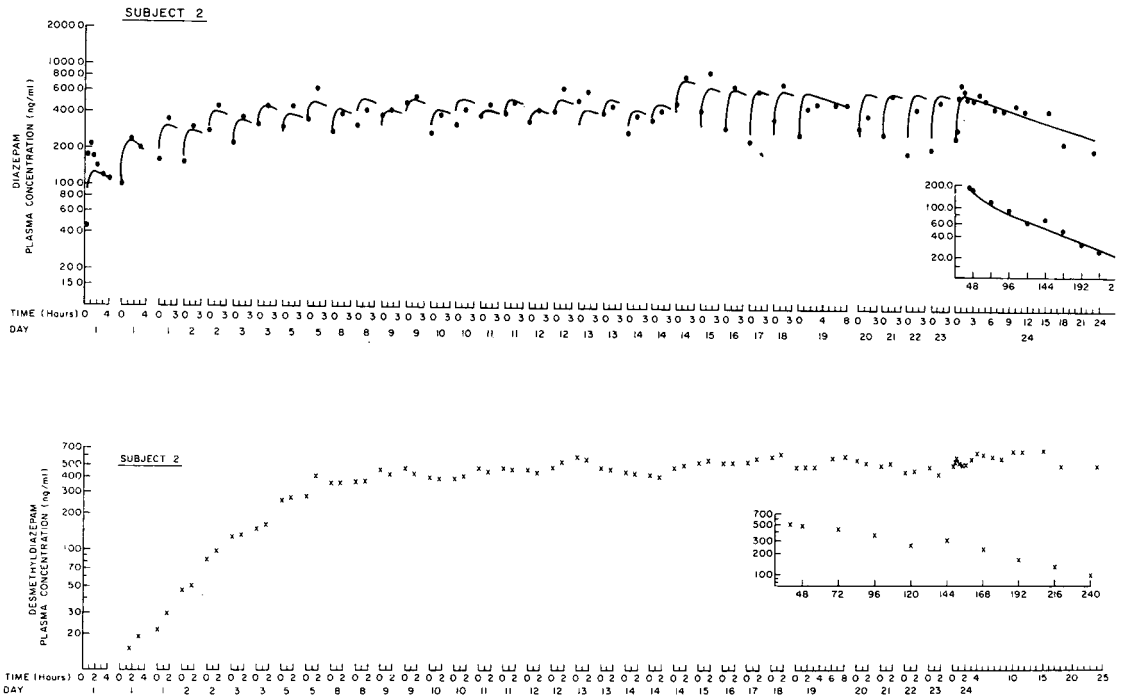

Fig. 8. Experimental plasma concentration—time profile curves of diazepam (upper) and nordiazepam (desmethyldiazepam, lower) following oral administration of Valium™ to one subject (No.2). On days 1 to 13, the dose was 5 mg at 0, 5, and 10 h (three times daily regimen). On day 14, at the time for the third dose, the regimen was crossed over to 15 mg every 24 h for an additional ten days. Abscissa represents time after each dose. Insert at bottom right portion of each curve illustrates the fall-off profile of diazepam and nordiazepam at times following the last administered dose. For diazepam, computer-generated theoretical curves assuming a linear two-compartment open model system with first order absorption, is also shown (see ref. 35 for complete details).

CONCLUSION

A rapid method for the determination of diazepam and nordiazepam in human plasma was developed. The method incorporates many desirable aspects of previously reported assays, but has significantly reduced the complexity of the sample preparatory steps by elimination of many sample manipulation steps, including multiple extractions, transfers, and solvent evaporation. The assay is essentially a one-step, one-tube extraction procedure utilizing methylnitrazepam as the internal standard, followed by GLC analysis through interfacing an electron capture linearizer, ^{63}Ni ECD, ALS and a mini-computer based data system to provide a nearly totally automated assay for diazepam and nordiazepam.

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Biomedical Applications

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ION-PAIR LIQUID CHROMATOGRAPHY OF STEADY-STATE PLASMA LEVELS OF CHLORIMIPRAMINE AND DEMETHYLCHLORIMIPRAMINE

BRITT MELLSTRÖM and GUNNEL TYBRING

Department of Clinical Pharmacology, Karolinska Institutet, Huddinge Hospital, S-141 86 Huddinge (Sweden)

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SUMMARY

A method for the determination of chlorimipramine and its metabolite demethylchlorimipramine in the plasma of depressed patients during treatment is described. The method involves extraction of the parent drug, its metabolite and the internal standard from plasma, back-extraction into an acidic aqueous phase and re-extraction into a small volume of organic phase. Separation and quantitation are carried out by ion-pair partition chromatography with UV detection. Accurate determination is possible down to levels of 30 and 60 nmole per liter of plasma for chlorimipramine and the metabolite, respectively, when 1 ml of plasma is used.

The coefficient of variation is 7.3% or less at different levels for chlorimipramine and demethylchlorimipramine. Plasma levels of the parent drug and the metabolite measured by this liquid chromatographic method and by a gas chromatographic procedure with electron-capture detection were in good agreement ($r = 0.98$).

The steady-state plasma level of the metabolite was always higher than that of the parent drug in the 34 depressed patients investigated. The mean ratio between the metabolite and the parent drug was 2.7 ± 1.1 (S.D.) Large inter-individual differences in the levels of the two compounds in patients receiving similar doses were found.

INTRODUCTION

The measurement of tricyclic antidepressant plasma levels is of significant clinical importance during therapy [1, 2]. When tertiary amines are given, the therapeutic effect will depend not only on the level of the parent drug but also on the level of the pharmacologically active demethylated metabolite formed. Tertiary tricyclics are more potent inhibitors of serotonin uptake than are their secondary amine metabolites, whereas the opposite is true for the uptake into noradrenergic neurons [3, 4]. Chlorimipramine is of special interest because it is the most potent serotonergic re-uptake blocker of the tricyclic antidepressants both in vitro [3, 4] and in vivo in man [5, 6].

Several methods have appeared for the simultaneous quantitation of amitriptyline or imipramine and their secondary amine metabolites. These methods include gas chromatography with flame-ionization [7, 8], alkali flame-ionization [9—11] or electron-capture [12] detection, mass fragmentography [13—15], thin-layer chromatography [16, 17] and liquid chromatography [18, 19].

Chlorimipramine and its demethylated metabolites have been determined by thin-layer chromatography [20] and mass fragmentography [21]. Recently, methods have been developed using ion-pair partition chromatography [22, 23]. This paper deals with the development and evaluation of a modified ion-pair liquid chromatographic method and its application to plasma samples from patients treated with Anafranil.

EXPERIMENTAL

Apparatus and materials

The chromatograph consisted of a pump (LDC 711-26 Solvent Delivering System), an injector (Model 905-19 Syringe Loading Sample Injector) and a UV detector (Model 153 Analytical UV Detector) operated at 254 nm (cell volume 8 μl, path length 10 mm). The injector and the detector were obtained from Altex Scientific, Berkeley, Calif., U.S.A. The separation column was a 75 × 3 mm I.D. stainless-steel column, packed with Partisil 10 (10 μm diameter, 400 m^2/g; Reeve Angel, Clifton, N.J., U.S.A.). For equilibration of the mobile phase, a 300 × 9 mm I.D. pre-column made of stainless steel was used. The pre-column was packed with Porasil C (37—75 μm diameter; Waters Assoc., Milford, Mass., U.S.A.). The whole system, except the pump, was thermostated at 23°.

The stationary phase consisted of 0.1 *M* hydrochloric acid containing 0.01 *M* tetrapropylammonium hydrogen sulphate, (Hässle, Mölndal, Sweden) and the mobile phase consisted of 13% 1-butanol in *n*-hexane. The phases were carefully equilibrated with each other at 23° by stirring overnight.

Preparation of columns

The separation column was packed by the balanced density slurry technique described by Majors [24]. The column was loaded with the stationary phase by precipitation from an acetone solution (75%, v/v) pumped through the column with saturated hexane [25]. The pre-column was dry-packed with the support previously equilibrated with 50% (w/v) of the stationary phase. The volume of the stationary phase (0.15 ml) in the separation column was determined by elution with methanol, followed by determination of the water content by Karl Fisher titration. The interstitial volume of the column (0.35 ml) was determined by injection of the non-retained solute benzene. With a flow-rate of 0.4—0.6 ml/min, the pressure never exceeded 100 p.s.i. for the entire system.

Chemicals

n-Hexane was of spectroscopic grade (Uvasol; E. Merck, Darmstadt,G.F.R.). All other solvents and chemicals were of analytical grade.

Stock solutions of chlorimipramine (CI) hydrochloride, demethylchlorimipramine (DMCI) hydrochloride (both from Ciba-Geigy, Basle, Switzerland) and trimipramine maleate (Leo, Helsingborg, Sweden) were prepared in 0.01 *M* hydrochloric acid.

Determination of partition coefficients and extraction constants

The partition experiments with the amines were performed with equal volumes of aqueous and organic phases (pre-equilibrated with each other [26]) in centrifuge tubes and with an equilibration time of 30 min at 23°. After centrifugation, the concentration of the amines was measured by photometry at the absorbance maximum in both the aqueous and the organic phase, either directly or after re-extraction into 0.1 *N* sulphuric acid.

Analytical method

To a plasma sample of 1 ml, 100 μl of the internal standard solution (containing 0.49 nmole of trimipramine maleate) and 0.2 ml of 2.5 *M* sodium hydroxide solution were added. The sample was extracted with 5 ml of diethyl ether for 40 min at 23° and centrifuged at 1000 *g* for 10 min.

About 4.5 ml of the organic phase were transferred into another tube containing 1 ml of 0.25 *N* sulphuric acid and the tube was shaken for 10 min and centrifuged. The ether layer was aspirated and 0.1 ml of hexane was added. The phases were mixed and the hexane phase was removed after centrifugation. (Instead of the extraction step with hexane the tube can be left uncapped overnight to remove trace amounts of ether.) The aqueous phase was transferred into a narrow 2-ml tube and was made alkaline with 0.2 ml of 2.5 *M* sodium hydroxide solution, then 75 μl of the mobile phase were added. The tube was slowly rotated for 10 min and centrifuged and an aliquot of the upper organic phase (40 μl) was injected into the column.

A standard graph was prepared by analysis according to the above procedure of 1-ml serum samples spiked with chlorimipramine and demethylchlorimipramine. Peak-height ratios with the internal standard were calculated.

Plasma samples

Blood samples were drawn from patients into heparinized plastic tubes immediately before the morning dose and centrifuged. The plasma was transferred into glass vials and stored at −20° until analysed.

The influence of storage time on the two compounds was checked when 295 plasma samples stored for 1—40 months had been analysed. No correlation was found between storage time and plasma level of either CI (r = 0.08) or DMCI (r = 0.01).

RESULTS AND DISCUSSION

The analytical procedure

The determination of chlorimipramine and demethylchlorimipramine in biological material is preferably effected by ion-pair partition chromatography as described in a preliminary report [12]. The ion pair consists of the protonated compound HA^+ and a chloride ion Cl^- which is partitioned between

the stationary and the mobile phase. The extraction of the cation HA^+ as an ion pair with the counter ion X^- can be quantitatively expressed by the distribution ratio, $D_{HAX} = E_{HAX} \cdot C_X$. The conditional extraction constant E^x is defined as $E^x_{HAX} = C_{HAX_{org}} \cdot (C_{HA} \cdot C_X)^{-1}$.

An increase in the detection sensitivity compared with the preliminary chromatographic system has been obtained by minor modifications. Changing the particle diameter of the support from 37—44 to 10 μm decreased the factor $N^{-1/2}$ and shortening of the column length decreased the ratio $V_m \cdot N^{-1/2}$, which resulted in an increase in the detectability according to the equation [27]

$$A = V_m \cdot (1 + k') \cdot N^{-1/2}$$

where

A = amount of sample giving a certain detector response;
V_m = volume of the mobile phase in the column;
k' = capacity factor;
N = number of theoretical plates.

Owing to tailing of the peaks and higher capacity factors obtained when using Partisil 10, an increase in the solvation of the ion pair in the mobile phase was necessary. The addition of methylene chloride to a mixture of hexane and alcohol results in a smaller separation factor between the amines than does an increase in the alcohol content. On the addition of butanol, the separation of a substance in front of DMCI present in the plasma of patients was achieved (Fig. 1). The identification of this peak as the didemethylated metabolite of chlorimipramine was supported by the following two experiments. Upon analysis of plasma samples by mass fragmentography after trifluoroacetylation, a small peak appears in front of DMCI when focusing the mass spectrometer on the ion of *m/e* 269 (cleavage in the α-position to the nitrogen atom). The relative retention in the liquid chromatographic system is about the same for the proposed didemethylated metabolite and demethylchlorimipramine as for demethylnortriptyline and nortriptyline.

The ratio between k'_f and k'_c was found to be slightly more than unity (Table I), indicating some influence of the support on the partition process in the column. Low values of the capacity factors are necessary in order to achieve high detection sensitivity [27] and a rapid separation (8 min) with a low flow-rate (0.45 ml/min), which improves the column efficiency [28].

The partition coefficients for chlorimipramine and demethylchlorimipramine as the free bases are very high with diethyl ether or hexane—butanol as the organic phase (Table II). The extraction of the amines from plasma still requires a long period, as mentioned previously [22] and later confirmed by Lagerström et al. [23].

Depressed patients are often treated with other drugs, e.g., benzodiazepines and barbiturates. Benzodiazepines were shown not to give any peaks in this system. Barbiturates and other acidic drugs cannot be extracted from an alkaline aqueous phase and therefore will not interfere.

Drugs that have chemical properties similar to those of chlorimipramine, e.g., other tricyclic antidepressants or phenothiazines, can be determined with this method. The separation of some common tricyclic antidepressants

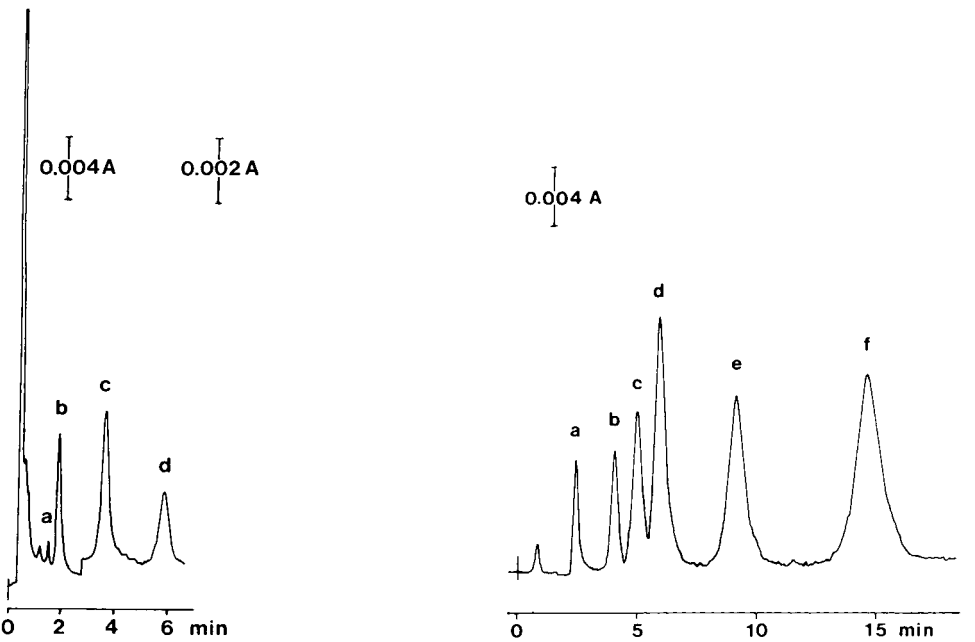

Fig.1. Chromatogram of a plasma sample from a patient receiving chlorimipramine hydrochloride. Peaks: (a) the didemethylated metabolite of chlorimipramine; (b) 456 nmole/l of demethylchlorimipramine; (c) 285 nmole/l of chlorimipramine; and (d) trimipramine (internal standard). The amplification was increased 2-fold after 2.5 min.

Fig.2. Separation of tricyclic antidepressant drugs and their demethylated metabolites. Reference compounds: (a) demethylchlorimipramine; (b) nortriptyline; (c) chlorimipramine; (d) desipramine; (e) amitriptyline; (f) imipramine. Chromatographic system as described under Experimental.

TABLE I

CAPACITY FACTORS AND EFFICIENCY OF THE CHROMATOGRAPHIC SYSTEM

Mobile phase: 1-butanol—*n*-hexane (13:87). Stationary phase: 0.1 *M* hydrochloric acid, containing 0.01 *M* tetrapropylammonium hydrogen sulphate. Support: Partisil 10. Column length: 75 mm. Flow-rate: 0.45 ml/min.

Amine	k'_c*	k'_f	k'_f/k'_c	H (mm)**
Demethylchlorimipramine	1.4	2.0	1.4	0.17
Chlorimipramine	3.0	5.0	1.7	0.09
Trimipramine	7.0	9.3	1.3	0.07

*$k'_c = V_s.(Vm \cdot E^x_{HAX} \cdot C_X)^{-1}$.
**H = theoretical plate height.

TABLE II

PARTITION AND EXTRACTION CONSTANTS

Amine	pK'_{HA}	log $k_{d(A)}$*		log E^x
		diethyl ether	1-butanol—*n*-hexane (13:87)	
Demethylchlorimipramine	10.2**	4.2**	4.3	0.55
Chlorimipramine	9.4**	4.9**	5.1	0.22
Trimipramine	9.5***	5.1	5.2	−0.15

*Calculated from log $k_{d(A)} \cdot K'_{HA}$ determined according to ref. 36.

$K_{d(A)} = \frac{[A]_{org}}{[A]_{aq}}$

K'_{HA} = dissociation constant of the acid.

$E^x_{HACl} = C_{HACl} \cdot (C_{HA} \cdot C_{Cl})^{-1}$ (conditional extraction constant of the amine as chloride ion pair).

**From ref. 23.

***From ref. 35.

is shown in Fig. 2. The reproducibility of the method was determined from measurements on 16 duplicate plasma samples. The coefficient of variation was 6.5% for CI at the lowest level (70—140 nmole/l) and 7.3% for DMCI (295—445 nmole/l). All other concentration levels gave better reproducibility.

The specificity of this liquid chromatographic method was checked by comparison with an electron-capture gas chromatographic method, based on the conversion of the two amines into trichloroethyl carbamates according to a method for amitriptyline and nortriptyline [12]. The two independent methods gave very similar plasma levels, as shown in Fig. 3.

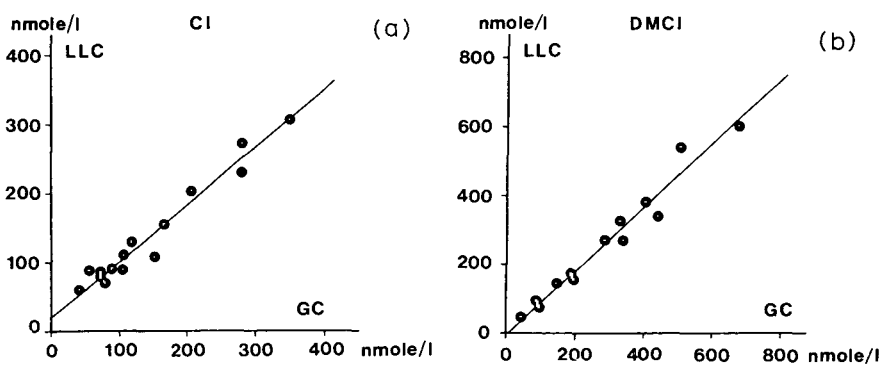

Fig.3. Comparison of results obtained by gas chromatography (abscissa) and liquid chromatography (ordinate) for chlorimipramine (CI) and demethylchlorimipramine (DMCI) in plasma. (a) CI (n= 15, r = 0.98). Line of best fit: y = 0.83 x + 16.4. (b) DMCI (n = 13, r = 0.98). Line of best fit: y = 0.94 x — 12.0.

Determination of plasma levels

When blood was collected in Vacutainer tubes, Cotham and Shand [29] found lower levels of propranolol in plasma compared with blood collected in an all-glass system. This result was explained by the presence of a substance in the rubber stopper that reduced plasma protein binding of the drug. They suggested that this effect might be seen with other highly protein-bound basic drugs that distribute significantly into red blood cells in proportion to the free fraction of the drug. Significantly lower plasma levels of chlorimipramine and demethylchlorimipramine (p = 0.032 and 0.021, respectively) were found when blood from five patients was drawn into Vacutainer tubes compared with blood collected in the usual plastic tubes from the other arm at the same time. The decrease in the mean ± S.D. was 28 ± 13% and 17 ± 10% for the two compounds, respectively. This drug-displacing substance has recently been proposed to be trisbutoxyethyl phosphate [30].

The plasma concentration graph for a patient treated with chlorimipramine (Anafranil, three 50-mg doses per day) for a period of 4 weeks is shown in Fig. 4. The steady-state level of CI was reached within 1 week, but the level of the metabolite had a tendency to increase during the 4-week period. This effect was also seen in some other patients. The plasma concentration of the metabolite was higher than that of the parent drug in all of the patients studied. The concentrations of CI and DMCI during the fourth week of treatment in 34 patients given a dose of 150 mg per day are shown in Fig. 5. The concentration of CI was 248 ± 91 (S.D.) nmole/l with a range of 63—900 nmole/l, which is a 14-fold variation, and the level of DMCI was 561 ± 288 (S.D.) nmole/l in the range 116—1178 nmole/l, a 10-fold variation. A large inter-

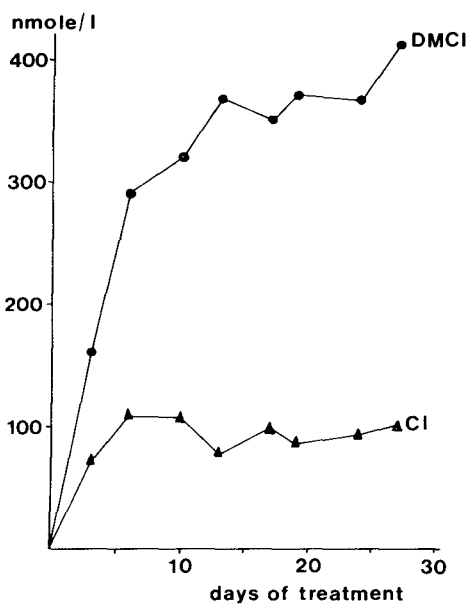

Fig.4. Plasma levels of chlorimipramine (CI) and demethylchlorimipramine (DMCI) during 4 weeks in a patient receiving 50 mg of CI·HCl three times a day.

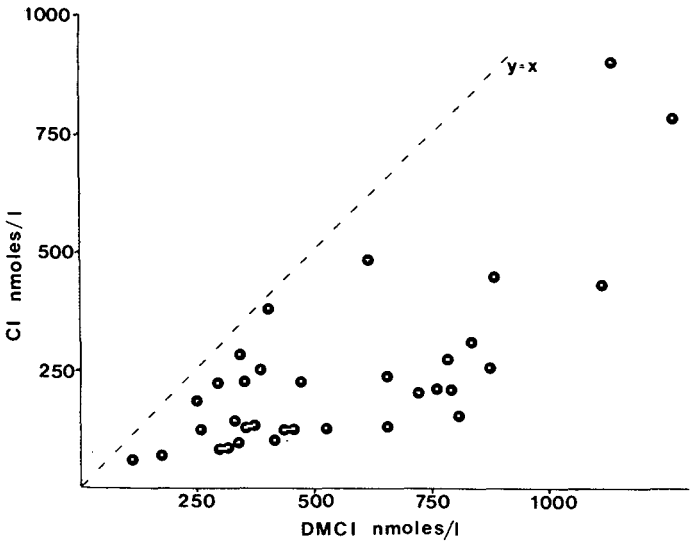

Fig.5. Fourth-week plasma levels of chlorimipramine (CI) and demethylchlorimipramine (DMCI) in 34 patients receiving 50 mg CI·HCl three times a day.

individual variation in plasma levels has been shown for other tricyclic antidepressant drugs: nortriptyline [31], amitriptyline [32], desipramine [33] and imipramine [34]. However, the ratio between metabolite and parent drug is always greater than unity (2.7 ± 1.1 (S.D.), range 1.05—5.24) for chlorimipramine, which differs from amitriptyline (1.0 ± 0.3 (S.D.), range 0.58—1.65) [32] and imipramine (3.4 ± 4.3 (S.D.), range 0.25—18.91) [34].

The relationship between the levels of CI and DMCI presented in this paper and the clinical effect of chlorimipramine in depressed patients is now under evaluation.

ACKNOWLEDGEMENTS

We are grateful to Dr. Marie Åsberg, the Psychiatric Clinic, Karolinska Hospital, for providing samples from patients for the study and to Miss Barbro Näslund, Department of Analytical Pharmaceutical Chemistry, University of Uppsala, for technical assistance with the gas chromatographic analysis. This research was supported by grants from the Swedish Medical Research Council (04X-03902), from the Bank of Sweden Tercentenary Fund (74/110) and by funds from the Karolinska Institute.

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Biomedical Applications

CHROMBIO. 066

FLUORIMETRISCHE BESTIMMUNG VON PROPRANOLOL UND SEINES METABOLITEN N-DESISOPROPYLPROPRANOLOL IN PLASMA UND URIN DURCH DIREKTE AUSWERTUNG VON DÜNNSCHICHTCHROMATOGRAMMEN

MONIKA SCHÄFER*, HEINRICH E. GEISSLER und ERNST MUTSCHLER**

Pharmakologisches Institut für Naturwissenschaftler der Johann-Wolfgang-Goethe-Universität, Robert-Mayer-Strasse 7—9, 6000 Frankfurt/Main (B.R.D.)

(Eingegangen am 7. Dezember 1976; geänderte Fassung eingegangen am 17. März 1977)

SUMMARY

Fluorometric determination of propranolol and its metabolite N-desisopropylpropranolol in plasma and urine by direct measurement of thin-layer chromatographic plates

The quantitative analysis of propranolol and its metabolite N-desisopropylpropranolol in plasma and urine is described. The drugs are extracted into 2-pentanol—heptane, and the solvent is concentrated. The whole residue is chromatographed on silica gel plates. The compounds are determined directly on the thin-layer plates without derivatization. The recovery of propranolol from plasma is 70%, with a standard deviation of ± 4%.

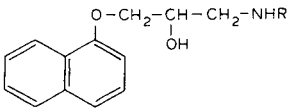
EINLEITUNG

Die quantitative Analyse von Propranolol (Dociton®, Inderal®) in Plasma ist nach verschiedenen Methoden möglich. Eine gaschromatographische Methode wurde von Di Salle et al. [1] entwickelt und von Walle modifiziert [2]. Propranolol wird dabei nach seiner Isolierung mit Heptafluorobuttersäureanhydrid [1], bzw. Trifluoressigsäureanhydrid [2] umgesetzt. Diese zuletzt genannte Methode ermöglicht auch eine gleichzeitige Bestimmung des Metaboliten N-Desisopropylpropranolol; mit Hilfe einer Variante dieser Methode kann der Hauptmetabolit 4-Hydroxy propranolol gemessen werden [3]. Die Verwendung eines Elektroneneinfangdetektors ermöglicht den Nachweis kleiner Substanzmengen. Der Nachteil der Methode liegt in dem grossen Zeitbedarf.

Eine fluorimetrische Methode wurde von Black et al. [4] erarbeitet und von Shand et al. [5] in grossem Umfang zur Bestimmung von Propranolol in Plasma

* Teilergebnisse der Dissertation M. Schäfer, in Vorbereitung.
** An den Anforderungen von Sonderdrucken gerichtet werden sollen.

herangezogen. Dabei wird die Eigenfluoreszenz des Naphthalinrings für die quantitative Analyse ausgenutzt.



R= $-C_3H_7$, Propranolol
R= –H, Desisopropylpropranolol

Propranolol wird aus alkalischer Lösung mit einem Alkohol—Heptan-Gemisch extrahiert und die Fluoreszenz nach einer Rückextraktion in verdünnte Salzsäure in der Küvette bestimmt. Der Nachteil dieser sehr einfachen und schnell durchführbaren Methode liegt in hohen Blindwerten und einer starken Störanfälligkeit [6]. Diese Methode ist ausserdem nicht sehr empfindlich. Vier Stunden nach Einnahme von 40 mg kann Propranolol nicht mehr mit Sicherheit im Plasma nachgewiesen werden [7].

Eine gewisse Verbesserung der Methode wurde durch Ambler erreicht [8], der durch Extraktion mit Pentylacetat die Wiederfindungsrate erhöhte und die Fluoreszenz in Citronensäure—Glycol bestimmte. Er berücksichtigt damit die pH-Abhängigkeit der Fluoreszenz (Fluoreszenzmaximum bei pH 4—5 [9]). Allerdings beobachten andere Autoren bei der Messung der Fluoreszenz in Citronensäure einen starken Anstieg der Blindwerte [5].

In jüngster Zeit wurde eine Propranololbestimmung durch Radioimmunoassay publiziert, die eine selektive Bestimmung der beiden Enantiomeren ermöglicht [10].

Uns gelang es nun, die fluorimetrische Methode zu verbessern, d.h. die Empfindlichkeit zu erhöhen sowie die Störanfälligkeit zu vermindern. Dies ist durch dünnschichtchromatographische Auftrennung des Plasmaextraktes möglich. Die quantitative Bestimmung erfolgt durch direkte Auswertung der Platte nach dem Besprühen mit einer 10%-igen wässrigen Citronensäurelösung anhand einer Eichgeraden. Propranolol und Desisopropylpropranolol zeigen eine starke Fluoreszenzemission bei 340 nm. Das Exzitationsmaximum liegt bei 290 nm (Fig. 1). Beide Stoffe sind nach dem Ausschütteln mit einem Gemisch von 1.5% 2-Pentanol in Heptan nebeneinander auf derselben Platte bestimmbar, da sich ihre R_F-Werte in dem gewählten Fliessmittel ausreichend unterscheiden (Fig. 2). Auf die Bestimmung von 4-Hydroxypropranolol wurde wegen der ausserordentlich grossen Oxidationsempfindlichkeit dieser Substanz verzichtet.

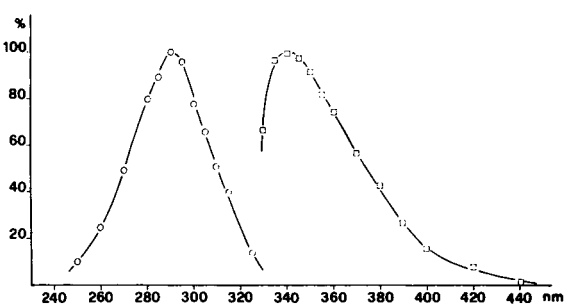


Fig. 1. Exzitations- (○) und Emissionsspektrum (□) von Propranolol und Desisopropylpropranolol.

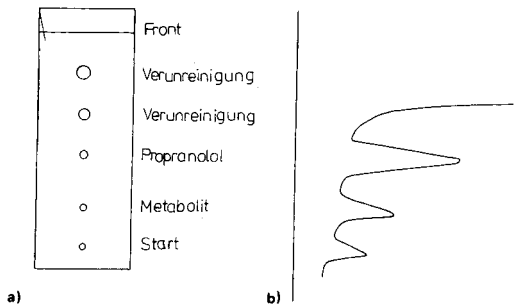


Fig. 2. Dünnschichtchromatogramm (a) und Fluoreszenzgrad-Ortskurve (b) von Propranolol und Desisopropylpropranolol aus Urin (50 ng/4 ml).

EXPERIMENTELLES

Geräte

Chromatogrammspektralphotometer KM3 (Carl Zeiss) mit Kompensationsschreiber Servogor Sb.

Chemikalien

Propranolol sowie sein Metabolit Desisopropylpropranolol wurden freundlicherweise von ICI (Plankstadt, B.R.D.), zur Verfügung gestellt. Die Chemikalien sowie die Dünnschicht-Fertigplatten (Kieselgel 60, 20 × 20 cm, ohne Fluoreszenzindikator) wurden von E. Merck (Darmstadt, B.R.D.) bezogen. Für die Eichgerade benötigten wir Propranolol-Base, die wir aus dem Hydrochlorid herstellten. Als Vergleichslösung I für die Chromatographie wurden 10 mg Base in 100 ml 2-Pentanol—Heptan (1.5 : 98.5) gelöst. Diese Lösung wurde noch einmal 1:10 mit 2-Pentanol—Heptan (1.5 : 98.5) verdünnt (Vergleichslösung II).

Extraktion

Aus Plasma. Zu 1 ml Plasma gibt man in einem verschliessbaren Reagenzglas 1 ml 1 *N* NaOH und schüttelt 30 min mit 7 ml 2-Pentanol—Heptan (1.5:98.5). Nach 5 min Zentrifugieren trennt man die organische Phase mit einer Pasteur-Pipette ab und gibt sie in ein 10-ml-Becherglas. Die Wände des Reagenzglases werden mit 0.3 ml 2-Pentanol—Heptan abgespült und die Waschlösung mit dem ersten Teil vereinigt. Die Probe wird unter Stickstoffbegasung und bei geringer Wärmezufuhr (bis zu 60°) zur Trockne eingedampft.

Aus Urin. Zu 4 ml Urin gibt man in einem verschliessbaren Reagenzglas 0.1 ml 10 *N* NaOH und schüttelt 30 min mit 10 ml 2-Pentanol—Heptan (1.5: 98,5). Sobald die beiden Phasen sich getrennt haben, nimmt man 9 ml der organischen Phase ab und dampft sie in einem 10-ml-Becherglas unter Stickstoffbegasung zur Trockne ein.

Chromatographie

Auftragen. Der gesamte Rückstand des Plasma- oder Urinextraktes wird auf eine Dünnschichtplatte aufgetragen, indem man ihn unter intensivem Schütteln (REAX I, Heidolph) in zwei Tropfen 2-Pentanol—Heptan löst und mit einem

Fettschmelzpunktröhrchen die gesamte Flüssigkeit punktförmig aufträgt. Dieser Vorgang wird noch zweimal wiederholt. Auf einer Platte können acht Extrakte im Abstand von 1.2 cm aufgetragen werden. Ausserdem trägt man für die Eichgerade jeweils 1, 2 and 5 μl der Vergleichslösung I sowie 5 μl der Vergleichslösung II (entsprechend 50, 100, 200 und 500 ng Propranolol-Base) mit Konstriktionspipetten auf.

Entwickeln. Die Platte wird in einer Desaga-Trogkammer entwickelt. Fliessmittel: Essigsäureäthylester—Benzol—Methanol (20:20:10) in Ammoniak-Atmosphäre. Die Entwicklung erfolgt bei Kammersättigung, die Laufstrecke beträgt 10 cm. Der R_F-Wert von Propranolol ist 0.42; der von Desisopropylpropranolol 0.17. Begleitfluoreszenzen aus Plasma oder Urin haben R_F-Werte > 0.6.

Messung

Die Messung erfolgt unmittelbar nach dem Entwickeln der Platte, da Propranolol und sein Metabolit oxidationsempfindlich sind. Die Platte wird mit einer Lösung von 10 g Citronensäure in 90 g Wasser—Äthylenglycol(1:1) besprüht. Die Fluoreszenz wird auf der feuchten Platte bestimmt, da das Trocknen der Platte mit einer starken Abnahme der Fluoreszenz verbunden ist. Die Verdunstung des Wassers wird dabei durch den Zusatz von Äthylenglycol verlangsamt.

Die Auswertung kann in verschiedener Weise erfolgen. Methode Probe—Monochromator (Pr—M): Anregung mit der Hg-Linie 313 nm der Quecksilber-Mitteldrucklampe ST 41 mit Quarzkondensor; Emissionsmessung beim Emissionsmaximum von 340 nm; Spaltbild: 0.5 × 8 mm; Hochspannung 2; Verstärkung 10—100 fach. Methode Monochromator—Probe (M—Pr): Anregung mit der Deuteriumlampe beim Exzitationsmaximum von 290 nm; Messung der Emissionsstrahlung nach dem Monochromatfilter M 365; Spaltbild: 10 × 8 mm; Hochspannung (~ 550V); Verstärkung etwa 5—50 fach.

In beiden Fällen wird die Platte auf einem Kreuztisch parallel zur Entwicklungsrichtung des Chromatogramms bewegt. Die Kreuztisch- und Schreibergeschwindigkeit beträgt dabei 120 mm/min. Man misst die Fluoreszenz des Propranolol-Standards (50, 100, 200, 500 ng) und die Fluoreszenz des aus Urin oder Plasma extrahierten Propranolols sowie die Fluoreszenz des Desisopropylpropranolols.

Auswertung

Die Flächen unter den Peaks, die in Integratoreinheiten erfasst werden, sind der Propranololmenge der Plasmaprobe direkt proportional. Die Auswertung erfolgt anhand einer Eichgeraden, die für jede Platte erstellt wird. Die Eichgerade verläuft linear im Bereich von 2 ng (Methode M—Pr) bzw. 4 ng (Methode Pr—M) bis ca. 1000 ng Propranolol-Base pro Fleck und geht durch den Ursprung des Koordinatensytems. Der Gehalt des Plasmas sowohl an Propranolol als auch an Desisopropylpropranolol wird auf die Propranolol-Base bezogen. Bei Einführung eines Korrekturfaktors von 1.11 (Mittelwert aus 4 Bestimmungen) entsprechen sich die Flächenintegrale von Propranolol-Base und Desisopropylpropranolol-Base bei gleichen Konzentrationen (in ng):

$A_P = 1.11\ A_D$

mit A_P = Fläche des Propranolol-Peaks, A_D = Fläche des Desisopropylpropranolol-Peaks.

DISKUSSION

Die Nachweisgrenze für Propranolol-Base liegt für Methode Pr—M bei 4 ng pro Fleck. Der 4-ng-Peak ist etwa dreimal so hoch wie das Grundrauschen, das sich aus dem elektronischen Geräterauschen sowie aus den Störungen durch die ungleichmässige Beschaffenheit des Plattenuntergrundes zusammensetzt. Bei Auswertung nach Methode M—Pr sind sogar noch 2 ng Propranolol-Base pro Fleck messbar, die Nachweisgrenze liegt bei 1 ng pro Fleck; das Grundrauschen beträgt für 2 ng Propranolol-Base 1/10 der Peakhöhe (Fig. 3).

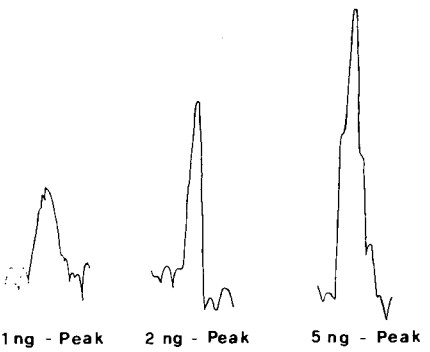


Fig. 3. Nachweis von Propranolol-Base bei verschiedenen Mengen pro Fleck.

Die Empfindlichkeit der fluorimetrischen Methode bei Auswertung mit dem Chromatogrammspektralphotometer ist vor allem dadurch bedingt, dass es gelingt, unter chromatographischer Abtrennung der mit dem Arzneistoff extrahierten Plasmabestandteile den gesamten Propranololgehalt von 1 ml Plasma auf einer sehr kleinen Fläche anzureichern. Auch kann bei dem pH-Optimum der Fluoreszenz gemessen werden, ohne dass es zu einer Erhöhung der Blindwerte kommt. Durch Besprühen mit Citronensäurelösung steigt die Empfindlichkeit der Methode etwa um das Achtfache. Die Fluoreszenzmessung muss unmittelbar nach dem Besprühen der Platte durchgeführt werden, da das Trocknen der Platte mit einem erheblichen Fluoreszenzverlust verbunden ist.

Die Extraktion mit 2-Pentanol—Heptan (1.5:98.5) liefert im Gegensatz zur Extraktion mit Diäthyläther oder Pentylacetat sehr reine Extrakte. Ausserdem hat Heptan einen niedrigeren Siedepunkt als Pentylacetat. Die thermische Belastung des labilen Arzneistoffes beim Eindampfen der Extrakte ist daher geringer. Um die Grösse der Verluste, die beim Eindampfen entstehen, abschätzen zu können, dampften wir eine Lösung von 500 ng Propranolol-Base in 7 ml Heptan—Alkohol ein. Durch Einengen und Auftragen auf die Dünnschichtplatte entstand ein Verlust von 14.5%.

Ein Vorteil der von uns entwickelten Methode liegt in der geringen Störanfälligkeit; eine besondere Behandlung der Glasgeräte (Einlegen in verdünnte Salzsäure), wie sie sowohl bei der gaschromatographischen als auch bei der fluorimetrischen Methode mit Messung in der Küvette unerlässlich ist, erfordert diese Methode nicht. Auch eine Störung durch Nahrungsaufnahme wurde nicht beobachtet.

Die Wiederfindungsrate des zu 1 ml Plasma zugesetzten Propranolols betrug 70% mit einer Standardabweichung von ± 4%. (Zur Überprüfung wurden zwölf Proben zu je 1 ml Plasma mit jeweils 50—500 ng Propranolol-Hydrochlorid versetzt und nach dem beschriebenen Verfahren analysiert.) Die Wiederfindungsrate des Metaboliten — seine Bestimmung erfolgt in einem Arbeitsgang mit der Propranololbestimmung — ist niedriger. Sie liegt bei 45% mit einer Standardabweichung von ± 4% (n = 8, 50—500 ng nach dem oben beschriebenen Verfahren). Fig. 4 enthält die Eichkurve und die Messwerte der wiedergefundenen Mengen von Propranolol und Desisopropylpropranolol. Da die Messwerte für die wiedergefundenen Mengen auf einer Geraden liegen, folgt, dass die Wiederfindungsrate von der zu bestimmenden Konzentration unabhängig ist. Damit sind nach diesem Verfahren therapeutische Blutspiegel von Propranolol messbar (maximal 250 ng/ml nach oraler Gabe von 80 mg an menschliche Versuchspersonen [5]). Über die zu erwartenden Blutspiegel des Metaboliten N-Desisopropylpropranolol sind uns keine Einzelheiten bekannt. Die Wiederfindungsrate für Propranolol aus Urin liegt bei 77 ± 3% (n = 8), diejenige seines Metaboliten beträgt 57.5 ± 4%. Da unverändertes Propranolol nur in Spuren ausgeschieden wird, sind 4 ml Urin zur Analyse notwendig. Über die Menge des im menschlichen Urin ausgeschiedenen N-Desisopropylpropranolols ist uns nichts bekannt. Eine Spaltung des konjugierten Propranolols durch Hydrolyse mit Sulfatase, Beta-Glucuronidase oder verdünnter Salzsäure wurde nicht versucht, da sie nach den Untersuchungen von Hayes und Cooper [11] und Paterson et al. [12] wenig aussichtsreich erschien.

Zusammenfassend ergibt sich, dass die von uns entwickelte Methode die Vorteile der fluorimetrischen Methode (geringer Arbeitsaufwand — eine Arbeits-

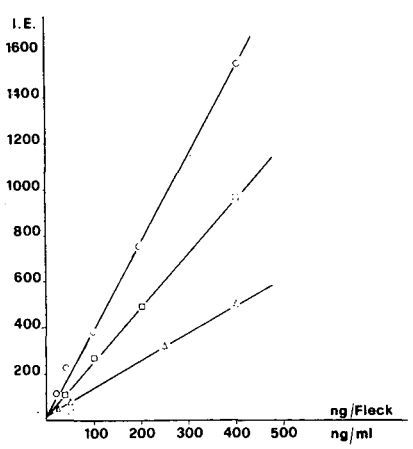


Fig. 4. Bestimmung von Propranolol und Desisopropylpropranolol aus Plasma. ○, Eichgerade; □, Propranolol; und △, Desisopropylpropranolol. I.E. = Integratoreinheiten.

kraft kann ca. 40 Proben am Tag bestimmen) mit der hohen Empfindlichkeit der gaschromatographischen Methode (geringere Belastung des Patienten auch bei häufiger Blutentnahme) verbindet, wobei zudem die Genauigkeit dieser Methode wesentlich grösser ist.

ZUSAMMENFASSUNG

Es wird die quantitative Analyse von Propranolol und seinem Metaboliten N-Desisopropylpropranolol aus Plasma und Urin beschrieben. Beide Stoffe werden mit einem 2-Pentanol—Heptan-Gemisch extrahiert. Der zur Trockne eingedampfte Extrakt wird quantitativ auf Kieselgel-Platten aufgetragen und chromatographiert. Die Messung erfolgt auf der Dünnschichtplatte ohne Derivatisierung. Die Wiederfindungsrate von Propranolol aus Plasma liegt bei 70 ± 4%.

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FLUORIMETRISCHE BESTIMMUNG VON HYDROCHLOROTHIAZID IN KÖRPERFLÜSSIGKEITEN DURCH DIREKTE AUSWERTUNG VON DÜNNSCHICHTCHROMATOGRAMMEN

MONIKA SCHAFER*, HEINRICH E. GEISSLER und ERNST MUTSCHLER**

Pharmakologisches Institut für Naturwissenschaftler der Johann-Wolfgang-Goethe-Universität, Robert-Mayer-Strasse 7—9, 6000 Frankfurt/Main (B.R.D.)

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SUMMARY

Fluorometric determination of hydrochlorothiazide in body fluids by direct measurement of thin-layer chromatographic plates

Two fluorometric methods for analysis of hydrochlorothiazide (HCT) are described utilizing direct measurement of thin-layer plates. The first method employs a modification of the Bratton—Marshall reaction and is therefore applicable to all aromatic primary amines. Following diazotation and azocoupling of the HCT hydrolysis product, a fluorescent group is added to the compound. For this purpose N-(1-naphthyl)ethylenediamine is first coupled with 4-chloro-7-nitrobenzo-2,1,3-oxadiazole. In the second method, the intrinsic fluorescence of underivatized HCT, following its extraction from plasma, urine or saliva, is used. It is shown that the sensitivity of this method is sufficient for estimating the kinetics following oral administration of 25 mg HCT.

EINLEITUNG

Die Bestimmung von (nicht radioaktiv markiertem) Hydrochlorothiazid (HCT) in Plasma bereitet ausserordentliche Schwierigkeiten. Bisher ist keine Methode bekannt, die empfindlich genug ist, Blutspiegel, wie sie nach Applikation therapeutischer Dosen auftreten, über einen ausreichend langen Zeitraum zu messen.

In den Jahren 1960 und 1961 wurden zwei Methoden veröffentlicht, welche die Verteilung von radioaktiv markiertem HCT im Organismus untersuchten. Dabei wurde [^{3}H] HCT Ratten [1] bzw. [^{14}C] HCT menschlichen Versuchspersonen [2] gegeben. Bei einer oralen Gabe von 65 mg [^{14}C]HCT wurden bei gesunden Versuchspersonen maximale Serumspiegel von durchschnittlich 800 ng HCT/ml Serum gemessen. Der maximale Serumspiegel war nach 4 h erreicht.

* Teilergebnisse der Dissertation M. Schäfer, in Vorbereitung.

** An den Anforderungen von Sonderdrucken gerichtet werden sollen.

Eine chemische Bestimmungsmethode wurde von Baer et al. [3] für Chlorothiazid entwickelt und von Yü und Sung [4] für die Hydrochlorothiazid-Bestimmung modifiziert. Sie beruht auf der alkalischen Hydrolyse des Moleküls zu 5-Chloro-2,4-disulfamidoanilin und seiner Bestimmung nach der Bratton—Marshall-Reaktion. Nur im Tierversuch können jedoch so hohe Dosen gegeben werden, dass die Empfindlichkeit für die Bestimmung von Serumspiegeln ausreicht. Bei Humanversuchen wurde deshalb Urin fraktioniert gesammelt und die Kinetik anhand der Urinausscheidung bestimmt [5].

In letzter Zeit wurden weitere Methoden veröffentlicht, welche durch die Fortschritte in der Instrumentalanalyse ermöglicht wurden. Bei zwei Methoden wird zur Trennung und quantitativen Bestimmung die Gaschromatographie eingesetzt [6, 7]. Das Tetramethylderivat wird dabei mit einem Elektroneneinfangdetektor erfasst. Da jedoch HCT nur ein Halogenatom enthält, ist die Empfindlichkeit nicht höher als beim Arbeiten mit einem Flammenionisationsdetektor. Die Nachweisgrenze liegt daher auch mit 50 ng/ml Plasma noch relativ hoch. Der Einsatz der Hochdruckflüssigkeitschromatographie zur Reinigung und die anschliessende Bestimmung mit einem UV-Detektor [8] ist nur arbeitsmässig einfacher. Bei Präparaten, die 25 mg HCT oder weniger pro Dosis enthalten*, reicht aber die Empfindlichkeit ebenfalls nicht aus, um die Plasmaspiegel nach einer einmaligen Gabe der Einzeldosis zu bestimmen.

Wir stellten uns daher die Aufgabe, eine Methode zu entwickeln, die es erlaubt, die Kinetik von HCT nach einer einmaligen Gabe von 25 mg zu bestimmen. Eine ausreichende Empfindlichkeit erwarteten wir von der Anwendung fluorimetrischer Methoden; für Hydroflumethiazid wurde bereits ein empfindliches Verfahren beschrieben [9]. Zwei fluorimetrische Verfahren zur Bestimmung von HCT in Plasma sollen im folgenden beschrieben werden.

Methode A beruht auf der oben genannten Bratton—Marshall-Reaktion. Über die Diazotierung und Azokupplung des alkalisch hydrolysierten Hydrochlorothiazids führen wir eine fluoreszierende Gruppe ein, indem wir die Kupplungskomponente in einer vorgelagerten Reaktion mit 4-Chloro-7-nitrobenzo-2,1,3-

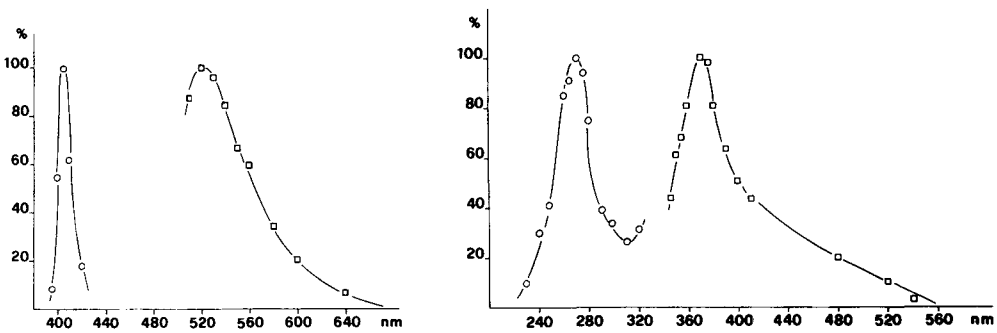


Fig. 1. Exzitations- (○) und Emissionsspektrum (□) HCT-Umsetzungsprodukt nach Methode A.

Fig. 2. Exzitations- (○) und Emissionsspektrum (□) von HCT.

* Adelphan—Esidrix® (Ciba), Dytide® H (Röhm-Pharma), Esimil® (Geigy), Moduretik® (Sharp & Dohme), Resaltex® (Röhm-Pharma).

oxadiazol (NBD-Cl) umsetzen. Ein Teil des Reaktionsgemisches wird durch Dünnschichtchromatographie gereinigt und die Fluoreszenz des Azofarbstoffs mit einem Chromatogrammspektralphotometer bei seinem Emissionsmaximum von 520 nm bestimmt (Fig. 1).

Nach Methode B wird die native Fluoreszenz des Hydrochlorothiazids nach dem Besprühen mit Triäthanolamin-Lösung ausgewertet. (Emissionsmaximum 370 nm; Fig. 2). Diese Methode erfordert einen höheren Reinheitsgrad der Plasmaextrakte. Da aber der gesamte Extrakt aus 0.5 ml Plasma auf die Platte aufgetragen werden kann, der Arbeitsaufwand für die Umsetzung entfällt und zudem die Streuung der Ergebnisse geringer ist, zogen wir diese Methode zur Bestimmung der Plasmaspiegel vor.

EXPERIMENTELLES

Geräte

Chromatogrammspektralphotometer KM 3 (Carl Zeiss) mit Kompensationsschreiber Servogor Sb.

Chemikalien

Alle verwendeten Chemikalien sowie die Dünnschichtplatten (Keiselgel 60, ohne Fluoreszenzindikator, 20 × 20 cm) wurden von E. Merck (Darmstadt, B.R.D.) bezogen. Hydrochlorothiazid stellte freundlicherweise Röhm-Pharma (Darmstadt, B.R.D.) zur Verfügung.

Das für die Analysenmethode A benötigte Hydrolyseprodukt stellten wir selbst im präparativen Massstab durch achtstündiges Kochen von HCT in 5 *N* Natronlauge her. Die Lösung wurde nach beendigter Reaktion angesäuert und mit Essigsäureäthylester extrahiert. Durch Einengen und Auskristallisieren erhielten wir das reine Hydrolyseprodukt (Schmelzpunkt 251°; Literatur: 251—252° [10]). Seine Identität wurde durch Infrarotspektroskopie und Elementaranalyse gesichert.

Das Umsetzungsprodukt von NBD-Cl mit N-(1-Naphthyl)äthylendiamin, ebenfalls für Methode A, stellten wir nach Vorschrift 2 von Reisch et al. [11] her: 1 mMol des Aminsalzes wurde in 5 ml destilliertem Wasser gelöst und die Base mit 0.5 ml 2 *N* NaOH in Freiheit gesetzt. Nach Zugabe von 1 mMol NBD-Cl in 20 ml Äthylacetat wurde das Zweiphasengemisch 6 h geschüttelt. Die organische Phase wurde abgetrennt und über Nacht bei 4° aufbewahrt. Die Kristalle wurden abfiltriert und aus Aceton—Wasser umkristallisiert. (Schmelzpunkt: 198° unter Zersetzung; Strukturbeweis: IR, Massenspektrum, Elementaranalyse).

Methode A

Erstellung der Eichkurve

Derivatisierung. Hydrochlorothiazid (0.5—20 μg) in Aceton wird zur Trockne eingeengt. Der Rückstand wird in 1 ml 0.25% $NaNO_2$ (gelöst in 0.2 *N* HCl) aufgenommen. Den Nitritüberschuss zerstört man durch Zugabe von 1 ml 2.5% Ammoniumsulfamidosulfonat-Lösung. Die Azokupplung erfolgt dann in der sauren Lösung innerhalb von 5 min nach Zugabe von 1 ml der 0.1%igen

Lösung des fluoreszierenden Naphtylaminderivats in Aceton. In einem Messkolben wird mit Aceton auf ein Volumen von 10.0 ml aufgefüllt.

Chromatographie. Jeweils 30 µl dieser Lösung werden mit einem Desaga-Microapplicator (10 µl) für organische Lösungen auf Dünnschichtplatten aufgetragen. Die Platten werden sofort entwickelt. Fliessmittel: Benzol—Methanol—Äthylacetat (20:30:100), Laufstrecke 10 cm, Kammersättigung, R_F = 0.41, überschüssiges Reagenz: R_F = 0.69. Nach dem Trocknen der Platte markiert man die Zone der roten Flecken und lässt die Platten 14 h liegen. Dabei zersetzt sich der rote Farbstoff und es entwickelt sich die gelbe Fluoreszenz. Diese wird mit dem Chromatogrammspektralphotometer gemessen.

Messung. Messanordnung Probe—Monochromator (Pr—M): Exzitation mit der Hg-Linie von 436 nm der Quecksilber-Mitteldrucklampe ST 41, mit Quarzkondensor; Emissionsmaximum: 520 nm; Spaltbild: 0.5 × 14 mm; Verstärkung 10—100 fach; Hochspannung 2.

Auswertung. Die Flächen unter den Peaks, die in Integratoreinheiten angegeben werden, sind der 5-Chloro-2,4 disulfamidoanilin-Menge pro Fleck direkt proportional. Dabei sind sogar die erhaltenen Ergebnisse auf verschiedenen Platten gleich, wenn die Zersetzungszeit gleich ist.

Bestimmung von HCT in Plasma

Extraktion. Zu 1—4 ml Plasma, die 50—500 ng HCT enthalten, werden 0.5 ml 2 *N* HCl sowie eine Spatelspitze Kochsalz gegeben. Mit 25.0 ml Essigsäureäthylester wird 30 min geschüttelt. Zwanzig Milliliter der organischen Phase werden zur Trockne eingedampft.

Hydrolyse. Zum Rückstand gibt man 2 ml 5 *N* NaOH und hydrolysiert 30 min im siedenden Wasserbad. Nach Zugabe von 1.5 ml konzentrierter Salzsäure extrahiert man mit 10 ml Äthylacetat. Die organische Phase wird mit einer Pasteur-Pipette quantitativ abgetrennt und eingedampft.

Derivatisierung. Die Umsetzung erfolgt wie bei der Erstellung der Eichgeraden beschrieben, nur werden jeweils 0.1 ml Reagenzlösung zugefügt und nach beendigter Reaktion auf ein Volumen von 0.50 ml aufgefüllt.

Chromatographie, Messung und Auswertung. Mit einem Microapplicator (10 µl) für organische Lösungen werden 30 µl pro Fleck aufgetragen. Die Platten werden zusammen mit den Platten, welche die Eichgerade enthalten, entwickelt. Die Auswertung erfolgt anhand der Eichgeraden.

Methode B

Nach folgender Arbeitsvorschrift kann HCT in Plasma, Urin und Speichel bestimmt werden.

Extraktion aus Speichel. Drei Milliliter Speichel werden 10 min bei 2000 *g* zentrifugiert, wobei Glycoproteide sedimentieren. Zwei Milliliter der überstehenden klaren Lösung werden in ein verschliessbares Reagenzglas gegeben und 30 min mit 7 ml Essigsäureäthylester geschüttelt. Zur vollständigen Phasentrennung wird anschliessend 5 min zentrifugiert. Der Essigester wird mit einer Pasteur-Pipette abgetrennt und in ein 10-ml-Becherglas gegeben. Die Wände des Reagenzglases werden mit 0.5 ml Äthylacetat abgespült und die Waschflüssigkeit wird mit dem ersten Teil vereinigt. Der Extrakt wird bei geringer Wärmezufuhr und unter Stickstoffbegasung zur Trockne eingedampft.

Extraktion aus Plasma. Zu 0.5 ml Plasma gibt man 0.5 ml Wasser und schüttelt in einem verschliessbaren Reagenzglas 30 min mit 5 ml Toluol. Zur vollständigen Phasentrennung wird 5 min zentrifugiert. Die organische Phase wird mit einer Pasteur-Pipette abgenommen und verworfen. Das so gereinigte Plasma wird analog wie der zentrifugierte Speichel weiterverarbeitet.

Extraktion aus Urin. Urin wird 1:5 mit Wasser verdünnt. Zu 1 ml dieser Lösung werden 200 mg $NaHCO_3$ gegeben. Die Aufarbeitung erfolgt dann wie es bei der Extraktion aus Speichel beschrieben wurde.

Chromatographie. Die Rückstände der Extrakte werden in zwei Tropfen Äthylacetat unter intensivem Schütteln (REAX I, Heidolph) gelöst. Die gesamte Flüssigkeit wird mit einem Fettschmelzpunktsröhrchen punktförmig auf eine Dünnschichtplatte aufgetragen. Der gleiche Vorgang wird zweimal wiederholt. Für die Eichgerade trägt man 20, 50, 100 und 200 ng HCT — gelöst in Aceton — mit Konstriktionspipetten auf. Die Platte wird mit einem Fön sorgfältig getrocknet. Die Entwicklung erfolgt nacheinander in zwei verschiedenen Fliessmitteln in derselben Richtung: Fliessmittel I: Aceton 70 ml, Laufstrecke 1 (!) cm; Fliessmittel II: Essigsäureäthylester 70 ml, Laufstrecke 10 cm. Um HCT von den mitextrahierten Plasmabestandteilen zu trennen, muss in Fliessmittel II ohne Kammersättigung chromatographiert werden. Der R_F-Wert von HCT in diesem System ist 0.52.

Messung und Auswertung. Nach dem Entwickeln wird die Platte getrocknet und mit einer 20%igen Lösung von Triäthanolamin in Methanol besprüht. Anschliessend wird 45 min mit UV-Licht von 365 nm bestrahlt. Die Messung erfolgt wieder mit dem Chromatogrammspektralphotometer (Fig. 3). Messanordnung Monochromator—Probe (M—Pr): Exzitation mit der Hg-Linie von 365 nm der Quecksilber-Mitteldrucklampe ST 41; Spaltbild: 0.3 × 8 mm; Hochspannung 2.

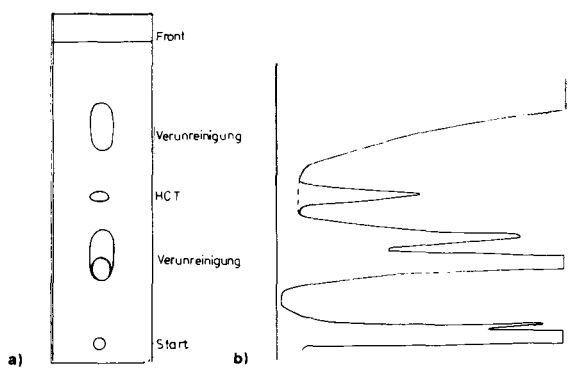


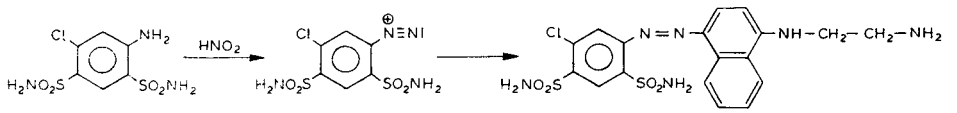
Fig. 3. Dünnschichtchromatogramm (a) und Fluoreszenz intensitäts-Ortskurve (b) von 50 ng HCT aus Plasma (Methode B).

Die Auswertung erfolgt anhand der Eichgeraden unter Berücksichtigung der Wiederfindungsraten für die verschiedenen Körperflüssigkeiten.

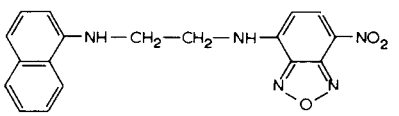
ERGEBNISSE

Methode A

Die von Bratton und Marshall [12] beschriebene Reaktion dient zum Nachweis primärer, aromatischer Amine. Die Aminogruppe wird mit salpetriger Säure diazotiert und das entstandene Diazoniumion mit N-(1-Naphthyl)äthylendiamin zu einem roten Farbstoff gekuppelt. Dabei entsteht quantitativ die *p*-Verbindung. Die Intensität der roten Farbe kann spektralphotometrisch in der Küvette oder mittels eines Chromatogrammspektralphotometers auf einer Dünnschichtplatte gemessen werden. Bei der HCT-Bestimmung muss dieser Reaktion eine Spaltung des Moleküls vorausgehen, damit die erforderliche primäre, aromatische Aminogruppe entsteht. Dies erzielt man durch Erwärmen in Natronlauge.

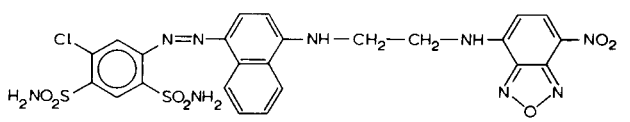


Ein entscheidender Fortschritt wird durch eine Variation der Kupplungskomponente erzielt, die so beschaffen sein muss, dass ein Azofarbstoff mit fluoreszierendem Molekülteil entsteht. Solch ein Reagenz stellten wir durch Umsetzung von N-(1-Naphthyl)äthylendiamin mit NBD-Cl her.



NBD-Cl dient als Reagenz zum Nachweis primärer und sekundärer aliphatischer Amine, mit denen es intensiv gelb fluoreszierende Produkte bildet. Die präparative Darstellung dieser Verbindungen wurde von Reisch et al. [13] beschrieben. Mit aromatischen Aminen reagiert NBD-Cl ebenfalls, die entstehenden Farbstoffe fluoreszieren allerdings nicht. Die Empfindlichkeit des Nachweises aromatischer Amine ist daher auch um eine Zehnerpotenz geringer als die Empfindlichkeit beim Nachweis aliphatischer Amine [11].

Kuppelt man das Diazoniumion des HCT-Hydrolyseprodukts mit dem modifizierten, fluoreszierenden Naphthylaminderivat, so erhält man folgendes Produkt:



Trägt man die Lösung, die das Kupplungsprodukt enthält, mit einer Konstriktionspipette punktförmig auf eine Dünnschichtplatte auf, so sind anhand der Fluoreszenz noch 0.6 ng HCT-Hydrolyseprodukt sichtbar. Das entspricht einer Empfindlichkeitssteigerung um das Achtfache gegenüber der klassischen Bratton—Marshall-Reaktion, da die Nachweisgrenze für den Azofarbstoff bei 5 ng pro Fleck liegt. Beim Auftragen mit einem Microapplicator können noch 3 ng

pro Fleck mit dem Chromatogrammspektralphotometer gemessen werden. Die Eichkurve verläuft linear im Bereich von 20—200 ng pro Fleck, die lineare Regression für die Eichgerade beträgt 0.9995 (Mittelwert aus zwei gleichzeitig durchgeführten Bestimmungen auf zwei Dünnschichtplatten).

Methode B

Bei der Auswertung der Eigenfluoreszenz des Hydrochlorothiazids nach der Messanordnung M—Pr können noch 2 ng HCT pro Fleck gemessen werden. Die Eichkurve verläuft dann linear bis ca. 500 ng pro Fleck (lineare Regression 0.9993). Durch längeres Liegen der Platte nimmt die Fluoreszenz ab. Sie kann jedoch durch erneutes Besprühen und Bestrahlen wieder erzeugt werden. Extrahiert man HCT aus 2 ml Speichel, so sind noch 5 ng/ml Speichel messbar. Die Wiederfindungsrate ist unabhängig vom HCT-Gehalt des Speichels, sie beträgt 99% mit einer Standardabweichung von ± 8% (n = 8). Für die Bestimmung von HCT in Urin liegt die Wiederfindungsrate bei 90%, die Standardabweichung beträgt ± 4% (n = 8).

Die Wiederfindungsrate für HCT aus Plasma ist ebenfalls vom HCT-Gehalt des Plasmas unabhängig. Sie liegt im Bereich von 20—500 ng/ml Plasma bei 90% mit einer Standardabweichung von ± 7.2% (n = 8). Eine exakte Messung ist wegen mitextrahierter Plasmabestandteile, die ebenfalls fluoreszieren, erst ab 20 ng/ml im Plasma möglich. Die vor der eigentlichen chromatographischen Trennung vorausgehende Entwicklung mit Aceton führt zu einer Verringerung des Fleckdurchmessers von HCT und damit zu einer erhöhten Empfindlichkeit.

Bestimmung der Hydrochlorothiazid-Kinetik

Um die Analysenmethode B zu überprüfen, wurden im Selbstversuch 25 mg HCT-Substanz eingenommen. Vor der Einnahme sowie innerhalb der ersten 24 h wurden sieben Blutproben entnommen. Das heparinisierte Blut wurde unter Zusatz von "Trennmittel Merck" zentrifugiert, das Plasma abgenommen,

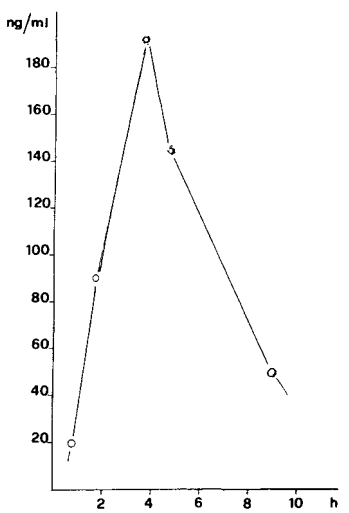


Fig. 4. HCT-Plasmaspiegel nach Einnahme von 25 mg HCT.

bei −18° eingefroren und erst unmittelbar vor Versuchsbeginn aufgetaut. Der HCT-Gehalt des Plasmas konnte über 9 h gemessen werden. Nach 24 h wurde kein HCT mehr im Plasma gefunden. Der maximale Plasmaspiegel war nach 3.5 h erreicht und betrug 192 ng/ml Plasma (Fig. 4). Dies entspricht den Beobachtungen von Anderson et al. [2], die mit ^{14}C markierten HCT gearbeitet hatten.

DISKUSSION

Die Ergebnisse zeigen, dass die von uns entwickelte Analysenmethode B in bezug auf die Empfindlichkeit den bisher bekannten Methoden, sofern nicht radioaktives Material verwendet wurde, überlegen ist. Sowohl mit der Hochdruckflüssigkeitschromatographie als auch mit den beiden gaschromatographischen Methoden, die eine Nachweisgrenze von 50 ng HCT/ml Plasma haben, kann nicht über einen ausreichend langen Zeitraum gemessen werden.

Ein gewisses Problem dieser Bestimmungsmethode liegt darin, dass die stark polare Substanz mit unpolaren Extraktionsmitteln, die Extrakte mit wenig Begleitstoffen ergeben, nicht extrahiert werden kann, während polare Extraktionsmittel, wie z.B. Essigsäureäthylester oder 4-Methyl-2-pentanon, auch andere Plasmabestandteile extrahieren. Arbeitet man nach der angegebenen Vorschrift, so ist die Empfindlichkeit gross genug und die Messgenauigkeit ausreichend, da durch die Chromatographie störende Substanzen weitgehend abgetrennt werden.

Der Vorteil der Methode A besteht darin, dass Störungen durch Plasmabestandteile nicht beobachtet werden. Jedoch streuen die Ergebnisse für die Extraktion von HCT aus Plasma stärker als bei Methode B. Die Hydrolyse-Reaktion verläuft bei dem Einsatz nur weniger Nanogramme HCT nicht immer mit der gewünschten Reproduzierbarkeit. Es wird vermutet, dass sich Zwischenstufen ausbilden, die zu einem erneuten Ringschluss befähigt sind [14].

Obwohl sie der Methode B in bezug auf die HCT-Bestimmung unterlegen ist, wird die Methode A in dieser Arbeit beschrieben, da sie sich nicht nur zur Bestimmung allein dieser Substanz eignet: Alle Stoffe, die bereits eine primäre, aromatische Aminogruppe enthalten, oder auch solche, die durch einfache chemische Umwandlung in primäre, aromatische Amine überführt werden können, sind auf diese Weise bestimmbar. Die modifizierte Bratton—Marshall-Reaktion ermöglicht somit die quantitative Bestimmung einer grossen Zahl von Arzneistoffen in Plasma oder anderen Körperflüssigkeiten.

ZUSAMMENFASSUNG

Zwei fluorimetrische Methoden zur Bestimmung von Hydrochlorothiazid (HCT) durch Auswertung von Dünnschichtchromatogrammen werden beschrieben. Die erste Methode stellt eine modifizierte Bratton—Marshall-Reaktion dar und ist somit für alle primären, aromatischen Amine anwendbar. Über eine Diazotierung und Azokupplung des HCT-Hydrolysats wird eine fluoreszierende Gruppe angeknüpft. Dazu wird in einer vorgelagerten Reaktion N-(1-Naphthyl)-äthylendiamin mit 4-Chloro-7-nitrobenzo-2,1,3-oxadiazol umgesetzt. Im zweiten Fall wird die Eigenfluoreszenz des nichtderivatisierten HCT nach seiner Ex-

traktion aus Plasma, Urin oder Speichel ausgewertet. Es wird gezeigt, dass die Empfindlichkeit dieser Methode zur Bestimmung der Kinetik nach oraler Gabe von 25 mg HCT ausreicht.

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Biomedical Applications

CHROMBIO. 074

Note

Use of high-pressure liquid chromatography in the assay of aldosterone in urine

C.P. DE VRIES, C. POPP-SNIJDERS, W. DE KIEVIET and A.C. AKKERMAN—FABER

Academisch Ziekenhuis der Vrije Universiteit, Laboratory for Endocrinology, Amsterdam (The Netherlands)

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It has been demonstrated that high-pressure liquid chromatography (HPLC) is suitable for the analysis of synthetic mixtures of steroids [1,2]. Up to now HPLC has not often been used in clinical steroid practice, although a few assays for cortisol and corticosterone have been described [3—5].

In our laboratory we are using HPLC with success in aldosterone assay in urine for the final separation and quantification. For several years we have estimated aldosterone in urine by means of gas—liquid chromatography (GLC), after conversion of aldosterone into a stable derivative, its corresponding γ-lactone [6]. We have now replaced GLC by HPLC, thus avoiding the oxidation of aldosterone. The two methods are compared, and HPLC is shown to be an improvement.

MATERIALS AND INSTRUMENTS

A Varian liquid chromatograph Model 8500 was used, equipped with a stop-flow injector, a 254-nm single-wavelength detector, and a 250 × 2.2 mm SI-10 MicroPak column (silica gel, particle size 10 μm). Samples were injected with a 10-μl Hamilton syringe.

Thin-layer chromatography (TLC) was performed on thin-layer plates pre-coated with silica gel 60 F_{254} (E. Merck, Darmstadt, G.F.R.). Components separated on the plates were revealed by short-wave UV irradiation, under a Camag UV lamp Type TL 900.

All solvents were of analytical reagent grade.

METHOD

(a) The acid-labile conjugate of aldosterone, its 18β-D-glucosiduronic acid [7], in 24-h urine is hydrolysed at pH 1 at room temperature for 24 h.

(b) Hydrolysed urine is split into two equal portions (duplicates), and each

portion is extracted three times with one-third volume dichloromethane. The pooled extract is washed successively with 1*M* sodiumhydroxide and 0.1*M* sodium hydroxide, both solutions being saturated with sodium chloride, and twice with water saturated with sodium chloride.

(c) The washed extract is dried over anhydrous sodium sulphate, filtered and evaporated to dryness in a rotary evaporator at 30°. The residue is transferred with acetone into a 10-ml centrifuge tube and the solution is evaporated to dryness under nitrogen.

(d) The residue is dissolved in 2 ml of 70% methanol in water and washed three times with 3 ml of toluene.

(e) The washed solution is split into two equal portions, and 10 μg of aldosterone is added to one portion.

(f) After being evaporated to dryness under nitrogen, the residue is purified by TLC. Three solvent systems are used, and after each TLC step the area corresponding to the area of the aldosterone standard is scraped off and eluted with acetone. The solvent systems are: (1) chloroform—methanol—water (90:10:0.8); (2) ethyl acetate—methanol—water (85:15:1); and (3) benzene—acetone—water (70:30:0.5) (twice in succession, on the same thin-layer plate).

(g) Quantification of aldosterone is performed by HPLC, after the addition of 5—10 μg of prednisolone to the eluate as internal standard. The amount of added prednisolone depends on the expected amount of aldosterone, as judged by viewing the last thin-layer plate under UV irradiation (254 nm). The eluate is evaporated to dryness under nitrogen, the residue is dissolved in 50 μl of dichloroethane; 1—10 μl of this solution is injected onto the column. The eluent used is 1.5% methanol in chloroform, half saturated with water, prepared by passing 2 l of 1.5% methanol in chloroform through a column 50 cm long and 1 cm I.D., filled with 20 g of silica gel, 30—120 mesh, that had been coated with 6 ml of water, and mixing this water-saturated solvent with an equal volume of water-free solvent. The flow-rate is 70 ml/h, which corresponds to a pressure drop of 1100 p.s.i.

RESULTS AND DISCUSSION

The separation of pure aldosterone and prednisolone, injected directly onto the column, is shown in Fig. 1; the shape of the aldosterone peak is symmetrical.

Fig. 2 shows the chromatogram of a urine extract, obtained according to the method described; here the aldosterone peak is not symmetrical as in Fig. 1, but shows a shoulder. The shoulder is caused by the effect of TLC on aldosterone; this is illustrated in Fig. 3, showing the chromatogram of pure aldosterone after TLC. Possibly a shift in the equilibrium between two tautomeric forms of aldosterone [8] (Fig. 3) is responsible for this effect.

To correct for the loss of material due to three elutions from the thin-layer plates, aldosterone standard was added to a part of each urine extract (see Method). The recovery was 63.1 ± 9.4% (n = 215).

The precision of both methods, GLC and HPLC, was estimated from duplicate assay by the equation [9]:

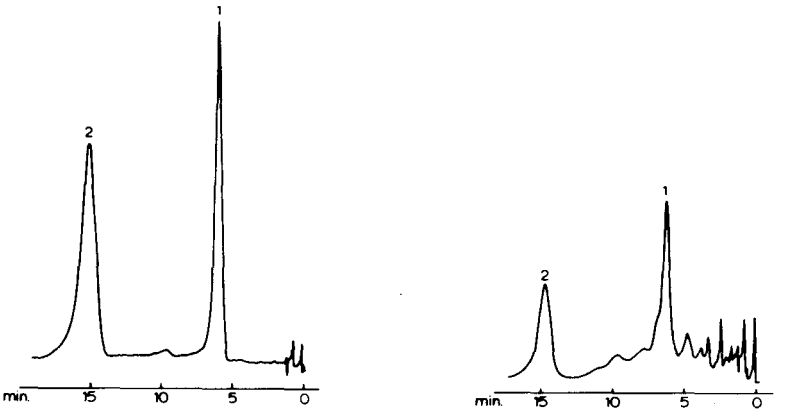

Fig. 1. Separation of pure aldosterone (1) and prednisolone (2). Column, SI-10 MikroPak; eluent. 1.5% methanol in chloroform half saturated with water; flow-rate, 70 ml/h; detection UV detector 254 nm.

Fig. 2. Chromatogram of a urine extract. 1 = Aldosterone, 2 = prednisolone (internal standard). For conditions see Fig. 1.

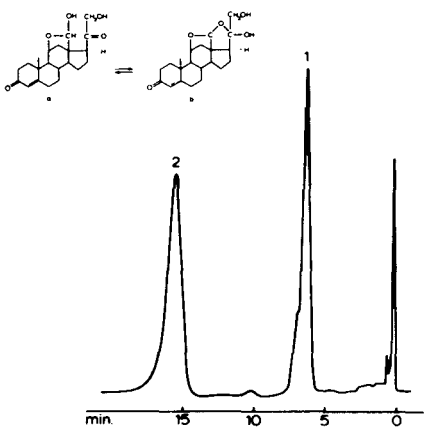

Fig. 3. Chromatogram of pure aldosterone after TLC. 1 = Peak + shoulder of aldosterone, possibly a mixture of two tautomeric forms of aldosterone a and b. 2 = prednisolone. For conditions see Fig. 1.

$$CV = \sqrt{\frac{\Sigma d^2}{2n}}$$

where CV = coefficient of variation; $d = \left(\frac{x_2}{x_1} - 1\right) . 100$,

with $x_2 \geqslant x_1$ (x_1, x_2 are duplicate values); and n = number of duplicate assays.

Results showed that for HPLC CV was 16 (n = 215), with a lower detection

limit of 5 nmoles/24 h whereas for GLC CV was 22 (n = 71), with a lower detection limit of 10 nmoles/24 hr. Normal values were 17.6 ± 12.6 nmoles/24 h (n = 15), range 5—45 nmoles/24 h.

Thus, in our hands, HPLC in aldosterone assay in urine appeared to be more precise and more sensitive than GLC. In addition, the conversion of aldosterone into a stable derivative, required for GLC, is avoided.

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Note

Simplified buffer system for accelerated column analysis of amino acids in physiological fluids

E. LEPOIRE and M. PIERSON

Laboratoire de Pédiatrie, Centre Hospitalier Universitaire, Avenue de Lattre de Tassigny, 27, 54 037 - Nancy (France)

(Received February 2nd, 1977)

The separation and determination of amino acids in biological fluids by elution chromatography on ion-exchange columns has developed considerably since the introduction of the first cation-exchange resin by Moore and Stein [1]. The method acquired great importance after its automation by Spackman and Moore [2] and later through the introduction of lithium citrate rather than sodium citrate buffers [3, 4], thus allowing the resolution of asparagine, glutamic acid and glutamine.

The most commonly used resins are made from sulphonated polystyrene crosslinked with 8—12% of divinylbenzene. They are characterized by their good stability but low reactivity, which necessitates the use of a high operating temperature; this in turn causes a loss of resolution for some amino acids. Hamilton et al. [5] showed the relationships between resin-bead diameter, buffer flow-rate and column length and diameter. The use of increasingly fine resin beads permits the operating temperature to be decreased, and also the column length and diameter [6], while maintaining the number of buffer plateaus needed for the fractionation of complex mixtures. This results in greater sensitivity and speed of analysis.

In the most recently described techniques, either a single column with a continuous buffer gradient or two columns with stepwise buffer changes are used. In the first instance, it is difficult to obtain satisfactory chromatogram reproducibility and, as Dautrevaux [7] pointed out, the identification of peaks is difficult, especially for biological fluids such as urine. In the second instance, two portions of the same sample must be used and the analysis takes 6 h.

As techniques improve, the demand for amino acid determinations constantly increase: for the diagnosis of aminoacidopathy resulting from a catabolic deficit; for the search of heterozygotes by tolerance tests; and for dietary surveillance of certain treated aminoacidopathies.

We describe here a modification of the Technicon technique [8] for the NC 11 P AutoAnalyzer. Koehl and Mandel [9] had already modified this technique by programming an automatic temperature change for the columns and by running two columns in parallel, thus doubling the capacity of the instrument and permitting simultaneous analyses of two different samples. Our modification substitutes three buffers for the seven previously used, and allows the separation of 40 amino acids and ninhydrin-positive substances of interest in human biology from as little as 50 μl of plasma or urine.

MATERIALS AND METHODS

Materials

Two columns (470 × 5 mm) mounted in parallel and fed by two high-pressure pumps, which are temperature-controlled by two Haake water-baths, are each packed with a 41-cm bed of Chromobeads C_3 resin beads (particle diameter 10 μm). These columns are linked to the NC 11 P Technicon system, consisting of a P 111 pump, a peristaltic valve and a programmer, a 95° water-bath with a double coil, two single-channel colorimeters for the AutoAnalyzer 11 equipped with a set of interference filters at 410 nm, and a double-pen recorder.

Reagents

The ninhydrin reagents are prepared as recommended by Technicon, ninhydrin and hydrazine sulphate being mixed as required. The pH of the ninhydrin reagent is adjusted to 6.30 for increased sensitivity [10].

After they have been used, the resins are regenerated in 0.3 *M* lithium hydroxide solution and re-equilibrated with the first buffer at pH 2.75.

The lithium citrate elution buffers [3, 11, 12] are prepared as shown in Table I.

Sample preparation

Plasma or urine samples are deproteinized with 5% or 2% sulphosalicylic acid, respectively. They are then diluted with an equal volume of the sample-dilution (pH 2.20) described by Benson et al. [3]. The final pH of the sample is 2.20.

Standard mixture

The amino acids in the standard mixture have concentrations of 2.5 μmoles/ml in 0.1 *M* hydrochloric acid, except β-alanine, sarcosine, β-aminoisobutyric acid and urea (10 μmoles/ml).

Methods

The flow diagram is shown in Fig. 1, and Table II gives the detailed programme of the peristaltic valve settings, showing all of the conditions for chromatographic elution.

Four positions (numbers 5—8) are used for transit of buffers and lithium hydroxide. The first buffer change takes place 96 min after the beginning of

TABLE I
BUFFER COMPOSITIONS

		Stock solution
Lithium concentration	(*M*)	3
Citrate concentration	(*M*)	0.5
Lithium tricitrate. $4H_2O$	(g)	150.5
Lithium chloride	(g)	59.05
Thiodiglycol	(ml)	25
Caprylic acid	(ml)	1
30% Brij-35	(ml)	20
Distilled water		to 1 l

		Buffer 1	Buffer 2	Buffer 3
Stock solution	(ml)	100	100	400
Methyl Cellosolve	(ml)	35	–	–
Distilled water		to 1 l	to 1 l	to 1 l
Final pH at 25°		2.75	3.50	4.10

the run, and the second 46 min later. The flow-rate is set at 0.47 ml/min. The pressure does not exceed 600 p.s.i. at the lower temperature and decreases to 400 p.s.i. at the higher temperature.

Two positions (numbers 21 and 22) are used for ninhydrin or methyl Cellosolve, the flow-rate of which is set at 0.8 ml/min.

Four positions (numbers 13—16) are used to change the temperature of the columns automatically. For the first 180 min, the effluent from the first water-bath holds both columns at 37° by means of peristaltic valve; meanwhile the water in the second water-bath does not circulate. Then the circuit switches to the second water-bath, raising the column temperature to 55° [13] and blocks the effluent from the first one.

This temperature programme, derived from that described by Koehl and Mandel [9], is very effective because the temperature change is effected very rapidly. We have not found any serious disadvantage in blocking the circulation of the unused water-bath.

After completion of the runs, the detection circuit is rinsed with a 50% solution of methyl Cellosolve in distilled water, replacing the flow from the column (positions 17—20) in order to maintain a constant flow-rate in the reaction water-bath and the colorimeter, while the column is regenerated and the samples are changed. Meanwhile, the regeneration eluate is by-passed into the drain (positions 2 and 4) without passing through the colorimeter.

Two chromatograms can be obtained in 5 h. It then takes 45 min to regenerate and re-equilibrate the columns and rinse the circuit, after which two new samples can be applied.

RESULTS AND DISCUSSION

Fig. 2 shows the results obtained from a standard solution of 40 amino acids

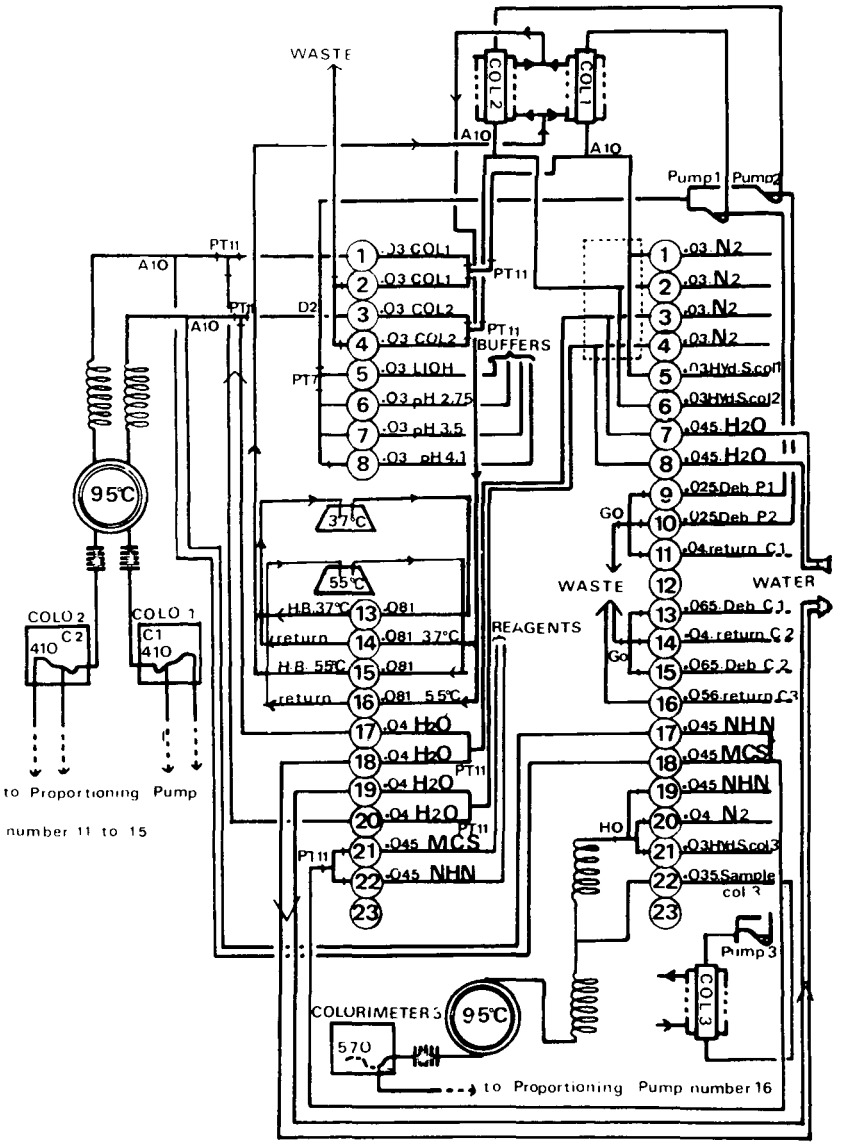

Fig. 1. Flow diagram.

and related substances and from biological samples. Chromatograms are shown for simultaneous runs on the plasma and urine from a child suffering from a liver disease and suspected to have tyrosinosis. In Table III we list the amino acids separated with each buffer under the described conditions of time, pH and temperature.

The first buffer (pH 2.75) elutes amino acids up to valine. However, phosphoserine and cysteinesulphinic acid are not resolved from cysteic acid. The sharpness in resolution of the amino acids from aspartic acid to citrulline de-

TABLE II

PROGRAMME OF THE NC II P PERISTALTIC VALVE FOR PHYSIOLOGICAL FLUIDS

L = large diameter space, S = small diameter space.

Running time (min)			2	49	96	122	142	182	222	262	302	310	330	346
Roller time (min)			2	47	47	26	20	40	40	40	40	8	20	16
Grip-ring washer			L	L	L	L	L	L	L	L	L	L	L	L
Analytical system Column 1		1		S	S	S	S	S	S	S	S	S		
Waste column 1		2	S										S	S
Analytical system Column 2		3		S	S	S	S	S	S	S	S	S		
Waste column 2		4	S										S	S
0.30 *M* LiOH		5										S		
Buffer, pH 2.75		6	S	S	S								S	S
Buffer, pH 3.50		7				S	S							
Buffer, pH 4.10		8						S	S	S	S			
Open		12			FIXED SPACER									
Water-bath 37°	Inlet columns	13	S	S	S	S	S	S						
	outlet columns	14	S	S	S	S	S	S						
Water-bath 55°	Inlet columns	15							S	S	S	S	S	S
	Outlet columns	16							S	S	S	S	S	S
Water system 1	Analytical circuit	17	S										S	S
	Waste	18		S	S	S	S	S	S	S	S	S		
Water system 2	Waste	19		S	S	S	S	S	S	S	S	S		
	Analytical circuit	20	S										S	S
Methyl Cellosolve (50%)		21	S										S	S
Ninhydrin		22		S	S	S	S	S	S	S	S	S		
Pump 1			S	S	S	S		S	S	S	S	S	S	S
Pump 2			S	S	S	S	S	S	S	S	S	S	S	S
Roller numbers			1	2	3	4	5	6	7	8	9	10	11	12

pends both on the precision of the buffer pH to ± 0.01 unit, and on the exact percentage of methyl Cellosolve: if the latter is below 3.5%, proline is not resolved from glycine; if it is above 3.5%, proline overlaps with glutamine, and asparagine is not resolved from glutamic acid.

The second buffer (pH 3.50) elutes amino acids up to tyrosine.

The change to the third buffer (pH 4.10) takes place during the elution of the leucine peak, 142 min after the beginning of the run. Maintaining the column temperature at 37° for the next 40 min gives a good resolution of tyro-

TABLE III

AMINO ACIDS OR COMPOUNDS ELUTED AS A FUNCTION OF THE THREE BUFFERS USED AND THE TEMPERATURE

Cysteic acid Taurine Phosphoethanolamine Urea Aspartic acid Hydroxyproline Methionine sulphone Threonine Serine Asparagine Glutamic acid Glutamine α-Aminoadipic acid Sarcosine Proline Glycine Alanine Citrulline α-Aminobutyric acid Valine	0.3 *M* Li buffer pH 2.75 + 3.5% methyl Cellosolve 96 min	37° 182 min
Cystine Cystathionine Methionine Isoleucine Leucine Norleucine	0.3 *M* Li buffer pH 3.50 46 min	
Tyrosine β-Alanine Phenylalanine Homocystine γ-Aminobutyric acid	1.2 *M* Li buffer pH 4.10 40 min	
Ethanolamine Ammonia Ornithine Lysine Histidine 1-Methylhistidine 3-Methylhistidine Arginine	120 min	55° 120 min

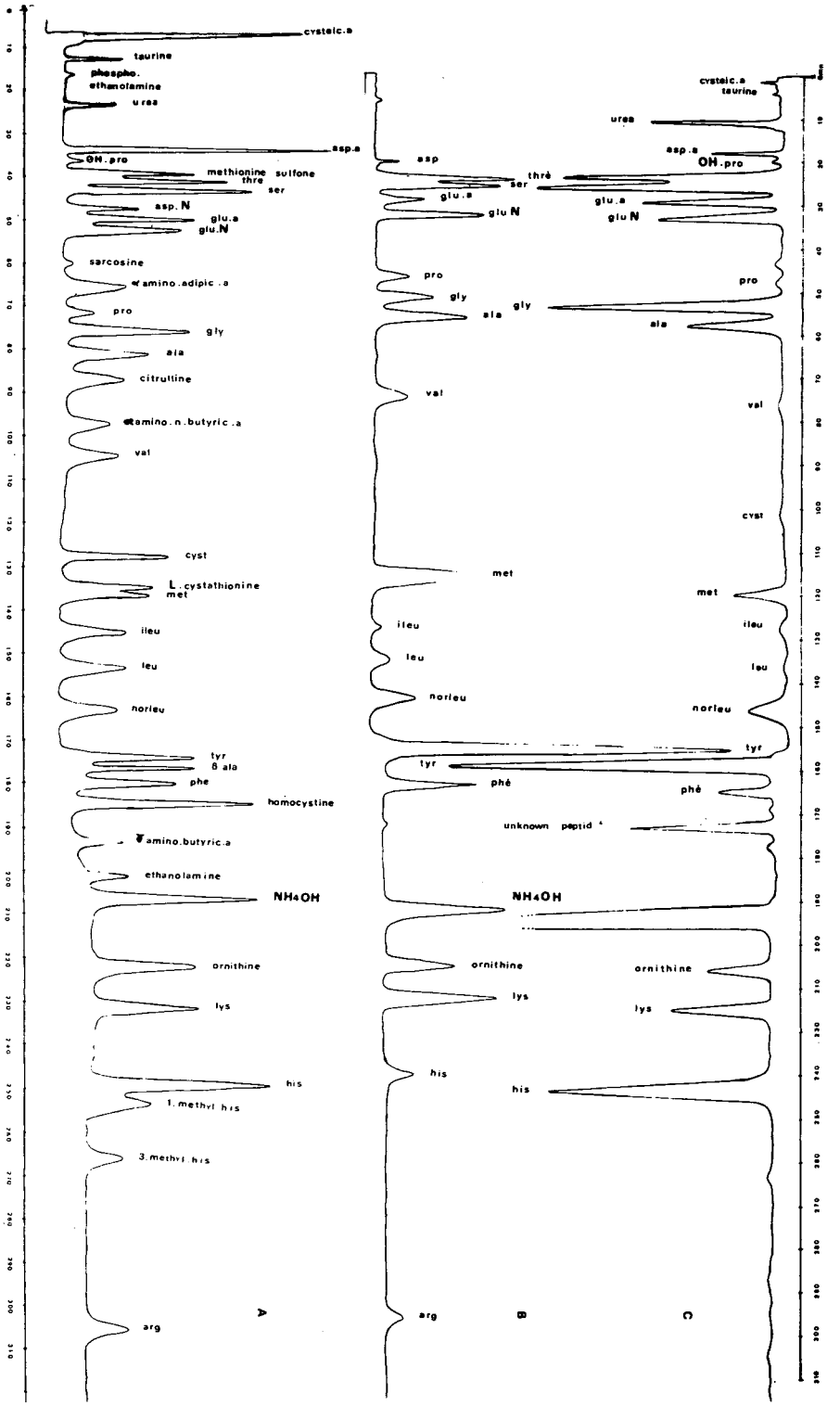

Fig. 2. Chromatograms of complex mixtures of ninhydrin-positive substances. (A) Chromatogram of a standard solution containing 0.2 μmoles of urea, sarcosine, and β-alanine, and 0.05 μmoles of the other compounds; (B) elution of 50 μl of a patient's plasma; and (C) simultaneous elution of 100 μl of urine from the same patient.

sine, β-alanine, phenylalanine, homocysteine and γ-aminobutyric acid. Only β-aminoisobutyric acid is not resolved from phenylalanine under these conditions. However, an excess of phenylalanine can be confirmed by simultaneous fluorimetric assay. Furthermore, β-aminoisobutyric acid is rare in biological fluids. Finally, if the pH of the pH 4.10 buffer is changed by 0.02 unit, β-aminoisobutyric acid is then separated from phenylalanine, but at the expense of β-alanine. Increasing the column temperature to 55°as the ammonia peak emerges allows the separation of the basic amino acids in 2 h.

The rapid preparation of the reagents and low sample volume (50 μl of plasma) required for a run permit micro-sampling. In addition, the daily output of the apparatus can be increased by using an automatic sample injector.

The system described here permits simultaneous dietary surveillance of treatment for leucinosis and phenylketonuria by assay on a 300 × 6 mm column packed with C_2 Chromabeads (13-μm particles). Small groups of amino acids such as valine, isoleucine and leucine can be resolved within 75 min and tyrosine and phenylalanine within 45 min [14]. As these partial chromatographic analyses are made independently of the system just described, the daily output of complete analyses is not reduced.

CONCLUSION

The use of three successive buffers for the simultaneous chromatography of two biological samples in 5½ h permits a good resolution and a good reproducibility of the results: variations in retention times are very small. Because the buffer changes, the temperature changes, the regeneration of the columns and the switching off are automated, up to six samples per day can be analyzed.

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We thank Miss E. Pierron for skillful technical assistance.

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Biomedical Applications

CHROMBIO. 072

Note

Fluorimetrische Bestimmung von Furosemid und 4-Chloro-5-sulfamoylanthranilsäure in Plasma durch direkte Auswertung von Dünnschichtchromatogrammen ★

MONIKA SCHÄFER, HEINRICH E. GEISSLER und ERNST MUTSCHLER

Pharmakologisches Institut für Naturwissenschaftler, Johann Wolfgang Goethe-Universität, Robert Mayer Strasse 7—9, 6000 Frankfurt/Main (B.R.D.)

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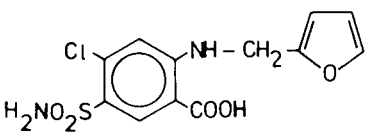
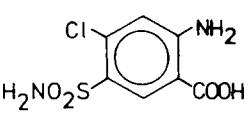
Furosemid (Lasix®) ist eines der am häufigsten verwendeten Diuretica. Jedoch ist bis heute keine Analysenmethode bekannt, die es erlaubt, Furosemid und seinen Metaboliten 4-Chloro-5-sulfamoylanthranilsäure (CSA) nebeneinander und mit ausreichender Empfindlichkeit in Plasma zu bestimmen bzw. zu zeigen, dass keine signifikanten Mengen an CSA im Plasma auftreten.



Furosemid



CSA

Die ersten Bestimmungsmethoden wurden von Hajdú und Häussler veröffentlicht [1, 2] . Nach Extraction aus Plasma oder Urin mit Äther wird Furosemid zu CSA hydrolysiert und nach Diazotierung und Kupplung kolorimetrisch bestimmt. Da sowohl Furosemid als auch CSA mit Äther extrahiert wird, ist es nicht möglich, mit dieser Methode den Gehalt des Plasmas an jedem der beiden Stoffe zu bestimmen. Ausserdem beschreiben Hajdú und Häussler noch eine fluorimetrische Mikrobestimmung von Furosemid, die auf der starken Eigenfluoreszenz des Anthranilsäurederivats beruht. Auch mit dieser Methode wird der Metabolit miterfasst [2]. Beide Methoden haben nicht die erforderliche Empfindlichkeit, um bei Gabe therapeutischer Dosen an menschlichen Versuchspersonen Blutspiegelkurven bestimmen zu können.

★ Teilergebnisse der Dissertation M. Schäfer, in Vorbereitung.

Für die Erzielung einer diuretischen Wirkung sind beim Menschen in der Regel 40 mg Furosemid ausreichend. Hajdú und Häussler messen mit den von ihnen entwickelten Verfahren die bei Tieren nach Gabe von 5 bis 25 mg/kg auftretenden Blutspiegel. Auch bei der Furosemidbestimmung in Urin, Kot, Galle und Milch sind solch hohe Dosen notwendig [2].

Ferner findet man bei diesen Analysenverfahren, insbesondere bei der fluorimetrischen Methode, hohe und schwankende Blindwerte (0.3 ± 0.1 µg/ml), die sich nachteilig auswirken. Da bei Gabe von 80 mg Furosemid an nüchterne Versuchspersonen der maximale Blutspiegel nur 2.2 µg/ml (Furosemid und Metabolit gemeinsam erfasst) beträgt [4], können bei Berücksichtigung einer Grundfluoreszenz der Leerprobe, die 0.3 ± 0.1 µg/ml entspricht, vor allem bei geringen Substanzmengen erhebliche Fehler entstehen.

Modifikationen der fluorimetrischen Methode wurden von Forrey et al. [3, 4] und Andreasen et al. [5] beschrieben. Sie führen zu nur noch schwach fluoreszierenden Leerwerten. Erfasst werden aber wiederum Furosemid und CSA zusammen. Forrey et al. führten auch die ersten pharmakokinetischen Untersuchungen am Menschen durch [4]. Der Furosemid-Gehalt des Serums konnte von ihnen nach oraler Gabe von 80 mg über 4 h gemessen werden. Ähnliche Ergebnisse erhielten sie bei Gaben von ^{35}S markiertem Furosemid. In der Literatur sind ferner Bestimmungsmethoden beschrieben, welche die Gaschromatographie [6] oder die Hochdruckflüssigkeitschromatographie [7,8] verwenden. Die Nachweisgrenzen liegen bei 0.1 µg [6] bzw. 1 µg/ml [7]. Die von MacDougall entwickelte Methode [8] erlaubt die Bestimmung von Furosemid und CSA nebeneinander. Die Nachweisgrenze liegt allerdings mit 0.2 µg/ml Plasma noch relativ hoch.

Das von uns entwickelte Verfahren ermöglicht es, Furosemid sowie seinen Metaboliten, ohne vorherige Reinigung des Plasmas, in einem Arbeitsgang zu bestimmen. Zur Trennung der beiden Stoffe verwenden wir die Dünnschichtchromatographie (DC). Die Messung erfolgt nach dem Besprühen der Platte mit einer Citronensäure-Lösung mit einem Chromatogrammspektralphotometer (Fig. 1). Das Emissionsmaximum liegt für beide Substanzen bei 420 nm.

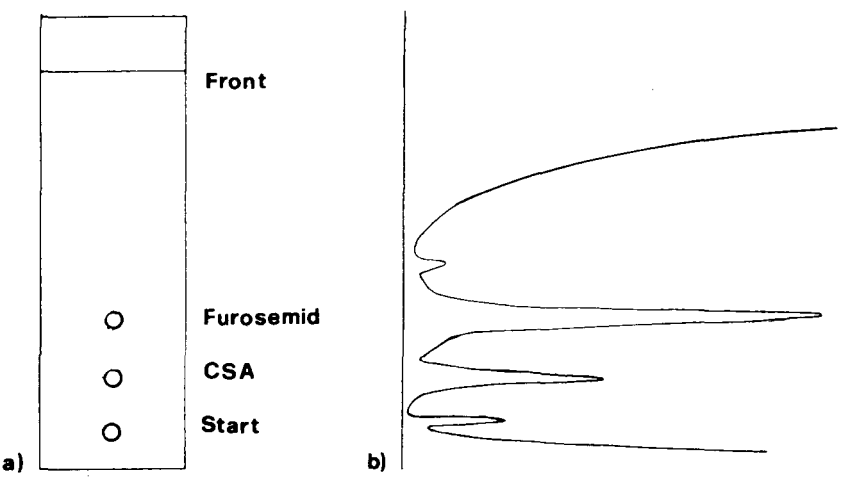


Fig. 1. Furosemid und CSA (200 ng/ml) aus Plasma. (a) Chromatogramm, (b) Auswertung.

EXPERIMENTELLES

Geräte

Chromatogrammspektralphotometer KM 3 der Firma Carl Zeiss (Oberkochen, B.R.D.), mit Kompensationsschreiber Servogor Sb.

Chemikalien

Die verwendeten Chemikalien und Dünnschichtplatten (Kieselgel 60, ohne Fluoreszenzindikator, 20 × 20 cm) lieferte Firma Merck (Darmstadt, B.R.D.).

Methodik

Probenvorbereitung und Chromatographie. 0.50 ml Plasma werden mit 1.00 ml Methanol versetzt. Die gefällten Proteine werden abzentrifugiert und der Überstand filtriert. 50.0 µl dieser Lösung werden mit einem Linomaten III (Camag, Muttenz, Schweiz) auf eine DC-Platte aufgetragen. Für die Eichgerade trägt man ausserdem 0.20, 0.50, 1.0, 2.0, 5.0 sowie 10.0 ng Furosemid und CSA (gelöst in Aceton) pro Fleck auf. Die Platte wird in Chloroform—Essigsäureäthylester—Ameisensäure (70:30:5) unter Standardbedingungen entwickelt (Fliessmittel modifiziert nach Lit. 9): R_F-Wert von Furosemid: 0.3 und von CSA: 0.15.

Messung und Auswertung. Nach dem Entwickeln wird die Platte getrocknet, zum Äquilibrieren mindestens 3 h liegengelassen und mit einer 10%igen Lösung von Citronensäure in Wasser—Äthylenglycol (1:1) besprüht. Die Messung erfolgt anschliessend ohne erneutes Trocknen der Platte mit dem Chromatogrammspektralphotometer. Die Platte wird parallel zur Entwicklungsrichtung mit einer Geschwindigkeit von 100 mm/min bewegt.

Messanordnung: M-Pr (Monochromator-Probe); Exzitation: Hg-Linie von 365 nm der Quecksilber-Mitteldrucklampe ST 41; Emission: Monochromatfilter M 436; Quarzkondensor; Hochspannung: −400 V; Spaltbild: 1.0 × 8 mm; Verstärkung: 1−100fach. Die Auswertung erfolgt anhand der Eichgeraden unter Berücksichtigung der Wiederfindungsraten.

ERGEBNISSE UND DISKUSSION

Nach Entwicklung mit dem oben beschriebenen Fliessmittel sind von den Reinsubstanzen noch 0.1 ng Furosemid und 0.5 ng CSA pro Fleck messbar. Die Eichgeraden verlaufen linear bis mindestens 1 µg pro Fleck (lineare Regression 0.9993) und gehen durch den Ursprung des Koordinatensystems.

Trägt man 50 µl des enteiweissten Plasmas auf, so können noch 10 ng Furosemid/ml Plasma gemessen werden. Die Wiederfindungsrate für Furosemid aus Plasma ist unabhängig von dessen Furosemid-Gehalt. Sie beträgt 104% mit einer Standardabweichung von ±4.7% (Zur Überprüfung wurden 8 Proben zu 0.5 ml mit 20—1000 ng Furosemid nach dem oben beschriebenen Verfahren aufgearbeitet.) Die untere Nachweisgrenze für den Metaboliten liegt bei 50 ng/ml. Die Wiederfindungsrate beträgt 86.5 ± 7.2% (n = 8).

Die Ergebnisse zeigen, dass die von uns entwickelte Methode zur Bestimmung von Furosemid in Plasma den bisher bekannten Verfahren überlegen ist. Sie erlaubt es, mit geringem Arbeitsaufwand Furosemid und seinen Metabo-

liten 4-Chloro-5-sulfamoylanthranilsäure in Plasma zu bestimmen. Die Nachweisgrenze liegt für Furosemid dabei um eine Zehnerpotenz niedriger als bei der von MacDougall beschriebenen Methode, bei der als einzige sowohl Furosemid als auch sein Metabolit in Plasma bestimmt werden kann. Bei der Bestimmung mit den anderen fluorimetrischen Verfahren werden immer Furosemid und CSA zusammen erfasst.

Es ist ausserdem von besonderer Bedeutung, dass durch die DC-Trennung alle durch Eigenfluoreszenz störenden Plasmabestandteile abgetrennt werden können. Leerproben (= Plasmaproben ohne zugesetztes Furosemid oder CSA) zeigen bei den Furosemid und CSA entsprechenden R_F-Werten keine Fluoreszenz. Veränderungen in der Zusammensetzung des Plasmas, wie sie z.B. durch eine Nahrungsaufnahme verursacht werden, können demnach nicht durch eine Schwankung in der Grundfluoreszenz stören. Der Analysenfehler ist daher geringer als bei den anderen Verfahren.

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Subject Index

Vol. 143, No. 6

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

APPARATUS

N-1067

FLUORESCENCE DETECTOR FOR LC

Available from Varian is a 4-page brochure discussing their Fluorichrom fluorescence detector for column liquid chromatography. Application areas include pollution detection, biological and pharmaceutical sciences, food and forensic analyses. Among the performance features are the tungsten-halide lamp which provides excitation from above 280 nm through the visible, a dual filter system to decrease background noise, and a microvolume (25 μl) flow cell for increased sensitivity.

N-1048

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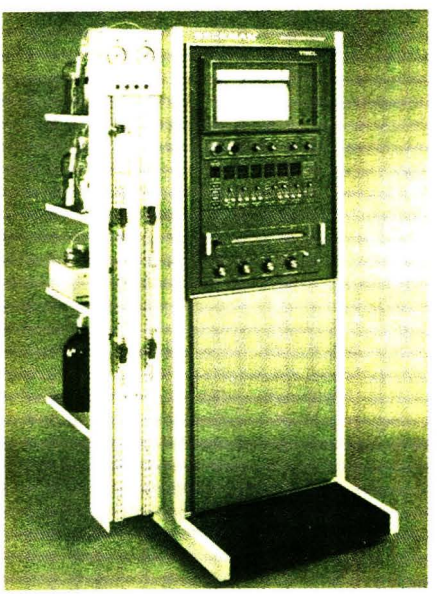

N-1051

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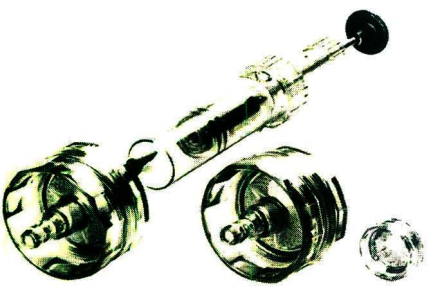
The Antlia™ pneumatic hand-pump system designed for pressure filtration with membranes, glass or paper filters, is available from Schleicher & Schuell. Useful for clearing solvents and solutions, sterile filtration, water sampling for trace metals, the pump can be used for a wide range of specialized applications. Separate screw-on 25- and 47-mm filter holders accomodate the filter media which are available in a range of pore sizes down to 0.01 μm. The action of two concentric polycarbonate pumps provides the necessary pressure. The outer cylinder holds up to 50 ml of solution, the inner functions like a hand-pump for tyres, exerting air pressures as high as 75 p.s.i. Large volumes can be filtered without having to disassemble the unit.



N-1054

SOFT LASER SCANNING DENSITOMETER

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N-1055

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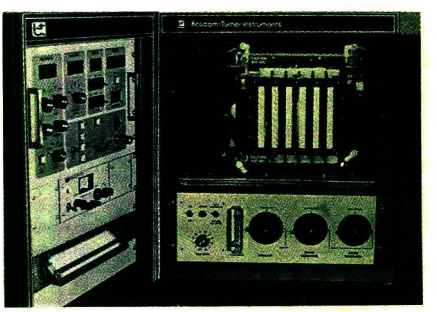

proteins, sub-cellular particles, and living cells. In addition to conventional measurements the instrument can be used to measure apparent zonal diffusion coefficients, resolution and plate height under various separation conditions, focusing times for reaching steady state in protein electrofocusing.

MEETING

BIOMEDICAL APPLICATIONS OF CHROMATOGRAPHY

The 6th International Symposium on Biomedical Applications of Chromatography will be held May 21–24, 1978 at the castle Hluboká, Czechoslovakia. The scope of the meeting will cover all types of chromatographic procedures devoted to metabolic profiles, chromatography in routine laboraty work, relation between chromatographic migration and biological activity, drug monitoring, and specialized chromatographic techniques for biomedical applications.

Papers discussing solutions of specialized problems with limited applicability to chromatography in general will be included in a poster session in order to encourage direct contacts between those most interested.

Participation in the symposium will be on the basis of invited papers, as well as unsolicited contributions. Authors desiring to present papers must submit 200–400 word abstracts by December 31, 1977. Full text of papers presented at the symposium will be published in a special issue of the Journal of Chromatography, Biomedical Applications.

All correspondence concerning the symposium should be directed to Assoc. Professor Dr. Karel Macek, Physiological Institute of the Czechoslovak Academy of Sciences, Prague 4, Budějovická 1083, Czechoslovakia.

CHEMICALS

N-1029

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N-1046

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N-1066

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NEW BOOKS

The tools of biochemistry, by T.G. Cooper, Wiley, Chichester, 1977, ca. 448 pp., price ca. US$21.25, £12.40, ISBN 0-471-17116-6.

Medicinal chemistry V (Proc. 5th Int. Symp., Paris, July 19–22, 1976), edited by J. Mathieu, Elsevier, Amsterdam, Oxford, New York, 1977, viii + 456 pp., price Dfl.130.00, US$52.95, ISBN 0-444-41594-7.

Methods of biochemical analysis, Vol. 24, edited by D. Glick, Wiley, Chichester, New York, Sydney, Toronto, 1977, ca. 496 pp., price US$33.00, £19.50, ISBN 0471-02764-2.

Biological separations in iodinated density-gradient media (Information Retrieval Symposium Series), edited by D. Rickwood, Information Retrieval Limited, London, 1976, 205 pp., price £ 5.50, US $ 12.00, ISBN 0-904-14702-9.

Quantitative analysis of drugs, by D.C. Garratt, Chapman & Hall, London, 3rd ed. (paperback), 1976, 940 pp., price *ca.* £ 12.00, ISBN 0-412-14810-2.

Concanavalin A as a tool, edited by H. Bittiger and H.P. Schnebli, Wiley-Interscience, London, New York, Sydney, Toronto, 1976, XV + 639 pp., price £ 19.50, US $ 38.50, ISBN 0-471-01350-1.

Rapid methods and automation in microbiology and immunology: A bibliography, edited by W. Palmer and S. Lequesne, Information Retrieval Limited, London, 1976, 250 pp., price £ 12.00, US $ 24.00, ISBN 0-904-14707-X.

Porphyrins in human diseases, (Proceedings of 1st International Porphyrin Meeting, Freiburg i. Br., May 1–4, 1975), edited by M. Doss, S. Karger, Basel, 1976, XX + 512 pp., 108 figs., 143 tables, price DM 192.00, ca. US $ 74.00, ISBN 3-8055-2259-2.

Analysis of drugs of abuse, by E. Berman, Heyden & Son, London, New York, Rheine, 1977, x + 80 pp., price £ 5.50, US $ 11.00, DM 35.00, ISBN 0-85501-226-9.

Clinical toxicology (Proc. 18th Meeting, European Society of Toxicology, Edinburgh, June 21–23, 1976), edited by W.A.M. Duncan, Excerpta Medica, Amsterdam, New York, 1977, ca. 350 pp., price Dfl. 87.50, US $ 35.75, ISBN 90-219-0333-4.

Quantitative mass spectrometry in life sciences (Proc. 1st Int. Symp., Ghent, June 16–18, 1976), edited by A.P. De Leenheer and R.R. Roncucci, Elsevier, Amsterdam, Oxford, New York, 1977, viii + 253 pp., price Dfl. 74.00, US $ 30.25, ISBN 0-444-41557-2.

Biomedical photography – A Kodak seminar in print, Kodak publication N-19, Eastman-Kodak, Dept. 454, Rochester, N.Y., 1977, price US $ 10.00.

Methods of protein separation, Vol. 2, edited by N. Catsimpoolas, Plenum Press, New York, London, 1976, xviii + 326 pp., price £18.60, US$ 35.40, ISBN 0-306-34602-8.

The hydrophobic fragmental constant; Its derivation and application; A means of characterizing membrane systems (Pharmacochemistry Library, Vol. 1), by R.F. Rekker, Elsevier, Amsterdam, Oxford, New York, 1977, xvii + 389 pp., price Dfl. 97.50, US $ 38.95, ISBN 0-444-41548-3.

Analytical biochemistry of insects, edited by R.B. Turner, Elsevier, Amsterdam, Oxford, New York, 1977, viii + 316 pp., price Dfl. 74.00, US $ 29.75, ISBN 0-444-41539-4.

Side effects of drugs annual 1977, edited by M.N.G. Dukes, Excerpta Medica, Amsterdam, New York, 1977, ca. 380 pp., price Dfl. 100.00, US $ 38.50, ISBN 90-219-3038-2.

Clinical pharmacy and clinical pharmacology (Proceedings of an International Symposium, Boston, Mass., September 17–19, 1975), edited by W.A. Gouveia, G. Tognoni and E. van der Kleijn, North-Holland, Amsterdam, New York, 1977, xiv + 470 pp., price Dfl. 136.00, US $ 52.50, ISBN 0-7204-0596-3.

Density gradient centrifugation (Laboratory Techniques in Biochemistry and Molecular Biology, edited by T.S. Work and E. Work, Vol. 6 Part I), by R. Hinton and M. Dobrota, North-Holland, Amsterdam, New York, 1976, iv + 290 pp., price Dfl. 47.00, US $ 17.95, ISBN 0-7204-4217-6.

Synthetic peptides, Vol. 4, by G.R. Pettit, Elsevier, Amsterdam, New York, xviii + 477 pp., price Dfl. 150.00, US $ 57.50, ISBN 0-444-41521-1.

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Advances in mass spectrometry in biochemistry and medicine, Vol. II (Proc. 3rd Int. Symp., Milan, June 1975), edited by A. Frigerio, Spectrum Publ./Halsted (Wiley), New York, 1977, XXII + 609 pp., price £ 35.00, US $60.00, ISBN 0-470-99039-2.

Biomedical and clinical aspects of coenzyme Q (Proc. Int. Symp. on Coenzyme Q, Lake Yamanaka, September 1976), edited by K. Folkers and Y. Yamamura, Elsevier, Amsterdam, Oxford, New York, 1977, xii + 316 pp., price Dfl. 80.00, US $ 32.75, ISBN 0-444-41576-9.

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2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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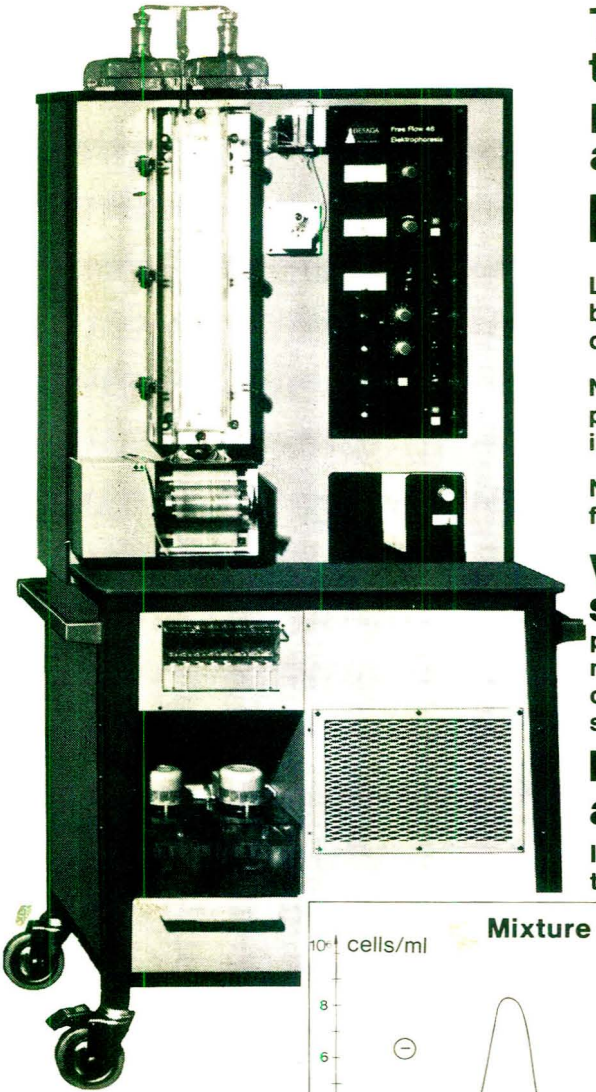

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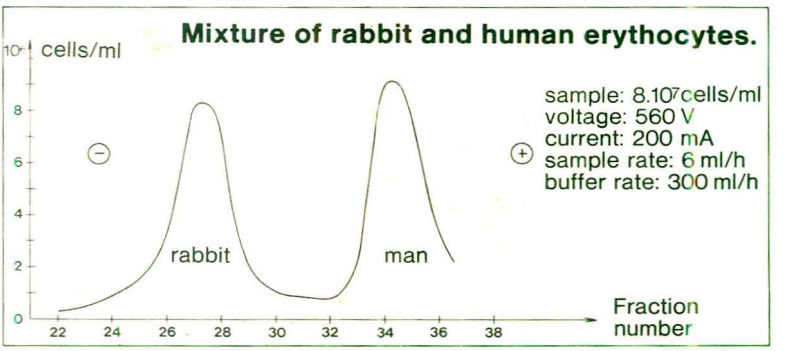
proteins, macromolecules, virus, membrane fragments, bacterial subcellular particles, nuclei, cells, cell sorting without loss of vitality.

Many fields of application:

Immunology, cancer research, hematology, molecular biology, cell research.

Mixture of rabbit and human erythocytes.



Please ask for detailed information.

DESAGA
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World Hallmark of Thin-layer Chromatography and Electrophoresis

C. DESAGA GmbH
Nachf. Erich Fecht
D 69 Heidelberg 1 · P. O. B. 10 19 69
Telephone (0 62 21) 8 10 13
Telex 04-61 736